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Experimental Methods to Study Protein Folding and Protein Dynamics

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References for Lectures on Experimental Studies of Protein Dynamics and Folding

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Eaton

Lecture I

Dynamics of Ligand Binding to Myoglobin†

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ABSTRACT: Myoglobin rebinding of carbon monoxide and dioxygen after photodissociation has been observed in the temperature range between 40 and 350 K. A system was constructed that records the change in optical absorption at 436 nm smoothly and without break between 2 μsec and 1 csec. Four different rebinding processes have been found. Between 40 and 160 K, a single process is observed. It is not exponential in time, but approximately given by $N(t) = (1 + t/t_0)^{-n}$, where t_0 and n are temperature-dependent, ligand-concentration independent, parameters. At about 170 K, a second and at 200 K, a third concentration-independent process emerge. At 210 K, a concentration-dependent process sets in. If myoglobin is embedded in a solid, only the first three can be seen, and they are all nonexponential. In a liquid glycerol-water solvent, rebinding is exponential. To interpret the data, a model is proposed in which the ligand molecule, on its way from the solvent to the binding site at the ferrous heme iron, encounters four barriers in succession. The barriers are tentatively identified with known features of myoglobin. By computer-solving the differential equation for the motion of a ligand molecule over four barriers, the rates for all important steps are obtained. The temperature dependences of the rates yield enthalpy, entropy, and free-energy changes at all barriers. The free-energy barriers at 310 K indicate how myoglobin achieves specificity and order. For carbon monoxide, the heights of these barriers increase toward the inside; carbon monoxide consequently is partially rejected at each of the four barriers. Dioxygen, in contrast, sees barriers of about equal height and moves smoothly toward the binding site. The entropy increases over the first two barriers, indicating a rupturing of bonds or displacement of residues, and then smoothly decreases, reaching a minimum at the binding site. The magnitude of the decrease over the innermost barrier implies participation of heme and/or protein. The nonexponential rebinding observed at low temperatures and in solid samples implies that the innermost barrier has a spectrum of activation energies. The shape of the spectrum has been determined; its existence can be explained by assuming the presence of many conformational states for myoglobin. In a liquid at temperatures above about 230 K, relaxation among conformational states occurs and rebinding becomes exponential.

. Myoglobin and Its Ligands

Myoglobin (Mb),[1] a globular protein of about 17200 molecular weight and 153 amino acids containing one protoheme, plays an important role in the mammalian cell where it stores (Theorell, 1934) and transports (Wittenberg, 1970) oxygen and possibly also carries energy (Hills, 1973). An understanding of the reactions of ferrous Mb with ligands, particularly dioxygen and carbon monoxide, is desirable because, as the simplest protein capable of reversible oxygenation, it can serve as a prototype for more complex systems. Dynamic studies are particularly meaningful because the primary and tertiary structures have been determined (Kendrew et al., 1958) and many properties of the active center are known (Weissbluth, 1974).

The reactions of various ligands with Mb have been investigated extensively with stopped-flow, flash-photolysis, and T-jump techniques. The pioneering work has been performed by Gibson (Gibson, 1956); his and later experiments are reviewed and referenced in the monograph by Antonini and Brunori (Antonini and Brunori, 1971). Our study of the binding of O_2 and CO to sperm whale Mb by flash photolysis extends earlier work in three directions, temperature, time, and dynamic range. Phenomena change so rapidly with temperature that measurements are needed at 10-K intervals between 40 and 350 K. Since processes can encompass more than nine orders of magnitude in time, we constructed a system capable of recording over this range in one sweep. Our equipment records data over more than three orders of magnitude in optical density and we thus can see even processes with relative intensities of less than 1%.

The experimental data are rich and complex but they can be unraveled to give a coherent description of the dynamics of ligand binding to Mb. The essential *experimental* fact is the discovery of four distinct processes (Austin et al., 1973). The central *interpretative* assumption is that ligand binding is governed by successive barriers (Frauenfelder, 1973). The four processes depend differently on temperature and concentration; thus all essential parameters of our model can be determined and all observed features can be understood.

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[1] Abbreviations, symbols, and units: Mb, ferrous sperm whale myoglobin; L, ligand molecule; PVA, poly(vinyl alcohol). Processes I–IV are defined in section 4, barriers I–IV and wells A–E in Figure 11. $N(t)$ denotes the fraction of Mb molecules that have not rebound a ligand at the time t after photodissociation. $N_a(t)$, for instance, gives the probability of finding a ligand molecule in well A at time t, k_{ab} is the first-order rate for transitions from well A to B. Second-order rates are denoted by primes, for instance k_{ed}' (eq 6). E_{ab} and A_{ab}, for instance, denote the activation energy and the frequency factor for the transition from well A to B. Energies are given in kcal/mol; 1 kcal/mol = 0.043 eV = 4.18 kJ/mol. Entropies are given in terms of the dimensionless ratio S/R, where $R = 1.99$ cal mol^{-1} K^{-1} is the gas constant.

Optical Spectra of Oxy- and Deoxyhemoglobin

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Abstract: The optical spectra of oxy- and deoxyhemoglobin are investigated over a wide frequency range using the techniques of polarized single crystal absorption spectroscopy and solution natural and magnetic circular dichroism. In addition to the porphyrin $\pi \rightarrow \pi^*$ transitions, seven transitions of oxyhemoglobin and four transitions of deoxyhemoglobin are characterized. Most of these transitions occur in the near-infrared spectral region. To make assignments extended Hückel calculations are carried out on the complex of iron-porphin with imidazole as a model chromophore for deoxyhemoglobin, with the addition of oxygen to obtain a model chromophore for oxyhemoglobin. Calculations are also carried out on the corresponding carbon monoxide complex to aid in the interpretation of the oxyhemoglobin spectra. Assignments are proposed for all of the transitions in terms of one-electron excitations between single configurations. Five of the seven bands of oxyhemoglobin are assigned to transitions into the lowest empty molecular orbital, which is delocalized over the FeO_2 unit. Three of the four bands of deoxyhemoglobin are assigned to porphyrin↔iron charge-transfer transitions. The remaining bands are interpreted as arising from $d \rightarrow d$ transitions of the iron.

In this paper we present a detailed experimental and theoretical investigation of the optical spectra of the heme complexes in oxy- and deoxyhemoglobin. The principal objectives of this study are to characterize experimentally as many electronic transitions as possible and to assign them to specific orbital promotions. Firm spectroscopic assignments are important, for the experimental parameters can then be used for testing theoretical descriptions of ground and excited state electronic structure in hemoglobin. The assignments are also required for interpreting spectral perturbations in terms of structural changes,[3a-c] and for understanding the mechanism of photolysis of oxy- and carbonmonoxyhemoglobin.[3d]

Our experimental approach has been to synthesize the results of optical absorption measurements that employ plane or circularly polarized light over a wide frequency range. The measurements include the polarized absorption of single crystals and the natural and magnetic circular dichroism of solutions (CD and MCD). From the single crystal spectra we obtain directions for the electric-dipole transition moments, while the CD and MCD spectra also depend on magnetic-dipole transition moments. At present the experimental information is limited to the "window" between about 4000 (2500 nm) and 33 000 cm^{-1} (300 nm). Transitions at lower frequencies are masked by solvent and protein vibrational absorption, while protein electronic absorption obscures heme transitions at higher frequencies. We present our experimental results in detail primarily for the near-infrared spectral region, since the visible and near-ultraviolet spectra using the three different techniques have been previously reported by us or by others. With the addition of the near-infrared spectroscopic data reported here, it is now possible to locate and characterize seven transitions in the optical spectra of oxyhemoglobin, in addition to the porphyrin $\pi \rightarrow \pi^*$ transitions. Four such transitions can be described for deoxyhemoglobin.

Our theoretical approach has been to use crystal field theory and the iterative extended Hückel molecular orbital method of Zerner, Gouterman, and Kobayashi to make the assignments.[4] For the extended Hückel calculations we have used the complex of iron-porphin with imidazole as a model chromophore for deoxyhemoglobin, with the addition of oxygen to obtain a model chromophore for oxyhemoglobin. Using the results of these calculations we attempt to assign the seven transitions of oxyhemoglobin and the four transitions of deoxyhemoglobin in terms of spin-allowed electronic promotions between single configurations. This is a difficult task for several reasons. A much larger number of transitions are predicted in the experimental frequency range than are observed. We have no good independent estimate of the reliability of the predicted frequencies, which appear to be in error by as much as 10 000 cm^{-1}. Finally, no absorption, MCD, or CD amplitudes have been calculated, so that our discussion of these must depend, in most cases, on semiquantitative or qualitative arguments. Nevertheless, we have been able to make reasonably convincing assignments of the observed transitions.

This study is part of a series of investigations on the interpretation of heme protein optical spectra using polarized light techniques. We have previously reported results of work on various complexes of myoglobin,[5] hemoglobin,[6] and cytochromes *c*,[7] *c'*,[8] and P-450.[9] A preliminary account of the present work on oxyhemoglobin has been reported elsewhere.[10]

Experimental Section

Concentrated solutions of horse and human hemoglobin were prepared by standard procedures.[11] Deoxygenation of solutions was carried out under nitrogen by the addition of excess sodium dithionite (Vine Chemicals Ltd., Widnes, Cheshire, England). All solution spectral measurements were carried out in 0.1 M potassium phosphate buffer, pH 7, at room temperature. For measurements at wavelengths longer than 1200 nm, the solutions were dialyzed against D_2O containing the same buffer. Crystallization of human deoxyhemoglobin A was carried out according to the method of Perutz,[11] except that sodium dithionite was used instead of ferrous citrate. Prior to spectral measurements the crystals were washed with a solution containing the same salt composition as the mother liquor (2.2 M ammonium sulfate, 0.3 M ammonium phosphate, 0.01 M sodium dithionite, pH 6.5).

Polarized single crystal absorption spectra were measured at room temperature with a microspectrophotometer described previously.[12] For measurements in the near-infrared to 1150 nm a tungsten source, Hamamatsu R196 photomultiplier tube (S-1 response), and a single ¼ m Ebert monochromator with 590 grooves/mm grating blazed at 1000 nm (Jarrell-Ash) were employed. The space group of human deoxyhemoglobin A crystals is $P2_1$ with two molecules (eight hemes) per unit cell.[11,13] Spectra were measured on the (010) crystal face with light polarized parallel to either the *a* or *c** axes. These axes had been previously identified from a combination of x-ray photography and optical measurements in the visible and near-ultraviolet spectral regions.[14] In the present study the *a* and *c** axes were found to remain the principal optical directions to within ±3° throughout the near-

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Spectroscopic studies of oxy- and carbonmonoxyhemoglobin after pulsed optical excitation

(picosecond spectroscopy/radiationless transitions/hemoglobin photolysis kinetics/hemoglobin excited states/hemoglobin molecular dynamics)

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ABSTRACT **The photolysis of HbO_2 and HbCO has been investigated with picosecond laser techniques. Transient absorption spectra were measured in the Soret and visible regions after excitation with 353- or 530-nm pulses. The photoproducts appeared within 8 psec and exhibited considerably broadened deoxyhemoglobin-like spectra, which persisted to 680 psec. The altered spectra are attributed to the production of deoxyheme conformational and spin states that might result from the intense excitation.**

Perutz introduced the approach to understanding the mechanism of cooperative oxygen binding by hemoglobin in terms of the molecular structure (1–4). More recently, Gelin and Karplus (5) have calculated a detailed reaction pathway for oxygenation of a single subunit, which involves conformational changes in localized regions of the globin in response to a change in heme geometry. Anticipating that some of the dynamics of the heme and globin structural changes may occur very rapidly, we have begun a spectroscopic investigation of the photolysis of HbCO and HbO_2 by using picosecond laser techniques.

Beginning with the studies of Gibson (6) there have been numerous kinetic studies of heme proteins by flash photolysis techniques (7–20). Most of this work was directed toward studying the rebinding of CO or O_2 to Hb or myoglobin on time scales of microseconds or longer (8, 9, 11, 14–16, 18, 20). Alpert *et al.* (10, 13) explored the time regime 50–300 nsec by using a 30-nsec laser pulse for photolysis and found a transient species that decayed at room temperature in about 100 nsec. Photolysis studies on Hb have been extended into the 1- to 20-psec regime by Shank *et al.* (17) who used subpicosecond pulses at 615 nm for excitation and probing. It was concluded that CO dissociation occurs in less than 1 psec, but that no photolysis of HbO_2 occurred under their conditions. A major aim of the present work was to measure transient spectra with sufficiently high accuracy that the structure of the initial photoproducts could be identified.

MATERIALS AND METHODS

Preparation of Materials. Human HbO_2 A was prepared according to a standard procedure (21). HbCO was formed by passing water-saturated CO over a thin layer of the dialyzed lysate. For dilution to the final concentration used in the experiments, the potassium phosphate buffer (0.15 M, pH 7.35) was saturated with CO.

Spectrometric Method. Our transient absorption spectrometer is of the excite and probe type in which a laser harmonic is used to create a sudden excitation of the sample; at a later, variable time, the absorption spectrum is measured with a broad-band picosecond continuum pulse (22). For a given delay time we obtain a full double-beam transient absorption spectrum from a single laser shot.

Both excitation and probe beams are derived from a single 8-psec pulse extracted from 1.06-μm output of a passively mode-locked Nd/glass laser. After amplification, this pulse carries 30 mJ, gives a second harmonic spectrum of 15 cm^{-1} width, and shows a TEM_{∞} transverse mode structure. Once the second and third harmonics at 530 and 353 nm, respectively, have been generated, the third harmonic pulse is isolated by a dichroic beam splitter and filter, passed through a wave plate to reorient its polarization, and focused through a 320-μm-diameter aperture into the sample. The residual first or second harmonic light is used to produce the probing continuum pulse by focusing it into a 5-cm cell of H_2O. The emerging continuum beam passes through a variable delay and is then split and focused into the sample cell through a dichroic beam combiner. One continuum beam passes through the aperture and excited volume collinearly with the excitation pulse while the second continuum beam traverses an unexcited region of the sample. A lens focuses these two beams onto different positions along the length of the entrance slit of a 0.75-m spectrograph used in first order with a 300-groove/mm grating.

The result is two parallel dispersed spectra on the focal plane of the spectrograph. Both of them are detected and recorded for each laser shot by a Princeton Applied Research Corp. model 1215/16/54 optical multichannel analyzer system. The two 250-channel tracks of spectral information are digitally processed in the following way: dark current spectra are subtracted from the raw data; one track is divided by the other; the result is divided by a corresponding ratio spectrum obtained with no excitation light present; and the logarithm of the new ratio spectrum is taken. This gives the change in absorbance in the excited sample volume as a function of wavelength for a single laser shot at the time delay determined by the setting of the optical delay line. Each of the spectra presented here has been averaged over several laser shots but has not been smoothed. The spectral resolution is 1.5 nm and the temporal resolution, determined by our pulse durations, is approximately 10 psec. The quantum yield for irreversible damage of HbO_2 under our irradiation conditions using 353-nm pulses was spectroscopically determined to be less than 10^{-4}.

RESULTS

Transient Spectra in the Soret Band Region. With our spectrometer adjusted to cover the range 398–453 nm, we measured the transient absorption changes induced in a 1-mm-thick sample of 42 μMHbCO solution by pulses of 353-nm light.§ Fig. 1 shows the initial absorbance spectrum of this

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§ At this concentration the system is almost saturated by our light pulse, and the signal is therefore nearly independent of light intensity.

to engender a hydrido–di-ironhexacarbonyl–π-allyl complex of structure (12) which tends to become stabilized by transformation into the crystalline non-linear di-π-allyl di-iron complex of structure (4). Of particular interest is the observed preference of the hydrogen to migrate to the distant vinylic methylene to produce a new methyl group rather than to an internal unsaturated carbon to form either a methine or a new methylene group. This sheds a new light on the well documented $Fe(CO)_3$-induced vinylcyclopropane→diene rearrangement.[5]

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Ultra-fast Recombination in Nanosecond Laser Photolysis of Carbonylhaemoglobin

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Summary The transient absorption change observed in nanosecond laser photolysis of carbonylhaemoglobin which was previously attributed to a tertiary structural change is shown to arise from ultra-fast ligand recombination.

ALPERT *et al.*[1] observed transient absorption changes immediately following the flash in nanosecond photolysis of liganded haemoglobin solutions. They concluded that these were due to tertiary structural changes and that for the carbon monoxide complexes, known to have high photodissociation quantum yields,[2] the final product on this time scale was completely ligand free. We have undertaken a detailed study of the spectra of this transient species and final product and their variation with temperature. The results show that the published interpretation cannot be correct.

50 μmol l^{-1} solutions of human carbonylhaemoglobin (HbCO) in 0·1 mol l^{-1} potassium phosphate buffer at pH 7 in a thermostatted cell were photolysed with 30 ns pulses from a frequency doubled ruby laser giving up to 0·5 J at 347 nm. Transient absorption changes were monitored with an oscilloscope.

At room temperature the kinetic trace observed is exactly as reported[1] but a change is observed when the temperature is varied. Whereas the initial transient absorption remains unchanged, within experimental error, the final level varies with temperature so that the difference between the initial and final levels increases with decreasing temperature. For illustration, the absorbances of the transient and final level, compared to that of HbCO, are given in the Table for wavelengths 416 and 438 nm. Traces are shown in the Figure. At 416 nm the transient absorption is lower than that of the ground state, *i.e.*, there is net transient bleaching whereas at 438 nm there is net transient absorption. In both cases the final level is intermediate between the ground state and the transient absorptions.

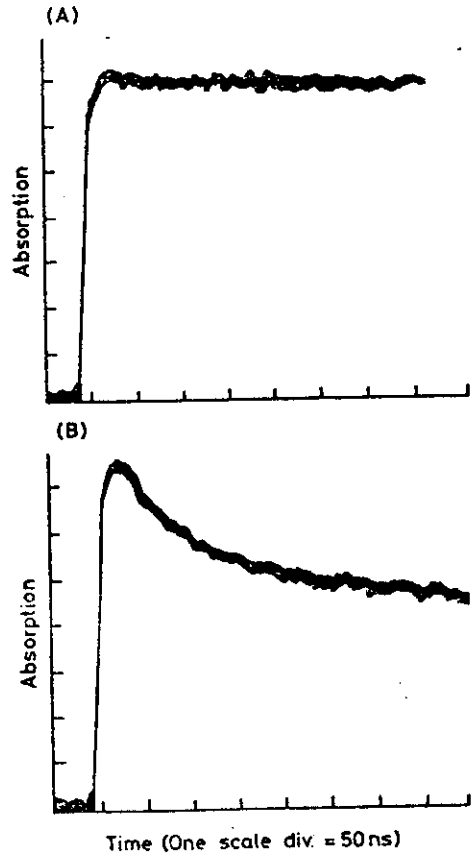

FIGURE. Kinetic traces observed in the 347 nm laser photolysis of carbonylhaemoglobin in aqueous buffer at pH 7·0. Wavelength 438 nm, temperature: (A) 328 K, (B) 277 K.

The fact that the magnitude of the transient absorption change decreases with increasing temperature is not in accord with it being due to conformational change. Evidence that the transient absorption change can be attributed

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Geminate recombination of O_2 and hemoglobin

(picosecond spectroscopy/radiationless transitions/hemoglobin photolysis kinetics/hemoglobin excited states/hemoglobin molecular dynamics)

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ABSTRACT The photolysis of HbO_2 and HbCO has been studied by measuring transient absorption spectra in the Soret region after excitation with picosecond pulses at 530 nm. Dissociation occurred promptly in both cases, followed (for HbO_2) by geminate recombination of *ca.* 40% of the photodissociated O_2 with a lifetime of 200 ± 70 psec (25°C). No recombination of Hb + CO was observed up to 1200 psec after photolysis. The HbO_2 and HbCO photoproduct spectra were broader, weaker, and red-shifted in comparison to the spectrum of stable Hb and Gibson's fast-reacting form, Hb*. For HbO_2 the spectrum was initially much broader to longer wavelengths but relaxed to a constant shape within 90 psec, whereas for HbCO there was no spectral evolution. The photophysics is analyzed by considering the effect of spin constraints as well as spin–orbit coupling and orbital correlation among the various electronic states of liganded and deoxy hemoglobins. The small quantum yield of HbO_2 dissociation is not primarily due to rebinding but rather to electronic relaxation to nonreactive states.

Photodissociation of liganded hemoglobins is a well-known phenomenon (1) used to study dynamics of ligand binding to deoxyhemoglobin (Hb) (2–8). The usefulness of the photoprocess for subsequent dynamical studies is restricted at present by a lack of knowledge of the mechanism of the photodissociation. Of particular interest is the quantum yield for dissociation under steady illumination, which is fairly high ($\phi \approx 0.5$) (9, 10) for carboxyhemoglobin (HbCO) but much lower ($\phi \approx 0.05$) for the oxy form (HbO_2) (9). Two limiting mechanisms can account for the relatively low yield of HbO_2 photolysis. First, energy relaxation to nonreactive states may be much faster than the dissociation. Second, although the initial dissociation step in HbO_2 may be just as efficient as that in HbCO, some released O_2 trapped nearby to the heme may be able to rapidly recombine. These mechanisms are evaluated here by examining the dynamics and identifying the products of the photolysis by picosecond spectroscopy. We have attempted to describe our spectroscopic and dynamical results in terms of recent experimental and theoretical assessments of the excited states of hemoglobins (11–15).

The time scale for studying photolysis and subsequent events in heme proteins has in recent years been pressed back to the picosecond regime, starting with the work of Shank *et al.* (5). Using subpicosecond pulses at 615 nm to excite and probe HbO_2 and HbCO, they found induced absorption build-up in less than 0.5 psec for both species, a decay of the HbO_2 signal in 2.5 psec, and no decay of the HbCO signal up to 20 psec. Rentzepis and coworkers (6, 7) studied HbCO and the myoglobin compounds MbCO and MbO_2 by exciting with 6-psec 530-nm pulses and using 440-nm interrogation over the period 0–300 psec. That group reported a significantly longer (11 psec) predissociation lifetime for HbCO and no subsequent signal change from 48 to 300 psec. The initial MbCO photoproduct signal at 440 nm was reported to undergo a 15% decay (lifetime $\tau = 125 \pm 50$ psec), but for MbO_2 no evidence of decay up to 450 psec was found. Greene *et al.* (8) conducted a study of HbO_2 and HbCO photolysis using 8-psec, 353-nm excitation. They obtained transient absorption spectra in the Soret (400–450 nm) and visible (535–575 nm) regions at 10 psec and 680 psec, finding a persistent, broadened deoxy Hb-like absorption. With their spectra, they observed less than 10% recombination in HbCO over this time interval and less than 20% recombination of photolyzed HbO_2. Greene *et al.* (8) found that HbCO responded similarly to 530- and 353-nm pulses. The present study of the 530-nm photolysis of HbCO and HbO_2 is aimed at identifying the species initially generated by light and how these species evolve.

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MATERIAL AND METHODS

Spectrophotometric Method. The excite-and-probe transient absorption spectrometer was described before in a related study (8, 16). A single 1.06-μm pulse (*ca.* 10 psec) was extracted from the mode-locked train of a TEM$_{00}$ Nd/glass laser, amplified, and then used to generate both a 530-nm excitation beam (fluence ≈ 0.1 J cm^{-2}) and a pair of much weaker, optically delayed probing continuum beams (17). The I and I_o beams, transmitted through excited and unexcited regions of the sample, were dispersed in a spectrograph and digitally recorded on separate tracks of an optical multichannel analyzer. The bandpass of the spectrometer was 6 nm and band positions could be measured to ±0.5 nm. This bandpass does not introduce any quantitatively important spectral distortions.

Although full conversion of the starting material contained within the probed region was not obtained due to imperfect beam overlap, the sample was essentially saturated at normal energies, and no correction was made for the energy of the excitation pulse. Nevertheless, shots for which the excitation energy varied by more than 40% from the norm were rejected. Typical transient difference spectra showed excursions of 0.3 A for bleaching or new absorption. In the range 410–440 nm, the photometric accuracy was ±0.015 A, whereas elsewhere it was poorer. Digitized spectra of the stable species Hb, HbCO, and HbO_2, obtained on our transient absorption spectrometer with continuous illumination, were used to construct stable difference spectra, Hb − HbCO and Hb − HbO_2.

Samples. Human HbO_2 and HbCO were prepared and handled by using common techniques (18). HbO_2 samples were prepared to a concentration of ≈0.63 mM (heme basis) in pH 7.35, 0.15 M potassium phosphate buffer for room temperature (24 ± 0.5°C) measurement using a 0.1-mm optical path length. We chose this high concentration in order to maximize the tetramer-to-dimer ratio (19). HbCO samples were used with a concentration of ≈52 μM and a path length of 1.0 mm.

Biochemistry

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Solvent Viscosity and Protein Dynamics†

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ABSTRACT: Proteins are dynamic systems. Recent evidence demonstrates that they exist in a large number of conformational substates and can continuously move from one substate to another; motion of a small ligand inside a protein may be possible only through these conformational fluctuations. To test this idea, we study with flash photolysis the binding of CO to protoheme and O_2 and CO to myoglobin in many different solvents. The standard evaluation of such experiments yields information only about the protein–solvent system. A novel approach is presented which permits conclusions concerning the protein: Data from all solvents are considered together, and the rates for transitions of the ligand over various barriers are studied as a function of temperature for fixed solvent viscosities. Results show that over a wide range in viscosity the transition rates in heme–CO are inversely proportional to the solvent viscosity and can consequently be described by the Kramers equation. The rates of O_2 and CO in myoglobin also depend on the solvent viscosity and are most sensitive to the solvent at the lowest viscosity. Viscosity influences protein reactions even in aqueous solutions. The data can be interpreted by a dynamic model in which transitions into and inside myoglobin are governed by fluctuations between conformational substates corresponding to closed and open pathways. Ligand motion thus is mainly controlled by gates and not by static potential barriers. Some characteristic parameters for the substates are determined, and they agree approximately with similar parameters found in Mössbauer experiments. As expected, the barrier parameters evaluated in the novel approach deviate markedly from the ones obtained by the conventional procedure. Comparison with model calculations or basic theories will be meaningful only with the new evaluation, and the method may be essential for many or possibly all biochemical reactions.

Dynamic Barriers in Proteins

A small ligand such as dioxygen (O_2) or carbon monoxide (CO), upon entering or leaving a protein, must overcome barriers. In previous papers (Austin et al., 1975; Alberding et al., 1976, 1978a) we have shown that binding over an extended temperature range, from ~60 to over 300 K, can indeed be described as motion of the ligand over a series of potential barriers, characterized by temperature-independent activation enthalpies and entropies. Below ~200 K, only one binding step is observed, and we interpret it as motion over the final potential barrier at the heme. This transition is not exponential in time and cannot be described by a unique activation enthalpy but can be explained if the protein exists in a large number of conformational substates with slightly different binding rates. Below ~200 K, each protein is frozen into a particular substate with a given activation enthalpy; the rebinding after photodissociation reflects the distribution in barrier heights. At high temperatures, the protein breathes and moves from one conformational substate to another; a ligand that binds sees an average barrier height, and the time dependence should become exponential. Our flash photolysis experiments thus lead to two salient concepts, description of the entire kinetics in terms of a sequence of static potential barriers and conformational substates. The two concepts are, however, incompatible: If the protein fluctuates, the barriers should be dynamic and not static. We are therefore compelled to examine the assumption of static barriers and the evidence for substates in more depth. The existence of conformational substates follows from the general principles of statistical mechanics, and evidence has been seen in many experiments [for a review, see Gurd & Rothgeb (1979)]. Perutz has pointed out that O_2 could not enter or leave Mb[1] if the atoms were fixed in their equilibrium positions (Alberding et al.,

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[1] Abbreviations and symbols used: Mb, ferrous sperm whale myoglobin; G_{ij}^*, H_{ij}^*, and S_{ij}^* denote activation Gibbs energy, enthalpy, and entropy for the transition $i \rightarrow j$, with the assumption that protein and solvent together form the complete system; G_{ij}^*, H_{ij}^*, and S_{ij}^* are the corresponding quantities for the protein alone; energies are given in kilojoules per mole; 1 kJ mol^{-1} = 0.239 kcal mol^{-1} = 0.010 eV; entropies are given in terms of the dimensionless ratio S/R, where R = 8.31 J mol^{-1} K^{-1}; CM, carboxymethyl.

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LETTERS TO THE EDITOR

Geminate Recombination of Carbon Monoxide to Myoglobin

Transient absorption spectra of myoglobin, following photolysis of the carbon monoxide complex at room temperature, were measured using a newly developed, sensitive nanosecond absorption spectrometer. The Soret spectrum of the immediate photoproduct is almost identical to that of deoxymyoglobin at equilibrium, suggesting that the heme group has changed from a planar to a domed structure in less than about 3 ns. About 4% of the photodissociated carbon monoxide molecules rebind to the hemes to which they were initially bound, with a relaxation time of 180 ns. Duddell *et al.* (1980) observed a geminate yield of 27% and a relaxation time of ~55 ns for the photolysis of oxymyoglobin. Comparison of the two results using the simplest kinetic model suggests that the 30-fold more rapid overall association rate for the reaction of oxygen with myoglobin compared to carbon monoxide results mainly from faster binding at the heme, with a small contribution from more rapid entry of oxygen into the protein from the solvent. The data on carbon monoxide are also compared with predictions from low-temperature studies of Frauenfelder and co-workers. This comparison points to the need for further experiments to demonstrate the correspondence between the ligand rebinding processes observed at high and low temperatures.

Photolysis of the oxygen and carbon monoxide complexes of myoglobin has provided a useful means for studying the detailed mechanism of the overall dissociation and binding reaction (Gibson, 1956; Antonini & Brunori, 1971; Austin *et al.*, 1975; Hasinoff, 1977,1981; Case & Karplus, 1979; Duddell *et al.*, 1980; Beece *et al.*, 1980; Doster *et al.*, 1982). Short laser pulses can be used to create an intermediate at room temperature in which the ligand is photodissociated from the heme iron, but has not yet escaped from the protein into the solvent. The ligand can either rebind to the heme in a unimolecular process called "geminate" recombination, or leave the protein and, eventually, bind to the heme of another molecule in a bimolecular process (Duddell *et al.*, 1979; Alpert *et al.*, 1979; Friedman & Lyons, 1980; Chernoff *et al.*, 1980). Although geminate recombination of oxygen (O_2) to myoglobin has been reported, kinetic studies, including measurements in the picosecond time regime, have failed to detect geminate recombination of carbon monoxide at room temperature (Duddell *et al.*, 1980; Cornelius *et al.*, 1981; Reynolds *et al.*, 1981). Here we report the measurement of both the geminate yield and relaxation time for CO, using a newly developed, sensitive nanosecond absorption spectrometer.

Sperm whale skeletal muscle metmyoglobin was purified by column chromatography on Sephadex G-25 and carboxymethyl-cellulose, equilibrated with 0·1 M-potassium phosphate (pH 7·0) containing variable concentrations of CO, and diluted into the same buffer containing 0·01 M-sodium dithionite. Protein concentrations were based on an extinction coefficient of 187,000 M^{-1} cm^{-1} for

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Femtosecond photolysis of CO-ligated protoheme and hemoproteins: Appearance of deoxy species with a 350-fsec time constant

(femtosecond spectroscopy/CO-heme photolysis kinetics/excited states of hemoglobin, myoglobin, and protoheme/molecular dynamics)

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ABSTRACT Photolysis of HbCO, MbCO, and CO-protoheme has been investigated by measuring transient differential spectra and kinetics of induced absorption after excitation with a 250-fsec laser pulse at 307 nm. Probing was performed by a part of a continuum pulse between 395 and 445 nm. Photodissociation of the three liganded species occurred within the pulse duration. By contrast, the formation of deoxy species appeared with a mean (± SD) response time of 350 ± 50 fsec. This time constant was identical for the three species and independent of the presence or absence of the protein structure. Our results suggest the formation of a transient high-spin in plane iron (II) species which relaxes in 350 fsec to a high-spin stable state with concerted kinetics of CO departure and iron displacement. The spin transition is suspected to occur via liganded excited states which relax in part to nonreactive states with a 3.2-psec time constant.

Picosecond spectroscopy has revealed that the dissociation of ligand from the heme in Mb and Hb occurs within 4 psec and that the nonliganded species (Mb or Hb) were formed with a time constant of 11 psec (1, 2). This has been confirmed recently by Cornelius *et al.* (3) who observed the appearance of Mb species after photodissociation of MbO_2 with a 12-psec time constant; their results on MbCO showed a "simultaneous development of both bleaching and absorption intensities." From results of studies with 0.5-psec optical pulses at 615 nm in a pump probe experiment, Shank *et al.* (4) deduced that the photolysis of CO from HbCO should occur in less than 0.5 psec.

More recently, Greene *et al.* (5) and Chernoff *et al.* (6), using 8-psec excitation pulses at 353 and 530 nm, obtained transient absorption spectra in the Soret and visible regions at 10 and 680 psec which indicated that deoxy species formed in a time shorter than their pulse duration. Terner *et al.* (7) used picosecond resonance Raman methods and found formation of HbCO photoproducts with spectra similar to those of deoxy Hb and corresponding to a high-spin at least partially out-of-plane Fe(II) state. These events occurred within 30 psec, their pulse duration.

Up to now, the kinetics of photodissociation of ligand from heme in hemoproteins has not been precisely resolved. Recent theoretical approaches dealing with the energies involved in ligand reactions to and from the heme and their relationship with the protein matrix surrounding the heme (8, 9) have led to tentative models describing the probable pathway(s) through energy barriers of the ligand in the heme pocket. It is of primary importance for such theoretical approaches to be supported by accurate quantitative measurements of the very early steps of the chemical events leading to the formation or dissociation of the ligand–heme complex.

In the present work, we have limited ourselves to the investigation of the kinetics of formation of the nonliganded species after dissociation of CO from MbCO and HbCO. In order to appreciate the possible influence of polypeptidic environment on the heme reactivity in these hemoproteins, we have also studied the kinetics of CO dissociation from protoheme and of deoxyprotoheme formation. These experiments were carried out with a newly developed spectroscopic technique operating with laser pulses of 250 fsec duration to excite the hemes at 307 nm.

MATERIAL AND METHODS

Preparation of the Hemoproteins and Protoheme Solutions. Purified human adult hemoglobin was prepared from fresh human blood by DEAE-Sephadex chromatography (10). The experimental solution was 0.1 mM on the basis of heme; it was diluted in 0.1 M K phosphate buffer at pH 7 or 8. The solution was first deoxygenated under moist argon before equilibration with CO at 1 atm (1 atm = 1.013×10^5 pascals). MetMb (horse heart type III, Sigma) was converted to $MbFe^{2+}$ after addition of a 5 molar excess of freshly prepared Na dithionite (Merck) under strict anaerobic conditions and then equilibrated with pure CO. This stock solution was chromatographed on an ion exchange resin column to remove dithionite and then diluted to 0.1 mM heme in the same buffer as HbCO. The purity of HbCO and MbCO was checked by isoelectric focusing which revealed a single band for both proteins. Protoheme was prepared from hemin [Fe^{3+}(protoporph)Cl] (bovine type I, Sigma) as follows. A stock solution (2 mM) was prepared in 1 M NaOH solution and then diluted in polyethylene glycol (PEG, Merck) to a final concentration of 0.1 mM. A 5 molar excess of Na dithionite was added under anaerobic conditions and a deoxy Hb spectrum (Cary 219, Varian) was recorded.

The deoxy solution was equilibrated with CO and kept in ice water until used. We observed that the deoxy spectrum of protoheme was much dependent upon the PEG/NaOH ratio (vol/vol), the highest value of $\epsilon_{420.5} = 138.6\ mM^{-1}cm^{-1}$ being obtained with a ratio of 94:6 or more. Changes in absorbance of deoxy protoheme solution and, to a lesser extent, of fully liganded protoheme with the nature of the solvent have been attributed to aggregation of the protoporphyrin molecules in aqueous media (11). In the CO-protoheme solutions ($\epsilon_{411} = 207.3\ mM^{-1}cm^{-1}$), the Na dithionite was not removed before the experiment because of the rapid oxidation of the iron complex during the ion exchange chromatography (12). However, at the concentration used in this study, the absorbance of the protoheme samples at 307 nm was not very different from that recorded in the MbCO or HbCO samples.

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CO binding to heme proteins: A model for barrier height distributions and slow conformational changes[a)]

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A model for the dependence of the potential energy barrier on a "protein coordinate" is constructed. It is based on a two dimensional potential energy surface having as variables the CO–iron distance and a conceptual protein coordinate. The distribution of barrier heights observed in kinetics follows from an initial Boltzmann distribution for the protein coordinate. The experimental nonexponential rebinding kinetics at low temperatures or large viscosities (when the protein coordinates can be assumed "frozen") can be fit with a simply parametrized energy surface. Using the same energy surfaces and the theory of bounded diffusion perpendicular to the reaction coordinate, we generate (in qualitative agreement with experiment) the survival probability curves for larger diffusivity, when the constraint on the protein coordinate is relaxed. On the basis of our results, the outcomes of new experiments which examine the concepts underlying the theory can be predicted.

I. INTRODUCTION

Heme proteins are perhaps the best understood class of protein molecules.[1] They are the most obvious candidate for trying to understand the effect of the overall protein on the "local" chemistry of small ligand binding. The kinetics of ligand binding to heme molecules has been studied[2,3] over a large range of temperatures and solvent viscosities. In a typical experiment[3] a sample of bound heme–ligand in a solvent saturated with ligand is cooled to the desired termperature, the ligand is flashed off by a strong light flash (of duration ~ 1 μs; the ns regime is just recently being investigated[4]) and the fraction of unbound heme is followed as a function of time (from 2 μs to a few seconds) by a monitoring beam in the Soret band.

At low temperatures, the same ligand molecule previously on a given heme recombines with that heme. The "survival probability" measured as above shows a power law time dependence, in contrast to the familiar exponential kinetics of elementary unimolecular reactions. As the temperature is increased the kinetics gradually becomes exponential. A similar transition from power law at high viscosities (small diffusivity) to exponential at low viscosities is observed at constant temperature as a function of solvent viscosity. But in spite of the viscosity dependence, the reaction rate does not tend to zero at high viscosities, in contrast to Kramers' model.

The observed nonexponential kinetics is apparently related to the fact that these large macromolecules have many conformations[5(a)] which can differ substantially in barrier height for ligand rebinding. If at low temperatures the rate of changing between such conformations is slow, an individual molecule will not be able to average the activation barrier over all conformations, resulting in an experimental observation of a distribution of barrier heights[2] instead of a sharp activation energy. Experiments have been fit by such distributions,[2] but a microscopic physical model from which these can be generated has not been proposed.

At higher temperatures (and lower viscosities) the observed kinetics was explained[2] in terms of sequential barriers. At the highest temperature, ligand escape into solution becomes dominant and the kinetics is exponential and bimolecular. The above experimental results spurred additional theoretical work,[7–12] in an effort to determine the ligand's path from the "heme pocket" into solution[7] and explain the temperature[8,10] and viscosity[11,12] dependence of the rate constants.

The present work has two objectives. First, a physical model for distribution of barrier heights for CO binding is constructed and fitted to the low temperature results. The model involves an energy surface for rebinding which depends on both the heme–ligand and the protein coordinates. Such a model provides a conceptual and quantitative framework for understanding the origin of such distributions. In addition, it is a significant guide to constructing new experiments.

Second, the theory of bounded diffusion perpendicular to the reaction coordinate[13] is used to project the kinetic results to higher temperatures (or lower viscosities). We observe that the turnover to exponential kinetics can be an effect of *parallel* reactions from the different conformations (even without invoking a *sequential*[2] kinetic scheme) and that a non-Kramers dependence on viscosity can result from diffusion perpendicular to the reaction coordinate (without the need to invoke a relation of "internal" and "external" viscosities[11]).

II. THEORY

The physical picture for geminate (unimolecular) ligand binding from the heme pocket is as follows: For each protein configuration x there is a different binding rate constant $k(x)$. At low temperatures (or high viscosities) when the protein coordinate is "frozen," each protein molecule i has a particular value x_i of its co-

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Molecular dynamics simulation of photodissociation of carbon monoxide from hemoglobin

(protein dynamics/Raman spectroscopy/transient spectroscopy/picosecond kinetics)

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ABSTRACT **A molecular dynamics simulation of the photodissociation of carbon monoxide from the α subunit of hemoglobin is described. To initiate photodissociation, trajectories of the liganded molecule were interrupted, the iron–carbon monoxide bond was broken, and the parameters of the iron–nitrogen bonds were simultaneously altered to produce a deoxyheme conformation. Heme potential functions were used that reproduce the energies and forces for the iron out-of-plane motion obtained from quantum mechanical calculations. The effect of the protein on the rate and extent of the displacement of the iron from the porphyrin plane was assessed by comparing the results with those obtained for an isolated complex of heme with imidazole and carbon monoxide. The half-time for the displacement of the iron from the porphyrin plane was found to be 50–150 fs for both the protein and the isolated complex. These results support the interpretation of optical absorption studies using 250-fs laser pulses that the iron is displaced from the porphyrin plane within 350 fs in both hemoglobin and a free heme complex in solution.**

Upon dissociation of O_2 or CO from Hb conformational changes of the heme are believed to initiate a sequence of conformational changes in the surrounding protein that are responsible for cooperativity (1–3). The principal heme conformational change is a displacement of the iron from the porphyrin plane toward the proximal histidine, which alters the stereochemical relation between the heme and the protein (Fig. 1). The resulting protein tertiary conformational change destabilizes the intersubunit bonding, causing a transition from the *R* quaternary structure to the *T* quaternary structure. A major objective of recent transient spectroscopic studies has been to measure the rates at which these structural changes occur following photodissociation of the ligand (4–8). As an aid to interpreting the results of these experiments we have begun a theoretical investigation of the kinetics of structural changes using the technique of molecular dynamics (9, 10). Here we report the results of our initial studies that focus on the influence of the protein on the rate and extent of displacement of the iron.

The most readily interpretable results have been obtained in experiments on the photodissociation of the CO complex. Photodissociation with 250-fs pulses showed that a species with a deoxy-like optical spectrum appears with a time constant of 350 fs and that no spectral change in the photoproduct occurs for 100 ps (7). The spectrum of the photoproduct was assigned to that of a high-spin ferrous heme, suggesting that the iron had been displaced from the porphyrin plane within 350 fs (7). In optical studies with 8-ps pulses the deoxy photoproduct appeared within the pulse width, consistent with the femtosecond studies, and no spectral change was observed for 1.2 ns (11, 12). In a resonance Raman study using 30-ps pulses to both photodissociate and measure the spectrum, the immediate photoproduct was found to have core size marker frequencies 2–4 cm^{-1} lower than those of deoxy-Hb but identical to a six-coordinate, ferrous high-spin model compound in which the iron is coplanar with the porphyrin (4, 5). These frequencies were unchanged at 20 ns but by 300 ns were the same as in deoxy-Hb (13). The interpretation of these results was that photodissociation produces a ferrous high-spin iron within 30 ps but that the iron does not reach the out-of-plane distance found in deoxy-Hb until 20–300 ns (4, 5, 13). It was assumed that relaxation of the iron to its position in deoxy-Hb would be coupled to a tertiary conformational change of the protein and could correspond to the structural change observed by optical absorption at 50 ns (8). Because of the 1.4 cm^{-1} experimental uncertainty in the measurement of frequency differences it could not be determined whether the iron was still in the porphyrin plane at 30 ps or had moved to an intermediate position between HbCO and deoxy-Hb (5). Other investigators have argued that a coplanar iron at 10 ns is inconsistent with the observation of an increased iron–histidine stretching frequency at 10 ns, since this frequency would be lowered by nonbonded interactions between the imidazole and porphyrin (6).

In the present molecular dynamics study we simulate the photodissociation process by interrupting a trajectory of the liganded complex and changing the potential function for the iron–carbon bond to one in which there is no attractive interaction; the parameters of the iron–nitrogen bonding are simultaneously changed to produce a deoxyheme conformation with the iron displaced 0.55 Å from the porphyrin plane in the absence of the protein. To assess the effect of the protein, the simulation was performed on a complete α subunit and compared with the results of a calculation on the isolated heme complex consisting of iron bonded to protoporphyrin IX, imidazole and CO. Because the results are critically dependent on the choice of the parameters involving the iron bonding, two sets of parameters were employed. These sets reproduce the energies and forces for the iron out-of-plane displacement that bracket values obtained from quantum mechanical calculations. We first describe the potential functions and selection of parameters and then present the results of the molecular dynamics simulations.

Heme and Protein Potential Function

The potential function used in the calculations, described in detail elsewhere (14), is:

$$\begin{aligned} E(\text{total}) = {} & \Sigma K_b(b - b_0)^2 + \Sigma K_\theta(\theta - \theta_0)^2 \\ & + \Sigma K_\phi[1 + \cos(n\phi + \delta)] + \Sigma(A/r^{12} - B/r^6) \\ & + \Sigma\{(A/r^{12} - B/r^6)\exp(-\theta^2_{O\text{-}H\text{-}N}/\sigma^2) \\ & + (A'/r^{12} - B'/r^6)[1 - \exp(-\theta^2_{O\text{-}H\text{-}N}/\sigma^2)]\}. \quad [1] \end{aligned}$$

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Rate Theories and Puzzles of Hemeprotein Kinetics

Hans Frauenfelder and Peter G. Wolynes

Summary. The binding of dioxygen and carbon monoxide to heme proteins such as myoglobin and hemoglobin has been studied with flash photolysis. At temperatures below 200 K, binding occurs from within the heme pocket and, contrary to expectation, with nearly equal rates for both ligands. This observation has led to a reexamination of the theory of the association reaction taking into account friction, protein structure, and the nature of electronic transitions. The rate coefficients for the limiting cases of large and small friction are found with simple arguments that use characteristic lengths and times. The arguments indicate how transition state theory as well as calculations based on nonadiabatic perturbation theory, which is called the Golden Rule, may fail. For ligand-binding reactions the data suggest the existence of intermediate states not directly observed so far. The general considerations may also apply to other biomolecular processes such as electron transport.

Chemical reactions govern all aspects of biological processes, from enzyme catalysis to transfer of charge, matter, and information. Any deep understanding of biological reactions must be based on a sound theory of chemical reaction dynamics. Most of the knowledge of reaction dynamics, however, has been deduced from studies of two-body interactions of small molecules in the gas phase (*1*). In contrast to this simple system, biomolecules provide a complex but highly organized environment that can affect the course of the reaction. Fortunately, the complexity imparts a richness of phenomena that allows the examination of fundamental aspects of reaction dynamics. Biomolecules, in particular heme proteins, are an excellent laboratory as shown, for example, by the observations of nuclear tunneling in them (*2–4*).

Most reactions involve motion of the nuclei of the reacting species and changes in their electronic structure. For many reactions the electronic structure adiabatically follows the nuclear motions, thus no explicit attention need be paid to the dynamics of the changing electronic structure. In that case, a picture of the reaction dynamics based on a single adiabatic potential-energy surface is appropriate. When the spins of the reactants change or when long-range electron transfer is involved, however, the changes in electronic structure may be slower than the nuclear motion. The characteristics of the electronic motion, then, are important in determining the reaction rate, and a theory of the nonadiabatic transition from one electronic state to another is needed. Such a theory has been used as the basis of most treatments of biological electron transfer (*5*). Since kinetic control is at the heart of many biological processes, an assessment of the relative importance of nuclear and electronic motions is desirable.

The binding of dioxygen (O_2) and carbon monoxide (CO) to heme proteins is a situation where the problem can be studied in detail. In this article, we discuss these reactions with the aim of constructing a qualitative framework for understanding the general issue of nuclear and electronic motions in biomolecular reactions. In this analysis, we consider recent ideas on the influence of dissipation and fluctuations on reaction dynamics and show the limitations of transition state theory in complex systems. Attempts to understand heme reactions have been made before, notably by Jortner and Ulstrup (*6*) and by Hopfield and his co-workers (*7*), who have used theories of nonadiabatic transitions. We show that an approximation of adiabatic behavior may be closer to reality. The analysis of the specific reactions leads to general conclusions that may be applicable to other reactions in biomolecules and in condensed phases.

Binding of O_2 and CO to Heme Proteins

The "laboratory" for studying the binding reaction is shown in Fig. 1, a schematic cross section of a heme protein, for instance myoglobin or a separated hemoglobin chain. Flash photolysis experiments suggest that ligand binding occurs through a complex path: the ligand, for instance O_2, enters the protein matrix from the solvent, moves through the matrix into the heme pocket, and binds covalently to the heme iron (*8*). The formation of the covalent bond between the ligand and the iron is the rate-limiting step (*9*). We will concern ourselves with this step, not with the motion from the solvent to the pocket.

The initial state (called state B) and the final state (called state A) in the bond formation are structurally and spectroscopically well characterized (*10*). The spatial structures of the CO- and O_2-bound species are similar. Before bond formation, the heme iron has spin 2; it lies about 0.5 Å out of the mean heme plane; and the heme is domed. In the bound state, A, the spin is zero; the iron has moved closer to the heme plane; and the heme is nearly planar. The best current descriptions of the electronic structure of the oxy and the carbon monoxy species are, however, different (*11*). The free CO molecule in state B has closed

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Molecular dynamics simulations of cooling in laser-excited heme proteins

(picosecond spectroscopy/intramolecular vibrational relaxation/Raman spectroscopy/hemoglobin/myoglobin)

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Contributed by Robin M. Hochstrasser, August 15, 1986

ABSTRACT **In transient optical experiments the absorbed photon raises the vibrational temperature of the chromophore. In heme proteins at room temperature conversion of a 530-nm photon into vibrational energy is estimated to raise the temperature of the heme by 500–700 K. Cooling of the heme is expected to occur mainly by interacting with the surrounding protein. We report molecular dynamics simulations for myoglobin and cytochrome *c in vacuo* that predict that this cooling occurs on the ps time scale. The decay of the vibrational temperature is nonexponential with about 50% loss occurring in 1–4 ps and with the remainder in 20–40 ps. These results predict the presence of nonequilibrium vibrational populations that would introduce ambiguity into the interpretation of transient ps absorption and Raman spectra and influence the kinetics of sub-ns geminate recombination.**

After molecules absorb visible or ultraviolet light, the deposited energy becomes distributed over a subset of the molecular motions and subsequently is exchanged with the surroundings. When ps or fs light pulses are used to probe photophysical or photochemical processes, it is necessary to consider that nonequilibrium distributions of vibrational energy may persist during the time scale of the experiment. Transient excesses of vibrational energy can alter the rates of chemical and physical transformations, and they may also introduce spectral changes that could easily be mistaken for chemical steps in a process.

Transient spectra of heme proteins have been studied with ps (1–7) and fs (8, 9) time resolution in experiments that have exposed a variety of spectral intermediates following light absorption by the heme group. The principal goal of such experiments is to understand the sequence of early structural alterations occurring after a diatomic ligand (O_2, CO, NO) is photodissociated from the heme iron. On these time scales the photolyzed sample may also contain electronically excited states, each of which may have a spectrum that changes with time as a result of changes in its vibrational energy content. The present paper is an attempt to predict, on the basis of molecular dynamics simulations, the rate at which the excess vibrational energy in the heme is dissipated into the protein.

To understand the detailed pathways for vibrational cooling of condensed-phase molecules, it is necessary to consider the intramolecular vibrational relaxation (10, 11) as well as the relaxation induced by coupling the molecule to its immediate environment. For large aromatic molecules, the intramolecular vibrational reorganization can be a sub-ps process so the molecule may quickly lose the memory of which levels were initially excited. However, a considerable body of work indicates that vibrational energy relaxation of highly excited solution phase molecules often occurs more slowly than the time resolution of conventional ps laser experiments (12–15). There is, therefore, a reasonable expectation that the heme group in the protein will retain vibrational energy in excess of equilibrium for measurably long times following optical excitation.

If one or more photons of wavelength λ were absorbed by a heme group that was initially at equilibrium at 300 K with average vibrational energy $\langle E \rangle$ exclusive of zero-point energy and if the excitation were distributed by the intramolecular vibrational relaxation over all the harmonic vibrational modes of the ground electronic state in accordance with Boltzmann statistics, the new molecular temperature T would be obtained by satisfying the relation

$$\langle E \rangle + nhc/\lambda = \Sigma\, h\nu_i [e^{h\nu_i/k_B T} - 1]^{-1}, \qquad [1]$$

where the sum is over all $(3N - 6)$ vibrational modes of frequencies ν_i and n is the number of photons absorbed. For a typical metal porphin having 37 atoms and 105 modes, the temperature for $n = 1$ and $\lambda = 530$ nm is 760 K. The increase of 460 K is substantial, but the critical aspect of its importance concerns the dynamics of the cooling process that results from the interactions between the heme and the protein surroundings. Molecular dynamics simulations seemed to be a natural approach to this question.

METHODS

Molecular dynamics simulations (16–18) were performed on both ferrocytochrome *c* (cyt *c*) and deoxymyoglobin (Mb) *in vacuo*. The basic procedure for both molecules was the same, using as starting points the x-ray crystallographic coordinates of all the heavy atoms in each molecule, to which were added coordinates of all hydrogen atoms that can participate in N-H · · · O hydrogen bonds. The x-ray coordinates for tuna heart cyt *c* were those from the 1.5-Å resolution structure by Takano and Dickerson (19) (Brookhaven Protein Data Bank file 4CYT‡) and the atomic coordinates for sperm whale Mb were from the 1.4-Å resolution structure by Phillips (Brookhaven Protein Data Bank file 1MBD‡). The potential function used to describe interatomic interactions in the protein matrix was essentially the same as that used in earlier simulations of hemoglobin photolysis (20), with the addition of electrostatic interactions between partial charges assigned to all the atoms (21). Partial charges for the protein atoms and the Mb heme atoms were taken from the parameter set TOPH19 of the program CHARMM (21, 22), while the partial charges for the heme atoms of cyt *c* were adapted from iterative extended Huckel molecular orbital calculations (L. K. Hanson, personal communication). In all the calculations, the dielectric constant ϵ was taken to be equal to the interatomic distance in Angstroms, making the electrostatic

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Abbreviation: cyt *c*; cytochrome *c*.
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Protein Fluctuations, Distributed Coupling, and the Binding of Ligands to Heme Proteins

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Abstract: A general model for the binding of small molecules to heme proteins is presented. The model is based on a potential surface involving the iron–ligand binding coordinate, r, and an internal protein coordinate, Q (e.g., iron–porphyrin out-of-plane displacement). A protein fluctuation coordinate, x, is used to modulate the coupling (iron out-of-plane equilibrium position) of the unligated state of the system. A Gaussian distribution in the out-of-plane equilibrium position has been previously shown to account for the non-Gaussian inhomogeneous broadening of the deoxy myoglobin Soret band (Šrajer et al. *Phys. Rev. Lett.* **1986**, *57*, 1267). We propose that this distribution is driven by protein conformational fluctuations that are frozen into the ensemble at low temperature (quenched disorder) leading directly to the inhomogeneous distribution in the geminate rebinding kinetics observed by Austin et al. (*Biochemistry* **1975**, *14*, 5355). Specific example calculations involving the low-temperature geminate recombination of CO to myoglobin and leghemoglobin are discussed in detail and a simple intuitive picture is presented that separates the activation enthalpy into distal pocket, H_D, and proximal, H_P, terms. The proximal term involves the work needed to bring the iron atom to the in-plane transition state. At physiological temperatures, when the fluctuations are rapid with respect to the kinetic time scales, the observed single exponential rate and the corresponding Arrhenius barrier height are predicted from the low-temperature kinetic parameters. These parameters, along with other experimental and theoretical constraints, are used to construct detailed potential energy surfaces that are useful in further investigations of ligand binding to heme proteins. As an example, we present an analysis of hemoglobin cooperativity in the Appendix. It is suggested that the protein conformations associated with the R and T states will couple differently to the unligated iron–porphyrin coordinate. Such differences in coupling can be envisioned as larger and more discrete versions of the fluctuations that drive the distributions in coupling found for the monomeric myoglobin system. It is shown that significant amounts of energy can easily be stored in the Stokes shift difference associated with the unligated T and R hemes. Such nuclear relaxation effects may have already been detected experimentally in magnetic susceptibility and kinetics experiments. The observed values for the relative "on" and "off" rates of the R and T states are in accord with the proposed potential surfaces.

I. Introduction

The protein structure–function relationships involved in the binding of ligands to heme proteins have been the focus of a wide variety of physical, biological, and chemical investigations during the last several decades. A well-studied process from an experimental point of view is the geminate recombination of CO to myoglobin (Mb) at low ($T \leq 160$ K) temperature.[1,2] Quantum effects, such as tunneling, are observed below ~60 K.[3,4] Above ~160 K, the CO ligand can begin to diffuse through the protein matrix and eventually into the solvent,[5] leading to more complicated effects. For the moment, we focus on the relatively simple geminate process:

$$\text{Mb-CO} \underset{\{k\}}{\overset{\gamma}{\rightleftharpoons}} \text{Mb} + \text{CO} \qquad (1)$$

Equation 1 denotes the photolysis of carbon monoxy myoglobin (Mb-CO) by light (γ) and the subsequent rebinding ($\{k\}$). The curly brackets around the $\{k\}$ indicate that a single rate is not sufficient to describe the rebinding and that a distribution in rates is necessary to explain the nonexponential kinetics observed at low temperature. The distribution of rates is thought[6] to arise from conformational substates of the myoglobin that are frozen into the ensemble below the phase transition of the solvent (quenched disorder). At physiological temperatures, when rapid fluctuations of the protein become possible, the system can be described by averaging over the fluctuation coordinates and the observed simple exponential relaxation should be predicted from the low-temperature distribution.

Several theoretical approaches have been previously applied to this problem,[7-11] and we will attempt to incorporate and discuss the various points of view as they pertain to the present treatment. We remark at the outset that the formal aspects of the model given here draw deeply from the previous work.[7-9] Nevertheless, fundamental differences arise due to the different dimensionalities of the treatments. Buhks and Jortner[9] calculate the low-temperature rates within a single-dimensional harmonic space and do not attempt to introduce the (protein) fluctuations. Agmon and Hopfield[7] and Bowne and Young[8] utilize a two-dimensional space composed of the iron–CO binding coordinate (r) and a generalized protein coordinate (X). In these models the generalized protein is treated with a *single* coordinate and it is not possible to consider the relaxation and energetics of the important iron–porphyrin coordinate separately from the rest of the protein. In the present model, we employ a three-dimensional approach that explicitly exposes the iron–porphyrin coordinate (Q) along with the rest of the generalized protein (x). We allow for coupling between these protein coordinates so that fluctuations in x can affect the equilibrium position of Q. We suggest that the ca. 0.45 Å distance that the iron must move (relative to the porphyrin) during the binding process contributes important terms to the free energy of the transition state. Moreover, we allow this coordinate to relax to an out-of-plane geometry, even at low temperature, when the rest of the protein is frozen (x held constant). This approach predicts the observed high-temperature single-exponential rebinding rate directly from the low-temperature distribution. In contrast, the Agmon–Hopfield (AH) model fails in the high-temperature prediction. This arises from the fact that

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Direct observations of ligand dynamics in hemoglobin by subpicosecond infrared spectroscopy

(transient IR spectroscopy/heme proteins/geminate recombination)

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Contributed by R. M. Hochstrasser, July 26, 1989

ABSTRACT **The photodissociation of CO from HbCO at ambient temperature is studied by means of a femtosecond IR technique. The bleaching of the FeCO absorption and the appearance of a new IR absorption near that of free CO are both observed at 300 fs after optical excitation. The bleach does not recover on the time scale of a few picoseconds but does recover by ≈4% within 1 ns, which suggests that a barrier to recombination is formed within a few picoseconds. The CO spectrum does not change significantly between 300 fs and 1 ns, suggesting that the CO quickly finds some locations in the heme pocket that are not more than a few angstroms from the iron. The de-ligated CO appears in its ground vibrational level. There is evidence that 85 ± 10% of this CO remains in the heme pocket at 1 ns; it probably resides there for 50 ns. The flow of excess vibrational energy from the heme to the solvent was directly observed in the IR experiments. The heme cools within 1–2 ps while thermal disruption of the surrounding solvent structure requires ≈30 ps.**

Ultrafast spectroscopic methods have contributed much to understanding the photophysics and structural dynamics that occur after the photodissociation of oxy- and carboxyhemoglobin (1–3). The motion of the iron out of the heme plane has been discussed in some detail already, and evidence from optical spectroscopy (3) and Raman scattering (4, 5) suggests that the heme is partially or fully domed within 350 fs and perhaps as rapidly as 50 fs. This time frame for motion of the iron and its associated proximal histidine is also found in theoretical simulations of the heme relaxation (6). On the other hand, very little is yet known about the properties of the ligand generated by photolysis in ambient temperature solution.

In the normal operation of hemoglobin, O_2 diffuses into the protein from the solvent and bonds to the iron. Questions regarding ligand diffusion into and escape from the heme pocket as well as binding within the protein therefore have central importance to the function of hemoglobin. The hemoglobin structure, as determined from x-ray analysis (7), has no channel large enough to facilitate passage of a ligand between the solvent and heme. Hence, diffusion to the heme requires significant globin motions (7–9). Although transient optical methods are sensitive, the electronic spectrum of hemes cannot respond to changes in the location of a relatively inert diatomic molecule within the protein. Thus, experiments that can observe the ligand directly and monitor any changes in its structure or environment are expected to extend significantly our understanding of hemoprotein function. Such measurements on free CO would enable us to address some new issues for hemoglobin under ambient aqueous conditions. For example, additional information could be obtained on the rate of generation of CO and on the pathways of photodissociation (10, 11), the efficiency of subnanosecond geminate recombination of CO to iron could be brought into relation with results from nanosecond (12) and microsecond (13) studies, properties of the energy and spatial distribution of CO molecules within the protein and the associated dynamics could be inferred from the spectral shapes and positions, and the departure of the CO from the pocket into the solution could be considered separately from the rebinding step. Unliganded CO from the photodissociation of MbCO was observed previously in low-temperature glasses (14, 15); however, the experimental techniques used lacked the time resolution needed to study the ligand dynamics at biologically relevant temperatures.

In an earlier paper, we introduced a method to study IR spectra and vibrational dynamics on the 10-ps time scale (16) and used this to determine the FeCO bond angles in MbCO, HbCO (17), and protoheme (18). More recently, the time resolution of the experiment was reduced to 300 fs (19), and it is this development that makes possible the present study of HbCO.

MATERIALS AND METHODS

Spectroscopic Method. Transient IR spectra were obtained by means of upconversion of a continuous wave (CW) IR probe (16, 19). The upconverted signal, which is detected with a photomultiplier, is related to the transmitted IR intensity during a slice in time defined by the gating pulse. Time-resolved IR spectra are recorded by varying the probe wavelength at a fixed delay between the pump and gate pulses. The time resolution is limited only by the excitation pulse duration, and the spectral resolution is limited only by the spectral bandwidth of the CW probe. Hence, all rise times that are slower than the integral of the pulse autocorrelation and all spectral features that are broader than the probing bandwidth arise from the molecular dynamics.

The optical pulses originate in a cavity dumped dye laser, which is synchronously pumped with the frequency doubled output of a CW mode-locked Nd:YAG laser. These pulses are shortened to 200–300 fs in an optical fiber-grating pulse compressor and amplified in a multipass dye cell that is pumped by the frequency doubled output of a CW Q-switched Nd:YAG laser. The amplifier output (580 nm, 5 μJ, 300 fs, 1 kHz) is split to derive the pump and gate pulses. The CW IR from a diode laser (Laser Photonics, Analytics Division, Bedford, MA) is focused to ≈100 μm in a sample flow cell composed of two CaF_2 windows and a 56-μm spacer. The pump beam is delivered to the sample colinearly with the CW IR, whose transmitted field is upconverted by the gating pulse in a crystal of $LiIO_3$.

The "free" CO spectra were accumulated under pulsed diode laser operation. Pulsed operation improves the sensitivity by boosting the diode laser intensity by a factor of

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Abbreviation: CW, continuous wave.

Ligand Binding to Heme Proteins: Connection between Dynamics and Function†

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ABSTRACT: Ligand binding to heme proteins is studied by using flash photolysis over wide ranges in time (100 ns–1 ks) and temperature (10–320 K). Below about 200 K in 75% glycerol/water solvent, ligand rebinding occurs from the heme pocket and is nonexponential in time. The kinetics is explained by a distribution, $g(H)$, of the enthalpic barrier of height H between the pocket and the bound state. Above 170 K rebinding slows markedly. Previously we interpreted the slowing as a "matrix process" resulting from the ligand entering the protein matrix before rebinding. Experiments on band III, an inhomogeneously broadened charge-transfer band near 760 nm (≈13 000 cm^{-1}) in the photolyzed state (Mb*) of (carbonmonoxy)myoglobin (MbCO), force us to reinterpret the data. Kinetic hole-burning measurements on band III in Mb* establish a relation between the position of a homogeneous component of band III and the barrier H. Since band III is red-shifted by 116 cm^{-1} in Mb* compared with Mb, the relation implies that the barrier in relaxed Mb is 12 kJ/mol higher than in Mb*. The slowing of the rebinding kinetics above 170 K hence is caused by the relaxation Mb* → Mb, as suggested by Agmon and Hopfield [(1983) *J. Chem. Phys. 79*, 2042–2053]. This conclusion is supported by a fit to the rebinding data between 160 and 290 K which indicates that the entire distribution $g(H)$ shifts. Above about 200 K, equilibrium fluctuations among conformational substates open pathways for the ligands through the protein matrix and also narrow the rate distribution. The protein relaxations and fluctuations are nonexponential in time and non-Arrhenius in temperature, suggesting a collective nature for these protein motions. The relaxation Mb* → Mb is essentially independent of the solvent viscosity, implying that this motion involves internal parts of the protein. The protein fluctuations responsible for the opening of the pathways, however, depend strongly on the solvent viscosity, suggesting that a large part of the protein participates. While the detailed studies concern MbCO, similar data have been obtained for MbO_2 and CO binding to the β chains of human hemoglobin and hemoglobin Zürich. The results show that protein dynamics is essential for protein function and that the association coefficient for binding from the solvent at physiological temperatures in all these heme proteins is governed by the barrier at the heme.

The binding of small ligands to heme proteins appears to be a simple reaction, described for the particular case of carbon monoxide binding to myoglobin by the one-step scheme Mb + CO ⇌ MbCO (Antonini & Brunori, 1971). In 1975 our group showed that data taken over wide ranges in time and temperature suggest a scheme in which a CO molecule, coming from the solvent, encounters, not one, but three or four potential barriers (Austin et al., 1975). In 1983, Agmon and Hopfield (1983) introduced a model which explained the low-temperature features observed by Austin et al. and described some of the features seen at high temperatures as a relaxation process. Here we show that ligand-binding and kinetic hole-burning experiments together lead to a model in which features of the Agmon–Hopfield model are kept, but where the reaction surface becomes time and temperature dependent. The model fits the low- and the high-temperature data using two sequential barriers.

Kinetics of CO Binding to Myoglobin

(1.1) Method and Results. In a flash photolysis experiment, a laser pulse strikes an MbCO sample and breaks the bond between the CO and the heme iron. The difference in the absorption spectra at a selected wavelength, $\Delta a(t)$, for the bound and the dissociated species monitors the subsequent CO rebinding. The survival probability, $N(t,T) \equiv \Delta a(t)/\Delta a(0)$, is the fraction of Mb molecules at temperature T that have not rebound CO at the time t after the flash. We have previously described the experimental technique and the rebinding data (Austin et al., 1975; Doster et al., 1982; Dlott et al., 1983; Ansari et al., 1986). We have repeated the flash photolysis experiments with an improved system.

(1.2) Reaction Energy Landscape. CO rebinding data to sperm whale myoglobin are shown in Figure 1. We call the faster process seen at all temperatures I for "internal"; it is nonexponential in time and independent of CO concentration. The slower process that appears above 200 K, denoted by S for "solvent", is exponential in time with a rate coefficient proportional to the CO concentration in the solvent. The processes I and S can be described in terms of the reaction potential $V(rc)$ sketched in Figure 2 (Austin et al., 1975;

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Ligand Binding and Protein Relaxation in Heme Proteins: A Room Temperature Analysis of NO Geminate Recombination†

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ABSTRACT: Ultrafast absorption spectroscopy is used to study heme–NO recombination at room temperature in aqueous buffer on time scales where the ligand cannot leave its cage environment. While a single barrier is observed for the cage recombination of NO with heme in the absence of globin, recombination in hemoglobin and myoglobin is nonexponential. Examination of hemoglobin with and without inositol hexaphosphate points to proximal constraints as important determinants of the geminate rebinding kinetics. Molecular dynamics simulations of myoglobin and heme–imidazole subsequent to ligand dissociation were used to investigate the transient behavior of the Fe–proximal histidine coordinate and its possible involvement in geminate recombination. The calculations, in the context of the absorption measurements, are used to formulate a distinction between nonexponential rebinding that results from multiple protein conformations (substates) present at equilibrium or from nonequilibrium relaxation of the protein triggered by a perturbation such as ligand dissociation. The importance of these two processes is expected to depend on the time scale of rebinding relative to equilibrium fluctuations and nonequilibrium relaxation. Since NO rebinding occurs on the picosecond time scale of the calculated myoglobin relaxation, a time-dependent barrier is likely to be an important factor in the observed nonexponential kinetics. The general implications of the present results for ligand binding in heme proteins and its time and temperature dependence are discussed. It appears likely that, at low temperatures, inhomogeneous protein populations play an important role and that as the temperature is raised, relaxation effects become significant as well.

The microscopic aspects of ligand binding in myoglobin and hemoglobin are not fully understood, although great progress has been made recently in their analysis. Structural disorder and its temporal evolution (Frauenfelder et al., 1979; Case & Karplus, 1979; Debrunner & Frauenfelder, 1982; Elber & Karplus, 1987a,b) apparently play an important role. The high-resolution X-ray structures of ligated and unligated myoglobin do not reveal any path by which ligands can move between the heme binding site and the outside of the protein (Perutz & Mathews, 1966; Takano, 1977). Since motion must therefore be involved in the ligand binding, myoglobin has become a model system for studying the relation of motion to function in proteins.

Much of what is known about the influence of protein fluctuations on heme protein reactivity is due to measurements of ligand recombination after photodissociation on the nanosecond to second time scales. A series of studies (Austin et al., 1975; Henry et al., 1983; Ansari et al., 1985) have suggested that a phenomenological description of the kinetics of photodissociation and rebinding of a ligand X to myoglobin (Mb) can be written as

$$MbX \rightleftarrows (Mb + X)_{pocket} \rightleftarrows (MB + X)_{protein} \rightleftarrows (Mb + X)_{solution}$$

where the subscript refers to the location of the ligand X. Each of the designated species may involve several different states or substates on a microscopic level. At low temperatures (below 200 K in ethylene glycol/water), it is found that the geminate rebinding of the CO ligand is nonexponential. This has been attributed to a distribution of barrier heights, associated with the different substates that equilibrate slowly relative to the rebinding. As the temperature is raised, the geminate recombination of CO becomes exponential and can be described by a single barrier.

In analyzing the origin of the nonexponential behavior and, more generally, the complexity of the rebinding kinetics in proteins, it is important to consider two types of motional phenomena (Ansari et al., 1985). One of these consists of the fluctuations that occur at equilibrium. These equilibrium fluctuations consist of motions within a potential well at low temperature. At higher temperatures transitions between wells are superposed on the harmonic fluctuations (Elber & Karplus, 1987; Smith et al., 1990). The other type of motion arises in a nonequilibrium system and corresponds to relaxation toward equilibrium. Since photodissociation creates a nonequilibrium

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The Energy Landscapes and Motions of Proteins

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Recent experiments, advances in theory, and analogies to other complex systems such as glasses and spin glasses yield insight into protein dynamics. The basis of the understanding is the observation that the energy landscape is complex: Proteins can assume a large number of nearly isoenergetic conformations (conformational substates). The concepts that emerge from studies of the conformational substates and the motions between them permit a quantitative discussion of one simple reaction, the binding of small ligands such as carbon monoxide to myoglobin.

PROTEINS ARE DYNAMIC AND NOT STATIC SYSTEMS (*1*). Indeed, Weber has characterized proteins as "screaming and kicking" (*2*). Our purpose in this article is not to prove again that proteins move. Excellent reviews of the experimental evidence exist (*3*), and results from molecular dynamics computations have been elegantly exposed (*4*). Rather, we want to show that (i) the "screaming and kicking" is not incomprehensible but that the motions can be characterized and classified, (ii) studies from other "complex" systems such as glasses yield information on how to describe the motions, and (iii) the relation between motions and function is beginning to be understood in some simple situations, such as the binding of small ligands to myoglobin (Mb). Studies of biomolecular dynamics today are in some sense where atomic physics was near 1885. A bewildering variety of protein motions has been revealed by fluorescence spectroscopy, nuclear magnetic resonance (NMR), hydrogen exchange, and Raman scattering. Can regularities be found and connected to the structure of proteins, and can the underlying concepts and laws be discovered? We try to show that some progress has been made.

Spectral lines are transitions between energy levels of atoms or molecules, and protein motions can be described as transitions between conformational substates of the protein. Thus, the characterization and classification of these substates is the first task. Different proteins most likely exhibit different sets of conformational substates, but we believe that the general concepts are likely to be universal. We have selected Mb, the protein that reversibly stores O_2 (*5*), as prototype. Mb is simple enough that dynamic concepts can be studied in detail and yet sufficiently complex that the concepts discovered may be globally valid.

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Our second goal is important because progress is often made by good use of analogies. Proteins and glasses share many properties. Because glasses are simpler, they can serve as guides to the formulation of concepts and theories. Two important features emerge from the comparison of proteins and glasses: (i) Although it is customary to describe the time dependence of protein reactions and motions by simple exponentials and their temperature dependence by the Arrhenius (transition state) expression, neither of these forms is adequate. Glasses suggest what to substitute. (ii) At the theoretical level many properties of the motions of glasses and proteins can be discussed in terms of the features of rugged energy landscapes, which thus provide a unifying language.

The third goal, the exploration of the relation of motions to function, is the most difficult one to reach. We sketch one case, in which a semiquantitative description of the role of motions in function exists, namely, the binding of small ligands to Mb.

Conformation and Energy Landscape

Even a monomeric protein as small as Mb can execute a large number of motions, and not all will be coupled to function. Functionally important motions can be studied only if they can be selected. In addition, the various motions must be temporally resolved. Originally, experiments at physiological temperatures suggested that the reaction of Mb + $O_2 \leftrightarrow MbO_2$ was a simple one-step process (*5*). Low-temperature flash photolysis of MbCO and MbO_2 showed that the rebinding of the ligand to the heme active center was nonexponential in time below ~200 K (*6*). This observation suggested that Mb did not have a single structure but could assume a large number of slightly different structures, each with a different rebinding rate. Because Mb has two globally distinct macrostates, ligand-bound (MbCO or MbO_2) and unbound (Mb), and there is a spectrum of conformations in either state, these microstates are called "conformational substates (CS)" (*7*). The cryochemical experiments reveal that a protein in a given state can assume a large number of CS, which form the scaffold for protein motions.

The organization of the CS in MbCO as presently known can be visualized as in Fig. 1 (*7, 8*). The energy landscape describes the potential energy E_c of the protein as a function of conformational coordinates; it is a hypersurface in the high-dimensional space of the coordinates of all atoms in Mb. The energy landscape (Fig. 1) has structure on several energy and length scales as illustrated by different one-dimensional cross sections through it. Figure 1 implies that the CS can be roughly classified into a hierarchy (*8*), where CS*i* denotes the substates in the *i*th tier in the organization. The top row of Fig. 1 depicts MbCO in the conventional conception with a unique structure corresponding to a unique energy valley. A hint that MbCO is not as simple comes from infrared (IR) spectra: The bound CO molecules display multiple stretch bands (*9*). Each band

Photoselection in polarized photolysis experiments on heme proteins

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ABSTRACT Polarized photolysis experiments have been performed on the carbon monoxide complex of myoglobin to assess the effects of photoselection on the kinetics of ligand rebinding and to investigate the reorientational dynamics of the heme plane. The results are analyzed in terms of the optical theory developed in the preceding paper by Ansari and Szabo. Changes in optical density arising from rotational diffusion of the photoselected population produce large deviations from the true geminate ligand rebinding curves if measurements are made with only a single polarization. The apparent ligand rebinding curves are significantly distorted even at photolysis levels greater than 90%. These deviations are eliminated by obtaining isotropically-averaged optical densities from measurements using both parallel and perpendicular polarizations of the probe pulse. These experiments also yield the optical anisotropy, which gives a novel method for accurately determining the degree of photolysis, as well as important information on the reorientational dynamics of the heme plane. The correlation time for the overall rotational diffusion of the molecule is obtained from the decay of the anisotropy. The anisotropy prior to rotational diffusion is lower than that predicted for a rigidly attached, perfectly circular absorber, corresponding to an apparent order parameter of $S = 0.95 \pm 0.02$. Polarized absorption data on single crystals suggest that the decreased anisotropy results more from internal motions of the heme plane which take place on time scales shorter than the duration of the laser pulse (10 ns) than from out-of-plane polarized transitions.

INTRODUCTION

Photodissociation experiments play an increasingly important role in the investigation of the kinetics and dynamics of ligand binding and conformational changes in heme proteins (1–16). In the most frequently performed experiment a linearly polarized laser pulse is used to photodissociate the heme-bound ligand, and a second linearly polarized pulse is used to probe the molecule at a variable time-delay after excitation. In most experiments photolysis is incomplete. This may occur simply because it is not possible to obtain sufficient laser energy to photodissociate all molecules in the sample, or it may be carried out by design. Partial photolysis experiments are particularly useful in investigating hemoglobin kinetics. Systematic changes in photolysis are used to create variable distributions of ligation states of the tetramer, making it possible to distinguish between tertiary and quaternary conformational changes (3, 8, 17–19). An important consequence of incomplete photodissociation is the creation of an anisotropic distribution of molecular species by photoselection (20). Photoselection is the preferential photodissociation (i.e., "selection") of molecules oriented with their transition moments parallel to the electric vector of the excitation pulse. Although, as we shall see in this work, the influence of photoselection on the measurement of ligand rebinding kinetics is quite substantial, its effect has largely been ignored.

In order to obtain accurate kinetic progress curves for geminate rebinding of oxygen and carbon monoxide on the picosecond to microsecond time scale, it is essential to understand the consequences of photoselection. A qualitative picture of these effects is shown schematically in Fig. 1. It is known from polarized absorption studies on single crystals that, to a good approximation, hemes behave like circularly-symmetric absorbers of linearly polarized light at the wavelengths used in photolysis experiments (21). Circular absorbers have the property that there is equal absorption of light polarized parallel to any direction in the plane defined by the circle, and no absorption of light polarized perpendicular to the plane.[1] The probability of photodissociation is therefore greater when the porphyrin planes of heme-ligand complexes are oriented parallel to the electric vector of a linearly polarized excitation pulse, than when they are oriented perpendicular. Immediately after photodissociation, the sample exhibits linear dichroism because the distribution of each species is no longer isotropic. If the sample is probed with light linearly polarized parallel (perpendicular) to the excitation polarization, the fraction of unliganded hemes will appear to be greater (less) than the true value (Fig. 1). As the molecular orientations randomize by rotational diffusion, the linear dichroism decays (Fig. 1). This decay contributes an apparent increase in the fraction of liganded hemes for measurements with the parallel orientation of the probe polarization. For the perpendicular orientation the apparent fraction of liganded hemes decreases. Thus, rotational diffusion interferes with an accurate measurement of the kinetics of ligand binding taking place on the same time scale.

The effect of photoselection can be eliminated in two ways.[2] One is to orient the polarization of the probe light

[1] Circularly-symmetric absorbers of linearly polarized light, or simply circular absorbers, are often called planar absorbers. The property of circular absorption requires that either the ground state or the excited state (but not both) be two-fold degenerate. Non-degenerate transitions exhibit linear absorption, while three-fold degenerate transitions exhibit spherically symmetric absorption.

[2] Photoselection also affects the distribution of ligation states in hemoglobin (18). If the probability of absorbing a photon by each heme of the tetramer were uncorrelated, the distribution of liganded hemes in the tetramer would be binomial. However, since the four hemes of hemoglobin are roughly parallel (21), excitation with linearly polar-

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The Role of Solvent Viscosity in the Dynamics of Protein Conformational Changes

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Nanosecond lasers were used to measure the rate of conformational changes in myoglobin after ligand dissociation at ambient temperatures. At low solvent viscosities the rate is independent of viscosity, but at high viscosities it depends on approximately the inverse first power of the viscosity. Kramers theory for unimolecular rate processes can be used to explain this result if the friction term is modified to include protein as well as solvent friction. The theory and experiment suggest that the dominant factor in markedly reducing the rate of conformational changes in myoglobin at low temperatures (<200 K) is the very high viscosity (>10^7 centipoise) of the glycerol-water solvent. That is, at low temperatures conformational substates may not be "frozen" so much as "stuck."

Important information on the mechanism and dynamics of a molecular process can be obtained by studying the dependence of its kinetics on solvent viscosity. Classic examples are the investigation of the role of diffusion in determining the rate of a bimolecular reaction (*1*) and the influence of solvent friction on a unimolecular reaction rate (*2*). Other examples include the effect of solvent viscosity on the motion of small molecules inside proteins (*3*) and the contribution of diffusive processes to the rate-limiting steps in protein folding (*4*). Here we show how such studies can be used to gain insight into the dynamics of conformational changes in proteins.

The carbon monoxide complex of myoglobin (MbCO) is dissociated by light. The conformation of the photoproduct is unstable, and the protein relaxes to the conformation of the unliganded molecule by a small but global displacement of protein atoms on one side of the heme. We have investigated the kinetics of this conformational change as a function of solvent viscosity by using high-precision, time-resolved absorption measurements after photodissociation by nanosecond laser pulses. A representative set of time-resolved absorption spectra in a 79% by weight solution of glycerol in water at 20°C is shown in Fig. 1. The time course of the decrease in the overall amplitude of the difference spectra measures the ligand rebinding kinetics, which take place in two phases (Fig. 2A). The first, nonexponential phase, with a half-time of about 200 ns, corresponds to geminate rebinding, that is, unimolecular rebinding of CO to the heme from which it was photodissociated (*5*). The second phase, at about 1 ms, corresponds to bimolecular rebinding of CO from the solvent. There are also changes in the shape of the spectra, which are primarily spectral changes of the deoxyheme photoproduct (Fig. 2B). The corresponding amplitudes (Fig. 2, B and C) monitor the extent of the deviations of the observed spectra from the average spectrum shown in Fig. 2A. We interpret these spectral changes as arising from protein conformational changes following photodissociation. Lambright *et al.* (*6*) have independently observed these kinetics but did not investigate their viscosity dependence.

The similarity of the deoxyheme spectral changes to those observed for hemoglobin suggests that they correspond to a displacement of the iron relative to the heme plane that is coupled to a protein conformational change on the proximal side of the heme (*7*). Comparison of the x-ray structures of Mb and MbCO shows a small global displacement of the protein atoms on the proximal side (*8*), and this is most likely the protein conformational change that is being monitored in our experiments. This conformational change is interesting because it could be responsible for slowing down the rate of ligand rebinding to produce the nonexponential geminate phase (*9*, *10*). Its global nature suggests that its rate would be influenced by solvent viscosity. There is also a localized rearrangement of side chains on the distal side of the heme observed in the x-ray studies (*8*) that may correspond to the 200- to 300-ps process observed in circular dichroism studies (*11*) or contribute to the sub–30-ps events detected in phase-grating spectroscopic experiments (*12*).

The data in Fig. 2, B and C, show that there is an initial large-amplitude process in the kinetics of the protein conformational change, which is followed by smaller amplitude processes (*13*). We shall be concerned here only with the large-amplitude initial process (indicated by the arrows in Fig. 2, B and C), for which relatively precise rate constants can be derived. The major result is that increasing the viscosity increases the amplitude of the initial process that was resolved with our 10-ns laser pulses, which indicates a slowing of the conformational change. Studies by Frauenfelder and co-workers (*3*) showed that ligand rebinding rates at a given viscosity and temperature are the same for different solvents, which implies that the effect of glycerol on the conformational kinetics in our experiments does not result from a solvent effect other than viscosity.

The data in Fig. 2, B and C, show that the kinetic progress curve for the initial conformational relaxation is at least biexponential. In order to obtain a single rate constant under each set of conditions, we

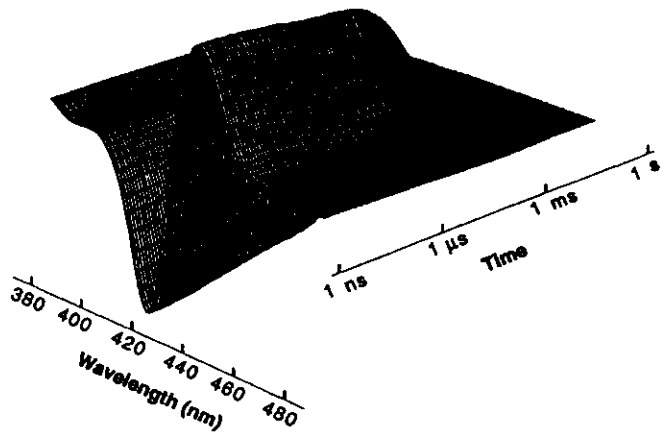

Fig. 1. Time-resolved optical absorption spectra following photodissociation of the CO complex of sperm whale myoglobin. Deoxy-minus-CO difference spectra of photodissociated MbCO in 79% by weight glycerol-water at 20°C are shown as a function of time. The experiments were carried out with the use of two Nd:YAG (yttrium-aluminum-garnet) lasers that produced 10-ns pulses for photolysis and optical absorption measurements as described (*21*). In order to eliminate effects due to rotational diffusion of the photoselected populations in incompletely photolyzed samples, we obtained isotropically averaged spectra by making measurements with the polarization of the photolysis pulse both parallel to and perpendicular to the polarization of the probe pulse. The rotational correlation time was determined from the decay in the optical anisotropy and exhibited Stokes-Einstein behavior, which indicates that increasing the glycerol concentration has no perceptible effect on either the size or the shape of the protein.

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Molecular Dynamics Simulation of No Recombination to Myoglobin Mutants*

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Molecular dynamics simulations on two coupled electronic surfaces are employed to investigate the geminate recombination of nitric oxide to mutants of sperm whale myoglobin. A model for the ground and the excited states is constructed based on experimental data. The crossing between the surfaces is treated using the Landau-Zener formula. The reaction probability and the recombination curves are calculated directly by histogramming the results of an ensemble of trajectories. The experimental trend is reproduced in which the picosecond recombination rate of different mutants increases in the order $Phe^{29} > Leu^{29} > Val^{29} > Ala^{29}$. Furthermore, in accord with the experiment on significantly longer time scales an opposite trend is obtained, in which the recombination rate for Ala^{29} is larger than for Phe^{29}. These results are explained by constrained diffusion of the ligand in the heme pocket. The average and the transient volume of the heme pocket is modified by the 29 mutants.

Computer simulations of the interactions of small ligands with heme proteins have advanced considerably during the last few years. This is due to the rapid growth in computer technology and to the parallel developments in new simulation techniques (1–4). Equally important are the significant enhancements of experimental methodologies that provided stimulating and detailed data. Most relevant to the present article is the introduction of short laser pulses and the availability of myoglobin mutants (5–9). The mutants make it possible to study the influence of individual residues on the recombination process.

The experimental set up of interest to us is that of geminate recombination. There, a short laser pulse is used to dissociate the ligand that is bound to the heme (here we consider only the diatomic ligand, nitric oxide), and the rate of ligand recombination to the heme is measured. The rate of recombination provides information (in an indirect way) on the ligand diffusion in the protein matrix and on the interactions of the ligand with the protein residues (10). Understanding of the geminate recombination process was advanced significantly by simulation of the ligand diffusion (1–4) and in one case by constructing a simplified model of the recombination (11). However, in order to fully appreciate what the geminate recombination can teach us about the motion of the ligand it is important to carry out simulations which can be related *directly* to raw experimental data; that is, to compute directly the recombination curves. This is the goal of the present paper in which a complete computational model for the geminate recombination process is presented.

Effort was made to evaluate the quality of the model directly against the recombination experiments without any further simplifications or approximations beyond the classical trajectory simulation techniques. For example, the equilibrium assumption of the transition state theory (2, 4) may be questionable for the process under investigation (10), and it is therefore avoided. Even approximations that were shown in the past to give useful results (such as the Locally Enhanced Sampling methodology (1, 11)) are not quantitative. Therefore, the rebinding curves were calculated in the most straightforward way that we could afford computationally. An ensemble of trajectories is initiated on the electronically excited state. The trajectories are propagated numerically in small time steps, and the number of trajectories that do not rebind is calculated as a function of time. These trajectories reflect the concentration of the dissociated heme that is measured experimentally. Since the calculations are pursued in a direct way, they can also serve as a future reference and as a test of approximate theories that may require considerably less computer resources.

In the following sections, we describe the parameters of the model together with the Landau-Zener scheme used to describe the jumps between the electronic surfaces and we outline the computational protocol.

EXPERIMENTAL PROCEDURES

Computational Model and Protocol—All the calculations presented in this paper were performed using the program MOIL.[1] The potential energy function of MOIL is the combination of AMBER/OPLS (13, 14) with improper torsions adopted from the CHARMM force field (15). The parameters of Kuczera *et al.* (16) (see Table I) for the heme force field were used, unless specifically stated otherwise in the text. Kuczera *et al.* provided the parameters for the six coordinated and five coordinated heme iron.

In constructing the ground and the excited state potentials between the nitric oxide and the heme we used (as much as possible) pieces of independent experimental data. Ab-inito calculations (17) can provide qualitative understanding of the coupling and of the ordering of

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Simulation of the kinetics of ligand binding to a protein by molecular dynamics: Geminate rebinding of nitric oxide to myoglobin

(picosecond kinetics/chemical dynamics/lasers)

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ABSTRACT **We have begun to use molecular dynamics to simulate the kinetics of nitric oxide rebinding to myoglobin after photodissociation. Rebinding was simulated using a potential function that switches smoothly between a nonbinding potential and a binding potential as a function of the position and orientation of the ligand, with no barrier arising from the crossing of potential surfaces of different electron spin. In 96 of 100 trajectories, the ligand rebound in <15 ps. The kinetic progress curve was obtained by determining the time in each trajectory at which the ligand rebound and then calculating the fraction of unbound ligands as a function of time. The curve can be well reproduced by a simple model based on the dynamics of a Langevin particle moving on a one-dimensional potential of mean force calculated from nonreactive protein trajectories. The rate of escape from the energy well adjacent to the heme is in good agreement with the value calculated from experimental data, suggesting that a multiple-well model provides a plausible explanation for the nonexponential rebinding kinetics. A transition-state analysis suggests that protein conformational relaxation coupled to the displacement of the iron from the heme plane is an unlikely cause for the nonexponential rebinding of nitric oxide.**

Advances in computer technology now make it possible to simulate the kinetics of ultrafast chemical reactions in solution by using the technique of molecular dynamics. One of the fastest known biochemical reactions is the geminate rebinding of nitric oxide to the hemes of hemoglobin and myoglobin. This reaction occurs on a tens of picoseconds time scale and was discovered by Hochstrasser and coworkers (1), after the introduction of picosecond laser technology to the study of proteins in the late 1970s (2). It is the very high speed of this reaction that makes it suitable for calculating the many trajectories that are necessary to obtain statistically meaningful kinetics. The attractive feature of simulating kinetics by classical molecular dynamics is that in principle a complete description of the process (apart from quantum effects) is contained in trajectories run on an accurate potential surface. It should therefore be possible to address important issues and gain insights into the chemical dynamics of ligand binding to myoglobin.

Molecular dynamics simulations of ultrafast laser photolysis experiments began with the work of Henry *et al.* (3). This study showed that the displacement of the iron to near its equilibrium position in deoxyhemoglobin was complete in <200 fs, supporting the interpretation of optical experiments (4). Longer trajectories have shown an additional slower displacement of the iron from the heme plane attributed to conformational relaxation of the protein (5, 6). Other simulations have focused on ligand motion in the protein (7–11).

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In this study we have simulated photodissociation and geminate rebinding of nitric oxide to myoglobin. The potential surface for rebinding is complex, because the change in spin from a quintet iron and doublet ligand to a doublet iron–ligand complex necessarily introduces an "electronic" barrier arising from the crossing of potential surfaces. Since nothing is known about these surfaces from quantum mechanical calculations, we have for simplicity ignored the electronic barrier and have used a Morse potential for the rebinding surface. This has the effect of artificially speeding up the reaction. Elber and coworkers (12) have independently carried out similar simulations in which an electronic barrier has been included.

METHODS

Molecular dynamics simulations using the program CHARMM23 (13) were performed on the system consisting of the complete myoglobin molecule (Brookhaven Protein Data Bank File 1MBO), including all hydrogen atoms, surrounded by 351 water molecules (14), plus nitric oxide. To simulate the dissociation of ligands and subsequent rebinding, three distinct potential functions for the heme group were employed at different stages of the simulations. These potential functions differ only in the description of interactions involving the set of eight atoms that includes the iron (Fe), the four pyrrole nitrogens (N_p), the nitrogen $N_{\epsilon 2}$ of the proximal histidine bonded to the iron, and the nitrogen (N) and oxygen (O) of the ligand. The general form of the potential function for these atoms is

$$\begin{aligned} V = {} & D\{1-[1-e^{-\beta(r-r_0)}]^2\} \\ & + s(r)K_\theta(\theta-\theta_0)^2 + s(r)K_\phi(\phi-\phi_0)^2 && \text{[term 1]} \\ & + \sum_{(\mathrm{Fe}-\mathrm{N_p})} K_{bp}(b_p-b_{p0})^2 + K_{b\epsilon}(b_\epsilon-b_{\epsilon 0})^2 \\ & + \sum_{(\mathrm{N_p}-\mathrm{Fe}-\mathrm{N_p})} K_{\alpha pp}(\alpha_{pp}-\alpha_{pp0})^2 \\ & + \sum_{(\mathrm{N_p}-\mathrm{Fe}-\mathrm{N_\epsilon})} K_{\alpha p\epsilon}(\alpha_{p\epsilon}-\alpha_{p\epsilon 0})^2 && \text{[term 2]} \\ & + [1-S(r,\theta,\phi)] \sum_{(\mathrm{N}-\mathrm{N_p})} \varepsilon\left[\left(\frac{\sigma}{r}\right)^{12}-\left(\frac{\sigma}{r}\right)^{6}\right] && \text{[term 3]} \end{aligned} \quad [1]$$

where r is the distance between the iron and the ligand nitrogen (N), θ is the angle Fe–N–O, and ϕ is the angle $N_{\epsilon 2}$–Fe–N.

The potential function for the liganded heme (6-coordinated iron) was used prior to dissociation. This potential includes iron–ligand bonding interaction terms [labeled term 1 in Eq. 1 with $s(r) = 1$], the first of which is a Morse potential involving the distance between the iron and the

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Complex nonexponential relaxation in myoglobin after photodissociation of MbCO: measurement and analysis from 2 ps to 56 μs

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Photodissociation of carbon monoxide myoglobin (MbCO) creates Mb in a nonequilibrium conformation and triggers a global conformational change. The driving force for this change is localized initially in the iron–porphyrin coordinate. The dynamics of the iron–porphyrin displacement at physiologically relevant temperatures have been followed by probing the evolution of band III, a weak iron–porphyrin charge-transfer transition near 13110 cm^{-1} (763 nm) that is sensitive to the out-of-plane position of the iron. This functionally important motion is highly nonexponential, stretching over 5 decades in time in 70:30 (w/w) glycerol/water mixtures at 301 ± 1 K. The relaxation is well described by a stretched exponential function that has been modified to account for the limiting rate associated with the primary relaxation process.

1. Introduction

Ligand association or dissociation can trigger a change in the global conformation of a protein and thereby alter its activity. Evidently, structural changes occurring at the binding site drive the protein toward a new equilibrium conformation with altered activity. We view this process as biomechanical motion with a functional purpose. If this biomechanical transduction were optimized, one might expect strong coupling between the structural change at the binding site and the global motion of the protein. Consequently, the relaxation along the coordinate driving the global conformational change would not be complete until the functionally important portion of the global motion is complete. Characterization of conformational relaxation dynamics in proteins should therefore provide important information regarding the relations between dynamics, structure, and function.

The dynamics of the approach to a new equilibrium conformation are expected to be nonexponential. This behavior is rationalized qualitatively using a hierarchical picture of coupled motions: the primary motion at the binding site is coupled to motions remote from the binding site through a hierarchy of couplings. Clearly, the relaxation at the binding site is not complete until the relaxation at the highest level to which it is coupled is complete. Because relaxation at each successive level of the hierarchy is expected to occur on a longer time scale, relaxation in strongly coupled systems is nonexponential, and the range of time scales involved is determined by the strength of the couplings. In contrast, weakly coupled systems have only one characteristic time scale and therefore exhibit exponential relaxation. The picture of nonexponential relaxation dynamics arising from a hierarchy of coupled motions [1] is analogous to the picture of nonexponential rebinding dynamics arising from a hierarchy of conformational substates [2]. The underlying physics is dynamical in the former but statistical in the latter. In either case, the experimental observable dies away nonexponentially, the dynamics of which may be modeled by the stretched exponential function $r(t) = \exp[-(kt)^{\beta}]$, where β is the Kohlrousch–Williams–Watt exponent. This phenomenological function has enjoyed wide success in parameterizing a host of nonexponential relaxation phenomena with disparate underlying physics [3]. To

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Theory of photoselection by intense light pulses

Influence of reorientational dynamics and chemical kinetics on absorbance measurements

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ABSTRACT The theory of absorbance measurements on a system (e.g., chromophore(s) in a protein) that undergoes a sequence of reactions initiated by a linearly polarized light pulse is developed for excitation pulses of arbitrary intensity. This formalism is based on a set of master equations describing the time evolution of the orientational distribution function of the various species resulting from excitation, reorientational dynamics, and chemical kinetics. For intense but short excitation pulses, the changes in absorbance (for arbitrary polarization directions of the excitation and probe pulses) and the absorption anisotropy are expressed in terms of reorientational correlation functions. The influence of the internal motions of the chromophore as well as the overall motions of the molecules is considered. When the duration of the excitation pulse is long compared to the time-scale of internal motions but comparable to the overall correlation time of the molecule that is reorienting isotropically, the problem of calculating the changes in absorbance is reduced to the solution of a set of first-order coupled differential equations. Emphasis is placed on obtaining explicit results for quantities that are measured in photolysis and fluorescence experiments so as to facilitate the analysis of experimental data.

1. INTRODUCTION

With advances in laser technology, photolysis experiments have emerged as the most powerful method for investigating the kinetics and dynamics of ligand binding and conformational changes in heme proteins. In these experiments photodissociation of the hemebound ligand is produced by excitation with a linearly polarized laser pulse. Subsequent events are usually monitored by optical absorption measurements of the heme chromophore. Photoselection and rotational diffusion influence the measured absorbances. Photoselection produces an anisotropic distribution of species in the solution because molecules having a larger projection of their transition moment onto the electric vector of the excitation pulse are preferentially photodissociated. The resulting optical anisotropy can be detected by measuring the absorbance with light linearly polarized both parallel and perpendicular to the electric vector of the excitation pulse. As in the case of fluorescence depolarization experiments, there is dynamical information contained in these measurements. The optical anisotropy, however, also interferes with accurate determination of ligand rebinding and conformational relaxation kinetics if data from only a single polarization is employed.

To understand these effects on time-resolved polarized absorption measurements we have developed a comprehensive theory of photolysis experiments. Our formulation is based on a set of master equations describing the rate of change of the orientational distribution functions of the various species resulting from excitation, reorientational dynamics, and kinetics (1). Special emphasis is placed on the influence of intense excitation pulses which, with some notable exceptions (2–4), has received relatively little attention. When the excitation pulses are sufficiently intense, the system no longer responds linearly and one cannot simply convolute the response with the pulse profile. In this paper, we obtain expressions for the change of absorption and the absorption anisotropy as a function of time and laser intensity for both δ-function and finite excitation pulses. We consider the effects of both overall motions of the molecule and internal motions of the chromophore and the non-ideal (i.e., deviations from linear or circular) absorption properties of the chromophore. In the following paper we apply these results to experimental measurements on myoglobin (5).

This paper is organized as follows. Section 2 discusses the general formalism in the absence of chemical kinetics. The observables are expressed in terms of orientational distribution functions that are a function of orientation dependent extinction coefficients and that satisfy master equations. In section 2.1 we show that for weak excitation pulses, the absorbance changes can be obtained by convoluting the intensity profile with a response that depends on certain reorientational correlation functions. In section 2.2 we consider intense excitation pulses that are sufficiently short so that no reorientational dynamics occurs. In section 3 explicit expressions for the orientation dependent extinction coefficients are invoked. We consider absorbers that are ellipsoids of revolution (linear and circular absorbers are special cases). General ellipsoidal absorbers are treated in the Appendix. In sections 3.1 and 3.2 we obtain explicit expressions for the change in absorbance and absorbance anisotropy for weak and intense but short excitation pulses. In section 3.3 we consider the influence of internal motions. Section 3.4 shows how to handle intense excitation pulses that are so long that significant overall rotational diffusion occurs during the pulse. In section 4 the formalism is extended to include chemical kinetics that occurs on a time-scale longer than the dura-

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Conformational Relaxation and Ligand Binding in Myoglobin

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ABSTRACT: Absorption spectroscopy with nanosecond time resolution shows that myoglobin undergoes conformational relaxation on the same time scale as geminate rebinding of carbon monoxide. Ligand rebinding following photodissociation of the heme–CO complex was measured from the amplitude of the average difference spectrum, while conformational changes were measured from changes in the detailed shape of the Soret spectra of the deoxyhemes. Experiments in which the solvent viscosity was varied between 1 and 300 cP and the temperature between 268 and 308 K were analyzed by fitting the multiwavelength kinetic data with both empirical and molecular models. Novel numerical techniques were employed in fitting the data, including the use of singular value decomposition to remove the effects of temperature and solvent on the spectra and of a Monte Carlo method to overcome the multiple minimum problem in searching parameter space. The molecular model is the minimal model that incorporates all of the major features of myoglobin kinetics at ambient temperatures, including a fast and slow rebinding conformation and two geminate states for each conformation. The results of fitting the kinetic data with this model indicate that the geminate-rebinding rates for the two conformations differ by at least a factor of 100. The differences between the spectra of the two conformations generated from the fits are similar to the differences between those of the R and T conformations of hemoglobin. In modeling the data, the dependence of the rates on temperature and viscosity was parametrized using a modification of Kramers theory which includes the contributions of both protein and solvent to the friction. The rate of the transition from the fast to the slow rebinding conformation is found to be inversely proportional to the viscosity when the viscosity exceeds about 30 cP and nearly viscosity independent at low viscosity. The viscosity dependence at high viscosities suggests that the two conformations differ by the global displacement of protein atoms on the proximal side of the heme observed by X-ray crystallography. We suggest that the conformational change observed in our experiments corresponds to the final portion of the nonexponential conformational relaxation recently observed by Anfinrud and co-workers, which begins on a picosecond time scale. Furthermore, extrapolation of our data to temperatures near that of the solvent glass transition suggests that this conformational relaxation may very well be the one postulated by Frauenfelder and co-workers to explain the *decrease* in the rate of geminate rebinding with *increasing* temperature above 180 K.

The binding of ligands by proteins is a fundamental process in biology. One aim of modern biophysical studies is to understand the molecular mechanism of this type of process in detail. There is generally rather little mechanistic information on ligand binding in conventional kinetic studies, such as experiments in which reactants are rapidly mixed. Simple behavior is usually observed, with both the binding and dissociation reactions appearing to take place in a single step. Important information, such as the rate of a conformational change of a protein, can be totally obscured in such experiments. In the case of heme proteins, kinetic studies using flash photolysis have provided a powerful method for investigating conformational changes and the mechanism of ligand binding, revealing them to be complex and interesting processes. The power of the flash photolysis experiment is that photodissociation of the heme–ligand complex produces a coherent population of molecules in which the protein has the conformation of the liganded molecule and the ligand is still inside the protein. The evolution of this unstable intermediate toward the protein conformation of the unliganded molecule, as well as ligand motion and rebinding, can then be monitored by various spectroscopies with time resolution as short as 10^{-13} s. Most studies have focused on myoglobin or hemoglobin because of the considerable amount of X-ray crystallographic information available for these molecules. In particular, the structures of both the unliganded molecules and the molecules liganded with carbon monoxide or oxygen are known at high resolution, so that it is, in principle, possible to relate the kinetics of spectral changes to the kinetics of well-defined structural changes.

New concepts of ligand binding and protein conformational changes relevant to all protein–ligand processes have emerged from the studies on myoglobin and hemoglobin. These include the discovery by Frauenfelder and co-workers of a continuous distribution of protein conformations, so-called "conformational substates", that produce a distribution of geminate-ligand-rebinding rates in solid solutions at low temperature (Austin et al., 1975; Beece et al., 1980; Steinbach et al., 1991) and the discovery of geminate ligand rebinding in liquid solutions at ambient temperatures by Duddell et al. (1979) (Shank et al., 1976; Greene et al., 1978; Alpert et al., 1979; Duddell et al., 1980a,b; Friedman & Lyons, 1980; Chernoff et al., 1980; Cornelius et al., 1981; Catterall et al., 1982; Morris et al., 1982; Hofrichter et al., 1983; Henry et al., 1983a). The work on conformational substates has stimulated the development of analogies between the behavior of proteins

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Protein Reaction Kinetics in a Room-Temperature Glass

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Protein reaction kinetics in aqueous solution at room temperature are often simplified by the thermal averaging of conformational substates. These substates exhibit widely varying reaction rates that are usually exposed by trapping in a glass at low temperature. Here, it is shown that the solvent viscosity, rather than the low temperature, is primarily responsible for the trapping. This was demonstrated by placement of myoglobin in a glass at room temperature and subsequent observation of inhomogeneous reaction kinetics. The high solvent viscosity slowed the rate of crossing the energy barriers that separated the substates and also suppressed any change in the average protein conformation after ligand dissociation.

A physical understanding of protein structure and function is becoming increasingly important to biology (*1*). Since the pioneering work of Austin and others (*2*), myoglobin (Mb) has been the paradigm for such studies. At temperatures below the glass transition of the solvent ($T_g \approx 180$ K for 3:1 glycerol-water mixtures), the geminate ligand rebinding kinetics of Mb after photodissociation of its carbon monoxide complex are widely distributed, extending from microseconds to kiloseconds. These kinetics have been explained by the simple idea that Mb molecules at low temperature are "frozen" into conformational substates, each binding with a different exponential rate (*2*). At room temperature in water, geminate rebinding is nearly exponential (*3*), which indicates that the energy barriers that separate substates are sufficiently low for thermal averaging to occur on the nanosecond time scale at which rebinding occurs. From a study of the viscosity dependence of the conformational relaxation after ligand dissociation in Mb, Ansari *et al.* (*4*) suggested that the trapping of conformational substates at low temperature may result more from high solvent viscosity than from energy barriers internal to the protein. Here, we confirm this hypothesis by showing that Mb embedded in a glass at room temperature exhibits ligand binding kinetics similar to those observed at low temperature. The ligand binding rates are distributed, and there is no evidence for conformational relaxation after ligand dissociation. The averaged geminate rebinding rates in the glass are much higher than those of the relaxed protein, which points to the functional consequence of conformational relaxation. These studies suggest a reinterpretation of previous low-temperature kinetic data as well as a clarification of the relation between kinetics, neutron-scattering experiments (*5*), and molecular dynamics simulations of "glass-like" transitions in proteins (*6*).

Conformational changes play an important role in the kinetics of Mb as well as of hemoglobin. In aqueous solution at room temperature, the geminate rebinding of carbon monoxide to Mb is much slower than predicted by an extrapolation of the low-temperature distribution of geminate rates (*3*). In addition, the average rate of geminate rebinding decreases as the temperature is increased through the solvent-glass transi-

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Nonexponential structural relaxations in proteins

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Conformational changes in proteins have been observed to exhibit a nonexponential time course. In myoglobin the conformational relaxation that follows photodissociation of the heme ligand is a very extended process that stretches from less than 1 picosecond to nearly 1 microsecond. We explain these kinetics with a model in which the initial protein conformational substates are connected to the final substates and to each other via transition states of a single energy. © *1996 American Institute of Physics.* [S0021-9606(96)03609-6]

Protein conformational changes often do not follow a single exponential time course. Like relaxations in glasses[1] they may instead be described by the "stretched" exponential function, $\exp[-(\kappa t)^{\beta}]$, where $0<\beta<1$.[2-5] The most striking example is the highly stretched ($\beta\sim0.1$) conformational relaxation recently observed in myoglobin by Anfinrud and co-workers.[5] Myoglobin is a small protein with an iron–porphyrin complex that binds small ligands such as carbon monoxide and oxygen; photodissociation of its CO complex at room temperature initiates a relaxation that extends from less than 1 picosecond to nearly 1 microsecond (Fig. 1). This structural change in the protein is functionally important because it slows the rebinding of the ligand.[6,7] A similar relaxation is observed in hemoglobin prior to the quaternary conformational change,[2] and its description as a non-exponential process is the key to understanding the kinetics of cooperative ligand binding.[8] Although it has been suspected[1] that theories of relaxation in glasses and spin glasses[9,10] may also be relevant to proteins, the origin of stretched relaxation in protein molecules has not yet been explained. Here we propose a simple model in which nonexponential relaxation results from transitions among two groups of protein conformational substates connected by transition states of a single energy.

X-ray crystallography shows that the average structure of the myoglobin molecule changes when the heme ligand is dissociated.[11] There is considerable evidence that the liganded and unliganded structures each consist of a near continuum of conformational substates, which have similar energies but exhibit very different rates for the geminate rebinding of ligands.[1,6,12] These substates represent small variations on the two average structures;[13] they may, for example, have slightly different packing of the helices. Interconversion between substates occurs rapidly at room temperature in water, although it is suppressed by low temperatures[6,12] or by high solvent viscosities at room temperature,[14] resulting in a wide distribution of geminate ligand rebinding rates. We model the conformational states of the protein molecule as two groups of substates, corresponding to liganded (Mb^*) and unliganded (Mb) forms of the molecule, with different mean energies (Fig. 2). If a ligand is bound to the heme iron, the substates of Mb^* have lower energy than the Mb substates, and protein molecules overwhelmingly populate these Mb^* substates. Photodissociation of the ligand causes a structural change of the heme[11] which lowers the energy of the unliganded conformations, Mb, relative to the liganded conformations, Mb^*. This initiates a redistribution of the population of molecules into the unliganded conformations at low energy. In this model, the observed conformational relaxation of the protein, $Mb^*\rightarrow Mb$, is this redistribution.

This model is based in part on theoretical descriptions of protein folding[15] and other relaxation phenomena in complex systems.[10,16,17] Such analyses treat the interconversion between a large number of states, and therefore require an assumption about the rate at which a molecule moves from one state to another; a reasonable assumption, suggested by Koper and Hillhorst,[16] is that the rate of leaving a state depends only on the energy of that state, i.e., all states of the system are connected via transition states of a single energy. In our model we also assume a common transition state energy $E^{\ddagger}$. One may imagine that the transition states correspond to less compact forms of the protein in which the helices are slightly separated and free to reposition. (This picture is supported by

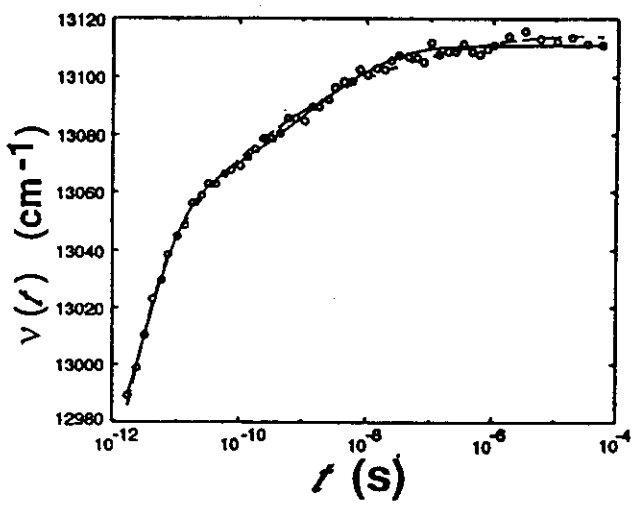

FIG. 1. The frequency shift of the near infrared optical band of horse myoglobin, resulting from motion of the Fe atom as the protein molecule relaxes after photolysis.[24] Data (circles) are from Jackson *et al.*,[5] curves are fits to $\nu(t)$ generated by the globally connected case of the model (solid line) and the diffusive case (broken line).

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Eaton

Lecture II

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A Mathematical Model for Structure–Function Relations in Hemoglobin

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A mathematical model is presented for utilizing the structural features of hemoglobin to determine its functional properties. A formulation containing the essential features of the Perutz mechanism is outlined; i.e. there are two quaternary conformations of the tetramer, two tertiary structures for each chain, and coupling terms arising from the interchain salt bridges. A diagrammatic representation is used to determine the contributions of individual structures to a generating function from which the equilibrium properties are obtained. It is shown that the model parameters have well-defined physical significance which determines their range of possible values. Consideration is given to the effect on the oxygenation curves of pH, 2,3-diphosphoglycerate, ionic strength and dissociation. Various modified, mutant and mixed-state hemoglobins are discussed. Limitations of the model found by comparison with the available data are described and the need for additional experiments is pointed out. Possible modifications and extensions of the model are suggested and it is indicated how to alter the specific assumptions within the general thermodynamic scheme to conform to new structural or functional information.

1. Introduction

The mechanism of the co-operative binding of oxygen by hemoglobin has been of interest for more than sixty years (Bohr, 1904; Hill, 1910). An almost overwhelming body of experimental data, most of which are summarized in comprehensive reviews (Wyman, 1948; Gibson, 1959; Rossi Fanelli, Antonini & Caputo, 1964; Antonini, 1965; Riggs, 1965; Antonini & Brunori, 1970), has been obtained concerning both equilibrium and kinetic effects. Interpretations of these data have in recent years focused primarily on two phenomenological models. The first, formulated by Koshland, Nemethy & Filmer (1966), who elaborated a proposal by Pauling (1935), is a sequential model defined in terms of the conformations and interactions of the individual chains of the hemoglobin tetramer; the second, introduced by Monod, Wyman & Changeux (1965), is defined in terms of two conformations of the entire molecule. Attempts to distinguish between these two models on the basis of the available information have not been successful, although a number of illuminating papers relating certain experimental results to one model or the other have been published (Ogawa & McConnell, 1967; Ogawa, McConnell & Horwitz, 1968; Ogawa & Shulman, 1971; Edelstein, 1971; Minton, 1971). However, even if it had been possible to show that one of these models is approximately correct, the molecular nature of the co-operative mechanism would

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Quaternary Conformational Changes in Human Hemoglobin Studied by Laser Photolysis of Carboxyhemoglobin*

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These experiments indicate that absorbance changes observed at the 425 nm isosbestic point of Hb and HbCO following laser photolysis of HbCO provide a direct measure of the rates of quaternary conformational changes between rapidly reacting Hb* (the immediate product of full photolysis) and slowly reacting normal deoxyhemoglobin. Hb*, first observed by Gibson (Gibson, Q. H. (1959) *Biochem. J.* 71, 293-303), has been interpreted as deoxyhemoglobin remaining in the liganded quaternary conformation following rapid removal of ligand by a light pulse. In borate buffers between pH 8.4 and 9.6 particularly simple pH-independent results were obtained which allowed the use of a Monod, Wyman, and Changeux model (Monod, J., Wyman, J., and Changeux, J. (1965) *J. Mol. Biol.* 12, 88-118) to fit the data. In this case Hb* is taken to be R state deoxyhemoglobin. Partial photolysis experiments at 425 nm show that the rate of the R → T conformational change at 20° decreases by about a factor of 2 for each additional bound ligand. The rate of the ligand-free conformational change is found to be 920 ± 60 s^{-1}, 6400 ± 600 s^{-1}, and 15,700 ± 700 s^{-1} respectively at 3°, 20°, and 30°. The previously uninterpreted effects of flash length and partial photolysis on the CO recombination kinetics can be explained in terms of the present model. Kinetic results obtained below pH 8 are found to be inconsistent with a two-state model. It appears that binding of inositol hexaphosphate produces a new rapidly reacting quaternary conformation of HbCO.

It is generally accepted that the cooperativity manifested in the reaction of hemoglobin with ligands is mediated by conformational changes of the protein. While the kinetic and equilibrium properties of the reactions of hemoglobin with ligands have been thoroughly studied, the rates of the protein conformational changes which produce cooperative effects have not been examined in detail because no direct experimental measure of conformational state applicable to kinetic measurements has been available. The experiments reported here indicate that the absorbance changes observed at the isosbestic point of deoxy- and carboxyhemoglobin, near 425 nm, following laser photolysis of carboxyhemoglobin provide such a measure of conformational state. A preliminary account of a part of this work has already been given (1) and similar changes have also been observed by Gray for sheep hemoglobin at pH 9 (2).

The effect of a protein conformational change on the rate of CO recombination following photolysis of HbCO was first recognized by Gibson (3). He found that the immediate product of full photolysis of HbCO, denoted by Hb*, reacted about 30 times faster with CO than normal deoxyhemoglobin. Hb* was also found to differ somewhat in absorbance from normal Hb. It has been suggested that Hb* is deoxyhemoglobin, left after rapid removal of ligand by photolysis, with the quaternary conformation of HbCO. Gibson (3) inferred the rate of relaxation of fast reacting Hb* to slowly reacting Hb from the effect of CO concentration on the biphasic recombination reaction. The model used assumed that the rate of conformational relaxation was independent of the degree of ligation of a molecule. This model, also used by Gray (2), sufficiently describes the CO recombination kinetics, but cannot account for the absorbance changes at 425 nm or for the effect of partial photolysis on the CO recombination reaction or the effect of flash duration on the distribution of fast and slow phases observed by Gibson (3).

In the present work we have observed the relaxation of the absorbance difference between Hb* and Hb at 425 nm and the kinetics of CO recombination at the 436 nm isosbestic point of Hb and Hb* following laser photolysis.[1] These kinetic data are interpreted by taking account of the tetramer-dimer equilibrium of HbCO and of the effect of the degree of ligation on the rate of conformational change.

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[1] Some of the data, including "Materials and Methods," are presented as a miniprint supplement immediately following this paper. Figs. 17 to 20 are found on p. 1542. For the convenience of those who prefer to obtain supplementary material in the form of full size photocopies, these same data are available as JBC Document No. 75M-1454. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.05.

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The effect of quaternary structure on the kinetics of conformational changes and nanosecond geminate rebinding of carbon monoxide to hemoglobin

(cooperativity/allosteric control/laser photolysis/singular value decomposition/tertiary structure)

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ABSTRACT **To determine the effect of quaternary structure on the individual kinetic steps in the binding of carbon monoxide to the α subunit of hemoglobin, time-resolved absorption spectra were measured after photodissociation of carbon monoxide from a hemoglobin tetramer in which cobalt was substituted for iron in the β subunits. Cobalt porphyrins do not bind carbon monoxide. Spectra were measured in the Soret region at room temperature after time delays that varied from a few nanoseconds to the completion of ligand rebinding at about 100 ms. The results show that the liganded molecule, $\alpha(Fe\text{-}CO)_2\beta(Co)_2$, is in the R state, but can be almost completely switched into the T state by the allosteric effectors inositol hexaphosphate and bezafibrate. The geminate yield, which is the probability that the ligand rebinds to the heme from within the protein, is found to be 40% for the R state and <1% for the T state. According to the simplest kinetic model, these results indicate that carbon monoxide enters the protein in the R and T quaternary conformations at the same rate, and that the 60-fold decrease in the overall binding rate, of carbon monoxide to the α subunit in the T state compared to the R state is almost completely accounted for by the decreased probability of binding after the ligand has entered the protein. The results further suggest that the low probability for the T state results from a decreased binding rate to the heme and not from an increased rate of return of the ligand to the solvent.**

The simplest and conceptually most appealing theory that relates the structure of Hb to its cooperative behavior is the two-state allosteric model (1–5). According to this model, Hb exists in two affinity states—a low-affinity state having the quaternary structure of fully deoxygenated Hb, called T, and a high-affinity state with the quaternary structure of fully liganded Hb, called R. The essential feature of the model is that the affinity does not depend on the number of ligands bound *per se*, but is determined only by the quaternary structure (4). The two-state allosteric model was first applied to the equilibrium ligand-binding properties of Hb but naturally has been extended to explain cooperativity in the kinetics of both O_2 and CO binding (6). Corresponding to the two affinities of the equilibrium treatment, there are four rates—one binding rate and one dissociation rate for each quaternary structure. For CO, the binding rate for the R state is about 60 times faster than for the T state (7), whereas the limited data suggest that the dissociation rate for the T state is about 10-fold greater than that for the R state (8). For O_2, cooperativity is mainly manifested in the dissociation rates (9), with the T state dissociating about 150 times faster than the R state (10).

Photodissociation experiments indicate that the mechanism is more complex than one in which binding of a ligand from the solvent or dissociation of a ligand from the heme into the solvent occurs in a single step (11, 12). Both CO (13) and O_2 (14, 15) undergo geminate recombination at room temperature. That is, ligands rebind to the same heme from which they were dissociated before escaping into the solvent. Geminate recombination of CO occurs on the nanosecond time scale at room temperature (13), whereas geminate recombination of O_2 has been observed on both picosecond (14, 16) and nanosecond (15, 17) time scales.

The minimal kinetic model that incorporates geminate rebinding is a two-step process for both overall binding and dissociation (18–20): (*i*) entry of the ligand into the protein followed by binding to the heme (or escape back into the solvent) in the overall association reaction and (*ii*) breaking of the heme–ligand bond followed by exit of the ligand from the protein into the solvent (or rebinding to the heme) in the overall dissociation reaction. The non-heme-bound ligand inside the protein presumably occupies the region on the distal side of the heme, referred to as the heme pocket. This model immediately raises the possibility that the rates of entering and leaving the protein may be altered by the quaternary structure and, therefore, contribute to kinetic cooperativity in the overall rates of binding or dissociation (18, 19). We already have analyzed the overall dissociation rate of CO from a comparison of geminate rebinding for R-state molecules and for samples in which a fraction of the molecules are in the T state prior to photodissociation (18, 19). For R-state Hb, the geminate yield is about 0.4, reducing the overall dissociation rate by about a factor of 2 relative to the bond-breaking rate. The geminate yield for the T state is <0.4, but a decrease in the geminate yield can increase the dissociation rate of the T state relative to the R state by a factor of at most 1.7, compared to the observed factor of about 10. Thus, cooperativity in the overall dissociation rate of carbon monoxide results primarily from differences in the rate of breaking the heme–ligand bond (18, 19).

Fig. 1 shows three extreme hypothetical cases that could explain the decrease by a factor of 60 in the overall binding rate to the T state compared to the binding rate to the R state. The decreased rate could result from a decreased rate of entry of the ligand from the solvent into the protein, an increased exit rate from the protein into the solvent, or a decreased rate of ligand binding to the heme from within the protein. The latter two possibilities are associated with extremely small geminate yields following photodissociation

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Abbreviation: BZF, bezafibrate.

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Stereochemistry of Cooperative Mechanisms in Hemoglobin

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Hemoglobin (Hb) is the respiratory protein of the red blood cells which carries O_2 from the lungs to the tissues and facilitates the return transport of CO_2 from the tissues to the lungs. The physicist J. J. Hopfield has called it the hydrogen atom of biochemistry because the understanding of its functions is so fundamental to proteins generally. One of these functions is allostery, the switching of proteins between active and inactive structures in response to chemical stimuli. Another is the formation of coordination complexes with transition metals for a great variety of catalytic and other actions. Hemoglobin may be unique in exploiting changes in Fe–N bond lengths accompanying the transition from high-spin to low-spin ferrous iron for the purpose of efficient oxygen transport: without use of that spin transition fast-moving animals could not have evolved. The isolation of the iron atoms in separate pockets of the globin prevents collisions between them, which means that their reactions with ligands can be studied without any of the elaborate apparatus of matrix isolation needed for investigating the reaction mechanisms of simpler metal complexes. Finaly, hemoglobin shows how Nature uses conjugated bases with pK's in the physiological pH range to sensitize proteins to small pH changes in their environment.

Hemoglobin (MW = 64 500) is a tetramer made up of two α-chains and two β-chains, each containing 141 and 146 amino acid residues, respectively. Each chain carries one heme. The α-chains contain seven and the β-chains eight helical segments, interrupted by nonhelical ones. Each chain also carries short nonhelical segments at the N and C termini. Myoglobin (Mb) is a similar protein consisting of a single chain of 153 amino acid residues and one heme. It is found in muscle where it stores the O_2 transferred to it from hemoglobin and liberates the O_2 to the mitochondria for oxidative phosphorylation of adenosine diphosphate. As shown in Figure 1a, the hemes in Mb and Hb are held in pockets formed by several helical and nonhelical segments CD and FG; their Fe's are 5-coordinated to N_ϵ of histidines F8, also known as proximal, and to the four porphyrin nitrogens (N_{porph}); the porphyrin is in van der Waals contact with another histidine on the

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Analysis of Proton Release in Oxygen Binding by Hemoglobin: Implications for the Cooperative Mechanism†

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ABSTRACT: The relationship in hemoglobin between cooperativity (dependence of the Hill constant on pH) and the Bohr effect (dependence of the mean oxygen affinity on pH) can be described by a statistical thermodynamic model [Szabo, A., & Karplus, M. (1972) *J. Mol. Biol. 72*, 163–197; Lee, A., & Karplus, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7055–7059]. In this model, salt bridges and other interactions serve to couple tertiary and quaternary structural changes. To test and refine the model, it is applied to the analysis of the pH dependence of the tetramer-Adair constants corrected for statistical factors (K_{4i}', $i = 1$–4). Attention is focused on the proton release of the first ($\Delta H_1^+ = \partial \log K_{41}'/\partial$pH) and last ($\Delta H_4^+ = \partial \log K_{44}'/\partial$pH) oxygenation steps, where K_{4i}' are the Adair constants corrected for statistical factors. Measurements of ΔH_1^+ and ΔH_4^+ under carefully controlled conditions are reported, and good agreement between the model calculation and these experimental results is obtained. The salt bridges are found to be partially coupled to the ligation state in the deoxy quaternary structure; it is shown that a Monod–Wyman–Changeux-type model, in which the salt bridges are coupled only to quaternary structural change, is inconsistent with the data for ΔH_1. The significance of the present analysis for an evaluation of the Perutz mechanism [Perutz, M. F. (1970) *Nature (London) 228*, 726–734, 734–739] and other models for hemoglobin cooperativity is discussed.

The dependence of the mean oxygen affinity of hemoglobin on pH in the alkaline range (Bohr effect) has been recognized since Bohr first reported it in 1904 [see Antonini and Brunori (1971) and references cited therein]. Wyman (1948) used linkage relationships to show that the Bohr effect is equivalent to the proton release per heme on oxygenation and ascribed it to one ionizable group changing its pK_a from 7.93 to 6.68. Although it was generally assumed that oxygenation-induced conformational changes in the vicinity of the Bohr group are responsible for the lowering in its pK_a, the manner in which these changes occurred was not known until high-resolution X-ray crystallographic data for unliganded and liganded hemoglobins become available in the 1960s and 1970s (Fermi & Perutz, 1981, and references cited therein). The X-ray data demonstrated that there exist two quaternary structures (deoxy and oxy) for the tetramer and two tertiary structures (liganded and unliganded) for each individual chain. On the basis of a detailed analysis of the contacts present in the crystal structures, Perutz (1970a,b) proposed a stereochemical mechanism to explain hemoglobin cooperativity in which the stepwise increase in oxygen affinity is closely related to the Bohr effect. In this model, ionic interactions involving eight salt bridges (four per $\alpha\beta$ dimer) play a dominant role in coupling the ligand-induced tertiary structural changes and the relative stability of the quaternary structures. Since some of the salt bridges involve ionizable protons, the Perutz model also predicts the manner in which the Bohr protons are distributed among the different oxygenation steps. These Bohr groups have been identified experimentally from structural studies of mutant and modified hemoglobins (Perutz & Ten Eyck, 1971; Fermi & Perutz, 1981; Perutz et al., 1984) and from NMR (Kilmartin et al., 1973; Brown & Campbell, 1976), hydrogen-exchange (Ohe & Kajita, 1980; Matsukawa et al., 1984), and chemical reactivity (Garner et al., 1975; Van Beek, 1979) measurements.

The Perutz mechanism was translated into a statistical mechanical model (Szabo & Karplus, 1972, 1975, 1976) to provide a quantitative interpretation of the available thermodynamic data, including the Bohr effect. The Szabo–Karplus formulation is mathematically related to the Monod–Wyman–Changeux (MWC) model (Monod et al., 1965) in which cooperativity arises solely from a ligation-induced shift in the equilibrium between the deoxy (low affinity, T) and oxy (high affinity, R) quaternary structures. However, in contrast to the assumptions of the MWC model, ligation-induced changes in the tertiary structure can occur in the absence of a quaternary transition. From a fit of calculated oxygenation curves to data for oxygen binding in human hemoglobin at pH 7 and 9.1 (Roughton & Lyster, 1965), a set of values was determined for the free energy parameters appearing in the model. As a consequence of the assumptions of the model and of the choice of parameter values, a relationship was shown to exist between the Bohr effect (change of oxygen affinity with pH) and the degree of cooperativity (change of Hill constant with pH).

Despite the elegance of the Perutz mechanism and the success of the Szabo–Karplus (SK) model, the molecular basis of the Bohr effect and its relationship to cooperativity have not been completely resolved. This is due in part to the lack of structural and thermodynamic characterization of oxy-

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Time-resolved optical spectroscopy and structural dynamics following photodissociation of carbonmonoxyhemoglobin

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Hemoglobin; Photodissociation; Time-resolved optical spectroscopy; Ligand-binding kinetics; Structural dynamics

A summary is presented of our current understanding of the kinetics of ligand rebinding and conformational changes at room temperature following photodissociation of the carbon monoxide complex of hemoglobin with pulsed lasers. The events which occur subsequent to excitation have been followed over 12 decades in time, from about 100 fs to the completion of ligand rebinding at about 100 ms. Experiments with picosecond and subpicosecond lasers by others, together with molecular dynamics simulations, indicate that by 1 ns the deoxyhemoglobin photoproduct is in a thermally equilibrated ground electronic state, so that subsequent processes are unaffected by the initial laser excitation. The principal results have been obtained from time-resolved optical absorption spectroscopy using a sensitive nanosecond laser spectrometer. Five relaxations have been observed which are interpreted as geminate rebinding at about 50 ns that competes with motion of the ligand away from the heme which produces a tertiary conformational change, a second tertiary conformational change at 0.5–1 μs, transition from the R to T quaternary structure at about 20 μs, and overall bimolecular rebinding of ligands from the solvent to the R and T quaternary structures at about 200 μs and 10 ms. Assuming that the dissociation pathway in photolysis experiments is the reverse of the association pathway, we find that for the R state there is a 40% probability that the ligand will bind to the heme after entering the protein, and a 60% probability that it will return to the solvent. Studies on the α-subunit of an iron-cobalt hybrid hemoglobin indicate that carbon monoxide enters the protein at the same rate for both R and T quaternary structures. For the α-subunit in the T state the probability of binding after entry is much lower, and the ligand returns to the solvent more than 99% of the time, accounting for the 60-fold overall lower association rate. This decreased probability of binding results from a decreased rate of binding to the heme from within the protein, and not an increased rate of return to the solvent. There are still unresolved problems on the basic structural description of carbon monoxide binding and dissociation, particularly the functional significance of the tertiary relaxations in both the R and T states, and the precise number of kinetic barriers within the protein.

1. Introduction

Dissociation of ligands from hemoglobin is a multistep process [1]. This has been demonstrated by experiments in which a short laser pulse is used to break the heme-ligand bond. Not all photodissociated ligands escape from the protein into the solvent. A certain fraction rebind from within the protein. This process is called geminate recombination. At room temperature geminate recombination of carbon monoxide occurs on a nanosecond time scale [2–18], while for oxygen geminate rebinding is observed on both nanosecond [8,9,19,20] and picosecond time scales [2,3,10,21,22]. A structural basis for geminate recombination has been known since the early X-ray findings that there is no open path from the heme iron to the solvent [23,24]. Residues on the surface of the protein would have to be displaced to allow the ligand to leave the protein after dissociation of the iron-ligand bond, or to enter the protein from the

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Biophysics

Application of linear free energy relations to protein conformational changes: The quaternary structural change of hemoglobin

(kinetics/dynamics/transition state theory/reaction path/allostery)

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ABSTRACT **The transition state for the R ⇌ T quaternary conformational change of hemoglobin has thermodynamic properties much closer to those of the R conformation than to those of the T conformation. This finding is based on a comparison of activation and equilibrium enthalpy and entropy changes and on the observation of a linear free energy relationship between quaternary rate and equilibrium constants. A previous theoretical study [Janin, J. & Wodak, S. J. (1985) *Biopolymers* 24, 509–526], using a highly simplified energy function, suggests that the R-like transition state is the result of a reaction pathway with the maximum buried surface area between $\alpha\beta$ dimers.**

Conformational changes play a critical role in the function of many proteins. In multisubunit proteins, the rearrangement of the packing of subunits, the quaternary conformational change, is responsible for cooperative behavior (1). In spite of the importance of quaternary conformational changes in protein chemistry, very little is known about their kinetics or molecular mechanisms. Hemoglobin remains the only protein for which there is a substantial body of kinetic data for a quaternary conformational change, beginning with the studies of Gibson performed over 30 years ago (2).

An essential element in the investigation of the molecular mechanism of a quaternary conformational change is the experimental characterization of the transition state. Knowledge of the transition state can explain systematic changes in rates and provides an important test of theoretical descriptions of reaction paths. In this paper, we describe results on hemoglobin which indicate that the transition state has properties much closer to those of the R quaternary structure than to those of the T quaternary structure. We also point out that an R-like transition state may be explained by a reaction path similar to the one Janin and Wodak (3) generated by maximizing the buried surface area at the subunit interfaces between $\alpha\beta$ dimers.

In a recent study on trout hemoglobin, time-resolved absorption spectroscopy was used to determine the activation parameters for the R → T transition of the molecule with no ligands bound (4). At 20°C, the conformational change was found to occur with a rate constant of 5×10^4 sec^{-1}, and with an activation energy, E_a, of 8.0 kcal/mol (1 cal = 4.184 J). Using the equation from transition state theory (TST),

$$k = \kappa k^{TST} = \kappa(k_B T/h)e^{-\Delta G^{\ddagger}/RT} = \kappa(k_B T/h)e^{\Delta S^{\ddagger}/R}e^{-\Delta H^{\ddagger}/RT}, \quad [1]$$

with $\Delta H^{\ddagger} = E_a - RT$, together with the equilibrium enthalpy and entropy changes (5, 6), the activation enthalpies and entropies were calculated for both the $R_0 \rightarrow T_0$ and $T_0 \rightarrow R_0$ quaternary structural changes (Fig. 1). The data in Fig. 1 show that the activation enthalpies and entropies for the $T_0 \rightarrow R_0$ transition are much more similar to the equilibrium enthalpies and entropies than the corresponding values for the $R_0 \rightarrow T_0$ transition. That is, the thermodynamic properties of the transition state are much more similar to those of the R quaternary conformation than to those of the T quaternary conformation (4).†

An R-like transition state for the unliganded molecule has important and interesting consequences. Adding ligands, or changing solution variables such as pH, is expected to change the free energy of the transition state by nearly the same amount as the R state. The free energy barrier for the R → T transition (and hence its rate) is therefore expected to change much less than the free energy barrier for the T → R transition (Fig. 2). This prediction is borne out by the published results summarized in Table 1 on the kinetics and thermodynamics of human hemoglobin analyzed in terms of the two-state allosteric model of Monod *et al.* (20).§ The results show that decreasing the allosteric equilibrium constant, $L_i = [T_i]/[R_i] = L_0 c^i = k(R_i \rightarrow T_i)/k(T_i \rightarrow R_i)$, by adding ligands such as carbon monoxide or oxygen to ferrous hemes, or cyanate and azide to ferric hemes, changes the R → T rates much less than the T → R rates (12, 18). Also, increasing the pH, which decreases L_0, changes the $R_0 \rightarrow T_0$ rate much less than the $T_0 \rightarrow R_0$ rate. Thus, changes in the R ⇌ T conformational equilibrium are manifested mainly as changes in the T → R rates, as predicted for an R-like transition state (Fig. 2).

We can take the analysis one step further and ask whether there is any simple quantitative relationship between the rate and equilibrium constants. The simplest of such relationships is a linear free energy relationship. A linear free energy relationship describes the situation in which the transition state has properties that are intermediate between reactants and products and in which, upon changing some variable, the change in the free energy of the transition state is a linear combination of the changes in free energy of the reactants and products (22, 23). Given this assumption, the variation in the

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†There are two reasons why the entropy of activation for $T_0 \rightarrow R_0$ is expected to be less than the equilibrium value even in the limit of virtually identical structures for the transition state and the R_0 state (4). One reason is that the transition state is missing one vibrational degree of freedom, corresponding to motion along the reaction coordinate. The entropy of the transition state is therefore lowered by $R \ln(k_B T/h\nu)$, where ν is the frequency of the missing vibration. The second reason is that in calculating the activation parameters the transmission coefficent, κ, in Eq. 1 was assigned a value of 1, which assumes that there are no recrossings of the barrier (8). For values of $\kappa < 1$, the calculated entropy of activation would be more positive.

§The interface between $\alpha\beta$ dimers (7, 21), which is the interface that changes with the quaternary transition, is sufficiently similar for trout I and human hemoglobin that it is reasonable to assume that the transition states are very similar for the two molecules (4).

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Speed of Intersubunit Communication in Proteins

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ABSTRACT: To determine the speed of communication between protein subunits, time-resolved absorption spectra were measured following partial photodissociation of the carbon monoxide complex of hemoglobin. The experiments were carried out using linearly polarized, 10-ns laser pulses, with the polarization of the excitation pulse both parallel and perpendicular to the polarization of the probe pulse. The substantial contribution to the observed spectra from photoselection effects was eliminated by isotropically averaging the polarized spectra, allowing a detailed comparison of the kinetics as a function of the degree of photolysis. These results show that prior to 1 μs both geminate ligand rebinding and conformational relaxation are independent of the number of ligands dissociated from the hemoglobin tetramer, as expected for a two-state allosteric model. After this time the kinetics depend on the ligation state of the tetramer. The conformational relaxation at 10 μs can be interpreted in terms of the two-state allosteric model as arising from the R to T quaternary conformational change of both unliganded and singly liganded molecules. These results suggest that communication between subunits requires about 1 μs and that the mechanism of the communication which occurs after this time is via the R to T conformational change. The optical anisotropy provides a novel means of accurately determining the extinction coefficients of the transient photoproduct. The decay in the optical anisotropy, moreover, provides an accurate determination of the rotational correlation time of 36 ± 3 ns.

Laser photolysis of hemoglobin provides a unique opportunity to investigate the dynamics of the interaction between protein subunits. In these experiments a short laser pulse is used to photodissociate one or more ligands from the carbonmonoxyhemoglobin tetramer, and the subsequent events are monitored by time-resolved optical absorption spectroscopy. Within 300 ns about 40% of the photodissociated ligands geminately rebind to the heme at room temperature; the remainder escape into the solvent and may rebind in a bimolecular process (Duddell et al., 1979; Alpert et al., 1979; Friedman & Lyons, 1980; Hofrichter et al., 1983). Spectral changes of the deoxyheme occur simultaneously with geminate rebinding, indicating evolution of the protein conformation toward that of deoxyhemoglobin at equilibrium (Lyons & Friedman, 1982; Hofrichter et al., 1983, 1985, 1991; Friedman, 1985; Sassaroli & Rousseau, 1987; Murray et al., 1988a,b). Both the kinetics of geminate rebinding and the kinetics of the deoxyheme spectral changes can be used as probes of subunit interaction. In this work we address two important questions concerning the dynamics of subunit interaction. First, how long does it take for the heme of one subunit of hemoglobin to respond to photodissociation of the heme complex on a neighboring subunit? That is, how fast is communication between subunits? Second, what is the mechanism of this communication?

To examine these questions we carried out a series of partial photolysis experiments on human hemoglobin in the R quaternary conformation using linearly polarized, 10-ns laser pulses. By varying the degree of photolysis, the distribution of tetramers having different numbers of photodissociated heme complexes was varied. The resulting kinetics were then compared in a model-independent analysis to determine when differences began to appear and in a model-dependent analysis to determine how the kinetics of the quaternary conformational change depend on the number of ligands bound. Since hemoglobin in the T quaternary conformation shows less than 1% geminate rebinding (Murray et al., 1988a), the geminate kinetics for hemoglobin initially in the R conformation, but evolving toward the T conformation following ligand photodissociation, provide a sensitive measure of communication between subunits.

Previous nanosecond experimental studies on trout hemoglobin suggested that photoselection effects have a major influence on measurements of geminate rebinding because rotational diffusion of hemoglobin occurs on the same time scale (Hofrichter et al., 1991). A theoretical analysis also showed that photoselection effects are significant even at degrees of photolysis greater than 90% (Hofrichter et al., 1991; Ansari and Szabo, in preparation; Ansari et al., in preparation). By making measurements with the probe pulse polarized both parallel and perpendicular to the photolysis pulse, we could obtain isotropically averaged spectra which contained no interference from photoselection effects. There were additional advantages in making the measurements with both polarizations. The optical anisotropy induced in the sample provided information on the reorientational dynamics of the heme group in addition to that due to the overall rotation of the protein. The measurement of the optical anisotropy, moreover, together with the theory of Ansari and Szabo (in preparation), provided a novel method for accurately determining the extinction coefficients of the photoproduct spectra and therefore for accurately determining the degree of photolysis.

MATERIALS AND METHODS

Sample Preparation. A lysate of human red cells was prepared from freshly drawn blood following the method of Perutz (1968). Oxyhemoglobin A was purified by DEAE-Sephacel (Pharmacia) anion-exchange chromatography using a pH gradient between 8.5 and 7.8 in 0.05 M Tris-HCl[1]

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Oxygen Binding by Single Crystals of Hemoglobin†

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ABSTRACT: Reversible oxygen binding curves for single crystals of hemoglobin in the T quaternary structure have been measured using microspectrophotometry. Saturations were determined from complete visible spectra measured with light linearly polarized parallel to the *a* and *c* crystal axes. Striking differences were observed between the binding properties of hemoglobin in the crystal and those of hemoglobin in solution. Oxygen binding to the crystal is effectively noncooperative, the Bohr effect is absent, and there is no effect of chloride ion. Also, the oxygen affinity is lower than that of the T quaternary structure in solution. The absence of the Bohr effect supports Perutz's hypothesis on the key role of the salt bridges, which are known from X-ray crystallography to remain intact upon oxygenation. The low affinity and absence of the Bohr effect can be explained by a generalization of the MWC–PSK model (Monod, Wyman, & Changeux, 1965; Perutz, 1970; Szabo & Karplus, 1972) in which both high- and low-affinity tertiary conformations, with broken and unbroken salt bridges, respectively, are populated in the T quaternary structure. Because the α and β hemes make different projections onto the two crystal axes, separate binding curves for the α and β subunits could be calculated from the two measured binding curves. The approximately 5-fold difference between the oxygen affinities of the α and β subunits is much smaller than that predicted from the crystallographic study of Dodson, Liddington, and co-workers, which suggested that oxygen binds only to the α hemes. This 5-fold difference is exactly compensated by a small amount of cooperativity to produce the crystal binding curve with a Hill n of 1.0. In terms of free energy, the cooperativity is only about 10% of that observed in solution. It therefore represents only a slight perturbation on the essential feature of an allosteric model that the binding curve for hemoglobin in the T quaternary structure be perfectly noncooperative. If the concentration of the crystallizing agent [poly(ethylene glycol)] and/or the fraction of oxidized hemes are not sufficiently high, oxygen binding is no longer reversible. Under these conditions the crystals crack and undergo a time-dependent increase in their saturation with oxygen. We tentatively assume that this change corresponds to the conversion of the low-affinity T quaternary structure to the high-affinity R quaternary structure, opening up the possibility of carrying out a detailed kinetic study of the quaternary conformational change in the crystal by monitoring the oxygen saturation.

Hemoglobin remains the paradigm for understanding the cooperative behavior of proteins. The reason is that a surprisingly simple model can explain the major features of a vast array of structural, spectroscopic, equilibrium, and kinetic data on its cooperative ligand binding and conformational changes. The model is a synthesis of the two-state allosteric model of Monod, Wyman, and Changeux (Monod et al., 1965; Shulman et al., 1975; Edelstein, 1975), the stereochemical mechanism of Perutz (Perutz, 1970; Perutz et al., 1987), and the statistical mechanical formulation of Perutz's mechanism by Szabo and Karplus (Szabo & Karplus, 1972; Lee et al., 1988). Figure 1 shows schematic diagrams of this MWC–PSK model. The novel features of the model, and also the most controversial, are the central role of the salt bridges and the existence of only two affinity states of the tetramer, each with noncooperative binding. In later work Perutz suggested that there is indirect evidence from studies on mutant hemoglobins for cooperative oxygen binding to the T quaternary structure (Perutz, 1976, 1989). Ackers and co-workers have also proposed that there is significant cooperativity within both the R and T quaternary structures. Their conclusion is based on an analysis of the dimer–tetramer equilibrium constants of model compounds for the intermediate ligation states (Daugherty et al., 1991; Ackers et al., 1992). Finally, Shulman et al. (1982) pointed out that the failure of the salt bridges to break upon binding of carbon monoxide in crystals of the T-state mutant hemoglobin Kansas (Anderson, 1975) argues against Perutz's proposal for the role of the salt bridges.

It appeared to us that much of the controversy arises from the fact that the structural studies have been carried out on crystals, while the functional studies have been carried out on solutions where the structures are unknown. This pointed to a critical need for functional studies on crystals, permitting a direct comparison with the results of X-ray crystallography. Such comparisons have been found to be useful in the study of structure–function relations in enzymes (Rossi & Bernhard, 1970; Mozzarelli et al., 1989; Rossi et al., 1992). Our interest in this problem was further sparked by the determination of the structure of a partially oxygenated hemoglobin in the T quaternary structure (Brzozowski et al., 1984a; Liddington et al., 1988). This X-ray crystallographic investigation was carried out on crystals grown from solutions of poly(ethylene glycol) (PEG).[1] Unlike crystals formed in concentrated salt solution, which crack and become disordered upon oxygenation

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[1] Abbreviations: oxyHb, oxyhemoglobin; deoxyHb, deoxyhemoglobin; metHb, methemoglobin; PEG, poly(ethylene glycol); DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate.

Eaton

Lecture III

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Fast events in protein folding initiated by nanosecond laser photolysis

(cytochrome *c*/kinetics/polypeptide dynamics/denatured state/optical spectroscopy)

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ABSTRACT **Initiation of protein folding by light can dramatically improve the time resolution of kinetic studies. Here we present an example of an optically triggered folding reaction by using nanosecond photodissociation of the heme–carbon monoxide complex of reduced cytochrome *c*. The optical trigger is based on the observation that under destabilizing conditions cytochrome *c* can be unfolded by preferential binding of carbon monoxide to the covalently attached heme group in the unfolded state. Photodissociation of the carbon monoxide thus triggers the folding reaction. We used time-resolved absorption spectroscopy to monitor binding at the heme. Before folding begins we observe transient binding of both nonnative and native ligands from the unfolded polypeptide on a microsecond time scale. Kinetic modeling suggests that the intramolecular binding of methionine-65 and -80 is faster than that of histidine-26 and -33, even though the histidines are closer to the heme. This optical trigger should provide a powerful method for studying chain collapse and secondary structure formation in cytochrome *c* without any limitations in time resolution.**

Protein folding generally occurs in two phases, one rapid and one slow. The rapid phase is the collapse of the unfolded polypeptide into a compact structure and the formation of secondary structure. The slow phase is the subsequent, often multistep rearrangement to the native conformation (1–7). Because of the limited time resolution (milliseconds) of conventional stopped-flow mixing experiments, the complete time course of protein folding has not yet been observed. A dramatic improvement in time resolution would result if protein folding could be initiated by light.

Here we present an example of an optically triggered folding reaction. We take advantage of the fact that under destabilizing conditions cytochrome *c* can be unfolded by preferential binding of carbon monoxide (CO) to the covalently attached heme group in the unfolded state. The folding reaction can thus be triggered by photodissociating the CO complex. In this study, we used time-resolved absorption spectroscopy with nanosecond lasers to monitor binding events at the heme (8–11). Although rebinding of CO prevents the complete formation of the native conformation, the rapid recovery of the sample permits repetitive photolysis and therefore the acquisition of high signal/noise transient spectra for investigating submillisecond events. Simulations of the multiwavelength data with kinetic models were carried out to generate the spectra of intermediates, as well as the rate constants connecting them. We find that before folding begins there is transient binding of both nonnative and native ligands from the unfolded polypeptide chain on a microsecond time scale.

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MATERIALS AND METHODS

Type VI horse heart cytochrome *c* from Sigma was purified by ion-exchange chromatography using carboxymethyl-cellulose (Whatman) (12), reduced with sodium dithionite anaerobically, and separated anaerobically from the sodium dithionite by gel filtration on Sephadex G-25M (Pharmacia). The solutions for fluorescence and circular dichroism measurements contained 15 μM cytochrome *c*, 0.1 M potassium phosphate buffer (pH 6.5), and 200 μM sodium dithionite. They were prepared anaerobically, equilibrated with either argon or CO, and sealed in 1-cm square cuvettes with dental wax and glyptal varnish. The fluorescence measurements were performed with an SLM 8000 fluorimeter (SLM Aminco, Urbana, IL) and were corrected for self-absorption by the heme and sodium dithionite. The circular dichroism measurements were performed with a Jasco 710 spectropolarimeter (Jasco, Easton, MD).

For time-resolved absorption measurements, the solutions contained 106 μM cytochrome *c*, 200 μM sodium dithionite, and 0.1 M potassium phosphate (pH 6.5) and were sealed under a CO atmosphere in 350-μm cuvettes. The measurements were made with a nanosecond spectrometer as described (10, 13, 14). The instrument is based on two Q-switched Nd/YAG (yttrium/aluminum garnet) lasers producing 10-ns (full width at half maximum) pulses. The 532-nm second harmonic of one laser photolyzes the sample. The 366-nm third harmonic of the second laser excites the spontaneous emission of a dye (stilbene 420), which is used as a broadband probe source for measuring the transient spectra. A silicon vidicon tube read by an optical multichannel analyzer detector controller is used for detection. The data were filtered by using singular value decomposition, and offsets due to shot-to-shot variations in laser intensity were removed by the procedure described by Jones *et al.* (10).

RESULTS AND DISCUSSION

The basis for our optical trigger is given in Fig. 1, which shows the guanidine hydrochloride (Gdn·HCl)-induced unfolding curve of reduced horse heart cytochrome *c* in the presence and absence of CO at 40°C, as measured by tryptophan fluorescence. There is a single tryptophan in horse cytochrome *c* at position 59 (Fig. 2). Its fluorescence is almost completely quenched in the native protein by energy transfer to the heme (15, 16). Unfolding results in an increase in the heme–tryptophan distance and, consequently, an increase in the fluorescence yield (15–20).

In the absence of CO, the unfolding transition begins at ≈4.5 M Gdn·HCl and is complete at ≈6.0 M Gdn·HCl with a midpoint concentration, c_m, of 5.1 M Gdn·HCl. In the pres-

Abbreviation: Gdn·HCl, guanidine hydrochloride.

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PATHWAYS OF PROTEIN FOLDING

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KEYWORDS: protein folding mechanisms, folding intermediates, mutagenic analysis of folding, partially folded proteins, spectroscopic studies of protein folding reactions

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RESEARCH ARTICLES

Funnels, Pathways, and the Energy Landscape of Protein Folding: A Synthesis

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ABSTRACT **The understanding, and even the description of protein folding is impeded by the complexity of the process. Much of this complexity can be described and understood by taking a statistical approach to the energetics of protein conformation, that is, to the energy landscape. The statistical energy landscape approach explains when and why unique behaviors, such as specific folding pathways, occur in some proteins and more generally explains the distinction between folding processes common to all sequences and those peculiar to individual sequences. This approach also gives new, quantitative insights into the interpretation of experiments and simulations of protein folding thermodynamics and kinetics. Specifically, the picture provides simple explanations for folding as a two-state first-order phase transition, for the origin of metastable collapsed unfolded states and for the curved Arrhenius plots observed in both laboratory experiments and discrete lattice simulations. The relation of these quantitative ideas to folding pathways, to uniexponential vs. multiexponential behavior in protein folding experiments and to the effect of mutations on folding is also discussed. The success of energy landscape ideas in protein structure prediction is also described. The use of the energy landscape approach for analyzing data is illustrated with a quantitative analysis of some recent simulations, and a qualitative analysis of experiments on the folding of three proteins. The work unifies several previously proposed ideas concerning the mechanism protein folding and delimits the regions of validity of these ideas under different thermodynamic conditions**

Key words: protein folding, energy landscape, folding pathway, folding funnel, lattice simulation, folding thermodynamics, folding kinetics, protein engineering

INTRODUCTION

The apparent complexity of folded protein structures and the extraordinary diversity of conformational states of unfolded proteins make challenging even the description of protein folding in atomistic terms. Soon after Anfinsen's classic experiments on renaturation of unfolded proteins,[1] Levinthal recognized the conceptual difficulty of a molecule searching at random through the cosmologically large number of unfolded configurations to find the folded structure in a biologically relevant time.[2] To resolve this "paradox," he postulated the notion of a protein folding pathway. The search for such a pathway is often stated as the motive for experimental protein folding studies. On the other hand, the existence of multiple parallel paths to the folded state has been occasionally invoked.[3] Recently, a new approach to thinking about protein folding and about these issues specifically has emerged based on the statistical characterization of the energy landscape of folding proteins.[4–6]

This paper presents the basic ideas of the statistical energy landscape view of protein folding and relates them to the older languages of protein folding pathways. The use of statistics to describe protein physical chemistry is quite natural, even though each protein has a specific sequence, structure, and function essential to its biological activity. The huge number of conformational states immediately both allows and requires a statistical characterization. In addition folding is a general behavior common to a large ensemble of biological molecules. Many different sequences fold to essentially the same structure as witnessed by the extreme dissimilarities in sequence which may be found in families of proteins such as lysozyme.[7] Thus for any given

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REVIEW

Principles of protein folding—A perspective from simple exact models

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Abstract

General principles of protein structure, stability, and folding kinetics have recently been explored in computer simulations of simple exact lattice models. These models represent protein chains at a rudimentary level, but they involve few parameters, approximations, or implicit biases, and they allow complete explorations of conformational and sequence spaces. Such simulations have resulted in testable predictions that are sometimes unanticipated: The folding code is mainly binary and delocalized throughout the amino acid sequence. The secondary and tertiary structures of a protein are specified mainly by the sequence of polar and nonpolar monomers. More specific interactions may refine the structure, rather than dominate the folding code. Simple exact models can account for the properties that characterize protein folding: two-state cooperativity, secondary and tertiary structures, and multistage folding kinetics—fast hydrophobic collapse followed by slower annealing. These studies suggest the possibility of creating "foldable" chain molecules other than proteins. The encoding of a unique compact chain conformation may not require amino acids; it may require only the ability to synthesize specific monomer sequences in which at least one monomer type is solvent-averse.

Keywords: chain collapse; hydrophobic interactions; lattice models; protein conformations; protein folding; protein stability

We review the principles of protein structure, stability, and folding kinetics from the perspective of simple exact models. We focus on the "folding code"—how the tertiary structure and folding pathway of a protein are encoded in its amino acid sequence. Although native proteins are specific, compact, and often remarkably symmetrical structures, ordinary synthetic polymers in solution, glasses, or melts adopt large ensembles of more expanded conformations, with little intrachain organization. With simple exact models, we ask what are the fundamental causes of the differences between proteins and other polymers—What makes proteins special?

One view of protein folding assumes that the "local" interactions among the near neighbors in the amino acid sequence, the interactions that form helices and turns, are the main determinants of protein structure. This assumption implies that isolated helices form early in the protein folding pathway and then assemble into the native tertiary structure (see Fig. 1). It is the premise behind the paradigm, primary → secondary → tertiary structure, that seeks computer algorithms to predict secondary structures from the sequence, and then to assemble them into the tertiary native structure.

Here we review a simple model of an alternative view, its basis in experimental results, and its implications. We show how the nonlocal interactions that drive collapse processes in heteropolymers can give rise to protein structure, stability, and folding kinetics. This perspective is based on evidence that the folding code is not predominantly localized in short windows of the amino acid sequence. It implies that collapse drives secondary structure formation, rather than the reverse. It implies that proteins are special among polymers not primarily because of the 20 types of their monomers, the amino acids, but because the amino acids in proteins are linked in *specific sequences*. It implies that the folding code resides mainly in global patterns of contact interactions, which are nonlocal, and arise from the arrangements of polar and nonpolar monomers in the sequence.

We review here the simple exact models that can address these questions of general principle. Such questions are often difficult to address by other means, through experiments, atomic simulation, Monte Carlo partial sampling, or approximate theoretical models. "Simple" models have few arbitrary parameters.

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Biochemistry

Submillisecond events in protein folding

(barstar/relaxation kinetics/cold denaturation/protein engineering)

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Contributed by Alan R. Fersht, August 7, 1995

ABSTRACT **The pathway of protein folding is now being analyzed at the resolution of individual residues by kinetic measurements on suitably engineered mutants. The kinetic methods generally employed for studying folding are typically limited to the time range of ≥1 ms because the folding of denatured proteins is usually initiated by mixing them with buffers that favor folding, and the dead time of rapid mixing experiments is about a millisecond. We now show that the study of protein folding may be extended to the microsecond time region by using temperature-jump measurements on the cold-unfolded state of a suitable protein. We are able to detect early events in the folding of mutants of barstar, the polypeptide inhibitor of barnase. A preliminary characterization of the fast phase from spectroscopic and Φ-value analysis indicates that it is a transition between two relatively solvent-exposed states with little consolidation of structure.**

The pathway by which a particular protein folds will be resolved experimentally when the structures of all stable, metastable, and transition states adopted by the protein during the process have been characterized structurally and energetically. The only way to analyze the structures of transition states is by kinetics, and the details of their structure and energetics can be gleaned from the kinetics of folding of proteins whose structures have been carefully altered by protein engineering (1–6). This protein engineering procedure has been used to characterize transition states and intermediates on a time scale of ≥1 ms (1–6), the time resolution of the stopped-flow and other rapid mixing techniques employed so far in these and most other studies (e.g., refs. 7–9). This is too slow to allow detection of the early events that initiate folding. To study faster events, kinetic techniques must be employed that eliminate the time delays arising from rapid mixing techniques. Relaxation methods, by which a preexisting equilibrium between denatured and folded states is rapidly perturbed by a change in physical or chemical conditions, may be used to extend the time range. It is not easy, however, to find methods that can readily cause a denatured state of a protein to renature. Millisecond time resolution has been achieved by using a repetitive pressure-perturbation method (10). Laser flash photolysis has been applied to dissociate CO from CO-bound cytochrome *c*, with concomitant denaturation (11). However, rapid CO rebinding, binding of non-native ligands, and possibly aggregation prevented the complete transition to the native state (11). A more generally applicable method for studying fast events would be to raise rapidly the temperature of a cold-denatured protein, since cold denaturation is a common phenomenon of globular proteins under suitable experimental conditions (12–15). Temperature jump (T-jump) by electrical discharge (16–20) and laser-induced heating would thus enable microsecond and nanosecond time resolutions, respectively.

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The structure of barstar (21), the inhibitor of the ribonuclease barnase from *Bacillus amyloliquefaciens*, has been solved by NMR spectroscopy in solution (22), and the gross features of its folding pathway have been determined by rapid mixing methods and equilibrium thermodynamics (23). The active barstar mutant C40A/C82A/P27A (pseudo-wild-type barstar), which was used in this study, contains no cysteines which may give rise to crosslinks in the denatured state and only one proline residue.

METHODS

Protein Expression and Purification. Site-directed mutagenesis of the barstar mutant C40A/C82A (23) was performed with the Sculptor kit (Amersham). Mutant plasmids, expressed in the *Escherichia coli* strain TG2, were identified by DNA sequencing. Expression at 28°C and purification of barstar mutants were performed as described (22). Since the protein is found in inclusion bodies, pellets of the lysed cells were dissolved in 7 M urea and dialyzed against 50 mM Tris Cl buffer, pH 8/0.1 M NaCl before purification. Pseudo-wild-type barstar (C40A/C82A/P27A) and the mutants I5V, L16V, L34V, and L51V of the pseudo-wild type were prepared.

Equilibrium Studies. Circular dichroism (CD) spectra of barstar in 50 mM Tris Cl buffer pH 8/100 mM KCl with urea concentrations and temperatures as stated were measured with a Jasco (Easton, MD) model J-720 spectrometer using 2-nm spectral bandwidth. The protein concentrations and pathlengths of the cell were 50 μM and 0.02 cm for the far-UV CD spectra, 30 μM and 1 cm for the near-UV CD spectra, and 20 μM and 0.1 cm for the temperature dependence of the CD signal at 222 nm. Barstar concentrations were determined by using an extinction coefficient at 280 nm of 22,690 M^{-1}·cm^{-1} (23). For measuring the temperature dependence of the CD signal at 222 nm, the sample was equilibrated at 0°C for 1 hr, then heated at 20°C·hr^{-1} to 25°C and then heated at 50°C·hr^{-1} to 80°C. Equilibrium fluorescence experiments were performed with a Hitachi model F-4500 fluorimeter with excitation at 280 nm, using a 0.4 cm × 1 cm cell containing 3 μM protein, 100 mM KCl, 50 mM Tris Cl buffer (pH 8), and 2 M urea. Samples were preequilibrated for 1 hr for experiments at 2°C, and for 20 min for experiments at other temperatures.

Kinetic Measurements. A T-jump apparatus from DIALOG (Dusseldorf, Germany) was equipped with a 0.7 cm × 0.7 cm cell of 0.8-ml volume and a 200-W mercury–xenon lamp. Fluorescence excitation was at 280 nm, and a cutoff filter at 295 nm was usually used for emission. Noise levels of <0.01% root mean square of the signal were achieved at a 5-μs response time. The buffer was degassed and the cell and sample were equilibrated at 2°C for 1 hr before the T-jumps. Signals of several T-jumps separated by 3-min equilibration time were accumulated. For double T-jump experiments, the cell and degassed buffer were precooled to 2°C prior to the addition of an appropriate amount of 100 μM barstar stock solution, which

Abbreviation: T-jump, temperature jump.

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Submillisecond folding of monomeric λ repressor

(dynamic NMR)

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ABSTRACT **The folding kinetics of a truncated form of the N-terminal domain of phage λ repressor [λ_{6-85}] has been investigated by using the technique of dynamic NMR. λ_{6-85} has been shown previously to fold in a purely two-state fashion. This allows the determination of folding and unfolding rates from simulation of the exchange-broadened aromatic resonances of Tyr-22. The folding kinetics were determined over a range of 1.35 to 3.14 M urea. The urea dependence of both folding and unfolding rate constants is exponential, suggesting that the rate-determining step is invariant at the urea concentrations studied. The folding and unfolding rates extrapolated to 0 M urea at 37°C are 3600 ± 400 s^{-1} and 27 ± 6 s^{-1}, respectively. The observed λ_{6-85} folding rate constant exceeds that of other fast-folding globular proteins by a factor of 14–54. The urea dependence of the folding and unfolding rate constants suggests that the transition state of the rate-determining step is considerably more exposed to solvent than previously studied protein-folding transition states. The surprising rapidity of λ_{6-85} folding and unfolding may be the consequence of its all-helical secondary structure. These kinetic results clearly demonstrate that all of the fundamental events of protein folding can occur on the submillisecond time scale.**

The elucidation of the mechanism by which proteins fold remains one of the most challenging problems of modern biology. The earliest events in protein folding are particularly difficult to study by current methods and can be important determininants of the overall reaction (1, 2). Time-resolved kinetic studies have established that many of these important early events occur in <1 ms and are complete within the dead time of the stopped-flow technique. This rapid folding phase has been detected by circular dichroism (CD), fluorescence, and NMR stopped-flow studies in many proteins and reflects the potential for proteins to fold on the submillisecond time scale (3–9). Ultrasonic attenuation and dielectric relaxation measurements indicate that isolated α-helices fold with relaxation times of 10^{-7} to 10^{-8} s, four to five orders of magnitude faster than rate constants observable by stopped-flow methods (10–12). This vast time-scale difference represents a major gap in our understanding of the fundamental early events in folding.

In principle, dynamic NMR methods can fill this time-scale gap. Exchange processes that interconvert chemically or conformationally distinct species on the 10-ms to 10-μs time scale can have profound effects on the shape and position of NMR peaks (13). Much faster processes, including motions of the fully native form, can be studied by relaxation time measurements (14–17). To date, dynamic NMR has not been used to measure overall protein folding rates, although it has been used to detect a folding intermediate of rat intestinal fatty acid-binding protein (18). Dynamic NMR-based protein folding studies are rare because the overall folding rates of most proteins are not fast enough to broaden NMR resonances (19–22).

However, there is a growing number of proteins observed to fold on the microsecond time scale (3, 23–25), and it is likely that their folding processes are similar to early folding events in proteins that fold more slowly. An example of a fast-folding protein is the N-terminal domain (residues 1–102) of bacteriophage λ repressor *c*I, which has been used extensively as a model system (26). The crystal structure of residues 1–92 bound to DNA has been determined to 1.8-Å resolution (27). A truncated form containing residues 6–85 (λ_{6-85}) is monomeric and has a solution structure and stability that are essentially identical to those of the longer versions (28). A structural model for λ_{6-85} based on NMR data and the cocrystal structure of the λ_{1-92}–DNA complex, is shown in Fig. 1. NMR and CD studies have shown that the protein folds in a two-state fashion when denatured thermally or in urea, thus making it possible to interpret the NMR line shapes in terms of two interconverting species (28). In this report we describe the application of the line-shape method to measure the overall folding rate of λ_{6-85}.

MATERIALS AND METHODS

λ_{6-85} protein was expressed and purified as described (28). NMR samples consisted of 500 μM protein dissolved in NMR buffer [~99% ^2H_2O/10 mM $C^2H_3COO^2H$/100 mM NaCl/3-(trimethylsilyl)propionic acid (17 μg/ml)/1 mM NaN_3, pH 5.6] with various concentrations of urea as determined by refractometry (30). Each sample was equilibrated at 37°C for at least 20 min prior to acquisition of the NMR spectrum.

Proton NMR spectra (1024 transients) were acquired at each urea concentration on a Varian Unity spectrometer at 500 MHz, by using presaturation for 1.5 s to suppress residual water. This recycle delay is sufficiently long to allow complete recovery of all protein signals. NMR data were processed by using FELIX 2.05 (Biosym Technologies, San Diego) and referenced to 3-(trimethylsilyl)propionic acid for each urea concentration. Line-shape simulations were computed by using a FELIX macro/accessory program (ZELIG) kindly provided by Michael Strain (University of Oregon).

RESULTS AND DISCUSSION

As demonstrated (28), the folding reaction of λ_{6-85} can be described by using the simple model:

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} D, \qquad [1]$$

where N and D represent the native and denatured states, respectively, and k_f and k_u are the first-order rate constants for folding and unfolding, respectively. When a protein folds in a two-state fashion, any NMR resonance can be used to determine k_f and k_u. In this study we use the aromatic region of the ^1H NMR spectrum because it shows good spectral dispersion and can be assigned in both the native and denatured states (28), as shown in Fig. 2. The aromatic spectrum of λ_{6-85} at various urea concentrations is shown in Fig. 3. At intermediate

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MOLTEN GLOBULE AND PROTEIN FOLDING

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I. INTRODUCTION

Shortly after Sanger determined the amino acid sequence of the first protein (Sanger, 1952), Anfinsen tried to find out whether protein sequence can spontaneously fold into the native three-dimensional (3D) structure with the corresponding function. The answer was "yes," as it has been shown that a protein with broken disulfide bonds unfolded by urea can spontaneously fold again, restoring its native disulfide bonds and full native activity (see Anfinsen, 1973, for a summary of these investigations).

Next the mechanism of protein folding was questioned. Does a protein fold by a complete search of all possible conformations looking for the structure with minimal energy or does it fall down into one of energy minima (not necessary the global one) by some mechanistic folding pathway coded in its sequence? The first possibility implies that protein

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Chemistry

Ultrafast thermally induced unfolding of RNase A

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Contributed by R. M. Hochstrasser, March 9, 1995

ABSTRACT **A temperature jump (T-jump) method capable of initiating thermally induced processes on the picosecond time scale in aqueous solutions is introduced. Protein solutions are heated by energy from a laser pulse that is absorbed by homogeneously dispersed molecules of the dye crystal violet. These act as transducers by releasing the energy as heat to cause a T-jump of up to 10 K with a time resolution of 70 ps. The method was applied to the unfolding of RNase A. At pH 5.7 and 59°C, a T-jump of 3–6 K induced unfolding which was detected by picosecond transient infrared spectroscopy of the amide I region between 1600 and 1700 cm^{-1}. The difference spectral profile at 3.5 ns closely resembled that found for the equilibrium (native − unfolded) states. The signal at 1633 cm^{-1}, corresponding to the β-sheet structure, achieved 15 ± 2% of the decrease found at equilibrium, within 5.5 ns. However, no decrease in absorbance was detected until 1 ns after the T-jump. The disruption of β-sheet therefore appears to be subject to a delay of ≈1 ns. Prior to 1 ns after the T-jump, water might be accessing the intact hydrophobic regions.**

Questions concerning the physical and chemical nature of protein folding are among the most challenging in biological research (1–4). Folding and unfolding events have seldom been studied on time scales shorter than milliseconds. Internal motions of macromolecules such as rotations about single bonds, chemical exchange reactions, diffusion over molecular dimensions, and barrier crossing processes can occur on nanosecond or even picosecond time scales, so protein structure reorganization might be expected to involve ultrafast intermediate steps. An example is the recent report of tens-of-microseconds folding in cytochrome *c* (5). Some of the faster processes in protein folding might involve relatively small alterations in electronic structure. Therefore the probes used to examine them must be sensitive to subtle changes in, for example, nonbonded interactions, weaker chemical bonds, charge distributions, and motions of pieces of the structure. For this reason we decided to use transient infrared (IR) spectroscopy (6–8), which is structure sensitive at a chemical-bond resolution, to identify any ultrafast folding steps.

The IR spectra of proteins in the region of the amide vibrations of the polypeptide structures are well known to be sensitive to the state of the protein. For example, there are distinct differences between the IR spectra of random coil, α-helical, β-sheet, β′-sheet, and turn structures of polypeptides (9). These differences arise from the dependence of interactions between the various amide groups on the local polypeptide structures. One can therefore conceive of carrying out time-resolved IR capable of following the kinetics of structure change as it affects these different spatial regions of the polypeptide backbone. Experiments on the kinetics of folding also require that the system be triggered to suddenly change. For this purpose we have developed an ultrafast temperature jump (T-jump) method.

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The protein RNase A was chosen for the present study because of the wealth of information available regarding its equilibrium properties around the denaturation temperature and its kinetic properties on the time scale of stopped-flow experiments. The crystal structure of RNase A is known (10). The unfolding process was studied previously by static temperature methods (11–13), fluorescence (14), x-ray scattering and Fourier-transform IR (15, 16), NMR (17–20) and CD (21) spectroscopy. In denaturing, the native protein first relaxes toward an unfolded intermediate state, which then slowly equilibrates with more fully denatured structures. The fast unfolding step(s) involves the dismantling of the protein secondary structure, whereas the slower ones have been attributed to specific isomerizations involving the proline residues. "Fast" in this context has previously meant tens of milliseconds; the "very fast" intermediate reported recently (14) also has a millisecond lifetime. The time resolution of solution mixing experiments is too slow to resolve the range of protein conformational states that might occur in the earlier stages of unfolding. Molecular dynamics simulations (22–24) suggest that picosecond resolution will be needed to characterize the relevant structural changes which include significant motions along dihedral angle coordinates of the polypeptides and the modification of hydrogen-bonded structures by water (25).

To obtain a T-jump in the solution on the picosecond time scale the protein is dissolved in a solution containing inert dye molecules having a large extinction coefficient and fast internal conversion. The temperature of the solution is raised quickly by irradiating the dye transducer with an intense laser pulse. Transient IR spectroscopy is then used to measure the protein spectral response to the T-jump and also the time dependence of the temperature of the medium.

MATERIALS AND METHODS

Sample Preparation. Bovine pancreatic RNase A was obtained from Sigma (catalogue no. R-5125) and used without further purification. The solution of protein and dye buffered at p^2H (pD) 5.7 by 2-(*N*-morpholino)ethanesulfonate (Mes) was thermostatted in a temperature-controlled bath. A peristaltic pump circulated the sample from the container to the sample cell. The sample temperature was maintained at 59°C.

Transient IR Method. To monitor changes in the IR portion of the spectrum, we constructed a neodymium–yttrium/aluminum garnet (Nd–YAG) laser-based T-jump apparatus. One 35-ps pulse from an active/passive mode-locked Nd–YAG laser (1064 nm) operating at 10 Hz was selected by a KD*P Pockels Cell (deuterated potassium dihydrogen phosphate; Medox) and amplified to 5 mJ in a three-stage Nd–YAG amplifier, then frequency-doubled in KDP (potassium dihydrogen phosphate) to produce 532-nm light of 0.6–1 mJ in energy. To generate the mid-IR at 3–5 μm, a portion of the 532-nm light was used to pump a traveling-wave dye laser whose output was mixed in $LiIO_3$ with the 532-nm pulse to produce the difference frequency. The 6-μm radiation was

Abbreviations: T-jump, temperature jump; CV, crystal violet; mOD, OD milliunits.

Fast Events in Protein Folding: Helix Melting and Formation in a Small Peptide†

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ABSTRACT: The helix is a common secondary structural motif found in proteins, and the mechanism of helix−coil interconversion is key to understanding the protein-folding problem. We report the observation of the fast kinetics (nanosecond to millisecond) of helix melting in a small 21-residue alanine-based peptide. The unfolding reaction is initiated using a laser-induced temperature jump and probed using time-resolved infrared spectroscopy. The model peptide exhibits fast unfolding kinetics with a time constant of 160 ± 60 ns at 28 °C in response to a laser-induced temperature jump of 18 °C which is completed within 20 ns. Using the unfolding time and the measured helix−coil equilibrium constant of the model peptide, a folding rate constant of approximately 6×10^7 s^{-1} ($t_{1/2}$ = 16 ns) can be inferred for the helix formation reaction at 28 °C. These results demonstrate that secondary structure formation is fast enough to be a key event at early times in the protein-folding process and that helices are capable of forming before long range tertiary contacts are made.

The question of how proteins fold from a random coil or quasi-random state to their final compact biologically active state (native state) is under intense theoretical and experimental investigation. It is evident that the native structure of the protein is coded within the primary amino acid sequence, but the fundamental physical and chemical processes which drive the sequence to a specific three-dimensional structure are not known. The folding process must somehow be guided, however, since a random search through all possible final states that the amino acid sequence might adopt to find the correct native state would take a very long time, certainly longer than the lifetime of an organism [the Levinthal paradox; see e.g. Creighton (1992) for a comprehensive review of the folding problem]. Because the precise structure of the folded protein is specifically linked to its biological activity, the protein-folding problem bears directly on practical issues, including structure−function relationships and the rational design of new proteins.

It has been known for about thirty years that many proteins will spontaneously unfold or fold reversibly in response to denaturing or renaturing conditions. A major stumbling block in experimental studies of protein-folding dynamics, however, is that these studies have generally employed techniques limited to millisecond or longer times and that key events occur faster than the millisecond time scale. In experiments based on the rapid mixing of solutions, for example, the reversibly denatured, unfolded protein in solution (e.g. at high concentrations of urea, extremes of pH, etc.) is rapidly diluted or mixed with reagents which reverse the denaturing conditions and chemically initiate folding. The best temporal response of studies based on mixing is typically one millisecond, the "dead time" of a stopped-flow apparatus, and the early events simply cannot be observed. Relaxation methods such as temperature jump (induced by capacitive discharge), electric field jump, and resonant ultrasound that access time scales as short as several nanoseconds have been applied to study the helix−coil kinetics of homopolymers consisting of several hundred units (see Discussion). However, the structural assignment of the kinetics observed by these approaches is not straightforward because of interference from molecular reorientation, solvation, charge transfer reactions, and/or proton transfer reactions, depending on the method. New experimental approaches are needed to study protein folding on fast time scales.

Because the α-helix is a common structural motif in proteins, it is important to understand the folding process of the α-helix in detail as a step toward understanding how proteins arrive at their native structures. For example, it has been proposed that short range interactions along the polypeptide chain of a forming protein induce regions of secondary structure which then act as nucleating regions for further collapse, leading to native tertiary structure [see e.g. Karplus and Weaver (1994) and Kim and Baldwin (1990)]. While there has been progress in understanding the thermodynamics of the helix−coil transition (Chou & Scheraga, 1971; Rialdi & Hermans, 1966; Scholtz et al., 1991), less is known about the kinetics of this process. In this paper, we report the unfolding kinetics of a small 21-residue α-helical peptide, the so-called suc-F_S 21-peptide: Suc-AAAAA-$(AAARA)_3$A-NH_2 (Suc = succinyl, A = alanine, and R = arginine). This peptide is reported to be greater than 90% helical in water at temperatures near 0 °C by Lockhart and Kim (1992, 1993). This peptide, like all small helical peptides, unfolds over a relatively large temperature range,

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