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WORKSHOP ON THE STRUCTURE OF BIOLOGICAL MACROMOLECULES

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"X-Ray Crystallography and Enzyme Chemistry"

presented by:

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X-ray Crystallography and Enzyme Chemistry

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Introduction

Enzymes are proteins specifically designed, by natural evolution, to catalyse the most diverse and difficult chemical reactions in the mild conditions needed to sustain life. The understanding of their function has been, and still is, one of the most strongly pursued goals of modern science.

Only a little more than one century has passed since the first cell-free enzyme extract was prepared (Büchner, 1897) giving start to modern enzymology, but only about four decades ago the first protein structure was revealed by X-ray methods. Since then the impressive amount of information already gathered on enzymes was only indirect. Actually, for many years the concept that enzymes were proteins has been controversial and only after that Sumner was able to crystallise urease from jack beans and to demonstrate the proteic nature of the enzyme, this debate was finally settled (Sumner, 1926). Then the rapid development of protein chemistry provided the means to obtain pure material for experimental work which resulted in the great successes of modern enzymology.

However, the scientific community had to wait until the 50's for a first look into the structure of proteins and DNA thanks to the pioneering work of the Cambridge crystallographic school.

The first structural determinations of DNA, myoglobin and hemoglobin have had an unprecedented impact on molecular biology bringing the discipline to a copernican revolution. Since then, the field of macromolecular crystallography has gone through enormous progresses by taking full advantages of the technological advances like the availability of the extremely intense X-ray sources provided by the modern synchrotrons, 2-D detectors and the speed of recent computers [for a complete overview of macromolecular crystallography see Methods in Enzymology vols. 114-115 (Wyckoff, Hirs & Timasheff eds.; 1985) and vols. 276-277 (Carter Jr. & Sweet eds.: 1997)].

Nowadays protein crystallography has achieved great successes in revealing the architecture of the molecules involved in biological processes. Nevertheless the static picture of the molecule resulting from the crystallographic experiment (averaged in space and time) is seldom providing a thorough understanding of the underlying chemistry.

Recently, with the availability of the modern synchrotron X-ray sources, intense white radiation has been utilised for time-resolved crystallography utilising Laue-techniques

[Methods in Enzymology, Vol 277, Chapts. 22-23 (1997)]. These experiments provide crystal structures on a millisecond time scale from which information on the mechanism of biochemical reactions may be obtained. This technique, however, suffers from experimental complexities limiting its application (narrow time window, substrate activation, data completeness etc.). In my opinion a different approach may be used to shed light on the mechanisms of biochemical catalysis. Indeed it is often easier to determine the crystal structure of the complexes between an enzyme and different ligands, specifically designed after a mechanistic hypothesis to simulate substrates, transition-states, intermediates and products of the reaction. The structural information from such 'snapshots' of the system along the reaction coordinate coupled with data from different techniques (kinetics, spectroscopy, etc.) may provide an accurate description of the microevents underlying the enzymatic reaction under investigation.

As a matter of fact, when high resolution crystal structures are available, it is possible to make direct observation of the non covalent interactions responsible of the binding and of the conformational changes induced on the enzyme native structure by the formation of the complex. Thus it becomes evident the role played by the active site amino acid residues in the enzyme chemistry. Consequently it is possible to understand the relationship between macromolecular structure and activity, making possible not only the design of potent inhibitors, but also to conceive the active site modifications that may lead to different catalytic activity and specificity towards 'unnatural' substrates.

In the lectures I will illustrate the technique by using working examples taken from studies done in my laboratory over the last few years on different metallo-enzyme systems.

Outline of the technique

- Protein quality for successful crystallisation
- Crystallisation techniques
- Protein complexes in the solid state: soaking and co-crystallisation
- Data collection
- Structure solution and refinement
- Data analysis

Protein quality

The protein sample should be of the highest purity. Optimally, the sample should pass through HPLC and isoelectric focussing in order to separate isozymes, if present. The requirement is not only for sequence homogeneity, but also for conformational homogeneity. Divide the protein sample in small, concentrated, aliquots and keep them frozen (-20°/-70° C) in order not to have to repeatedly freeze-thaw the entire sample. Be aware that freezing is counter-indicate in same case.

Crystallisation

Vapour-diffusion methods

Hanging-drop. Pros: small quantity of protein needed to screen many different conditions. Cons: small crystals, waste of protein.

Sitting-drop. Pros: larger drops, i.e. larger crystals. Cons: waste of protein.

Techniques for growth of X-ray quality crystals

Micro- and Macro-seeding. This techniques allow to grow few large, flawless crystals from the crystallisation conditions found by the first screening. The concept is that in order to grow large crystals we must meet conditions were nucleation is rare and the growth is slow. Seeding provide the starting crystallisation nuclei and, most important, seeds will usually grow in conditions were spontaneous nucleation does not occur.

Soaking

Once diffraction quality crystals of the native protein are obtained, the complex with the molecule of interest can be prepared just by soaking the crystals in a slightly more concentrated solution (≈10%) of the precipitant used to obtain the crystals containing the ligand molecule at the right concentration. Remember that the binding of the ligand to the protein is regulated by the K_{inst} of the complex. Pros: Isomorphism of the complexed crystals with the native protein, i.e. no need to solve the structure. Cons: Partial occupation of ligand sites, cracking of crystals, redissolving of the crystals.

Co-crystallyzation

Prepare the ligand-protein complex in solution and proceed with crystallisation trials. Pros: Full occupancy of ligand sites. Cons: Probable lack of isomorphism, i.e. need for independent structure solution of the complex.

Data Collection

In order to observe the binding of the ligand to the protein, the experiment should aim to obtain the highest resolution (better than 2.0 Å). The quality of the crystals is obviously the limiting factor to achieve this goal, but resolution can be improved by using bright X-ray sources (synchrotron) and/or data collection at cryogenic temperatures (100 K). Using a conventional laboratory equipment with X-rays from a rotating anode generator an extension of the diffraction by 0.2-0.5 Å it is often observed by collecting data at 100 K. Larger effects are obtained with synchrotron radiation.

Diffraction

Analogy between crystal structure determination from diffraction amplitudes and image reconstruction by optical lenses. Atomic electrons are the particles which interact with the X-ray photons causing their coherent scattering. An ordered arrangement of electrons, like that present in crystalline matter, makes the scattered photons to interfere in such a way to have enhancement of radiation only in certain directions, i.e. diffraction is observed. Diffraction from a crystal can be simply described in terms of reflection by crystal planes. This is the origin of the term reflection to indicate the effect of X-ray diffraction by the crystal. The spatial disposition of the reflections depends on the metric of the crystal (unit cell). The intensity of the reflections depends on the electron distribution within the crystal unit cell. The reflections contain information on the electronic distribution within the cell and hence on the positions of the atoms of the crystal. The observed diffraction pattern is the convolution between the Fourier transform of the crystal lattice with that of the object (atom, molecule) that makes it. The process of structure solution is then to reconstruct the image of the object from the measured reflection intensities:

$$\rho(\mathbf{r}) = 1/V \, \Sigma_{\mathbf{h}} \, \mathbf{F_h} \cdot \exp[-2\pi i(\mathbf{h} \cdot \mathbf{r})]$$

$$\mathbf{F_h} = 1/V \, \Sigma_{\mathbf{r}} \, \rho(\mathbf{r}) \cdot \exp[2\pi i(\mathbf{h} \cdot \mathbf{r})]$$

$$\text{i.e. } \rho(\mathbf{r}) = \mathbf{T} \, \mathbf{F_h}$$

and the experimentally available reflection intensity is:

$$I \propto |F^2|$$

Structure solution

If the crystals of the complexes have been obtained by soaking, they are isomorphous with the native protein (i.e. they have the same unit cell and space group). In this case, if the native protein structure is known, the starting phases for Fourier calculations and the starting model for least-squares refinement are provided by the atomic positions of the native model. The process of refining the model and to calculate Fourier maps to observe the complex structure and to improve the atomic model should be repeated until convergence is obtained.

Thermodynamics of the Protein-Ligand Complex

The crystal structure of a complex unambiguously reveals the interactions which are responsible of the formation of the complex by identifying the residues involved and the type of the interaction (covalent, van der Waal's, hydrogen-bond, etc.). Comparison with the native structure allows to monitor the conformational changes occurring upon ligand binding and to see the water molecules occupying the binding site. When the ligand and the protein

associate the entropic contribution dominates the free energy of binding since the ligand loses translation and rotation entropy, but in turn the system gains entropy as water molecules are released from the cavity where the ligand binds. Furthermore, in the case of binding to a metal cofactor, when the ligand displaces more than one water molecule from metal coordination sphere a stabilization of the complex due to a chelate effect is observed. Indeed the ligand loses only one set of translational and rotational entropies. The crystal structures of protein-ligand complexes show that the covalent and non-covalent interactions of the ligand with the protein are mainly responsible for the specificity and directionality of the interaction.

The entropy of a molecule is the sum of internal (rotations, vibrations), translational and rotational entropies. The translational entropy is high (120 J deg^{-1} mol^{-1} for 1M solution of a small molecule, ≈40 kJ mol^{-1} at 25°C). The translational entropy decreases with concentration because the average volume occupied by a molecule is inversely proportional to the concentration. Its dependence from the mass is small. The rotational entropy is also large , but it is independent from concentration and slowly increases with mass. When two molecules condense in one a set of rotational and translational degrees of freedom is lost which is not compensated for by the increase of mass. This is the reason why unimolecular reactions are favoured with respect to bimolecular ones (≈55-60 kJ mol^{-1}, 25°C) and an important aspect of catalytic efficiency of enzymes.

Enzymes kinetics:

$$E + S \underset{\Delta G^S}{\overset{K_m}{\leftrightarrow}} ES \underset{\Delta G^{\#}}{\overset{k_{cat}}{\rightarrow}} EP$$

K_m is the dissociation constant of the ES complex (Michaelis constant).

$$E + S \xrightarrow[\Delta G^{\#T}]{k_{cat}/K_m} ES^{\#} (EP)$$

where k_{cat}/K_m is the rate constant of the free enzyme and substrate to give products.

If the binding between enzyme and substrate is strong, K_m is low, but if the binding between transition state end enzyme is strong, then k_{cat} is high.

Suggested reading

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Working Examples

1. UREASE (Benini S., Ciurli S., Rypniewsky W., Wilson K.S. & Mangani S.- *Acta Cryst. D* in press, *JBIC* in press)

Urease (urea aminohydrolase E.C. 3.5.1.5), the enzyme that catalyses the hydrolysis of urea at a rate 10^{14} times faster than the rate of the uncatalysed reaction (Hausinger, 1993), may be thought to represent the paradigm in the development of biological inorganic chemistry. The first enzyme to be crystallised, from the plant source *Canavalia ensiformis* (jack bean) (Sumner, 1926), was the first protein shown to contain nickel in the active site (Dixon, Gazzola, Blakeley & Zerner, 1975). This discovery stimulated efforts to unravel the chemistry of such a rare microelement in other biological settings, leading to the discovery of the presence of nickel in CO-dehydrogenase (Drake, Hu & Wood, 1980), methyl coenzyme M reductase-bound factor Ni-F430 (Diekert, Klee & Thauer, 1980, Whitman & Wolfe, 1980),

and Ni,Fe-hydrogenase (Lancaster, 1982). In the absence of structural models of the urease active site, the synthetic analogue approach was utilized to obtain small coordination compounds of known structure featuring spectroscopic and functional properties as similar as possible to the enzymatic system (Stemmler, Kampf, Kirk & Pecoraro , 1995, Wages, Taft & Lippard, 1993).

Recently, the report of the X-ray structure of urease from a bacterial source, *Klebsiella aerogenes* (Jabri, Carr, Hausinger & Karplus, 1995), together with the structure of the apoenzyme and three active site mutants (Jabri & Karplus, 1996, Park et al., 1996), has finally provided a detailed picture of the active site. In *K. aerogenes* urease, an heteropolymeric protein with an $\alpha_3\beta_3\gamma_3$ quaternary structure, the two Ni(II) ions, known to be present in the active site, (Todd & Hausinger, 1987) are in the α subunit: one Ni cation appears to be bound to $His^{\alpha 246}$ through the Nδ atom and to $His^{\alpha 272}$ through Nε, while the other Ni cation is bound to $His^{\alpha 134}$ and $His^{\alpha 136}$ through Nε, to $Asp^{\alpha 360}$ through Oδ1, and to one water molecule. The two Ni ions are held close to each other (at 3.5 Å) by a bridging carboxylate group of the carbamylated $Lys^{\alpha 217}$ residue (Jabri et al., 1995). The structure of native *K. aerogenes* urease suggests the presence of one *tricoordinated pseudotetrahedral* Ni cation (with low occupancy of the fourth site by a water molecule) and one *pentacoordinated distorted trigonal bipyramidal* Ni cation. This conclusion is in disagreement with all previous spectroscopic data on *K. aerogenes* and jack bean ureases (Alagna, Hasnain, Piggot, & Williams 1984, Blakeley, Dixon & Zerner 1983, Clark & Wilcox, 1989, Clark, Wilcox & Scott, 1990, Finnegan et al., 1991, Hasnain & Piggot, 1983, Wang et al., 1994), which pointed to the presence of *hexa- or pentacoordinated slightly distorted octahedral* Ni cations.

Urease from *Bacillus pasteurii*, a highly ureolytic and alkaliphilic soil bacterium (Gibson, 1935), was the first urease isolated from bacterial sources (Larson & Kallio, 1954). This enzyme is heteropolymeric (Benini, Gessa & Ciurli, 1996) ($M_r(\alpha)$ = 61.4 kDa; $M_r(\beta)$ = 14.0 kDa; $M_r(\gamma)$ = 11.1 kDa (Moersdorf, Weinmann & Kaltwasser, 1994)) and features an active site containing two Ni cations (Benini et al., 1996). The primary structures of *B. pasteurii* (Moersdorf et al., 1994) and *K. aerogenes* (Mulrooney & Hausinger, 1990) ureases are highly homologous (63% (α), 46% (β) and 61% (γ), respectively), and the amino acid residues ligating the nickel cations in *K. aerogenes* urease are conserved in the enzyme from *B. pasteurii* (Moersdorf et al., 1994). In contrast to the reported structure of native *K. aerogenes* urease, Ni-edge X-ray absorption spectroscopy (XAS) studies, carried out on *B. pasteurii* urease, suggest an average coordination environment of the Ni ions represented by five or six N/O ligands arranged in a pseudo-octahedral geometry, (Benini, Ciurli, Nolting & Mangani, 1996), with two of the ligands being histidine imidazole side chains.

The inconsistencies between the spectroscopic and structural analyses of urease might be resolved by determining the number and position of light, non-protein ligands in the

vicinity of the metal ions, which could be represented by water molecules or by hydroxide ions bound to the Ni cations either in an end-on or a bridging mode. Given the relatively low resolution (2.2 Å) and completeness (92 %) of the structure of urease from *K. aerogenes* (Jabri et al., 1995), it is possible that such ligands could not be clearly detected in the proximity of the electron-rich dinuclear Ni center. This point is of fundamental importance in understanding the catalytic mechanism of urea hydrolysis, for which the role of water molecules (or hydroxide ions) present in the active site may be crucial (Zerner, 1991). Data with higher resolution and completeness should also be useful in resolving the discrepancies between the coordination geometry of Ni(II) found in urease and the stereoelectronic behavior of this metal cation in synthetic coordination compounds, for which no cases of coordination geometry

analogous to that proposed for *K. aerogenes* urease are known. Another reason to establish with high accuracy the structure of ureases isolated from different sources is to allow a comparison, essential in establishing the existence of common features. These will provide a better understanding of the chemistry of the catalysis and also allow rational design of inhibitors capable of functioning with a broad range of microbial ureases, in order to decrease the negative effects of urea hydrolysis in both medical and agricultural applications (Mobley & Hausinger, 1989).

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2. SUPEROXIDE DISMUTASE (Ferraroni M., Rypniewski W., Wilson K.S., Orioli P., Bruni B., & Mangani S.- *JBIC.*, In press)

Cu,Zn superoxide dismutase (Cu,ZnSOD ; E.C. 1.15.1.1) is an enzyme ubiquitous in living organisms where it catalyzes the disproportionation of the superoxide radical to dioxygen and hydrogen peroxide thus protecting the cells from the toxic effects of the O_2^- radical anion [1-3]. The recent demonstration of the involvement of Cu,ZnSOD point mutants in FALS syndrome [4-6] has refueled the interest on the enzyme and on its function.

The enzyme from bovine erytrhocytes is a homodimer of molecular weight of approximately 32 kDa. Each monomer contains one Cu(II) and one Zn(II) ion in the active site where the only copper ion is essential for catalysis [2,3,7]. Cu,ZnSOD is characterized by very high catalytic rates which are diffusion controlled [8,9]. It is generally accepted that

the reaction mechanism proceeds in two steps [9,10] with approximately equal rate constants for the two reactions ($k_r = k_O \approx 2\text{-}3 \cdot 10^9\ M^{-1}s^{-1}$) [10-12]:

$$\text{E-Cu(II)} + O_2^- + H^+ \xrightarrow{k_r} \text{EH-Cu(I)} + O_2$$

$$\text{EH-Cu(I)} + O_2^- + H^+ \xrightarrow{k_O} \text{E-Cu(II)} + H_2O_2$$

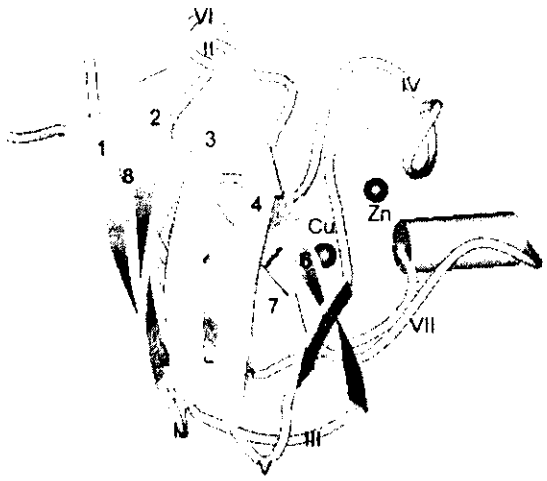
During the catalytic cycle the copper ion alternates between the +2 and +1 oxidation states as indicated by the bleaching of the enzyme green-blue color at steady state [13,14]. The oxidized enzyme has been well characterized by X-ray studies and by several chemical and physical methods [3](and references therein). The crystal structures of Cu,ZnSOD from bovine, human and amphibian erythrocytes have been reported as well as Cu,ZnSOD from yeast, spinach and a prokaryote [15-20]. All the above structural determinations show that each Cu,ZnSOD monomer maintains the characteristic tertiary structure consisting of a flattened, antiparallel β-barrel of eight strands joined by seven turns and loops (numbered I to VII following Getzoff et al., 1989 [21]). The active site is located in between the external wall of the β-barrel and the extended loop VII at the bottom of a conical narrow cavity shaped to accommodate the superoxide substrate. The cavity is surrounded by charged residues which are supposed to provide electrostatic guidance to the substrate [22,23]. The cavity hosts one copper and one zinc ions. The copper ion is coordinated by the nitrogen atoms of four histidines and by a loosely bound water molecule. One of the histidines (His61 in the bovine SOD sequence) bridges copper and zinc. The coordination of zinc is completed by two other histidines and an aspartate group in a tetrahedral geometry. Sketches of the Cu,ZnSOD monomer fold and of the active site cavity displaying the secondary structure numbering and the relevant active site residues respectively, are reported in the Schemes I and II below.

bond of the bridging His61 and simultaneous protonation of its Nε2 atom as requested by the mechanism proposed by Tainer et al. (1983). A similar arrangement of the copper coordination has been recently observed in the crystal structure of the reduced yeast enzyme from *S. cerevisiae* [30]. An EXAFS study, which appeared just at the time of the present paper preparation, has repeated the measurements published thirteen years before [26] and has confirmed the Cu(I) tri-coordination in the bovine Cu(I),ZnSOD solution [31].

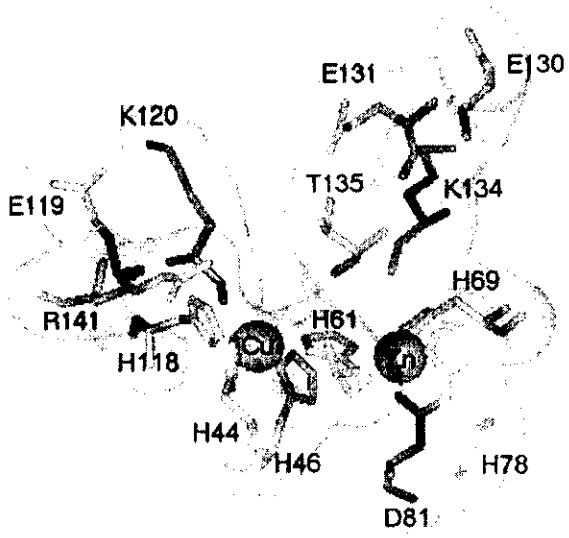
In view of the extremely fast rate of the enzyme catalyzed reactions and of experimental evidence like the equivalence of rate constants for the enzyme in both oxidation states which are pointing towards small energy differences between the oxidized and reduced enzyme, several authors have proposed different reaction mechanisms implying outer sphere electron transfer without His61 protonation and the consequent Cu - His61 bond breaking [32-34].

We have recently determined the crystal structure of the reduced bovine enzyme crystallized in space group $P2_12_12_1$. Our X-ray investigations on a $P2_12_12_1$ crystal form grown at pH 7.5 [35,36] revealed that, contrarily to spectroscopic and to the recent X-ray data, no major structural changes take place upon reduction of the enzyme, in other words, the cuprous ion still appeared to be bound to His61 in bovine Cu(I),Zn SOD.

These new crystallographic findings about the reduced form of Cu,ZnSOD may have mechanistic implications because they imply that, at least under certain circumstances, the enzyme is able to maintain the histidinato bridge between Cu(I) and zinc. It is possible that the crystal structure does not show an active state of the enzyme, nevertheless the possibility for Cu,ZnSOD to function in this form may settle the debate about how the high catalytic rate of the enzyme can be consistent with a proposed mechanism [37] involving large and energy demanding, active site rearrangements like the breaking and reforming of the His61 bridge between Cu and Zn and concomitant protonation and deprotonation of the His61 Nε2. At low substrate concentrations and consequently lower turnover numbers, the enzyme can work with a mechanism involving the breaking of the imidazolate bridge, whereas under substrate saturating conditions a mechanism with a bridging histidine in both oxidation states would be more satisfying [35].



Scheme I



Scheme II

Due to the inaccessibility of Cu(I) to EPR and electronic spectroscopy a lesser amount of data is available on the reduced form of the enzyme. Uv/vis spectroscopy on the cobalt derivative both in solution and in the solid state [24,25], EXAFS data [26] and NMR spectroscopy [27-29] on the Cu(I) containing enzyme have provided evidence for the presence of a tri-coordinate cuprous ion resulting from the breaking of the copper-nitrogen

Besides, since the enzyme shows identical efficiency towards superoxide dismutation in both its oxidized and reduced forms [10-12], it remains the problem to understand the interaction between Cu(I) and the substrate which in principle should be different from that of Cu(II) in presence of the imidazolate bridge. For this reason we have decided to determine the crystal structure of complexes of Cu(I),Zn SOD with small mononegative anions like the isoelectronic azide and thiocyanate, which are inhibitors of the enzyme and have charge and the electronic structure similar to the substrate. Hence they should bind to the enzyme in a way resembling that of the superoxide anion and provide clues about its binding to Cu,ZnSOD. Our results will be discussed and compared with those of the recently reported crystal structures of the complexes of oxidized Cu(II),Zn SOD with azide and cyanide anions [38,39] and with the solution studies on the same complexes [40-42].

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