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international centre for theoretical physics

SMR: 1098/3

WORKSHOP ON THE STRUCTURE OF BIOLOGICAL MACROMOLECULES

(16 - 27 March 1998)

"NMR Metalloproteins"

presented by:

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Cytochrome b_5

An electron transfer low-spin hemoprotein involved in

- Fatty acid desaturation (interacting with NADH cytochrome *b*5 reductase)
- The reduction of myoglobin (in erythrocytes)
- Different hydroxilation reactions (interacting with cytochrome P450)

contains a b-type heme, whose iron ion is coordinated by two histidines

Structures available for Cytochrome b_5

X-Ray Structures

Oxidized Rat Outer Mitochondrial Membrane Cytochrome b_5
resolution 2.7 Å
M.J.Rodriguez-Maranon, F.Qiu, R.E.Stark, S.P.White, X.Zhang, S.I.Foundling, V.Rodriguez, C.L.Schilling Iii, R.A.Bunce, M.Rivera
***Biochemistry*, 1996**

Oxidized Bovine Liver Microsomal Cytochrome b_5
resolution 1.5 Å
R.C.E.Durley, F.S.Mathews
Acta Cryst., 1996

NMR Solution Structures

Rat Apocytochrome b_5
C.J.Falzone, M.R.Mayer, E.L.Whiteman, C.D.Moore, J.T.Lecomte
***Biochemistry*, 1996**

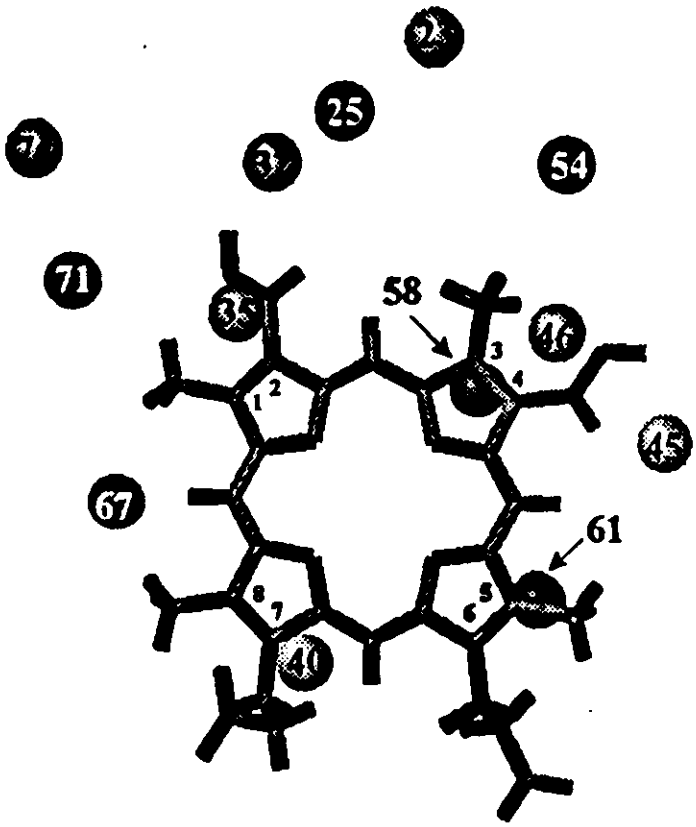
Oxidized Bovine Cytochrome b_5
F.W.Muskett, G.P.Kelly, D.Whitford
J. Mol. Biol., 1996

Reduced Microsomal Rat Cytochrome b_5
L.Banci, I.Bertini, F.Ferroni, A.Rosato
Eur. J. Biochem., 1997

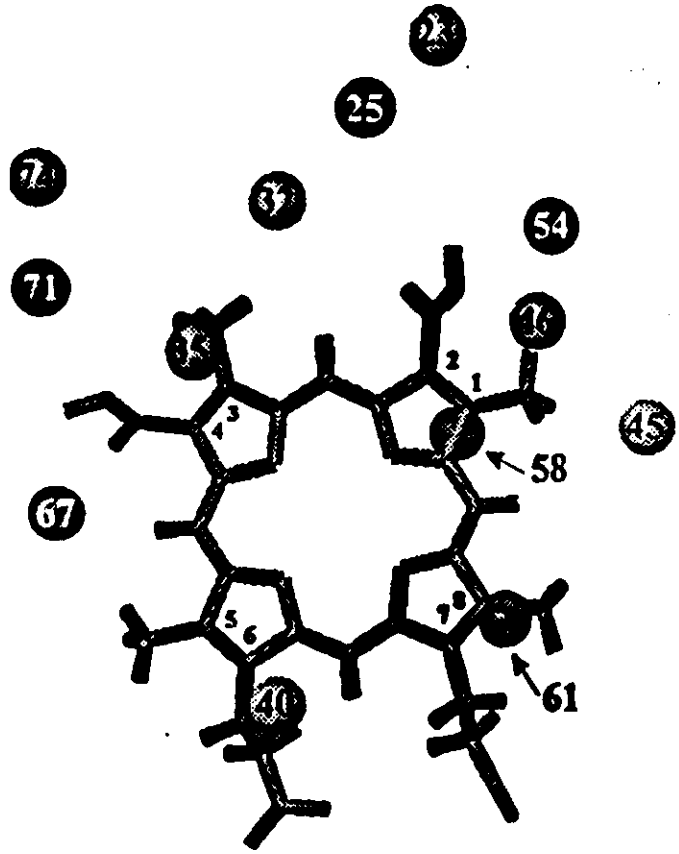
Oxidized Rat Microsomal Cytochrome b_5
F.Arnesano, L.Banci, I.Bertini, I.C.Felli
Biochemistry, 1998

B Form of Oxidized Rat Microsomal Cytochrome b_5
F.Arnesano, L.Banci, I.Bertini, I.C.Felli
Biochemistry, submitted

The two heme conformations in Cytochrome b_5



A form



B form

Organism	A:B ratio
Rat	6 : 4
Bovine	9 : 1
Calf	9 : 1
Rabbit	9 : 1
Chicken	20 : 1

Protein: Oxidized Rat Microsomal Cytochrome b_5

Cofactor:		***b*-type heme (LS Fe(III))**
AA:		**98**
% of assigned AA		**95%**
NOE's:		**1816 (1378)**
Pseudocontact Shift Constraints:		**235**
RMSD/40 str. (Å)	**BB -**	**0.58 ± 0.10**
	HA -	**1.05 ± 0.11**

Calculation procedure: PSEUDODYANA

Refinement procedure: PSEUDOREM$_v$

Reference:

Arnesano F., Banci L., Bertini I., Felli I. C., (1998) *Biochemistry 37*, 173-184

Protein: Reduced Rat Microsomal Cytochrome b_5

Cofactor:		***b*-type heme (Fe(II))**
AA:		**98**
% of assigned AA		**95%**
NOE's:		**1722 (1203)**
RMSD/40 str. (Å)	**BB -**	**0.78 ± 0.18**
	HA -	**1.29 ± 0.16**

Calculation procedure: DYANA

Refinement procedure: REM$_v$

Reference:

Banci L., Bertini I., Ferroni F., Rosato A., (1997) *Eur. J. Biochem. 249*, 270-279

Protein: B Form of Oxidized Rat Microsomal Cytochrome b_5

Cofactor:		***b*-type heme (LS Fe(III))**
AA:		**98**
% of assigned AA		**95%**
NOE's:		**1765 (1302)**
Pseudocontact Shift Constraints:		**220**
RMSD/40 str. (Å)	**BB -**	**0.55 ± 0.09**
	HA -	**1.03 ± 0.11**

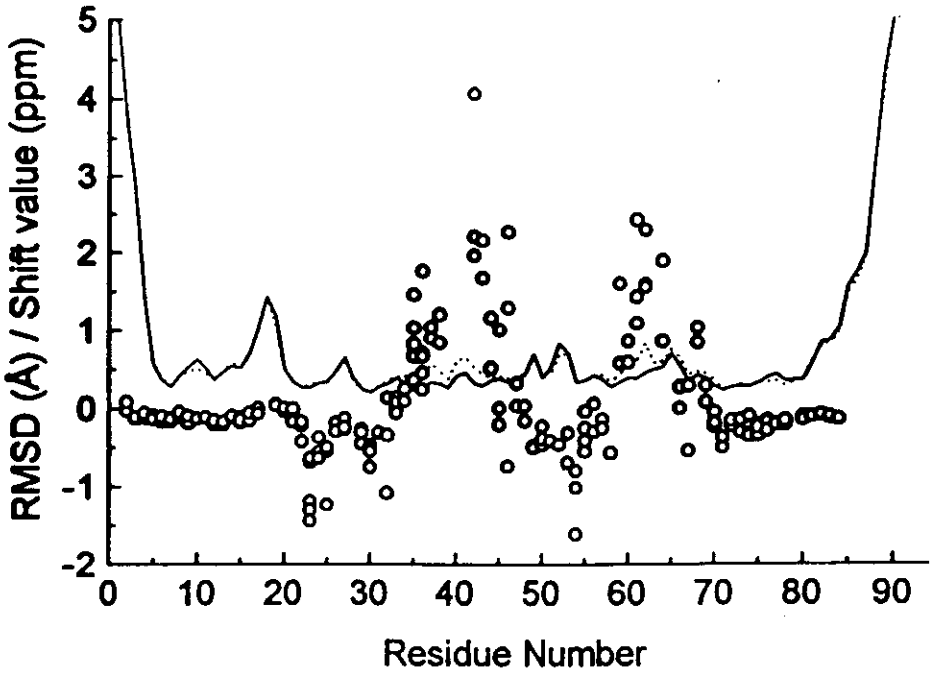
Calculation procedure: PSEUDODYANA

Refinement procedure: PSEUDOREM$_v$

Reference:

Arnesano F., Banci L., Bertini I., Felli I. C., (1998) *Biochemistry* (submitted)

RMSD [illegible]
OF OXIDIZED CYt b5
AND P.C.S. CONSTRAINTS

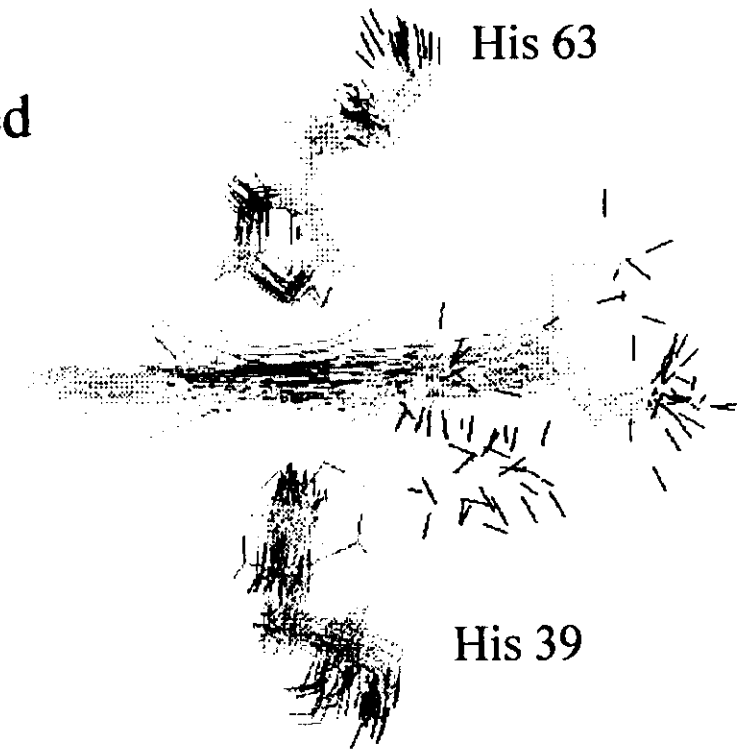


ARNESANO, BANCI, BERTINI, FELLI, 1997

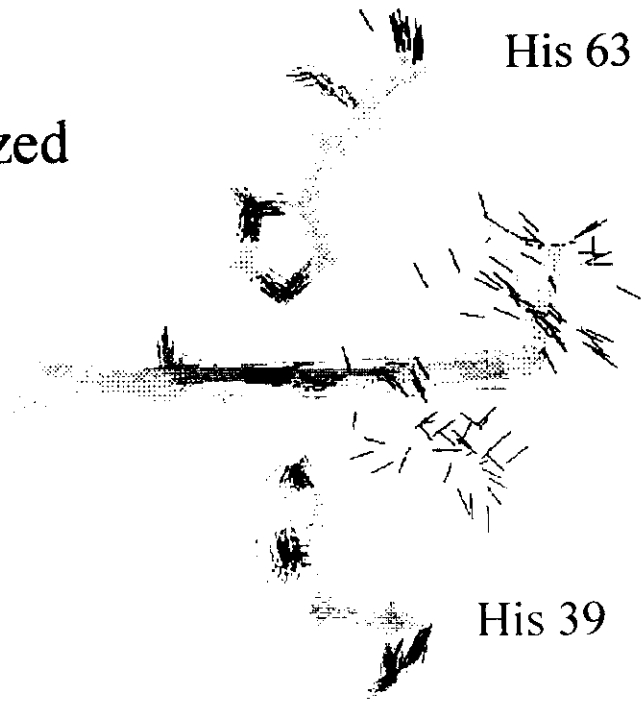
COMPARISON BETWEEN OXIDIZED AND REDUCED CYT b_5

Redox state dependent conformational change of propionate 7 in rat microsomal cytochrome b_5 (form A)

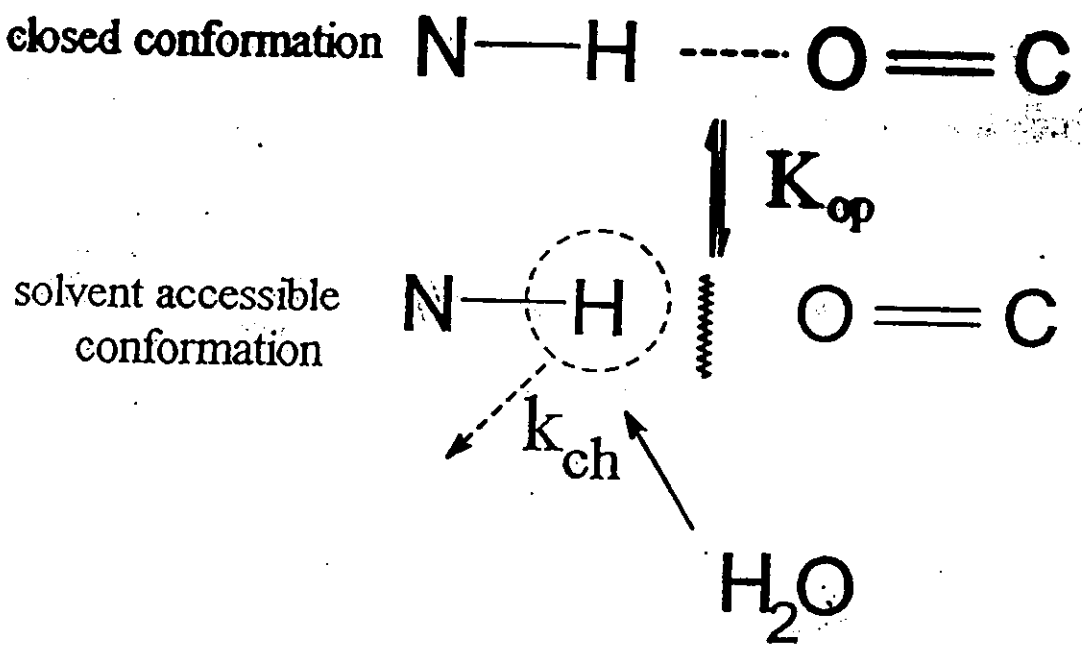
Reduced



Oxidized

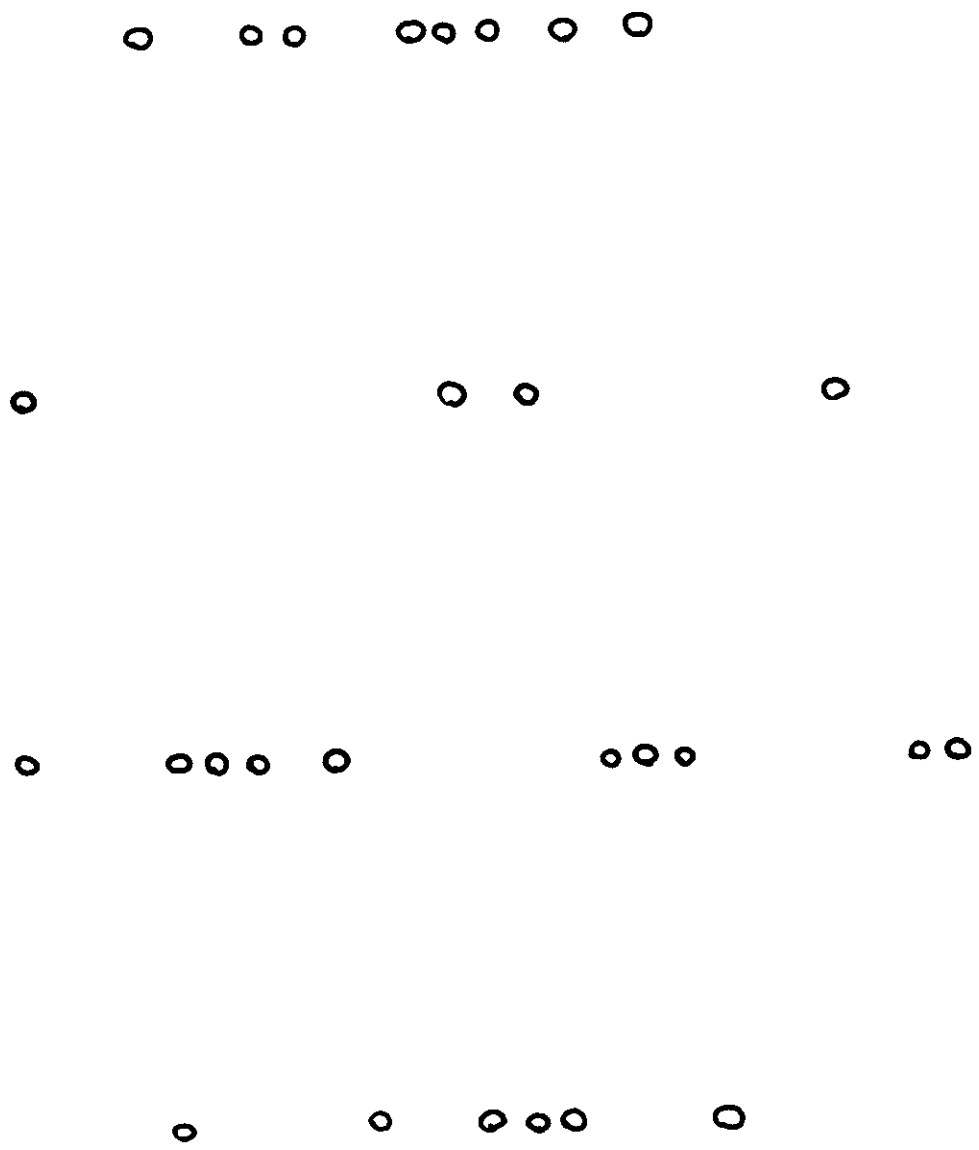


Exchange mechanism for amide protons

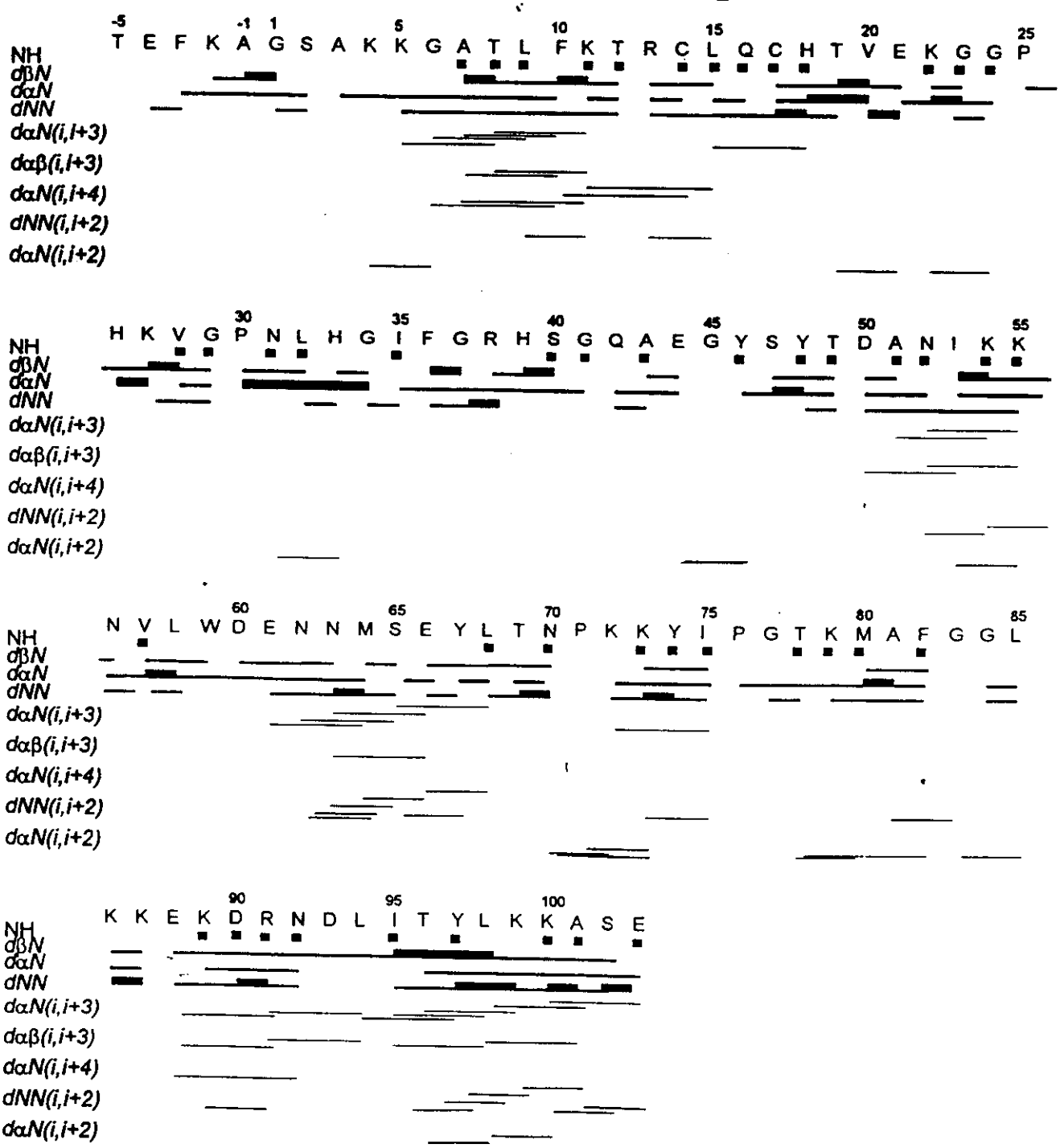


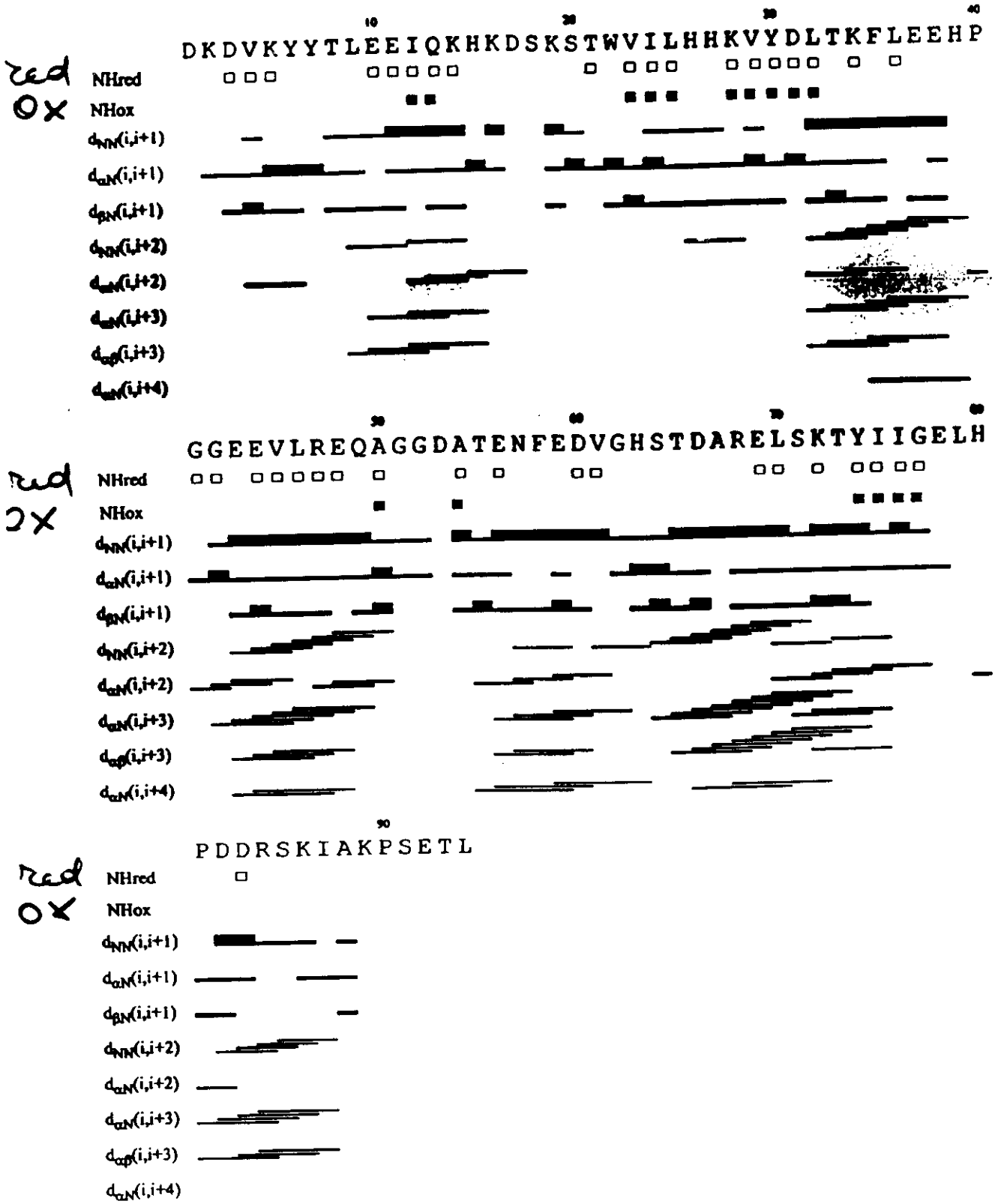
17 amide protons are exchanging slower in the reduced cytochrome *c* than in the oxidized cytochrome *c*

Reference: Bai, Y. et al., *Science* (1995), **269**, 192.



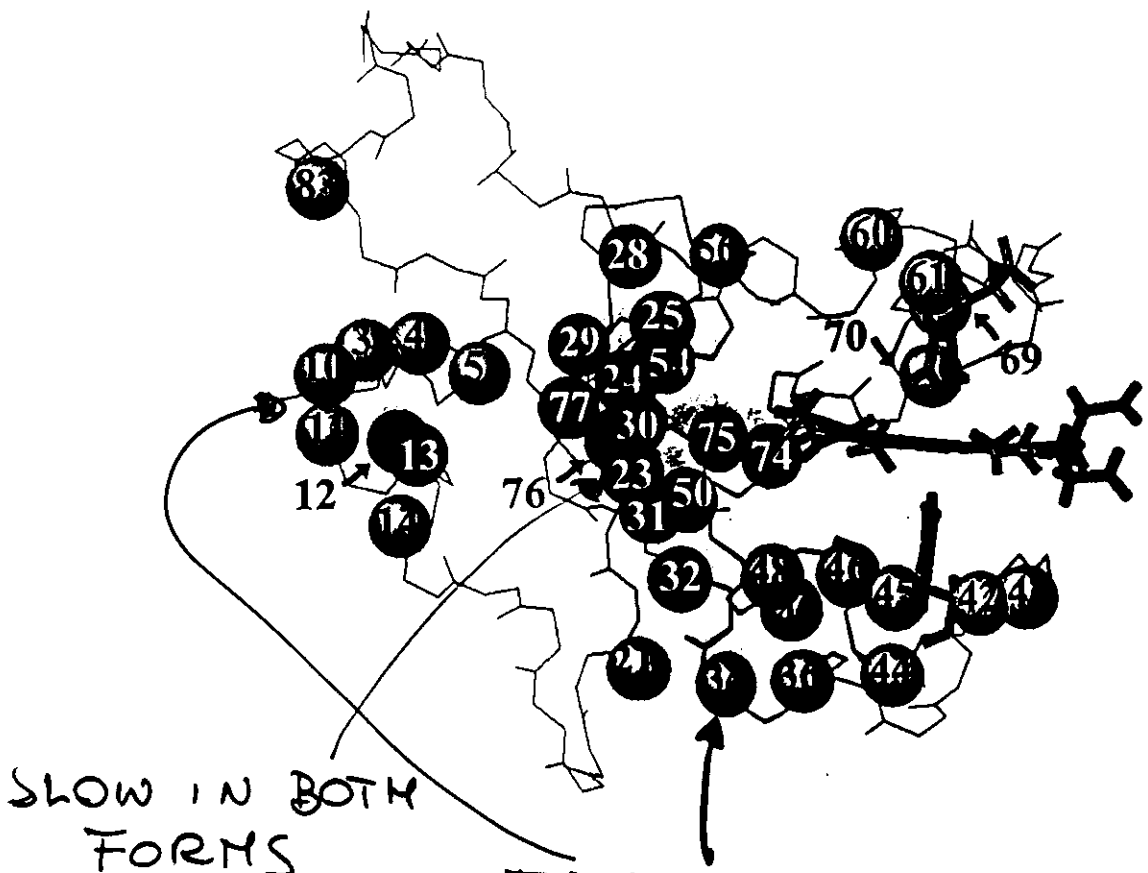
SEQUENCE-SPECIFIC ASSIGNMENT IN ISO-1 YEAST CYT C (REDUCED)





NH EXCHANGEABILITY IN CYT b5

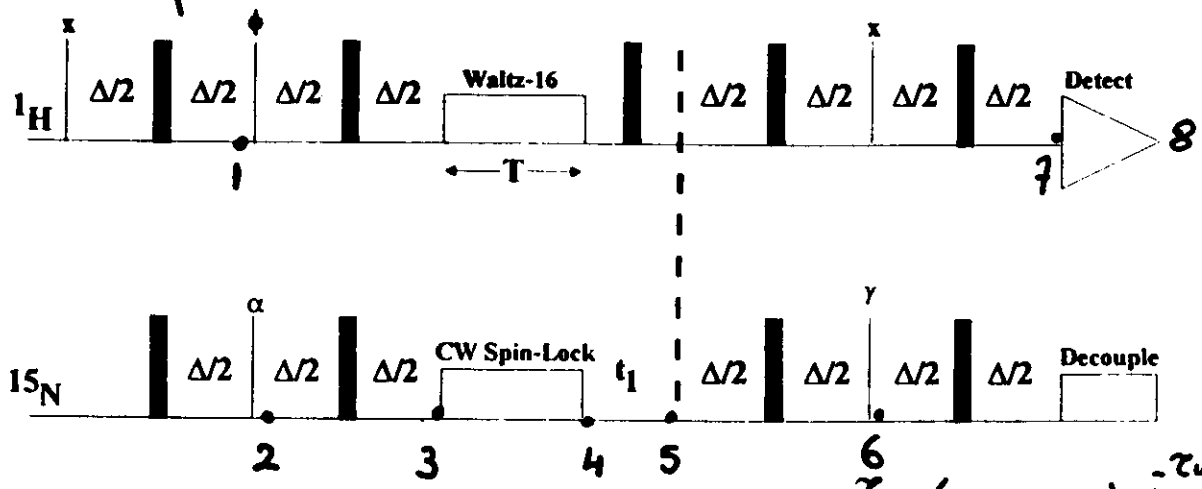
OXIDATION-STATE DEPENDENCE OF NH EXCHANGEABILITY



ARNESANO, BANCI, BERTINI, FELLI BIOCHEMISTRY, in press

PULSE SEQUENCES FOR $T_{1\rho}$ MEASUREMENTS

A $T_{1\rho}$ ON RESONANCE

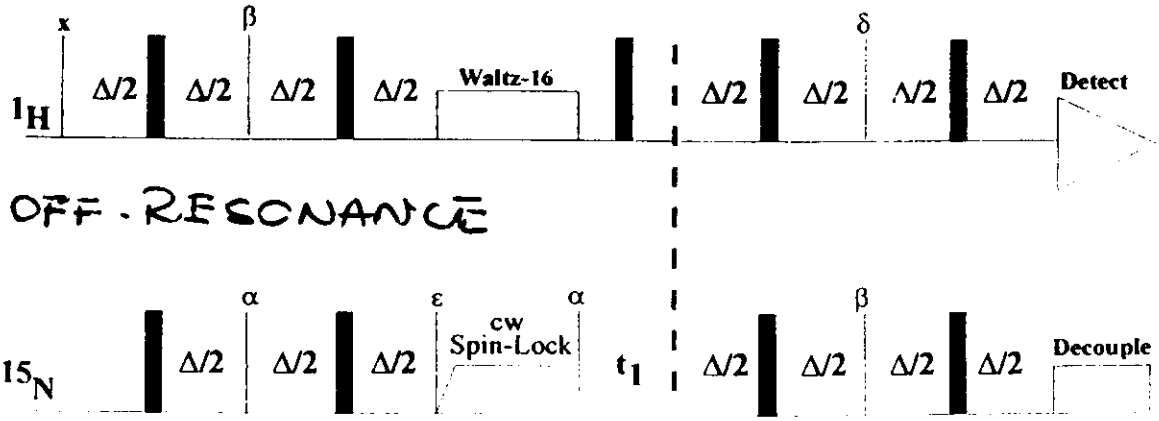


1 $2H_yN_z$; 2, $2H_zN_y$; 3 N_x ; 4 $N_x e^{-\tau_m/T_{1\rho}}$; 5 $N_x e^{-\tau_m/T_{1\rho}} (\cos \omega_N t_1)$

6 $2N_z H_y e^{-\tau_m/T_{1\rho}} \cos(\omega_N t_1)$; 7 $H_x e^{-\tau_m/T_{1\rho}} (\cos \omega_N t_1)$

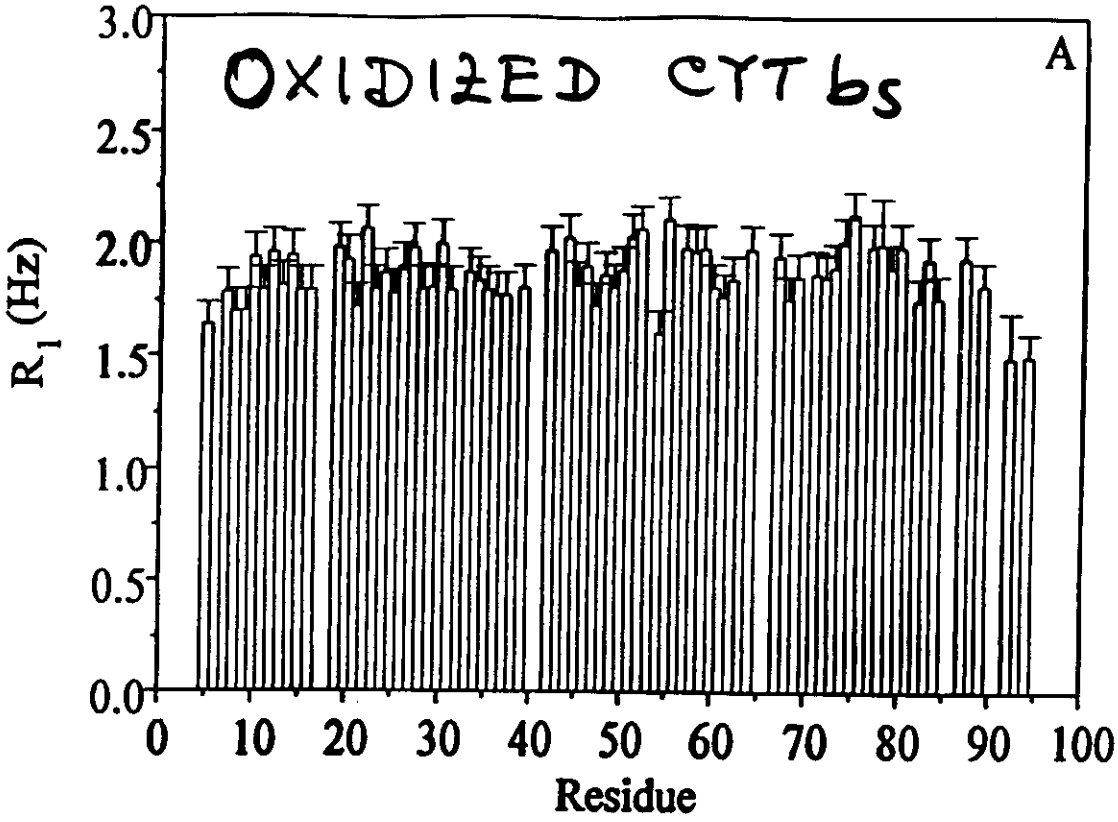
8 $H_x e^{-\tau_m/T_{1\rho}} \cos(\omega_N t_1) (\cos \omega_H t_1)$

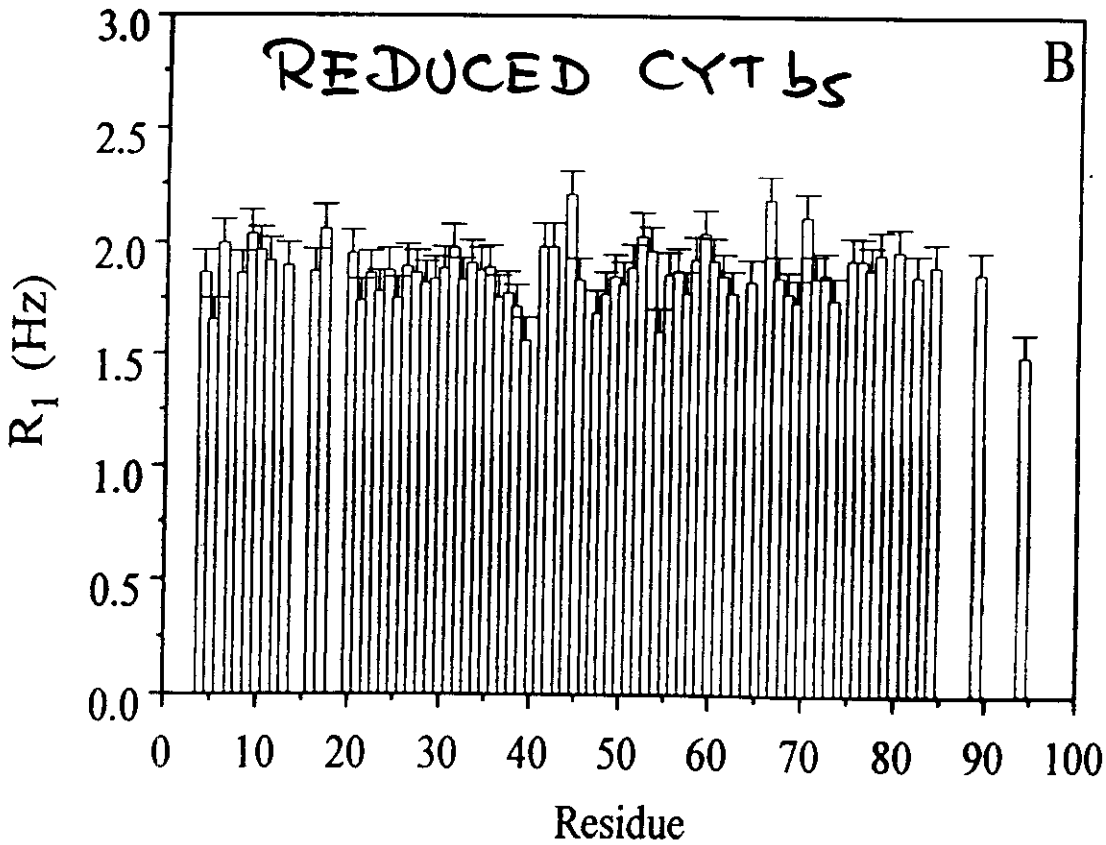
B



$T_{1\rho}$ OFF-RESONANCE

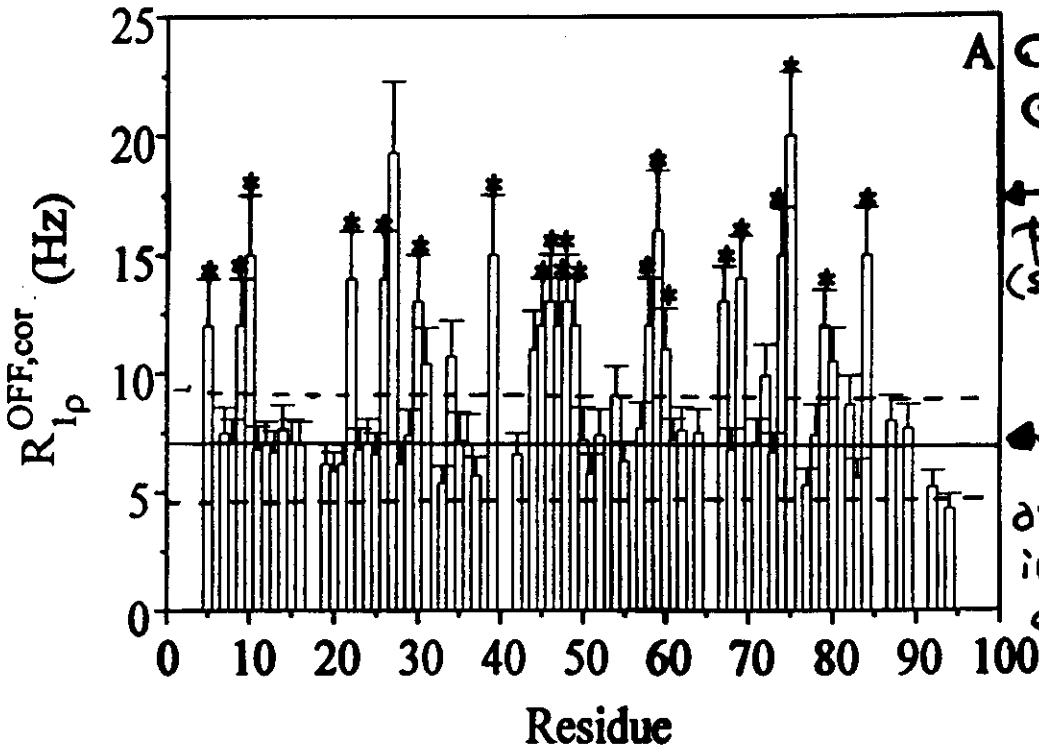
LONGITUDINAL RELAXATION RATES OF BACK BONE NHs





ROTATING FRAME RELAXATION RATES OF BACKBONE NHs

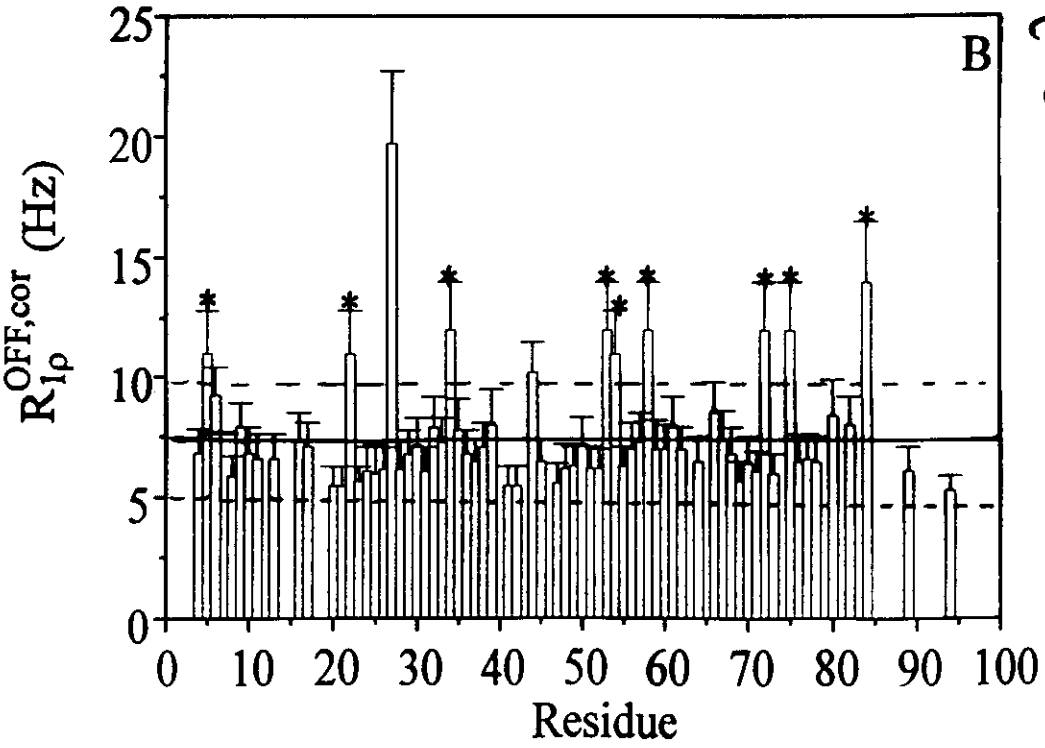
INDICATE PRESENCE OF EXCHANGE PROCESSES



OXIDIZED CYT b5

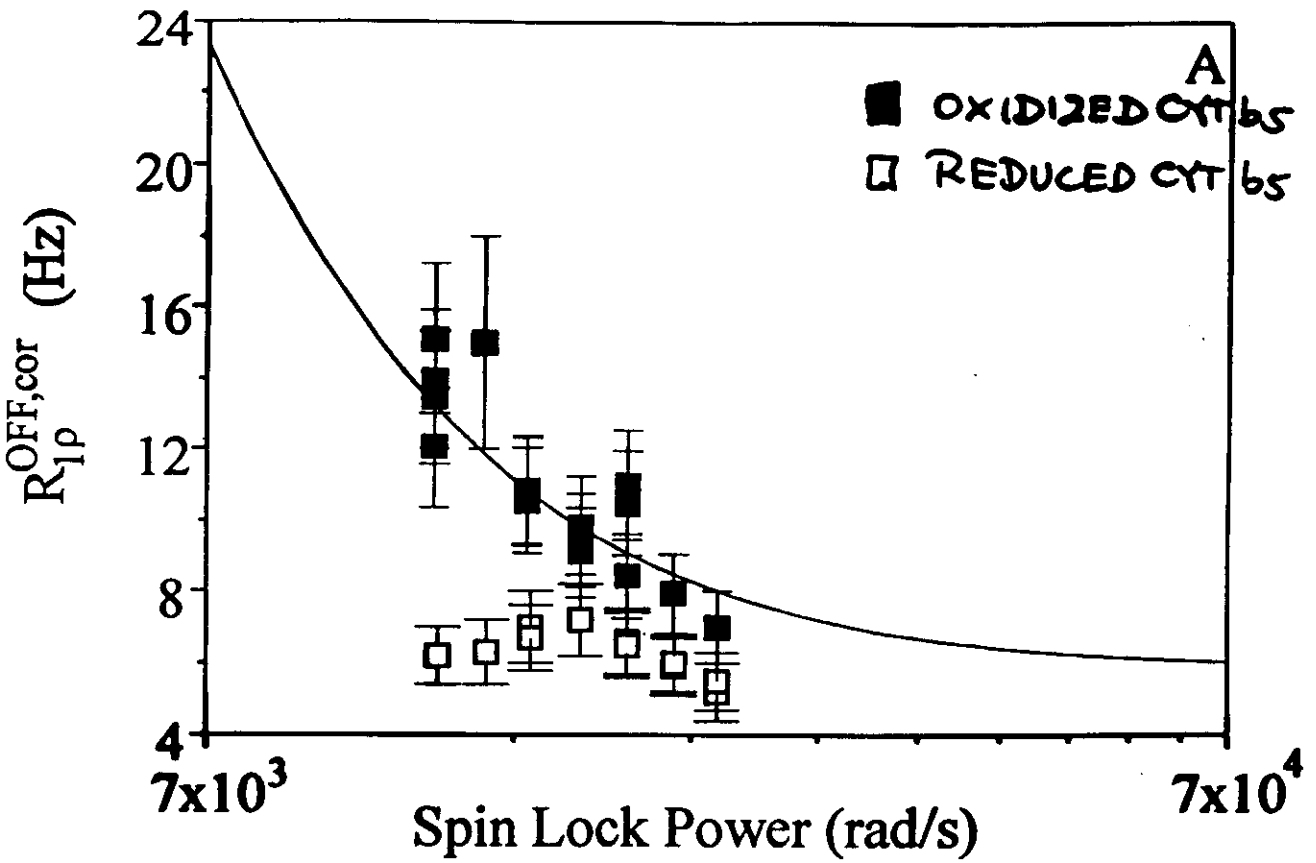
← exchange processes (slow time scale) ($\tau_{ex} > 10^{-5}$ s)

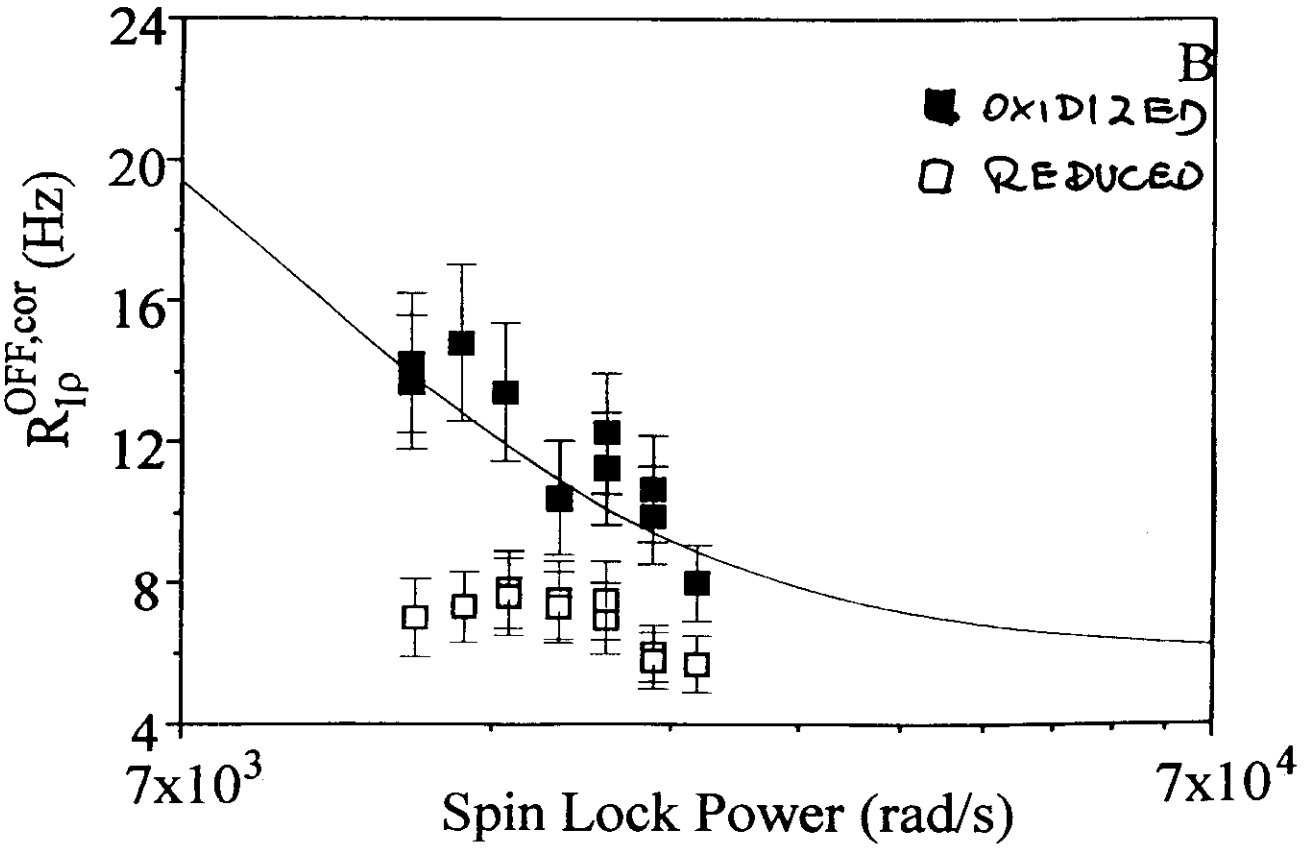
← average $R_{1\rho}$ in absence of exchange phenomena



REDUCED CYT b5

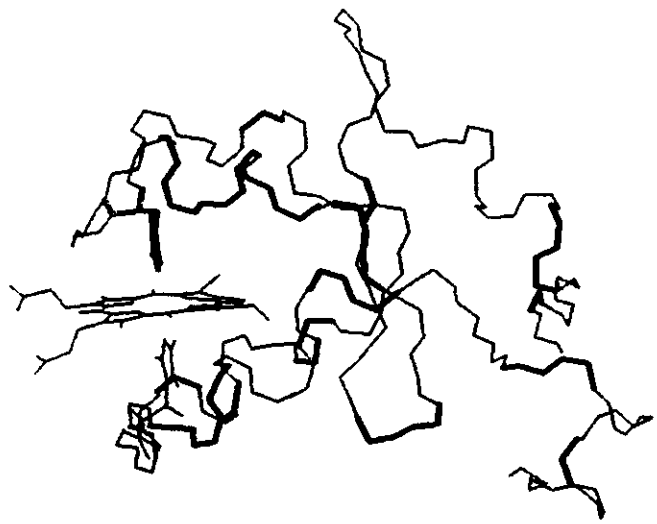
DEPENDENCE ON SPIN-LOCK POWER OF $R_{1\rho}$ RATES



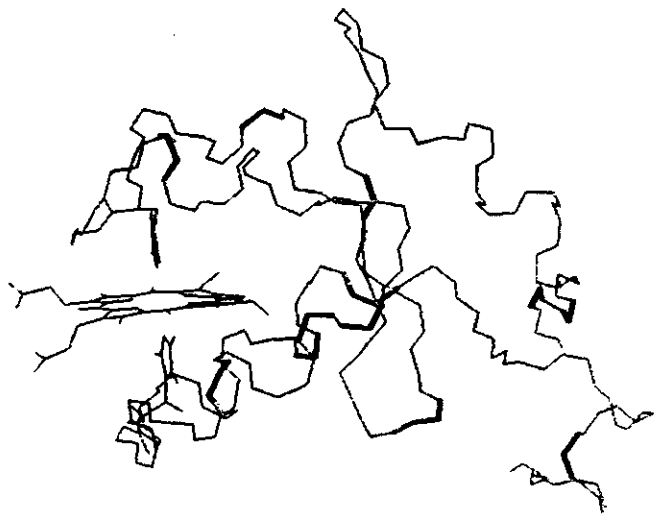


Redox state dependent conformational exchange in rat microsomal cytochrome b_5

Oxidized

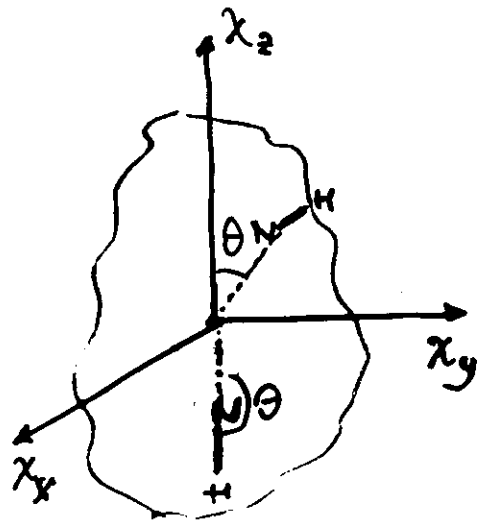


Reduced



RESIDUAL DIPOLAR $^1J_{^{15}N-^1H}$ COUPLING IN FIELD-ORIENTED MOLECULES AS STRUCTURAL CONSTRAINTS

Getting oriented. By aligning proteins (red), liquid crystals (green) sharpen NMR's atomic mapping.



$$^1J_{^{15}N-^1H}(B_0) = {}^1J_{^{15}N-^1H}(0) - K B_0^2 [\Delta\chi_z(3\cos^2\theta - 1) + 3/2\ \Delta\chi_{zh}(\sin^2\theta \cos 2\phi)]$$

OXIDATION-STATE DEPENDENT CHANGES

- REORIENTATION OF PROPIONATE 7
  → POSSIBLE PATHWAY FOR ELECTRON TRANSFER

- DIFFERENT MOBILITY OF SOME PROTEIN PARTS

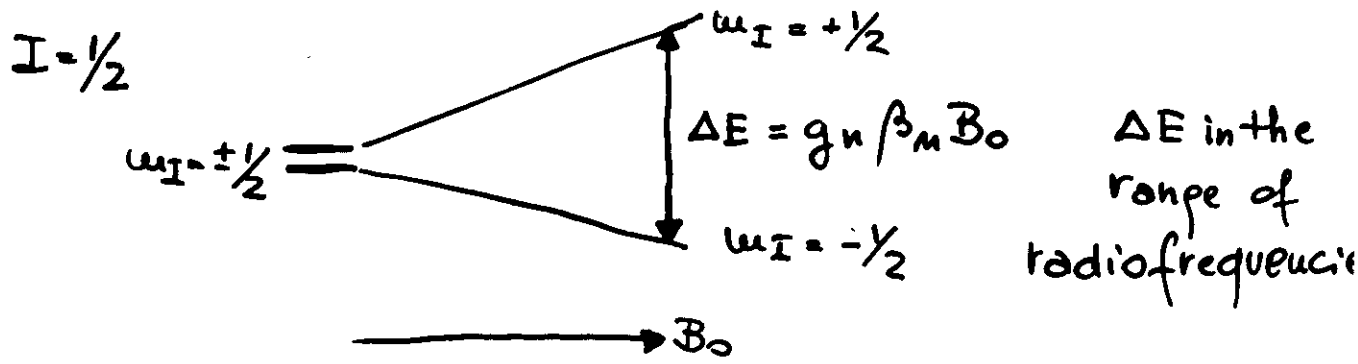
- CHANGE IN CONFORMATION OF SURFACE RESIDUES

  → RELEVANT FOR MOLECULAR RECOGNITION

NUCLEAR MAGNETIC RESONANCE - NMR

IT DEALS WITH NUCLEAR SPINS IN A MAGNETIC FIELD

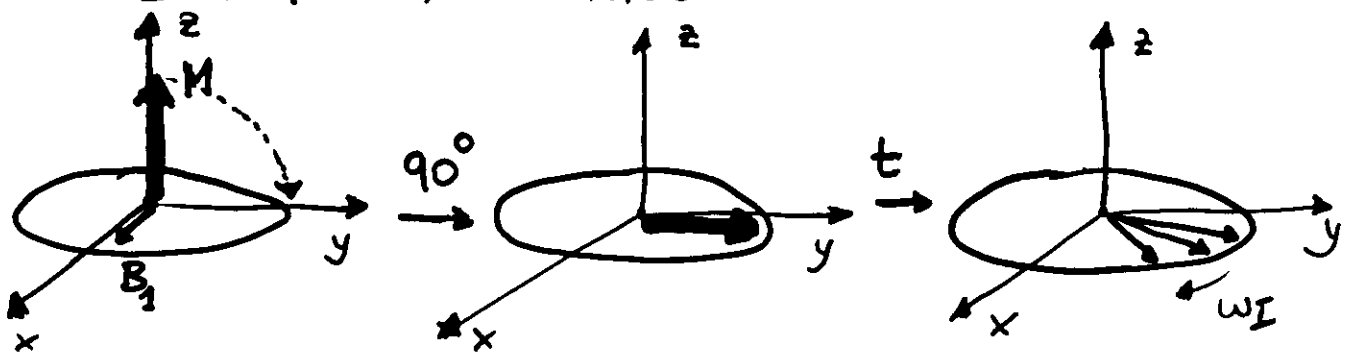
A RADIOFREQUENCY IS APPLIED TO INDUCE THE TRANSITION BETWEEN THE SPIN STATES



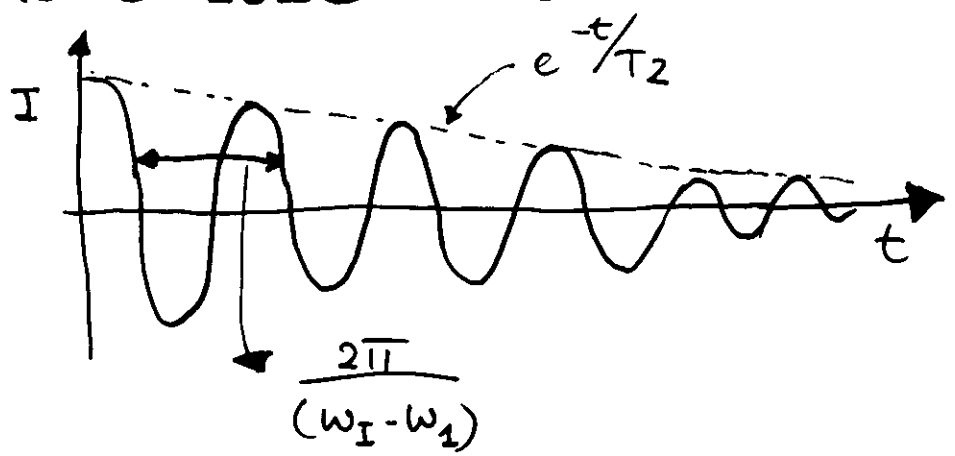
WHEN AN ENSAMBLE OF SPINS IS CONSIDERED THE SPIN MAGNETIC MOMENTS SUM UP TO GIVE A MACROSCOPIC MAGNETIZATION, **M**, ALONG THE z AXIS

THE NMR EXPERIMENT

A SHORT, STRONG RADIOFREQUENCY (B_1ou
PULSE IS APPLIED TO THE MAGNETIZATIO
TO BRING IT IN THE XY PLANE.
AFTER THE PULSE IS SWITCHED OFF,
THE MAGNETIZATION PRECESSES IN
THE XY PLANE AND RELAXES TO EQUILIBRIU



THE CURRENT INDUCED IN A COIL BY THE
MAGNETIZATION PRECESSING IN THE XY
PLANE IS RECORDED.
IT IS CALLED FID

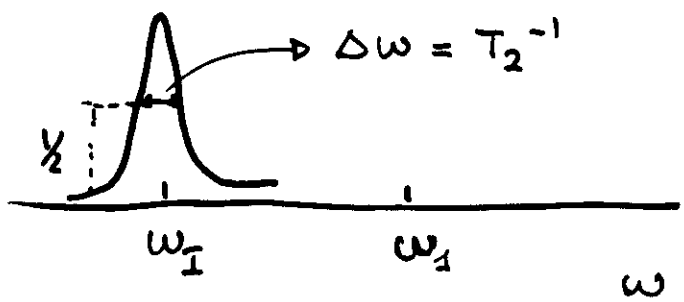


THE NMR SPECTRUM

THE FOURIER TRANSFORM OF THE FID PROVIDES THE NMR SPECTRUM

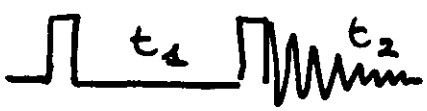
FID $f(t) \propto \left(\cos \omega_1 t + i \sin \cos \omega_1 t\right) e^{-t/T_2}$

SPECTRUM $F(\omega) \propto \dfrac{T_2}{1+(\omega_1-\omega_1)^2 T_2^2}$

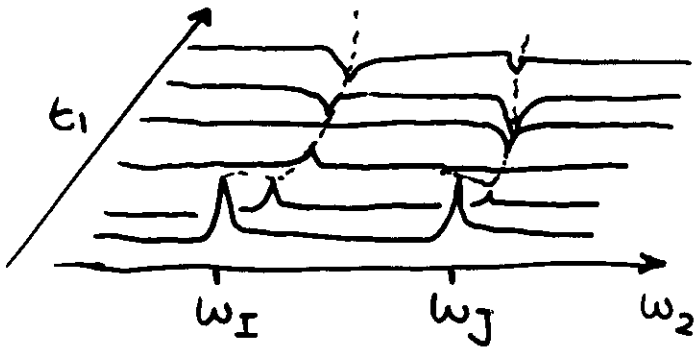


MULTIDIMENSIONAL NMR

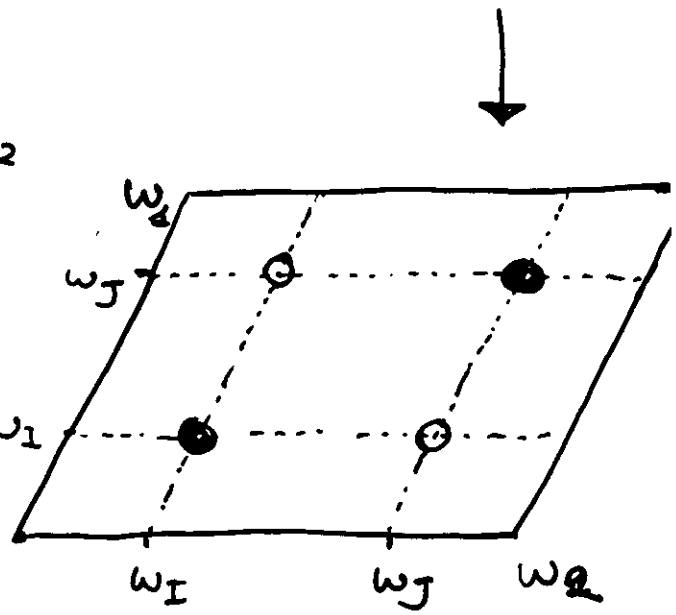
IF A SERIES OF PULSES ARE APPLIED AND MORE THAN ONE TIME INTERVAL IS PRESENT, FOURIER TRANSFORMATION OVER ALL THE VARIOUS TIME DIMENSION PROVIDE MULTIPLE FREQUENCY DIMENSIONS



IF A SERIES OF FIDS (all with the same t_2 range) ARE MEASURED WITH DIFFERENT t_1 VALUES, FT ALONG t_2 PROVIDES



FT ALONG t_1

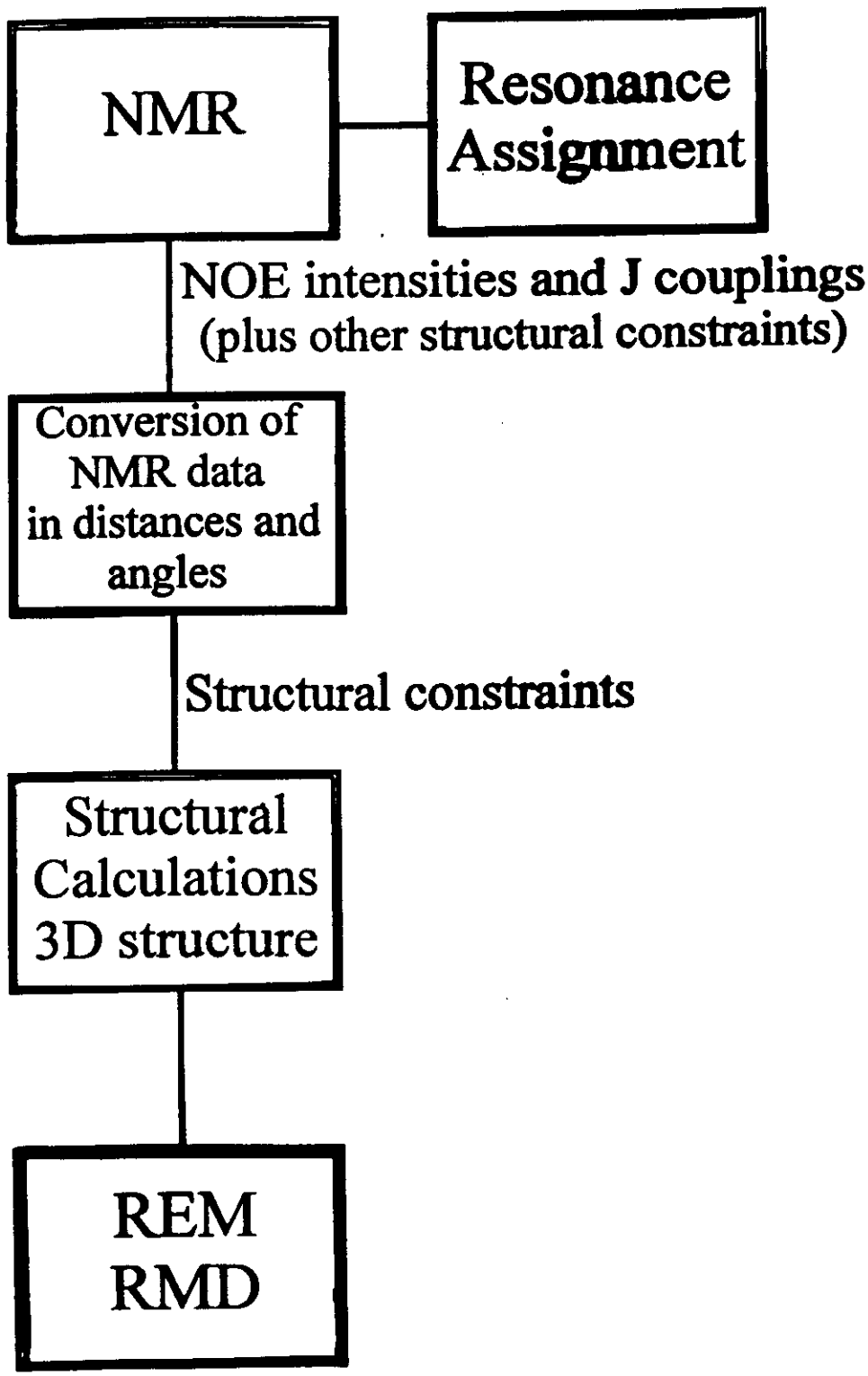


PEAKS AT FREQUENCIES $\omega_2=\omega_I$ AND $\omega_1=\omega_J$ (PLUS the symmetric) ARE PRESENT WHEN COUPLING BETWEEN THE TWO SPINS IS OPERATIVE

NMR ON PROTEINS

- NEED OF HIGHER AND HIGHER MAGNETIC FIELS
  - * INCREASED RESOLUTION
  - * INCREASED SENSITIVITY
- RELATIVELY HIGH CONC. SOLUTIONS
  - ($\leq$ 2 mM x STRUCTURAL STUDIES)
  - ($\geq$ 2 mM x STUDIES WHERE RESOLUTION IS NOT CRITICAL)

STRUCTURE DETERMINATION THROUGH NMR



ASSIGNMENT STRATEGY

1. IDENTIFICATION OF THE SPIN SYSTEMS

the scalar connectivities (through chemical bonds) are analyzed to assign the spin systems of the aminoacidic residues.

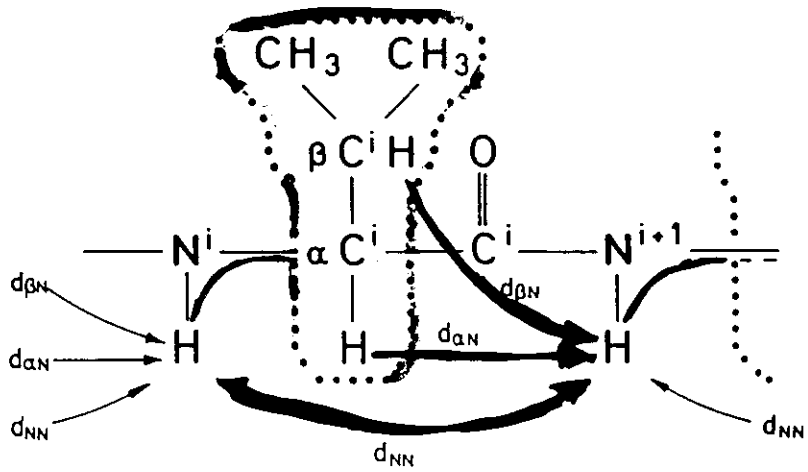
- scalar connectivities of geminal and vicinal protons with 2D COSY
- long range scalar connectivities (more than 3 bonds) with 2TOCSY

FOR LARGER PROTEINS NEED OF HETCOR AND 3D-HETCOR EXP

2. IDENTIFICATION OF DIPOLAR CONNECTIVITIES

the dipolar connectivities (through space) are analyzed in NOESY spectra to identify protons close each other.

- sequencial assignment: Hα(i)-NH(i+1) e HN(i)-NH(i+1)
- long range connectivities and secondary structure (α elices)
- assegnment of all NOESY cross-peaks



CLASSICAL STRUCTURAL CONSTRAINTS

- Distance constraints

NOESY volumes are proportional to the sixth power of the interproton distance and to the correlation time for the dipolar coupling

$$V \propto (K/r^6) \cdot \tau_c$$

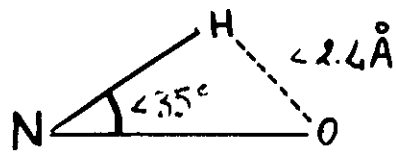
- Dihedral angle constraints

Derived from the ^3J coupling through the Karplus equation

$$^3J(H_\alpha\text{-}N) = A \cos^2(\psi + 60°) + B \cos(\psi + 60°) + C$$

- Hydrogen bonds

Identification of slow exchanging protons and of the presence of H-bonds



STRUCTURAL CALCULATIONS THROUGH TORSION ANGLE DYNAMICS

- USE LAGRANGE EQUATION OF MOTIONS
- INTERNAL COORDINATES ARE CHANGED FOR RIGID BLOCKS (COVALENT STRUCTUR IS KEPT FIXED)

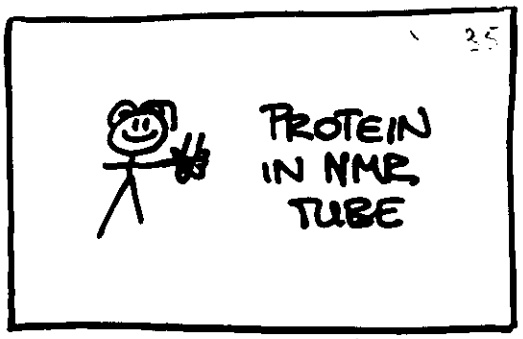
  ⇓

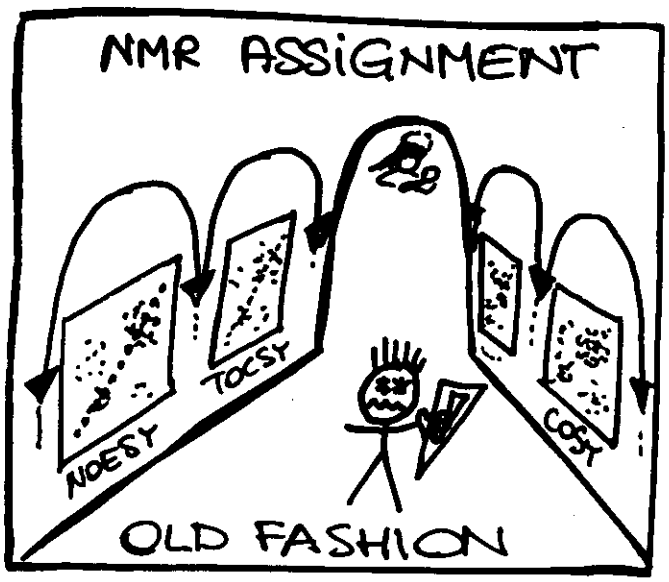
  1/10 DEGREES OF FREEDOM WITH RESPECT TO DYNAMICS IN CARTESIAN COORDINATES

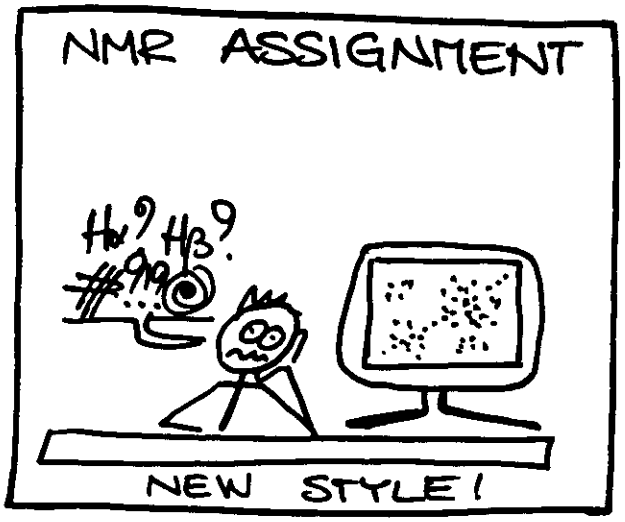
- NO NEED FOR STRONG POTENTIALS TO CONVERGE TO THE FOLDED STRUCTUR

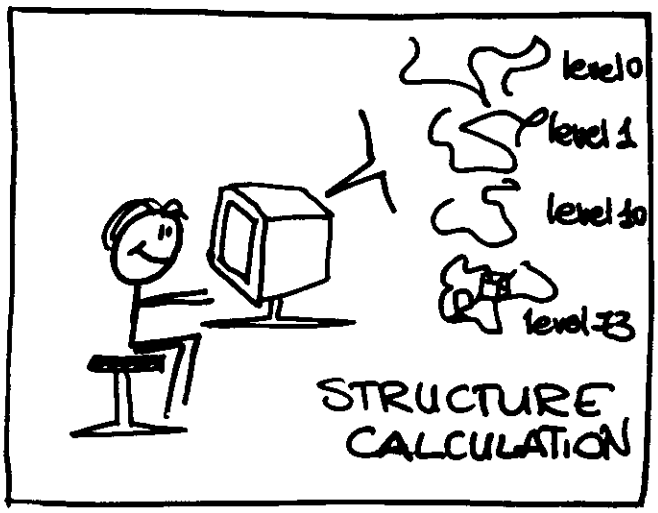
PROTEIN STRUCTURE DETERMINATION

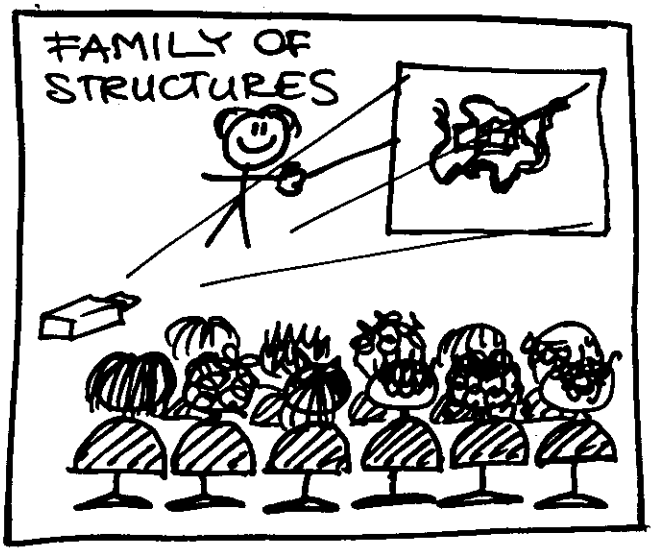
NMR lab, University of Florence





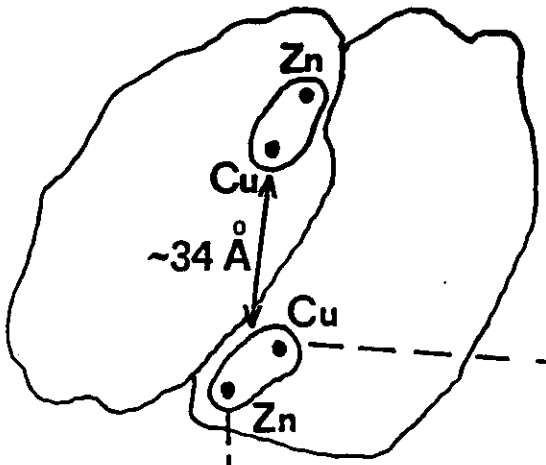






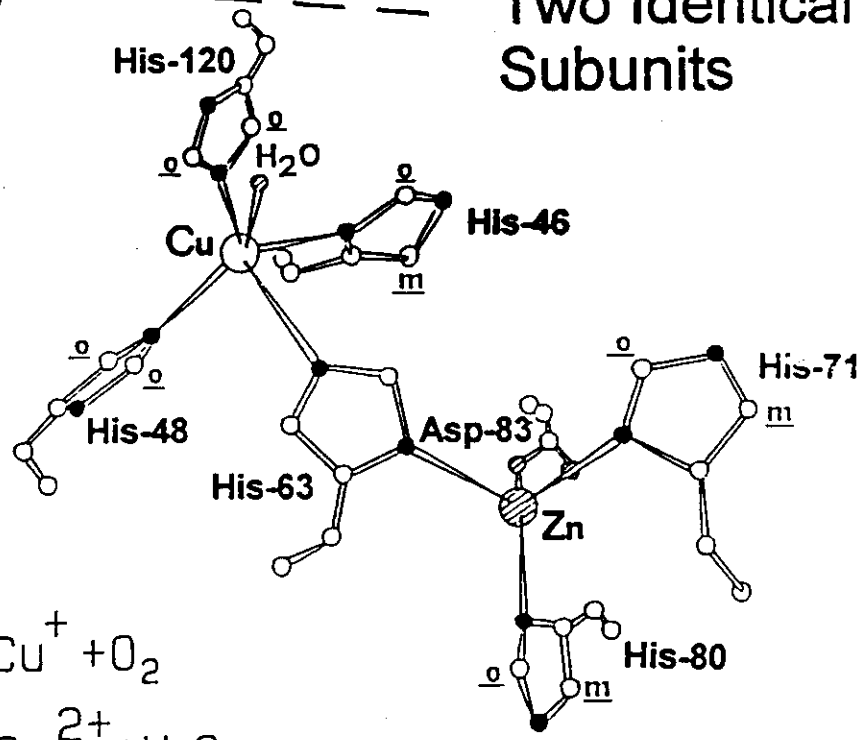
B4

SUPEROXIDE DISMUTASE



Molecular Weight
31,200

Two Identical
Subunits

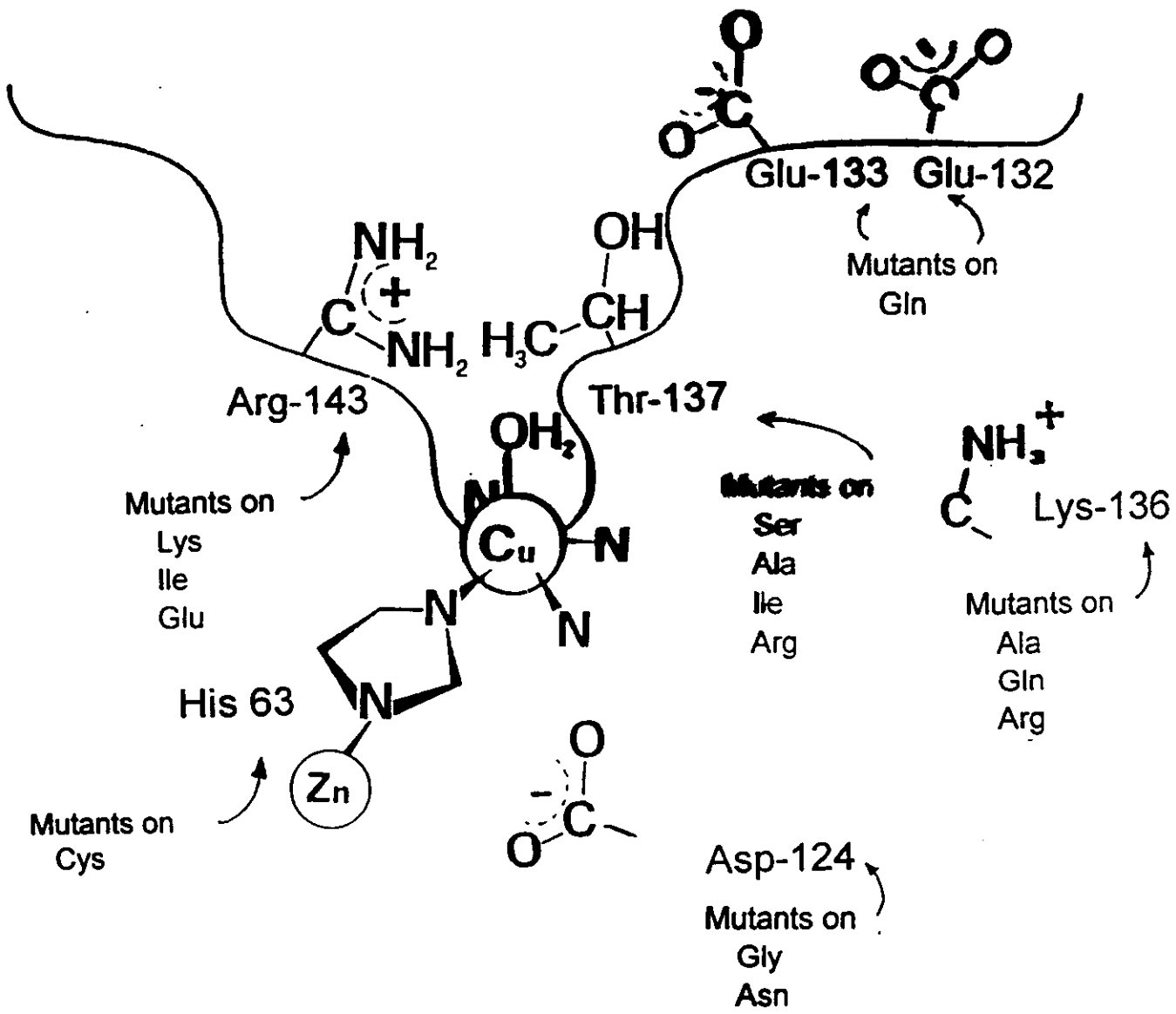


$$Cu^{2+} + O_2^- \longrightarrow Cu^{+} + O_2$$

$$Cu^{+} + O_2^- + 2H^{+} \longrightarrow Cu^{2+} + H_2O_2$$

$K_{obs} \sim 2\text{-}3 \times 10^9\ M^{-1} s^{-1}$

THE CAVITY OF SOD



Beyond the Diffusion Limit

Increased activity in SOD

	Activity (%)	Affinity for N_3^- (%)
SOD WT	100	100
SOD Gln132	191	**141**
SOD Gln133	222	**387**
SOD Gln132, Gln133	230	**561**

Getzoff, E. D., Cabelli, D. E., **Fisher,** C. L., Parge, **H. E., Viezzoli,** M. S., Banci, L., Hallewell, R. A., **Nature,** 358:347-351, **1992**

Getzoff, E. D., Cabelli, D. E., **Viezzoli,** M. S., Banci, **L., Hallewell,** R. A., Journal of Inorganic Biochemistry, 50, 89-100, 1993

The subunit-subunit interface in dimeric SOD

Tainer, Getzoff, Beem, Richardson, Richardson , J. Mol. Biol. 1982.

The obtainement of a monomeric SOD

Substitution	at Phe 50 -- **Glu 50** at Gly 51 -- **Glu 51**
Activity	10% than that of WT SOD
Affinity for anions	8% than that of WT SOD

Some small rearrangements occur at the copper site.

Bertini, Piccioli, Viezzoli, Chiu,
Mullenbach, Eur. J. Biophysics, 1994

The SOD mutants on the Glu 133 residue

	Activity (%)	Affinity for anions (%)
Dimeric SOD		
Glu 133 SOD	100	100
Gln 133 SOD	220	390
Monomeric SOD		
Glu 133 SOD	10	8
Gln 133 SOD	20	30

Getzoff, Cabelli, Fisher, Parge, Viezzoli, Banci, Hallewell, Nature, 1992

Banci, Bertini, Mullenbach, Viezzoli, Eur. J. Biochem., 1995

Solution Structure Determination of Reduced Monomeric E133Q Copper Zinc Superoxide Dismutase

Assignment:

^{13}C, ^{15}N sample, 3D triple resonance experiments at 800 MHz

151 out of 153 aminoacids assigned, 93% assigned protons

Solution Structure Determination of Reduced Monomeric E133Q Copper Zinc Superoxide Dismutase

Cofactor:		Cu(I), Zn(II)
AA:		153
NOE's		2550 (2300)
Dihedral angles		^{3}J HN-Hα 65
constraints:		
H-bonds:		16
	BB-	0.81 ± 0.13
RMSD/36 str, (Å)		
	HA-	1.23 ± 0.14

Refinement procedure: the calculations are in progress.

Reference: L. Banci, M. Benedetto, I. Bertini, R. Del Conte, M. Piccioli, M.S. Viezzoli, submitted

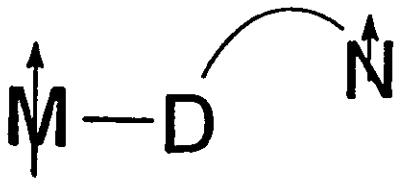
NMR OF PARAMAGNETIC MOLECULES

Coupling between the resonating nucleus and the unpaired electrons produces large effects on NMR parameters of the nuclei:

- Chemical shifts
- Relaxation rates

The interaction can be through the chemical bonds (contact) or through space (dipolar)

The electron-nucleus coupling



$$H = \hat{\underline{I}} \cdot \underline{\underline{A}} \cdot \hat{\underline{S}} = a \cdot \hat{\underline{I}} \cdot \hat{\underline{S}} + \hat{\underline{I}} \cdot \underline{\underline{B}} \cdot \hat{\underline{S}}$$

$a \cdot \hat{\underline{I}} \cdot \hat{\underline{S}}$
CONTACT

$\hat{\underline{I}} \cdot \underline{\underline{B}} \cdot \hat{\underline{S}}$
DIPOLAR (ENDOR)

for rotation faster than $\approx 10^5\ s^{-1}$

$b \cdot \hat{\underline{I}} \cdot \hat{\underline{S}}$
PSEUDOCONTACT (in solution)

The Effect of Paramagnetism on NMR Parameters

1) Hyperfine shifts - Contact ($\langle S_z \rangle$, A_c)

- Pseudocontact ($1/r^3$, $\Delta\chi$, θ, φ)

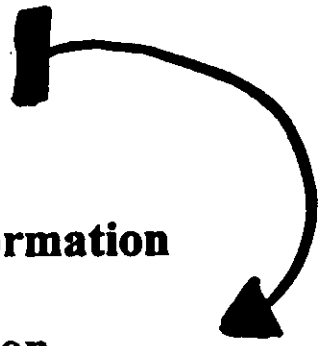
2) Relaxation rates - Dipolar ($1/r^6$, τ_c)
Contact (A_c^2, τ_e)
Curie ($1/r^6$, $[S(S+1)]^2$, τ_r, B^2)

T_1, T_2 shortening

ADVERSE EFFECTS ON DETECTABILITY OF SIGNALS .....BUT.....

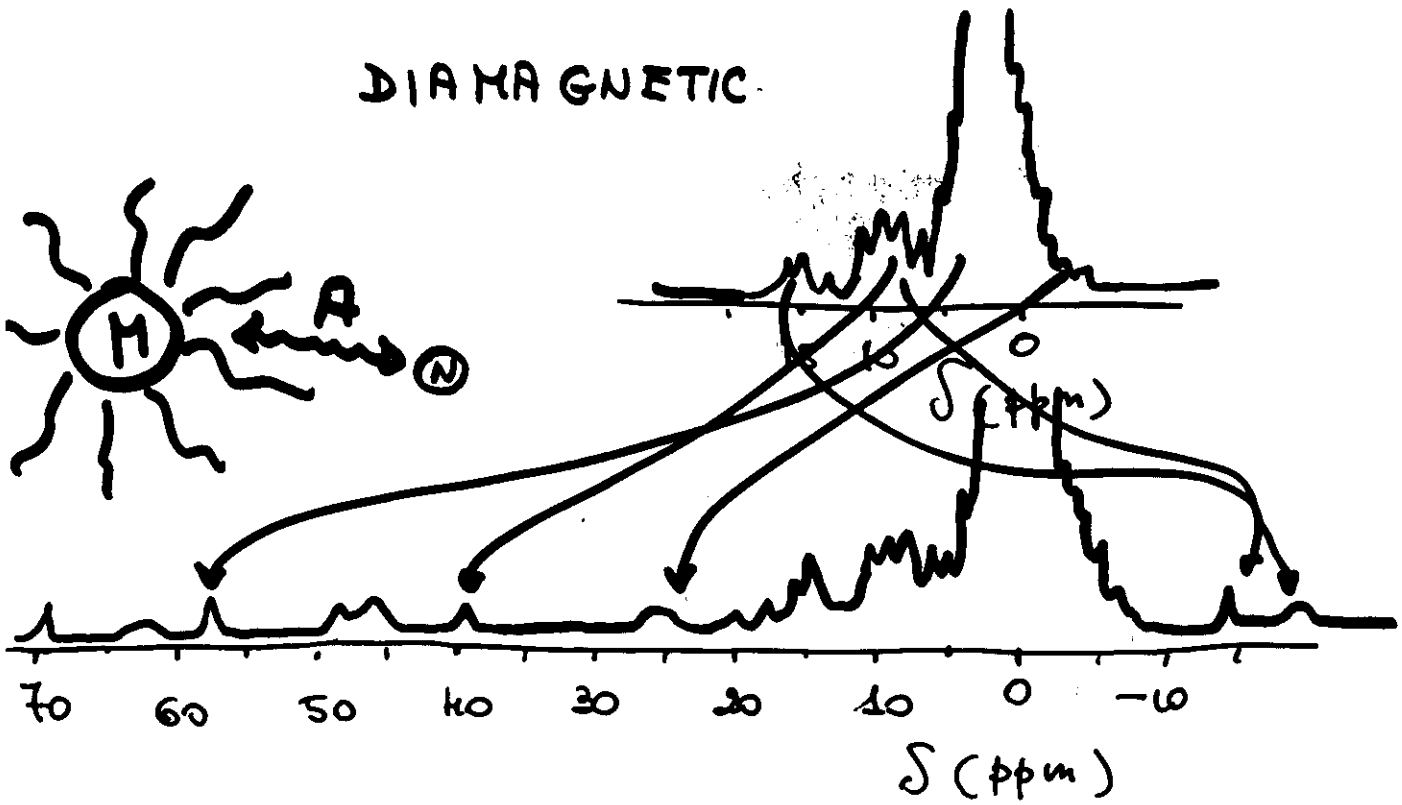
ALSO FAVORABLE EFFECTS:

T_1, T_2 shortening
Hyperfine shifts



Structural and dynamic information
electronic information
bulk susceptibility information

THE ISOTROPIC SHIFT



$$\left(\frac{\Delta\nu}{\nu}\right)_H \propto A \langle S_z \rangle$$

CONTACT SHIFT

$$\frac{-\Delta B}{B_0} = \frac{\Delta \nu}{\nu_0} = -\frac{A_C}{\hbar \gamma_N B_0}\langle S_z \rangle = \frac{A_C}{\hbar}\ \frac{g\,\mu_B S(S+1)}{3\gamma_N kT}$$

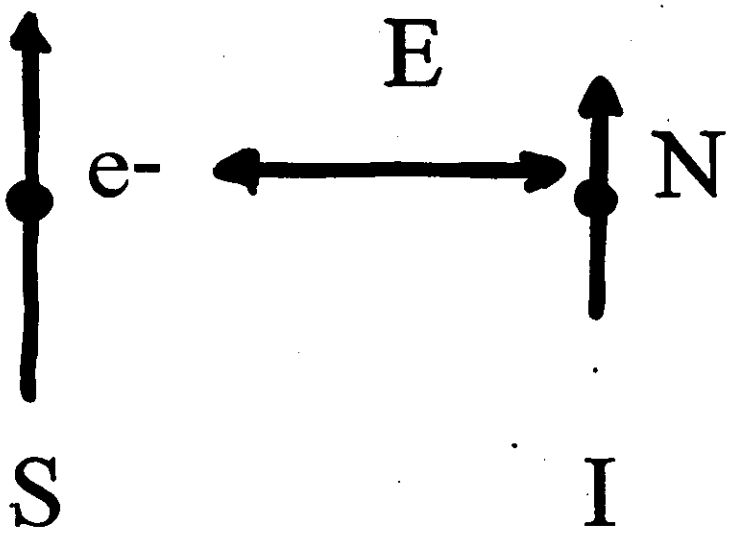
PSEUDOCONTACT SHIFT

$$\frac{-\Delta B}{B_0} = \left(\frac{\Delta \nu}{\nu_0}\right) = \frac{\mu_0}{4\pi}\frac{\mu_B^2 S(S+1)}{18kTr^3}\{[2g_{zz}^2 - (g_{xx}^2 + g_{yy}^2)](3\cos^2\theta - 1) + 3(g_{xx}^2 - g_{yy}^2)\sin^2\theta\cos 2\Omega\}$$

$$\frac{-\Delta B}{B_0} = \left(\frac{\Delta \nu}{\nu_0}\right) = \frac{1}{4\pi 2r^3}\left[(3\cos^2\theta - 1)\left(\frac{2}{3}\chi_{zz} - \frac{1}{3}\chi_{xx} - \frac{1}{3}\chi_{yy}\right) + \sin^2\theta\cos 2\Omega\,(\chi_{xx} - \chi_{yy})\right]$$

NUCLEAR RELAXATION

CAUSED BY THE ELECTRON



T_{1e} 10^{-13}–10^{-7} s

T_{1N} 10^{-2}–10^{2} s

$$T_{1,2}^{-1} \propto E^2 \, f(\tau_C, \omega)$$

In proteins $\tau_c = \tau_s = T_{1e}$

Paramagnetic Relaxation

Dipolar Relaxation

$$R_2^{dip} = \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left(4\tau_c + \frac{\tau_c}{1+(\omega_I-\omega_S)^2\tau_c^2} + \right.$$

$$\left. + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_I+\omega_S)^2\tau_c^2} + \frac{6\tau_c}{1+\omega_S^2\tau_c^2}\right)$$

$$\tau_c^{-1} = \tau_S^{-1} + \tau_R^{-1} + \tau_M^{-1}$$

Contact Relaxation

$$R_2^{cont} = \frac{1}{3}\left(\frac{a_c}{\hbar}\right)^2 S(S+1)\left(\frac{\tau_c}{1+(\omega_I-\omega_S)^2\tau_c^2} + \tau_c\right)$$

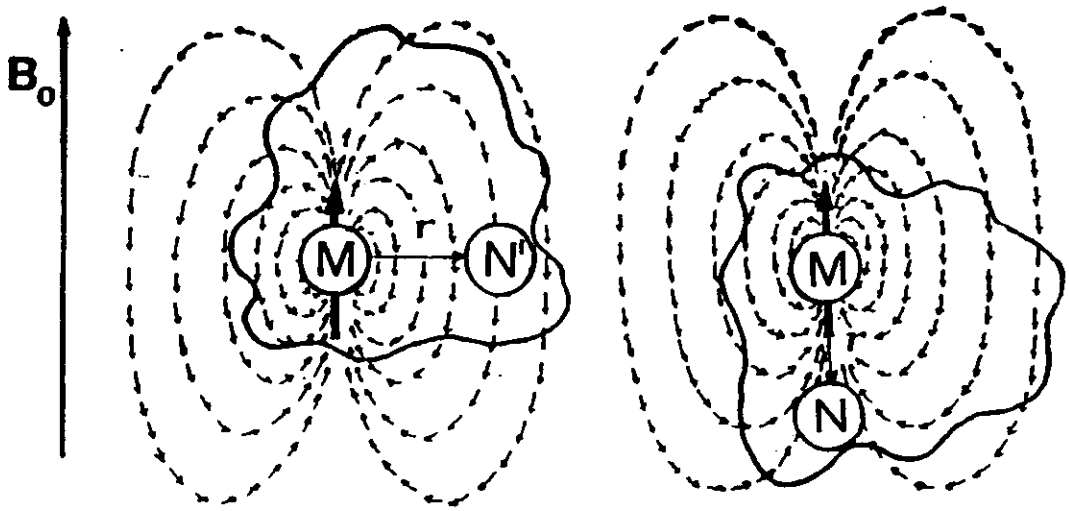
$$\tau_c^{-1} = \tau_S^{-1} + \tau_M^{-1}$$

CURIE RELAXATION

$$T_{2M}^{-1} = \frac{1}{5}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{1}{r^6} \frac{\gamma_I^2 B_0^2 \mu_{eff}^4}{(3kT)^2} \cdot 4\tau_r$$

The effect is sizeable when $\tau_r >> \tau_s$ and γ_I is large

$$\mu_{eff} \propto \langle S_Z \rangle^2$$



NMR of Paramagnetic Metalloproteins

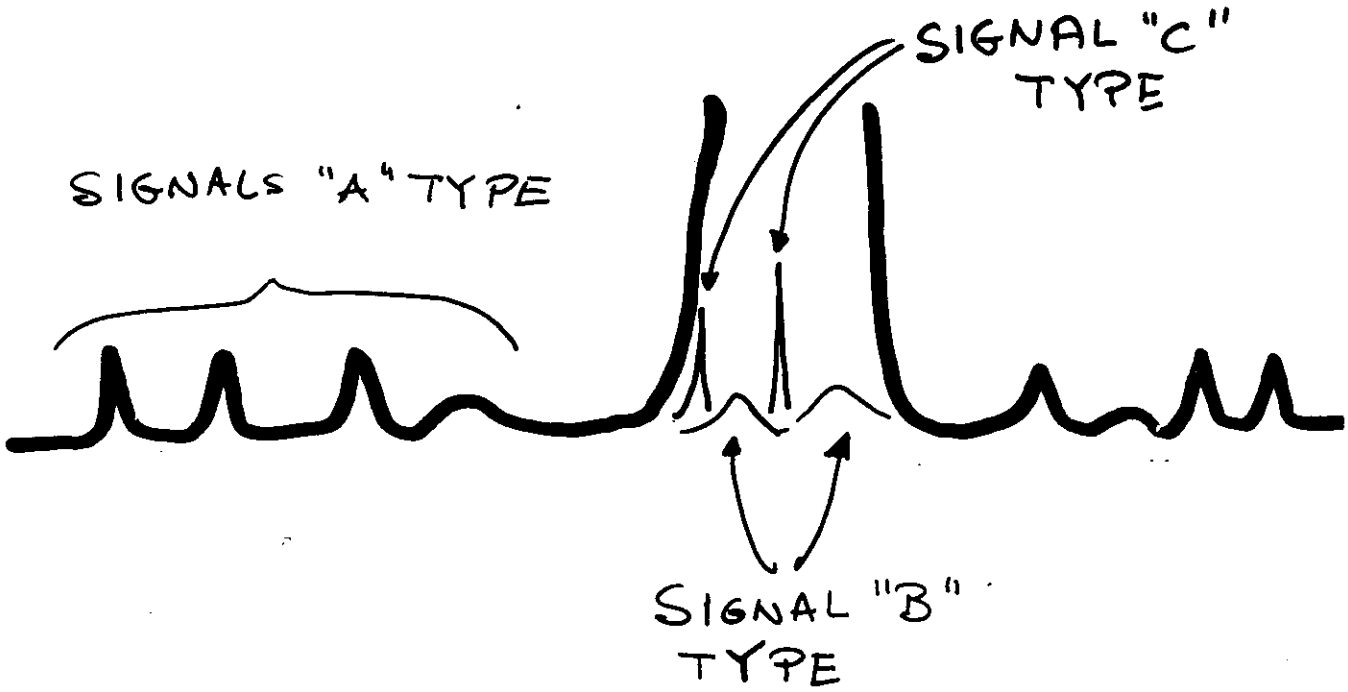
Three types of signals:

A) Signals hyperfine shifted and hyperfine broadened

B) Signals hyperfine broadened but not hyperfine shifted

C) Signals essentially diamagnetic

Signals of type B are hidden in the diamagnetic region of the spectrum, and are most difficult to detect and to assign



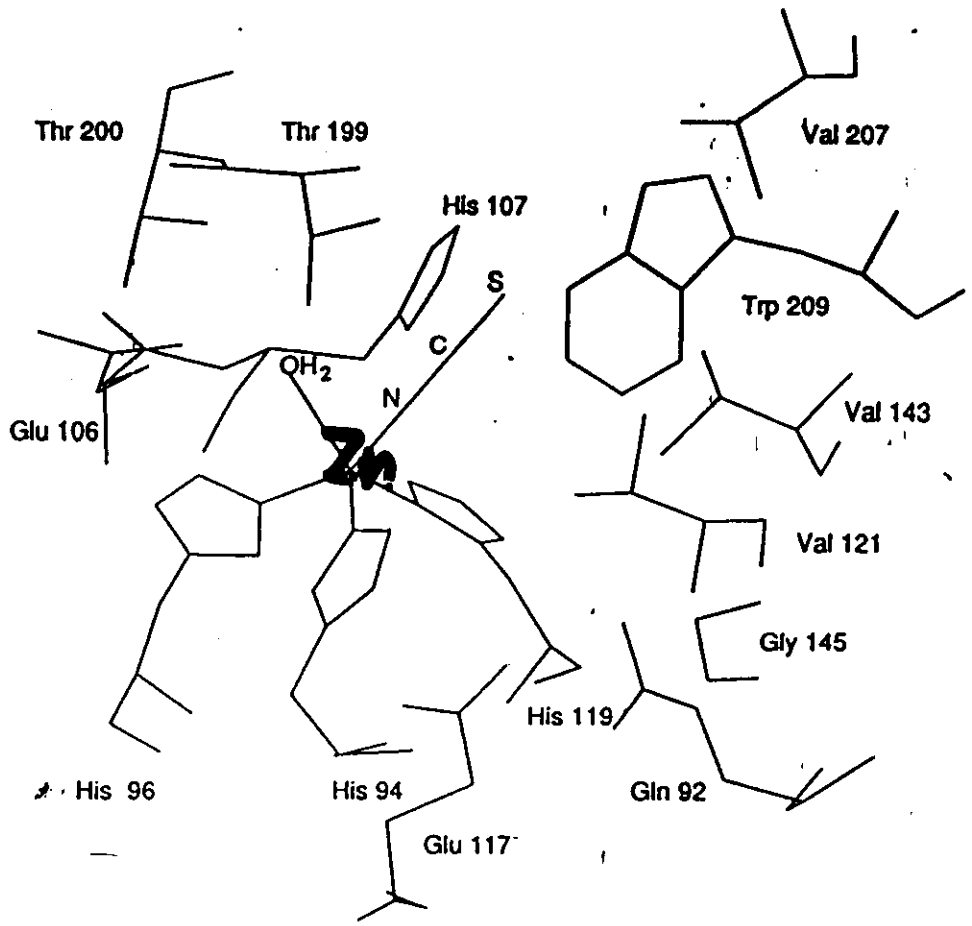
CARBONIC ANHYDRASE

NATIVE FORM CONTAINS ZINC

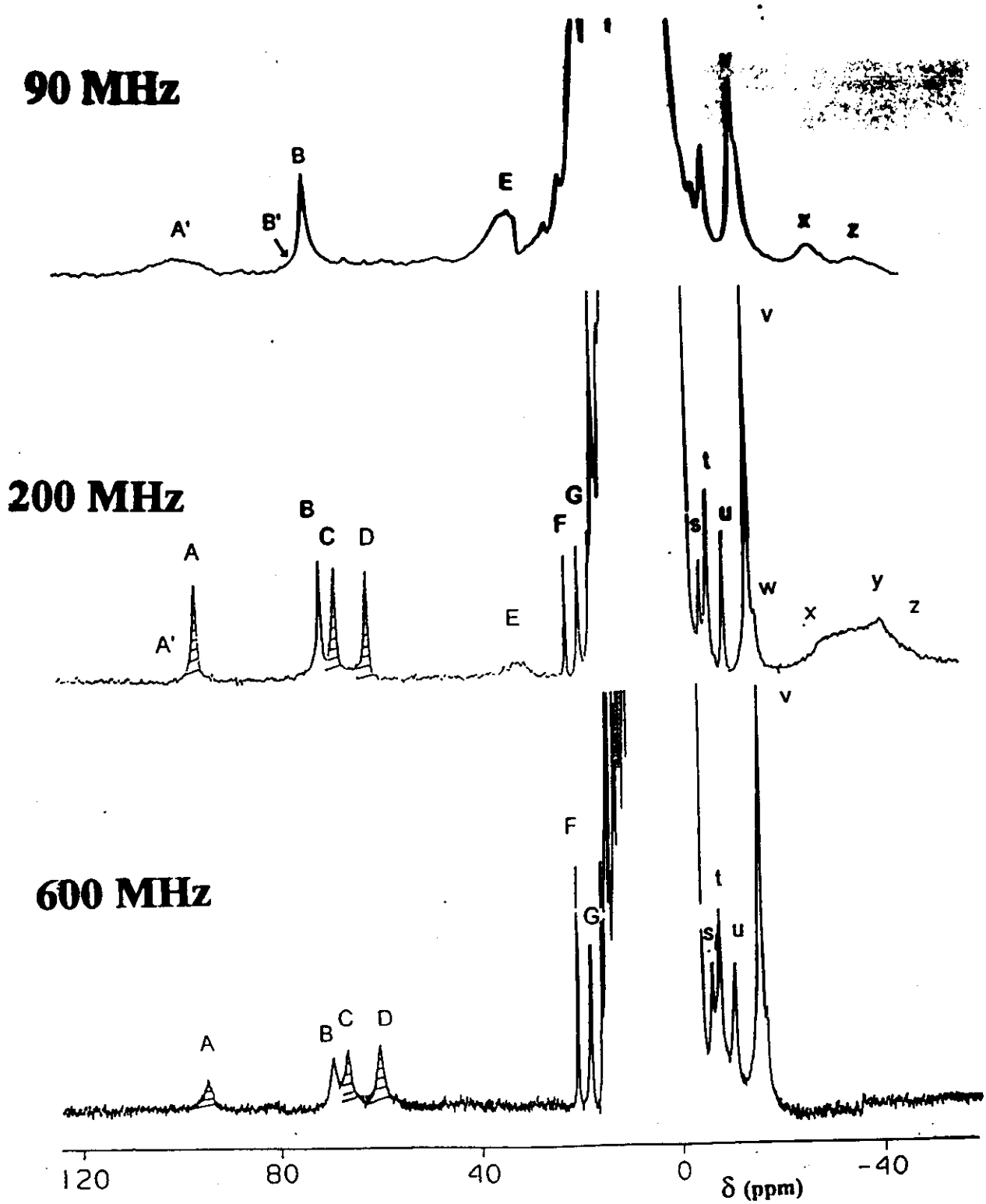
MW 30,000

cobalt(II) high spin

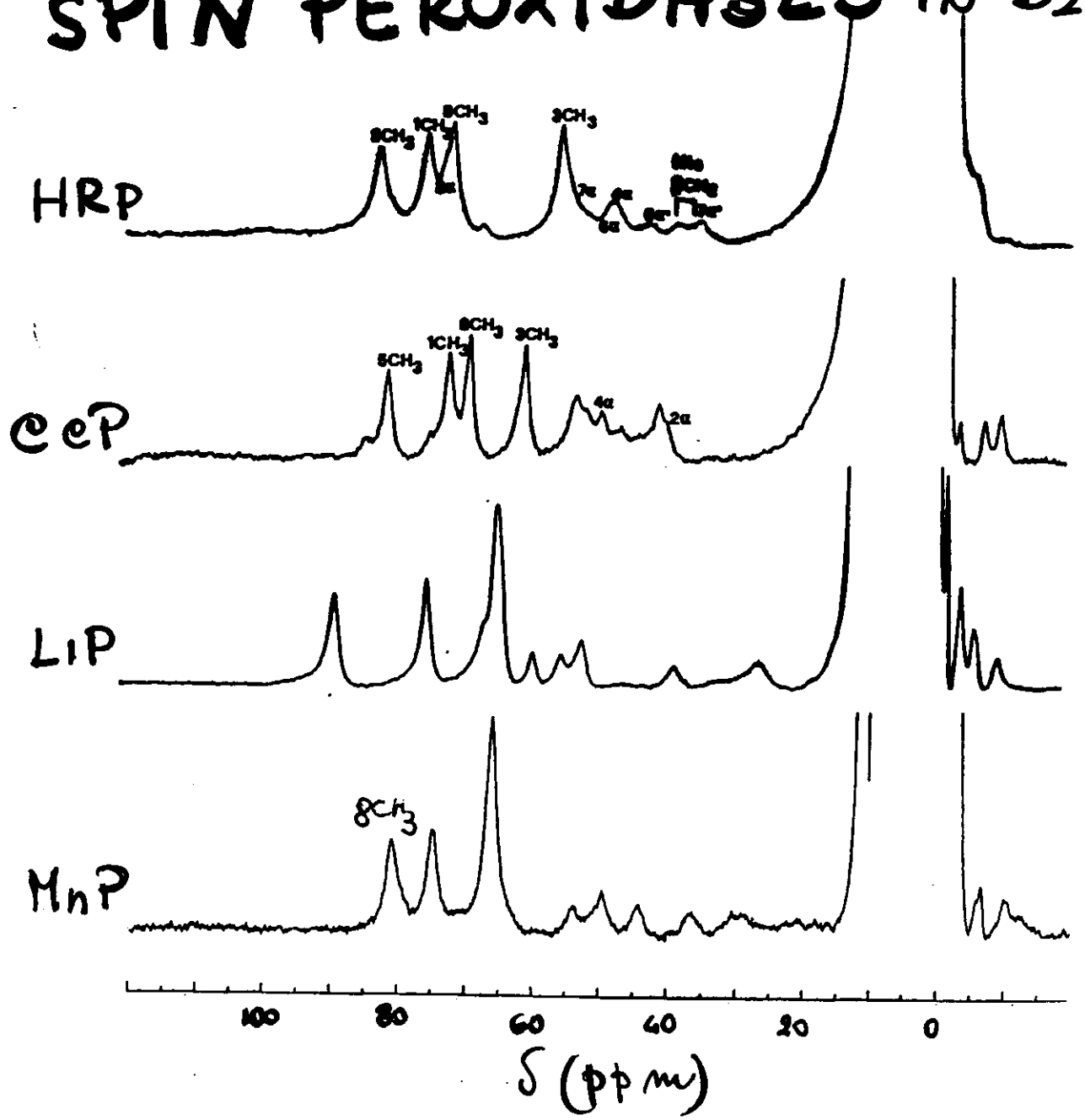
S=3/2



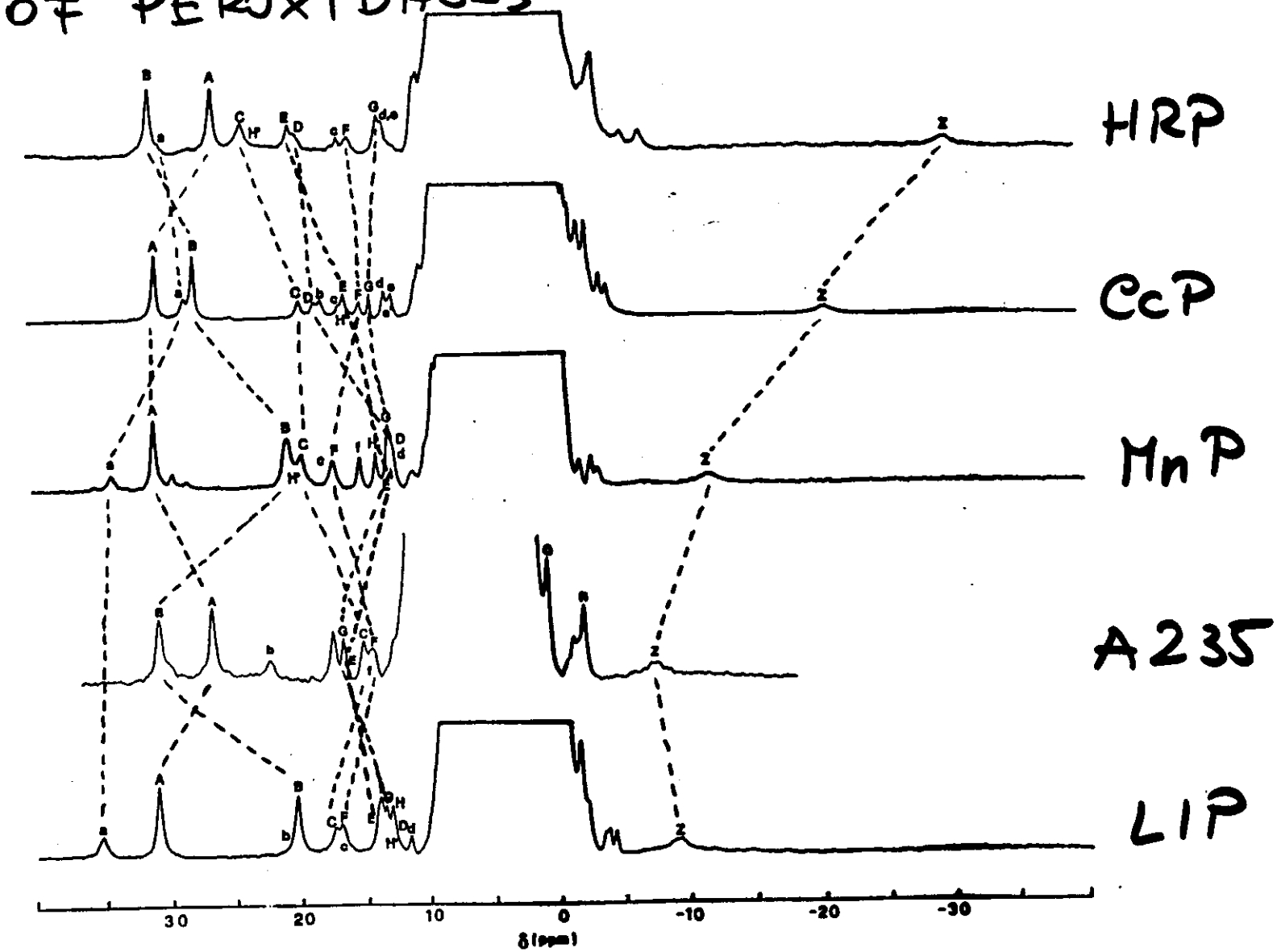
^1H NMR SPECTRA of Co(II)CA, thiocyanate adduct



^1H NMR SPECTRA OF HIGH-SPIN PEROXIDASES IN D_2O

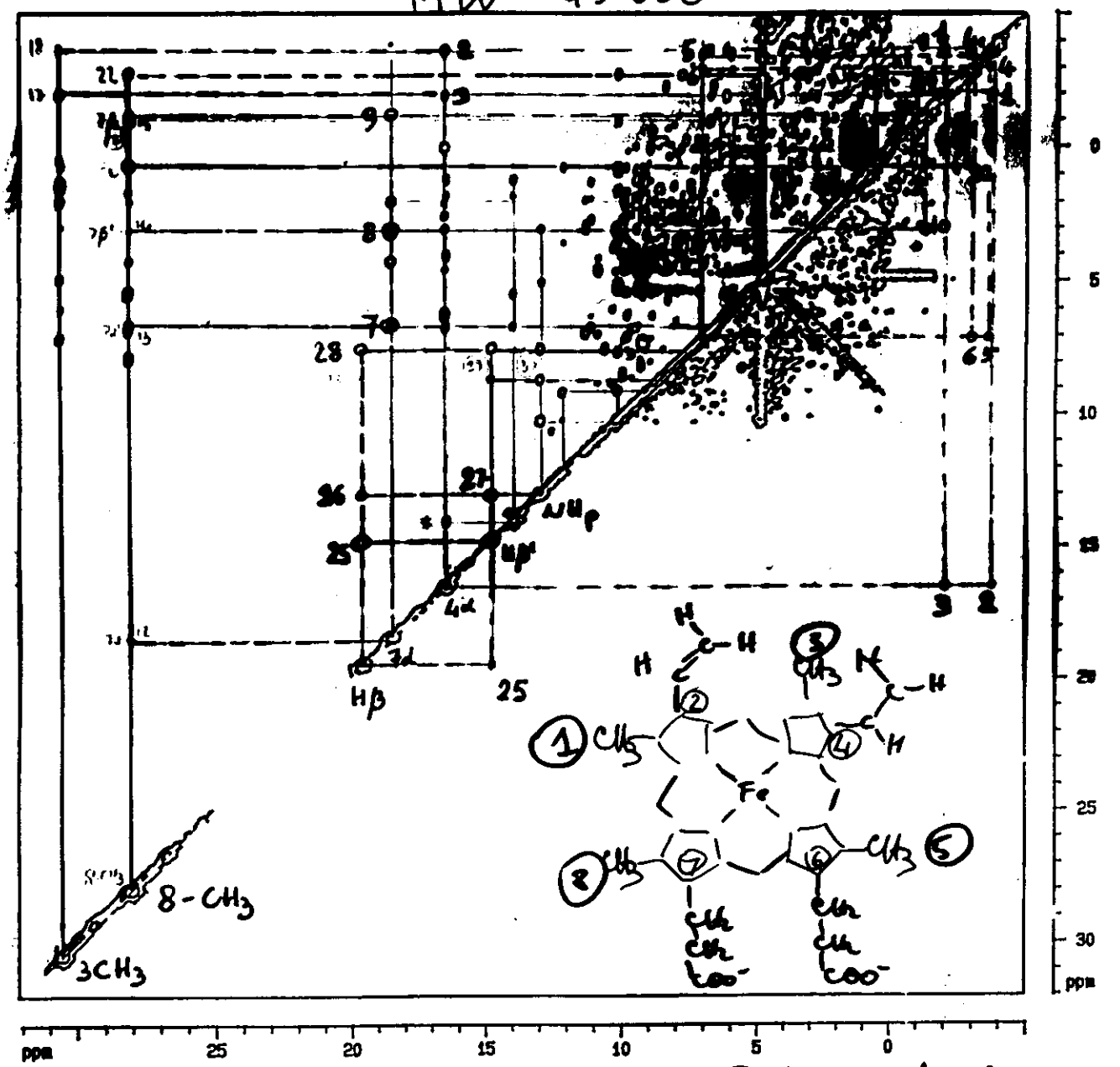


NMR SPECTRA OF CN^- ADDUCTS OF PEROXIDASES



600 MHz NOESY and COSY spectra of CcP-CN⁻

MW 45000



BANCI, BERTINI, MAUK, TURANO INORG. CHEM 1991

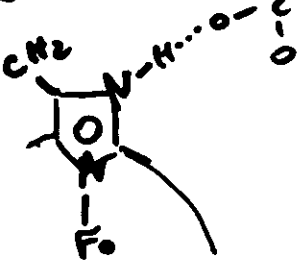
- 2-vinyl
- 4-vinyl
- 7-propionate
- proximal histidine
- Leu-232

●● 3-CH_3 / 4-vinyl

●● 8-CH_3 / 7-propionate

●● 8-CH_3 / Leu-232

PROXIMAL HISTIDINE IN PEROXIDASES



SANGI, BERTINI, MING, HAUK, TURANO

BIOCHEMISTRY 1992

Solution Structure of Paramagnetic Metalloproteins

Why is it seldom attempted?

- The line broadening of signals makes it more difficult to detect and to assign spin patterns

- 2D dipolar connectivities in the neighborhoods of the metal ion(s) may sometimes be lost

- The metal ion(s) and its (their) ligands are not directly observable through NMR

The regions of the protein closest to the metal ion(s) are likely to be less resolved than the rest of the protein

STRUCTURE DETERMINATION THROUGH NMR

Since 1984, NMR spectroscopy has been used to determine 3D protein structures in solution.

In 1990 it is still believed that **it is not possible to** solve a 3D structure of a **paramagnetic protein** through NMR:

"Structures of proteins containing paramagnetic centers may never be completely determined by NMR unless an isomorphous replacement of the paramagnetic group with a diamagnetic one can be achieved. The paramagnetic center usually broadens the proton resonances in its immediate environment by dipole-dipole interactions of the electron spin with the nuclear spins such that these cannot be detected."

G. Wagner, *Science*, 1990.

In 1994 the Florence NMR lab solved the first solution structure of a paramagnetic protein through NMR.

L. Banci, I. Bertini, L. Eltis, I.C. Felli, D. Kastrau, C. Luchinat, M. Piccioli, R. Pierattelli, M. Smith, *Eur. J. Biochem.*, 1994

Solutions

- Optimize 2D experiments for the detection of paramagnetically broadened signals

- Run different 2D experiments optimized for the detection of dipolar connectivities between signals of type A and signals of type B, between signals of type C, and between signals of type A/B and signals of type C

- Run 1D NOE experiments to detect dipolar connectivities from signals of type A

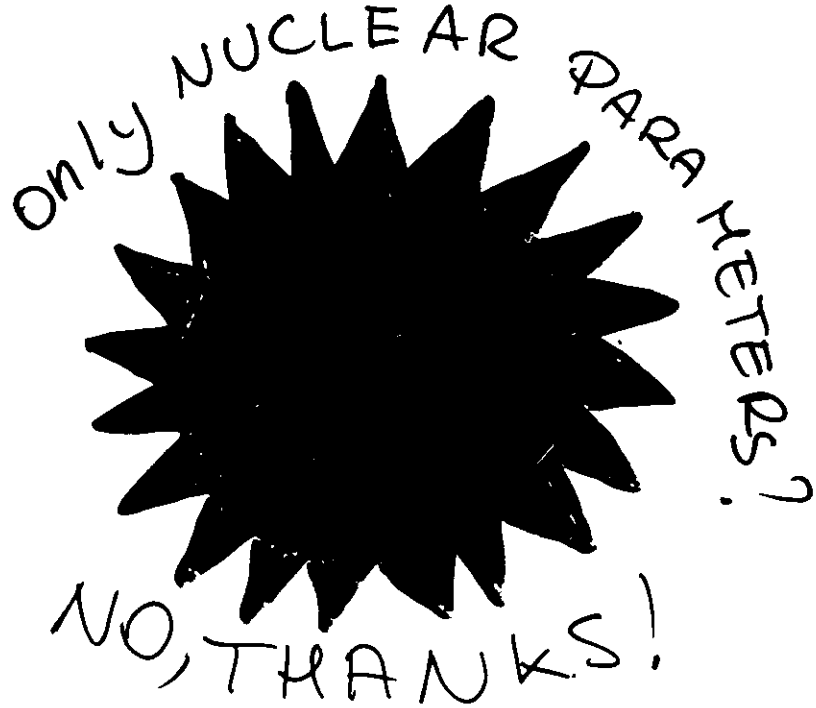
- Use pseudocontact shifts and/or nuclear relaxation rate enhancements as constraints

- Use different magnetic fields to have optimal S/N in the different shells of the protein

The resolution of the structure is comparable in all regions of the protein, regardless of the different influence of the paramagnetic center(s)

STRUCTURE DETERMINATION OF PARAMAGNETIC PROTEINS

WE CAN TAKE ADVANTAGE OF THE PARAMAGNETIC CENTER



WE NEED ELECTRON SPINS, TOO!!

STRUCTURAL CONSTRAINTS IN PARAMAGNETIC MOLECULES

1) NOE CONSTRAINTS
2) J COUPLINGS
3) FIELD DEPENDENT EFFECTS

PSEUDO CONTACT SHIFTS
PARAMAGNETIC RELAXATION
METAL-PROTON DIHEDRAL ANGLES

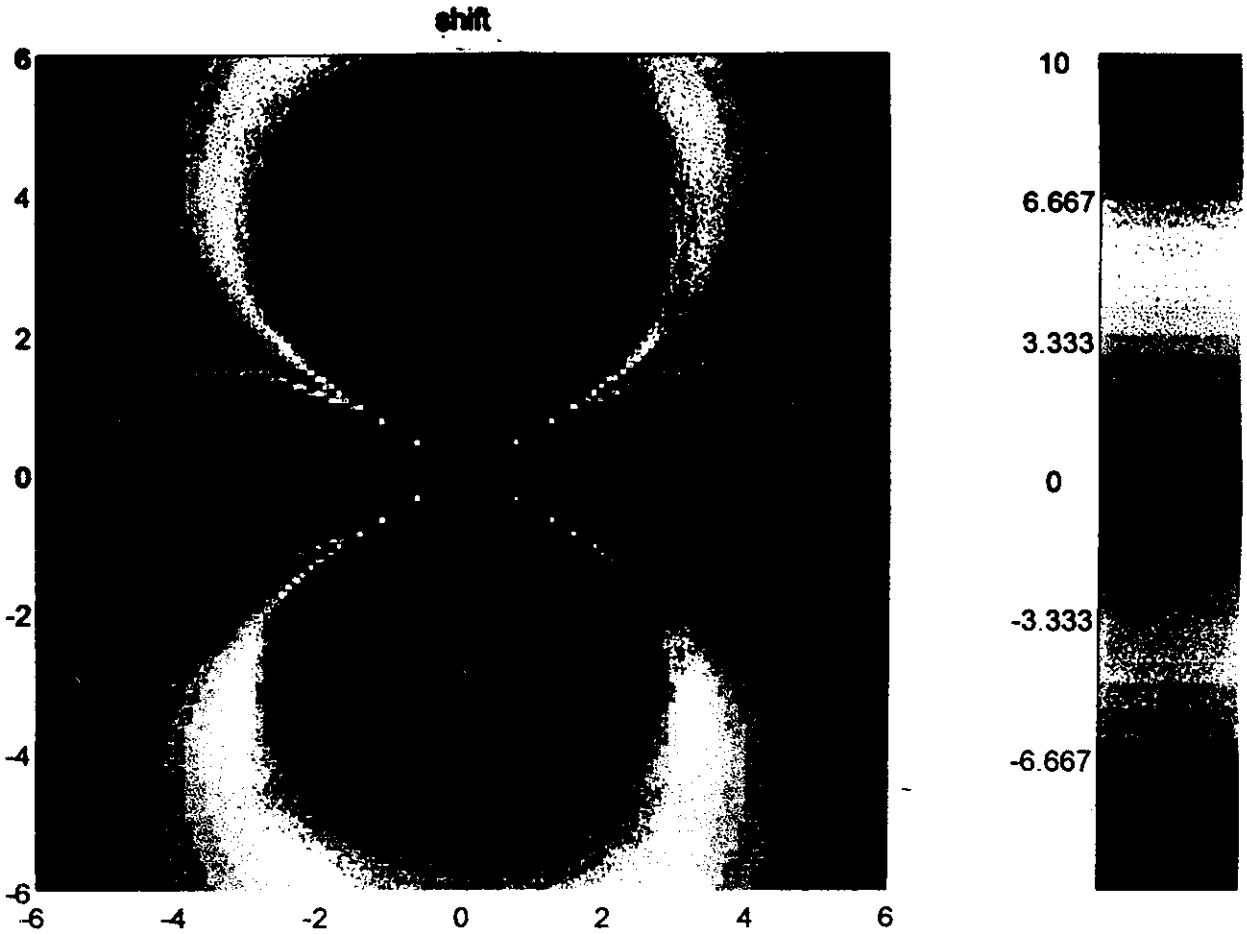
Advantages of paramagnetic constraints

- **Constitute an independent source of information on the metal center**

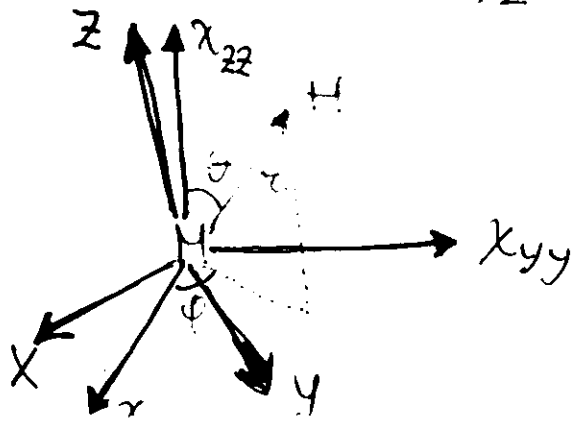
- **Link the metal ion to the protein frame**

- **Provide additional structural constraints in the regions close to the metal center**

THE PSEUDOCONTACT SHIFT



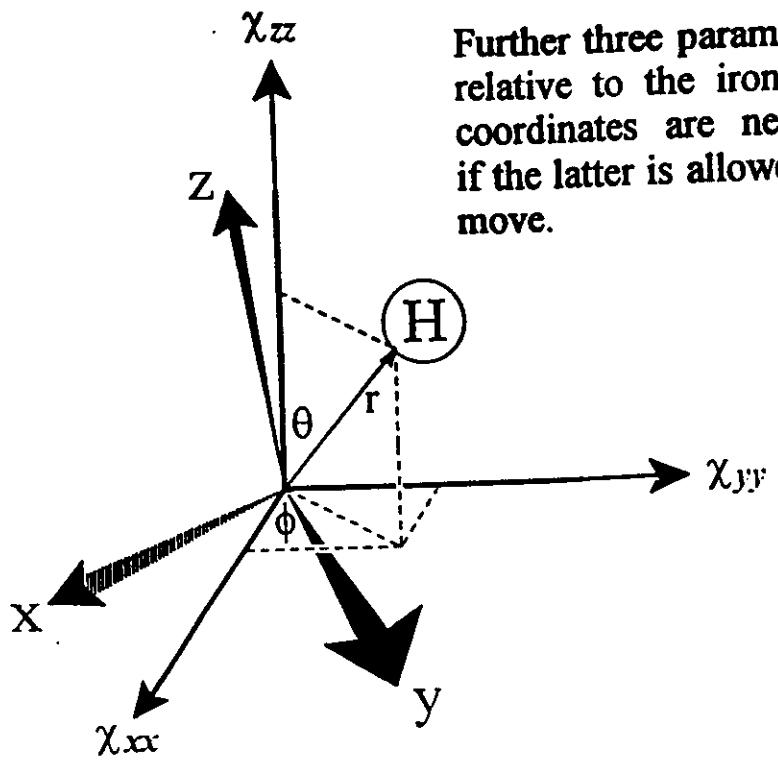
$$\delta_{pc} = \frac{1}{k\pi}\frac{1}{3N r^3}\left\{\Delta\chi_{ax}\left(3\cos^2\theta - 1\right) + \frac{3}{2}\Delta\chi_{rh}\cdot\sin^2\theta\cos 2\varphi\right\}$$



FROM THE STRUCTURE TO Δχ

AND MOLECULAR AXES

We know the coordinates r, θ, φ of H with respect to x, y, z. We have to define $\Delta\chi_{ax}$, $\Delta\chi_{rh}$ and three independent director cosines with the internal axis system.



Further three parameters relative to the iron ion coordinates are needed if the latter is allowed to move.

$$\delta_{pc} = \frac{1}{4\pi}\frac{1}{3N_A r^3}\left\{\left[\chi_{zz} - \frac{1}{2}\left(\chi_{xx} + \chi_{yy}\right)\right]\left(3\cos^2\theta - 1\right) + \frac{3}{2}\left(\chi_{xx} - \chi_{yy}\right)\sin^2\theta\cos 2\varphi\right\}$$

PSEUDYANA

A MODULE OF DYANA

$$T^{pc} = \sum_i \omega_i \left[\max\left(\left| \delta^{pc}_{i_{calc}} - \delta^{pc}_{i_{obs}} \right| - t, 0 \right) \right]^2$$

USED AS PSEUDOPOTENTIAL

where:

t = tolerance

ω = proper weight

Banci, Bertini, Cremonini, Gori Savellini, Güntert, Luchinat, in preparation.

SIMULATED ANNEALING TORSION ANGLE DYNAMI
STARTING FROM RANDOM COIL STRUCTURES,
THE DYNAMICS ON THE DIHEDRAL ANGLES IS
CALCULATED AT DECREASING TEMPERATURES
AS DRIVEN BY THE PSEUDOPOTENTIAL
CONSTITUTED BY NOE AND PCS CONSTRAINTS

THE USE OF PSEUDOCONTACT SHIFTS AS FURTHER CONSTRAINTS IN STRUCTURE CALCULATIONS

THE STRUCTURE IS FOLDED USING NOE AND PCS CONSTRAINTS

$$\delta_{pc} = \frac{1}{4\pi}\frac{1}{3N_A r^3}\left\{\left[\chi_{zz} - \frac{1}{2}(\chi_{xx}+\chi_{yy})(3\cos^2\vartheta - 1) + \frac{3}{2}(\chi_{xx}-\chi_{yy})\sin^2\vartheta\cos 2\phi\right]\right\}$$

2) Perform simulated annealing torsion angle molecular dynamics including δ_{pc} constraints (if the convergence of the χ tensor is not reached repeat steps 1-2).

A PSEUDO RESIDUE DEFINING THE χ TENSOR CAN BE ALLOWED TO MOVE FREELY IN STRUCTURE CALCULATIONS
IN THIS WAY THE POSITION OF THE METAL ION AND THE MOLECULAR AXES CAN BE DETERMINED ab initio.
NO INITIAL STRUCTURE OR TENSOR ARE NEEDED
NO ASSUMPTION IS MADE

ABOUT USING PSEUDOCONTACT SHIFTS

AS STRUCTURAL CONSTRAINTS

ADVANTAGES:

1) constraints around the metal

2) anchorage the metal

PROBLEMS:

1) definition of pseudocontact shift
(diamagnetic reference, tolerance)

2) do we need the starting structure?

3) computational strategies

THE USE OF PSEUDOCONTACT SHIFTS AS FURTHER CONSTRAINTS FOR ENERGY MINIMIZATION AND MD CALCULATIONS IN STRUCTURE REFINEMENT

THE PSEUDOPOTENTIAL TERM

$$U_{pc} = \sum_i K_{pc}\left[\max\left(\left|\delta_c^i - \delta_o^i\right| - T_i, 0\right)\right]$$

where:

K_{pc} = 10-100 kcal $mol^{-1} ppm^{-2}$
T_i = relative tolerance

STRATEGY

1) Obtain a χ tensor from a 5 (or 8) parameters fit of $\Delta\delta_{pc}$ and a model structure.
2) Perform EM and classical molecular dyanamics calculations including the pseudopotential energy term in the canonical potential energy function (bond, angle, torsion, LJ and electrostatic interactions).

Banci, Bertini, Cremonini, Gori Savellini, Gray, Luchinat, Romagnoli, Turano, *PROTEINS Structure, Function and Genetics*, in press. 1997

IT WILL BE A MODULE OF AMBER 4.2 (JULY 1997)

STRUCTURE AND DYNAMICS OF CYTOCHROMES

(HEME PROTEINS)

THE SOLUTION STRUCTURE AND BEYOND

- CHANGES INDUCED BY OXIDATION
- MOBILITY
- SOLVATION PROPERTIES

TO UNDERSTAND ELECTRON TRANSFER PROCESSES AND MOLECULAR RECOGNITION

SOLUTION STRUCTURES OF CYTOCHROMES

OXIDIZED YEAST CYT C BIOCHEMISTRY 1997
REDUCED YEAST CYT C BIOCHEMISTRY 1996

OXIDIZED CYT C_6 FROM M. BRAUNI SUBMITTED
REDUCED CYT C_6 FROM M. BRAUNI JBIC 1996

OXIDIZED HORSE HEART CYT C BIOCHEMISTRY 1997
REDUCED HORSE HEART CYT C IN PROGRESS

OXIDIZED RAT CYT b_5 BIOCHEMISTRY 1998
REDUCED RAT CYT b_5 EUR. J. BIOCHEM. 199?

OXIDIZED CYT C_7 (3 HEMES) from D. Acetoxidans
P.N.A.S. 1996

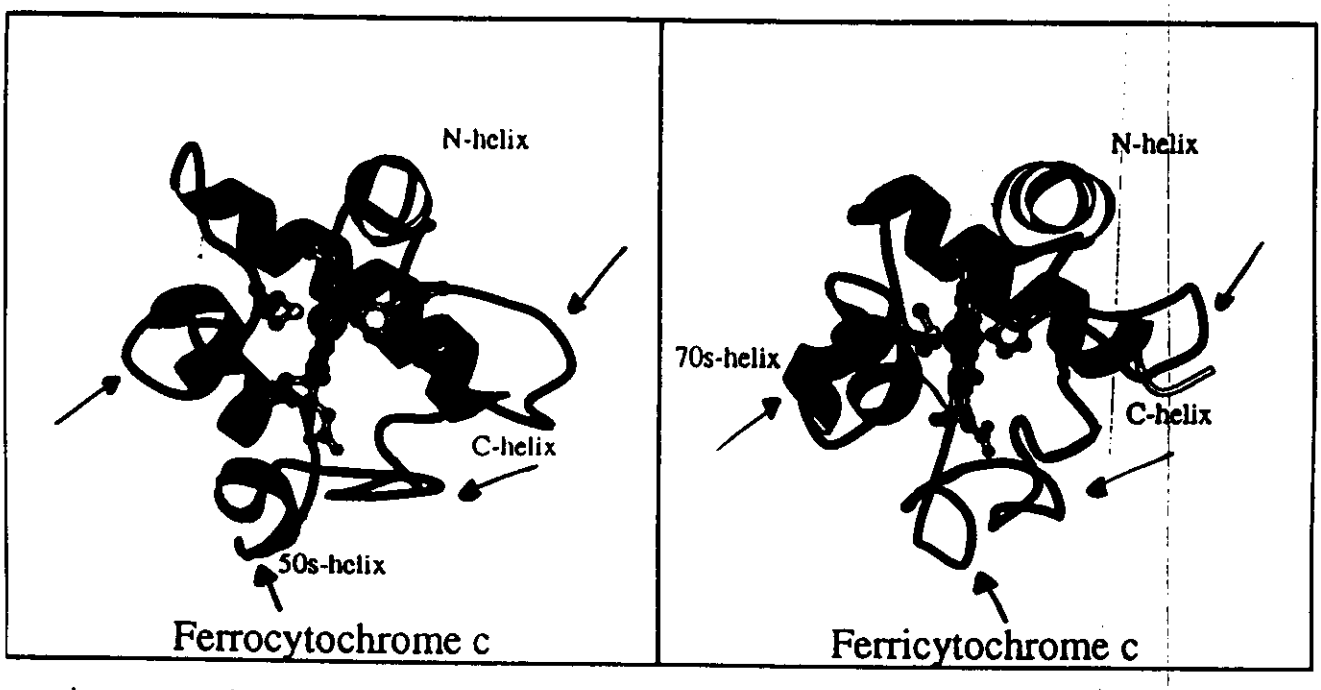
REDUCED CYT C_7 (3 HEMES) in progress

OXIDIZED CN^- ADDUCT METBO→ALA CYT C Biochemistry 1995

Constraints and final data relative to horse heart ferricytochrome *c*.

Number of amino acids	103
Metal cofactor	one *c*-type heme
number of assigned amino acids	103
% assignment of ^{1}H resonances	75%
Type of constraint:	
2D NOESY	2250 (1729)
1D NOE	28
Pseudocontact shifts	241
H-bonds	14
Refinement procedure	PSEUDO-REM
Number of structures	35
RMSD (Å) (from the mean structure, residues 5-100)	backbone: 0.70 ± 0.11 heavy atoms: 1.21 ± 0.14
NOEs' target function (Å^2)	0.97 (average value)
δ_{pc} target function (Å^2)	0.20 (average value)

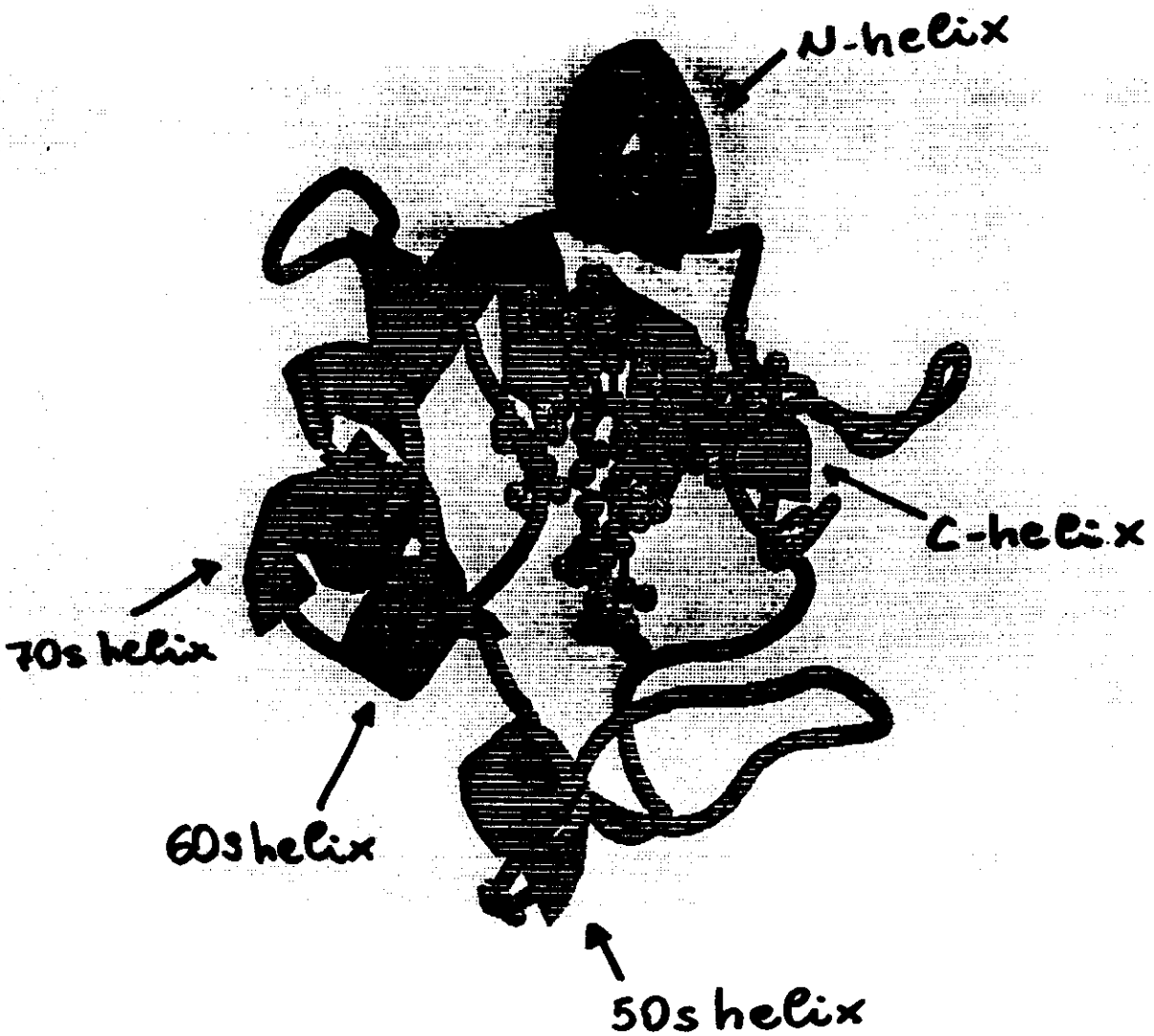
SOLUTION STRUCTURES OF HORSE HEART CYT C



Qi, Di Stefano, Wand
Biochemistry 1994

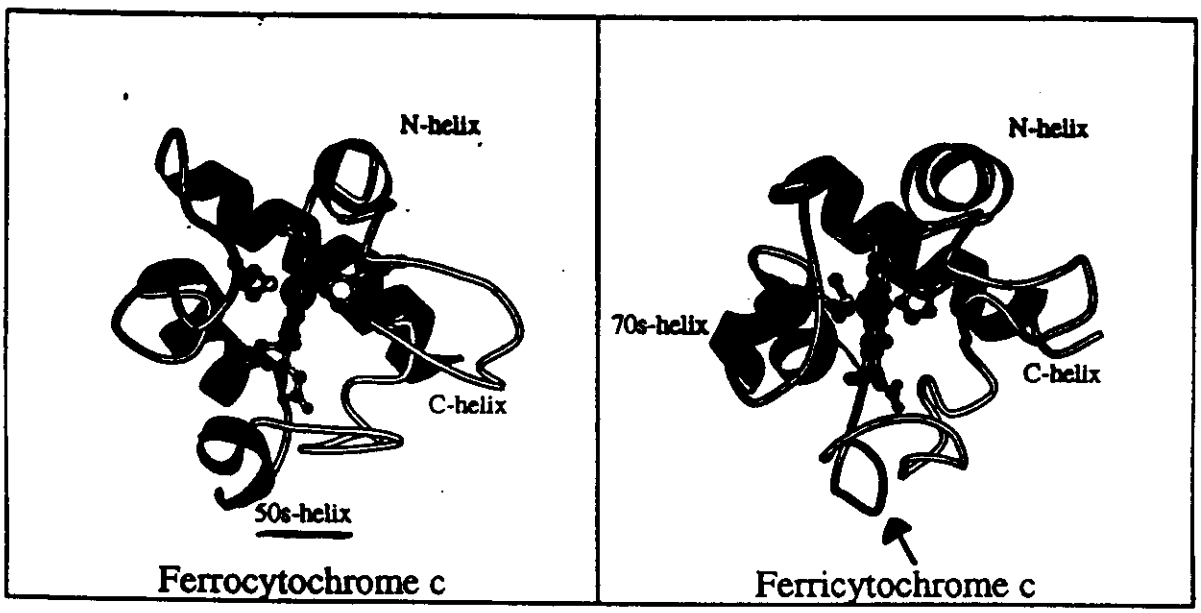
Qi, Beckman, Wand
Biochemistry 1996

Solution Structure of Oxidized Horse Heart Cytochrome *c*



L. Banci, I. Bertini, H. B. Gray, C. Luchinat, T. Reddig, A. Rosato, P. Turano, BIOCHEMISTRY, in press

SOLUTION STRUCTURES OF HORSE HEART CYT C



Qi, P. X., Beckman, R. A., Wand, A. J. (1996) *Biochemistry 35*, 12275-12286

Banci, L., Bertini, I.,Gray, H. B., Luchinat, C., Reddig, T., Rosato, A., Turano, P. *Biochemistry*, in press

PSEUDYANA VERSUS DYANA CALCULATIONS ON HORSE HEART CYTOCHROME C

A FAMILY OF 20 STRUCTURES

	$E^{NOE+vdW}$	E^{PCS}	RMSD HA	RMSD BE
DYANA CALCULATIONS	1.24-2.24	-	0.80	1.2
PSEUDYANA CALCULATIONS	1.26-2.32	0.39-0.43	0.69	1.1

L. BANCI, I. BERTINI, C. LUCHINAT, T. REDDIG, P. TURANO, Biochemistry 1997

RMSD OF RESIDUES CLOSE TO THE HEME IN OX CYT C

(in Å)

RESIDUES ATTACHED TO THE HEME	BB	HA
CYS 14	.35	.28
CYS 17	.36	.34
HIS 18	.24	.54

AVERAGE RMSD OVER ALL THE FAMILY

BB	0.70
HA	1.32

RESIDUES WITHIN 6 Å FROM THE IRON	BB	HA
GLY 29	.34	.34
PRO 30	.37	.41
LEU 32	.46	.63
TYR 67	.34	.93
LEU 68	.36	.86
PRO 71	.36	.38
MET 80	.52	.56
PHE 82	.90	1.43
WITHIN 7 Å FROM THE IRON		
ARG 13	0.34	1.51
VAL 28	.31	.33
THR 78	.46	.50
ALA 81	.73	.86

Protein: Reduced cytochrome c_6 from *Monoraphidium braunii*

Cofactor:		**c-type heme (L.S. Fe^{2+})**
AA:		**89**
NOE's:	**1D -**	**-**
	2D -	**1776 (1278)**
Dihedral angle constraints:	**ϕ**	**20 ($^3J_{HNH\alpha}$)**
RMSD/15 str. (Å)	**BB -**	**0.34 ± 0.06**
	HA -	**0.67 ± 0.06**
H-bonds:		**15**
Metal cluster links:		**3**

Refinement procedure: REM, RMD

References:

L. Banci, I. Bertini, G. Quacquarini, O. Walter, A. Díaz, M. Hervás, M. A. de la Rosa, (1996) JBIC, 1,330-340.

Protein: Oxidized cytochrome c_6 from Monoraphidium braunii

Cofactor:		**c-type heme (L.S. Fe^{3+})**
AA:		**89**
	1D -	**11**
NOE's:		
	2D -	**1657 (1100)**
Pseudocontact shifts		**288**
	BB -	**0.57 ± 0.08**
RMSD/ 40 str. (Å)		
	HA -	**0.94 ± 0.09**
Metal cluster links:		**3**
Refinement procedure		**PSEUDOREM**

References:

L. Banci, I. Bertini, M. A. De La Rosa, D. Koulogliotis, J.A. Navarro, O. Walter, submitted

Protein: yeast iso-1-cytochrome c (reduced)

Cofactor:		**c-type heme (Fe^{2+})**
AA:		**108**
NOE's:	**2D -**	**1702 (1442)**
	BB -	**0.97 ± 0.14**
RMSD/20 str. (Å)		
	HA -	**1.57 ± 0.16**
H-bonds:		**13**
Metal cluster links:		3
Refinement procedure:		**REM_V**

Reference: P. Baistrocchi, L. Banci, I. Bertini, K. Bren, H. B. Gray, P. Turano, BIOCHEMISTRY, 1996

Protein: Oxidized cytochrome *c* from *Yeast*

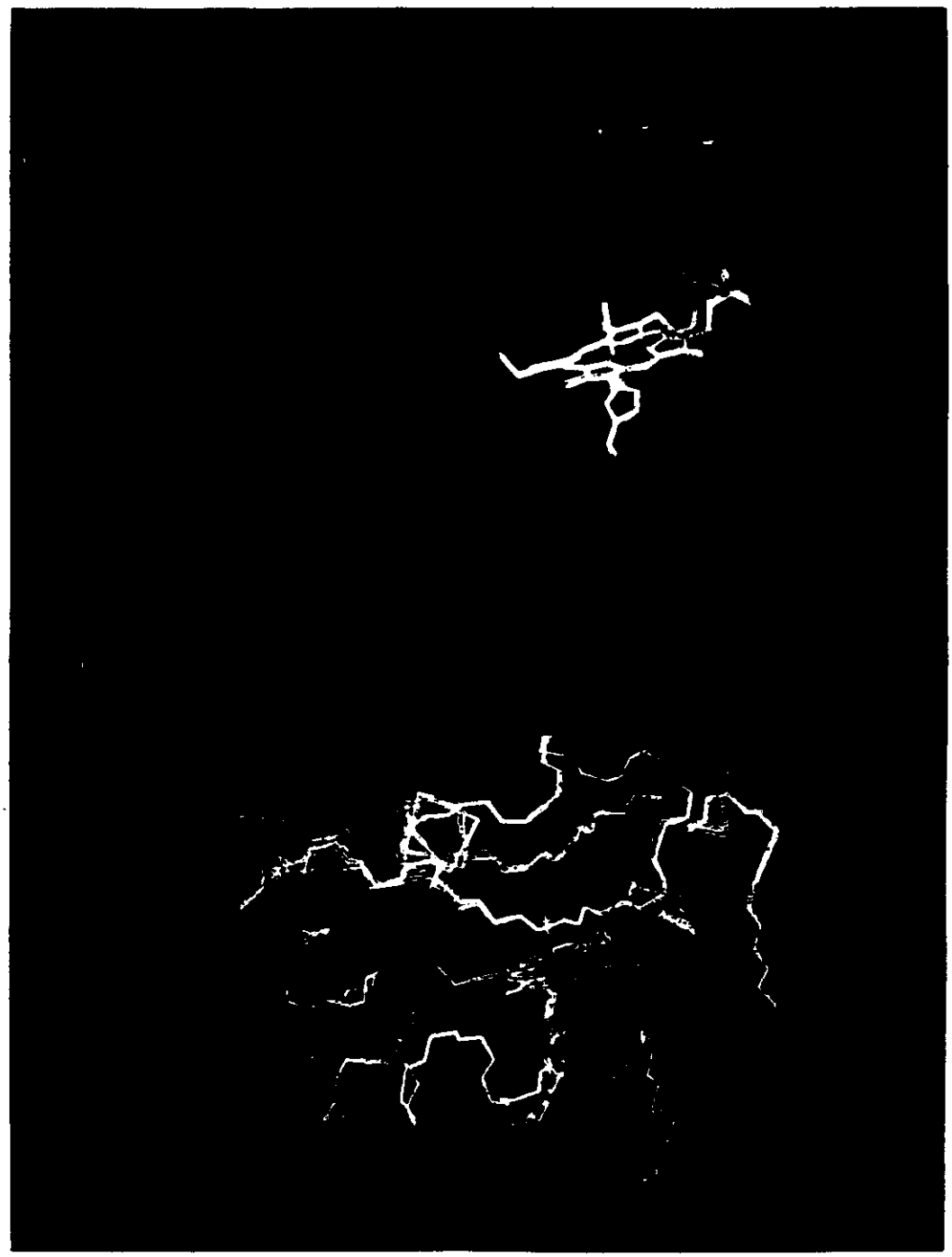
Cofactors:		**c-type hemes (L.S. Fe^{3+})**
AA:		108
NOE's:	1D	5
	2D	1671 (1361)
Pseudocontact shifts (tolerance ± 0.3 ppm)		256 (117)
H-bonds:		14
Metal cluster links:		3
RMSD/20 str. (Å)	BB	0.58±0.07
	HA	1.05±0.07
REFINEMENT		PSEUDO REM

Reference: L.Banci, I.Bertini,K.L.Bren,H.B.Gray,P.Sompornpisut, P.Turano. BIOCHEMISTRY, 1997

Protein: Ala80-cyt c-CN from *Yeast*

Cofactor:		**c-type heme (L.S. Fe^{3+}) (shortest $T_1 \approx 3$ ms)**
AA:		**108**
NOE's:	**1D -**	**8**
	2D -	**1834 (1418)**
RMSD/17 str. (Å)	**BB -**	**0.51 ± 0.07**
	HA -	**0.94 ± 0.08**
H-bonds:		**17**
Metal cluster links:		**24**
Refinement procedure:		**REM_V**

Reference: L. Banci, I. Bertini, K. Bren, H. B. Gray, P. Sompornpisut, P. Turano, *Biochemistry* 1995, 34, 11385-11398.



DG
FAMILY

DE + TSEUD
- CONTI
FAMILY

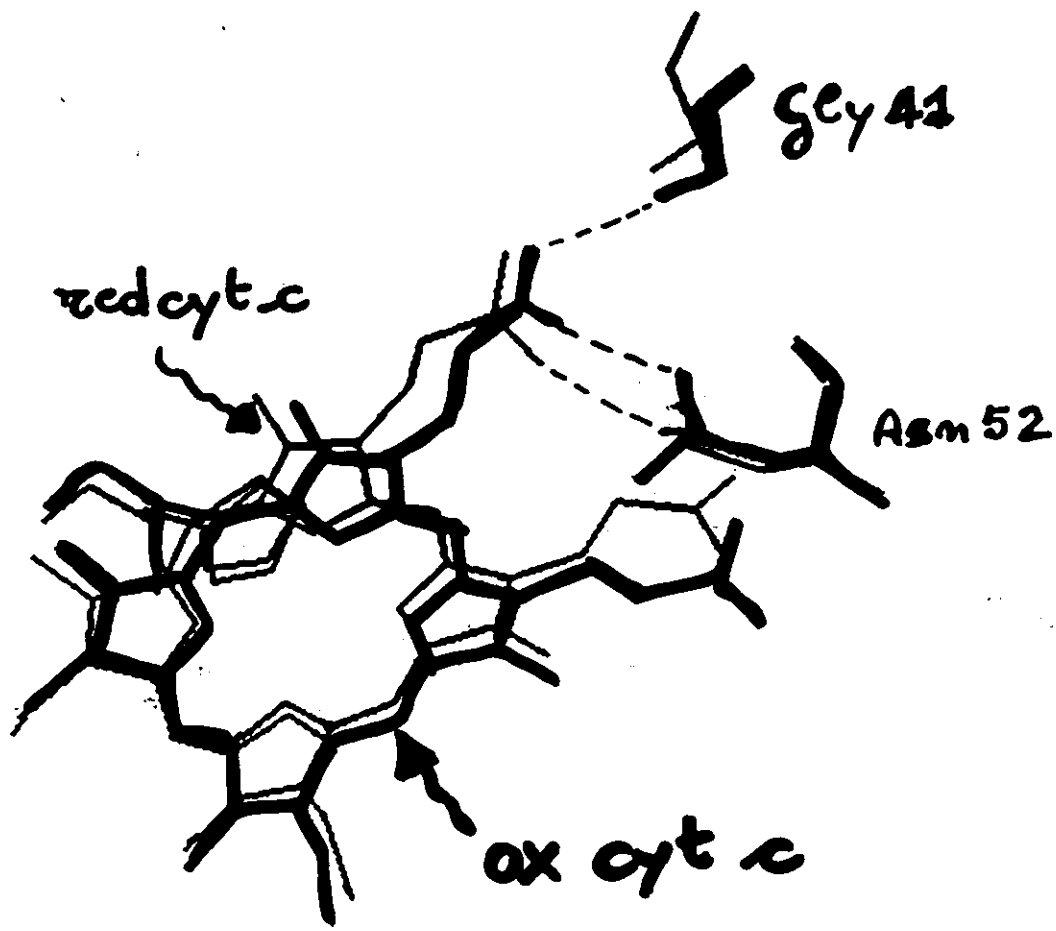
BANCI, BERTINI, BREN, CREMONINI, GRAY
LUCHINAT, TURANO, JBIC 1(2), 117-126, 1996

DIFFERENCES BETWEEN THE OXIDIZED AND REDUCED FORMS CAN GIVE HINTS ON THE ELECTRON TRANSFE PATHWAY

DIFFERENCES BETWEEN CRYSTAL AND SOLUTION FORMS CAN GIVE INFORMATION ON THE INTERACTIO WITH THE PARTNERS

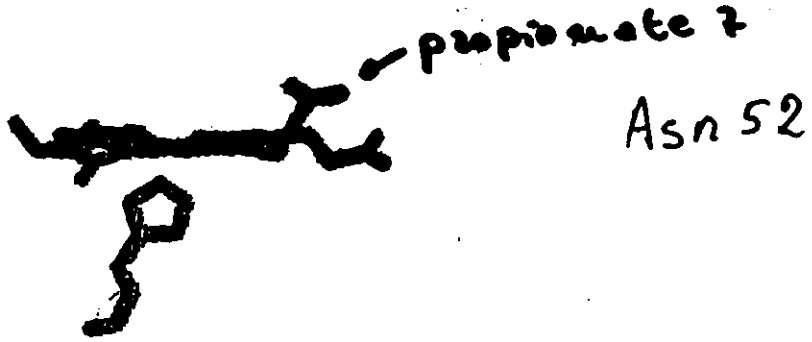
STRUCTURAL CHANGES OBSERVED UPON OXIDATION OF YEAST CYTOCHROME c

H-bond		X-ray	NMR
NH Gly 41	O2 7propronate	+	+
ND2 Asn 52	O1 7-propionate	−	=



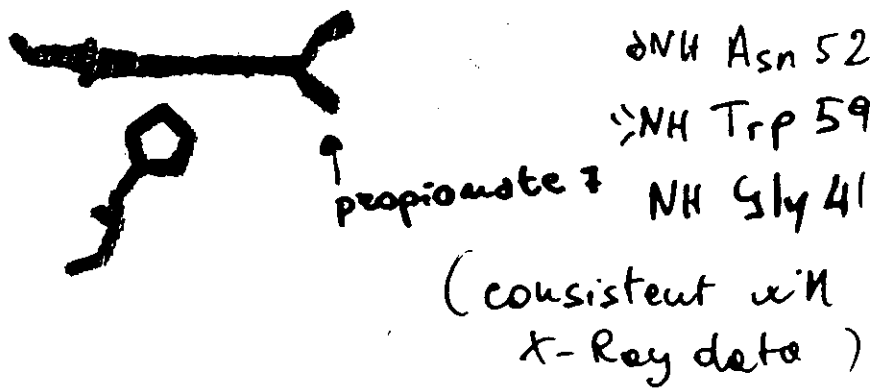
The 7-propionate in horse heart cytochrome *c*

Solution structure of the reduced form



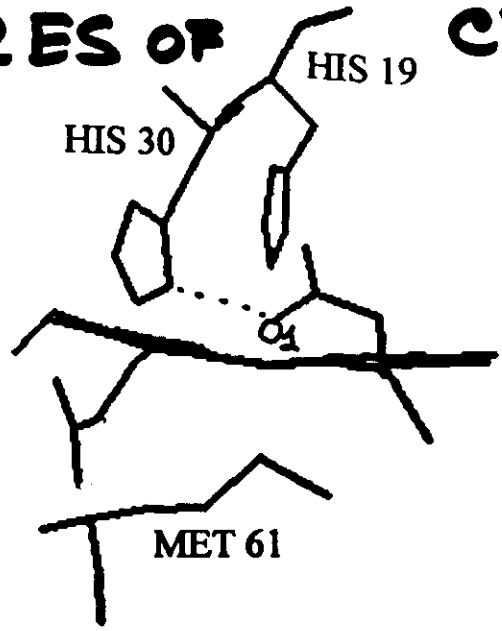
Qi, Di Stefano, Wand (1994) Biochemistry

Solution structure of the oxidized form

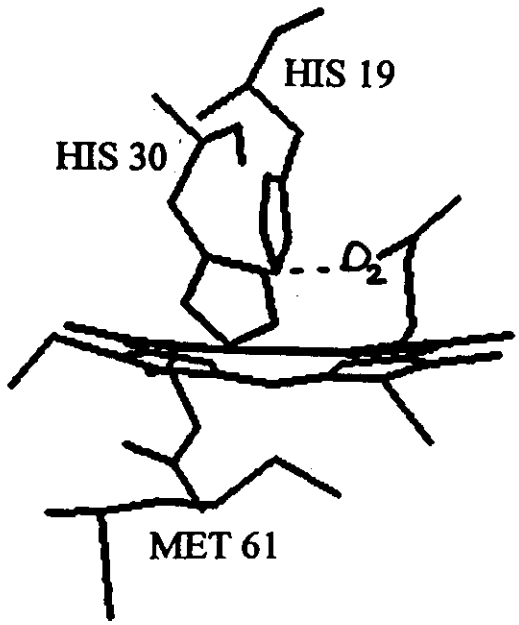


Banci, Bertini, Gray, Luchinat, Reddig, Rosato, Turano, [redacted] BIOCHEMISTRY, 1997

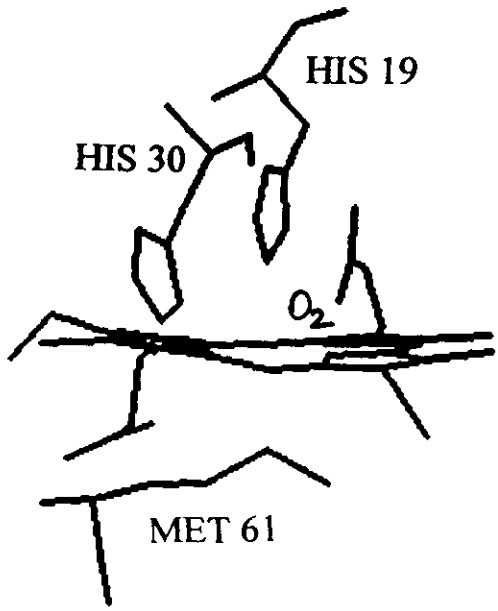
STRUCTURES OF CYT C_6



OXIDIZED IN SOLUTION



REDUCED IN SOLUTION



REDUCED IN THE CRYSTAL

Intermolecular distances of interest in the CcP:cyt *c*

CcP	**cyt *c***
Cytochrome c heme and surrounding area	
Ala193	Heme, Cys17, Gln16
Ala194	Heme
Potential hydrogen bonds	
Asp34	Lys87
Glu290	Asn70, Lys73
Van der Waals interactions	
Arg31	Lys87
Tyr39	Leu9, Arg13
Val197	Ala81, Phe82, Gly83
Gln120	Gly83

Ref. H.Pelletier & J.Kraut, *Science* (1992), **258**, 1748.

DIFFERENCES BETWEEN THE OXIDIZED AND REDUCED FORMS CAN GIVE HINTS ON THE ELECTRON TRANSFER PATHWAY

Structural changes observed upon oxidation of yeast iso-1-cytochrome *c*

X-ray thermal factor parameters of main chain atoms (X-ray)

1. Lower values for residues 37-39 (Arg38)
2. Higher values for residues:
   47-59 (Asn52)
   65-72 (Tyr67)
   81-85 (Phe82)

Amide proton exchange rates (NMR)

Higher values for residues:
14-25
73-82

Heme structure and ligands (X-ray)

1. Increased distorsion of heme planarity
2. Readjustment of propionate 7 and its H-bonds
3. Higher thermal parameters for the Met80 side chain
4. Rotation of the imidazole ring plane of His18

Internal water structures

X-ray: Wat166 moves towards the heme iron atom

NMR: Maintains the same position

Hydrogen bond interactions

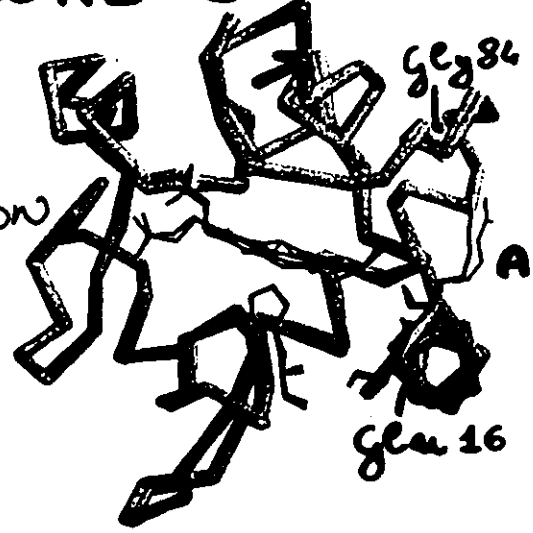
	X-ray	*NMR*
Gly41 N - Heme O2A	stronger	stronger
Asn52 ND2 - Wat166	lost	present
Wat121 - Heme O2A	new	
Gly84 O - Arg13 NH1	new	different conformation of Arg13
Asp60 OD1 - Wat124	new	

Berghuis & Brayer, *J. Mol. Biol.*, 1992
Baistrocchi, Banci, Bertini, Turano, Bren, Gray, ~~submitted~~ BIOCHEMISTRY 1996
Banci, Bertini, Bren, Gray, Sompornpisut, Turano, ~~submitted~~ BIOCHEMISTRY 1997

THE DIFFERENCES BETWEEN THE CRYSTAL AND THE SOLUTION CAN GIVE INFORMATION ON

CYTOCHROME C

THE SITES OF INTERACTION WITH CcP



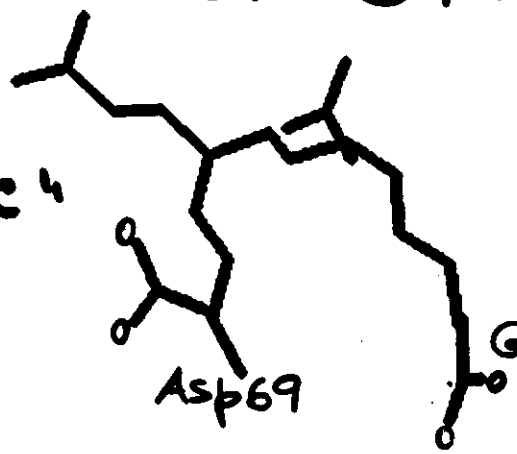
X-ray

NMR

MD_w

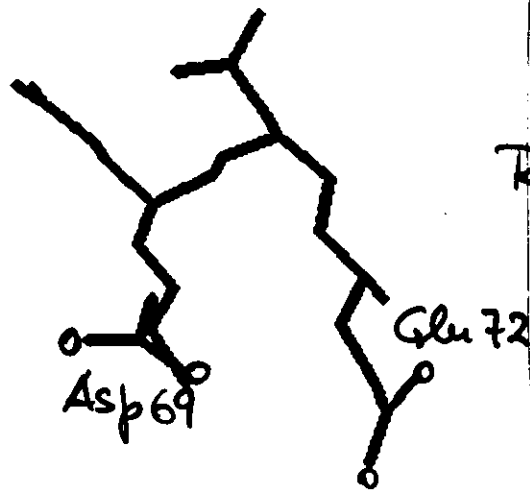
STRUCTURES OF CYT C_6

THE "ACIDIC" PATCH



OXIDIZED
IN SOLUTION

LARGER MOBILI



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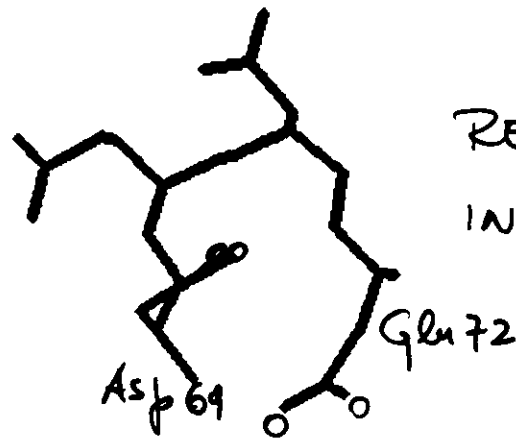
IN SOLUTION



REDUCED
IN THE CRYSTAL

BANCI, BERTINI, QUACQUERINI, WALTER, DIAZ, HERVAS, DELA ROSA, JBIC, 199(

BANCI, BERTINI, DE LA ROSA, KOULOUGLIOTIS, NAVARRO, WALTER, submitte