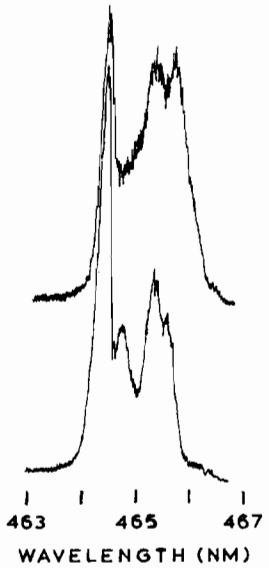


Fig. 2. $^7F_o \rightarrow {}^5D_2$ excitation spectra of Eu(III) bound to calmodulin in pH 5.7 in the presence of 4.0 (upper trace) or 2.0 (lower trace) equivalents of added Eu(III).

evident, consistent with the X-ray findings. Furthermore, lifetimes measured on crystals grown from both H_2O and D_2O are virtually identical indicating the absence of coordinated water in the crystalline state. This corroborates the X-ray diffraction results [12] which reveal a peak in the Fourier map which suggests that a sulfate ion has replaced the coordinated water molecule at the EF site. The excitation spectra (not shown) of crystals grown in the presence of larger amounts of Eu(III) show evidence for the occupancy of both CD and EF sites, but not for a third site. Dissolving the Eu(III)-substituted crystals results in the reformation of the solution state spectra.

Calmodulin. This important regulatory protein contains four Ca(II) ion binding sites, one each in domain numbers I–IV. Studies of tyrosine-sensitized Tb(III) luminescence [13–15] suggest that Ln(III) ions bind initially at sites I and II and subsequently at sites III and IV. The $^7F_o \rightarrow {}^5D_o$ excitation spectra of Eu(III) during a titration are consistent with the binding of four Eu(III) ions, but do not resolve differences between the two classes of sites [15]. Figure 2 shows, however, that there are significant differences between the excitation spectra of the $^7F_o \rightarrow {}^5D_2$ transition depending upon whether two or four equivalents of Eu(III) ions are bound. This illustrates the potential of excitation spectroscopy of higher transitions in the study of multiple classes of binding sites.

Using the same protocol as employed earlier with parvalbumin [8], Förster-type non-radiative energy transfer distance measurements were carried out between sites I and II of calmodulin. In the absence of an energy acceptor ion the reciprocal excited state lifetime of Tb(III) in these sites, τ_o^{-1}, is 0.94 ms^{-1}. In the presence of Ho(III) or Nd(III) in the adjacent site respective τ^{-1} values of 1.18 and 1.25 ms^{-1} were obtained. Using the Förster theory parameters established for parvalbumin [8], these results lead to respective distances of 11.6 and 10.8 Å between sites I and II. These values are close to the CD–EF site separation in parvalbumin (11.8 Å) and support the idea that the calmodulin structure is closely related to that of parvalbumin.

Additional results regarding Ln(III) ion binding to these proteins, including relative binding constants across the Ln(III) series will be presented.

Acknowledgment. This research was supported by the National Institutes of Health through grant GM 23599.

1 W. DeW. Horrocks, Jr., *Advan. Inorg. Biochem., 4,* 201 (1982).
2 W. DeW. Horrocks, Jr. and D. R. Sudnick, *Acct. Chem. Res., 14,* 384 (1981).
3 R. B. Martin and F. S. Richardson, *Quart. Rev. Biophys., 12,* 181 (1979).
4 W. DeW. Horrocks, Jr. and M. Albin, *Progr. Inorg. Chem., 31,* in press.
5 W. DeW. Horrocks, Jr. and D. R. Sudnick, *Science, 206,* 1194 (1979).
6 W. DeW. Horrocks, Jr. and D. R. Sudnick, *J. Am. Chem. Soc., 101,* 334 (1979).
7 W. deW. Horrocks, Jr., M.-J. Rhee, A. P. Snyder and D. R. Sudnick, *J. Am. Chem. Soc., 102,* 3650 (1980).
8 M.-J. Rhee, D. R. Sudnick, V. K. Arkle and W. DeW. Horrocks, Jr., *Biochemistry, 20,* 3328 (1981).
9 A. P. Snyder, D. R. Sudnick, V. K. Arkle and W. DeW. Horrocks, Jr., *Biochemistry, 20,* 3334 (1981).
10 R. H. Kretsinger, *CRC Crit. Rev. Biochem., 8,* 119 (1980).
11 W. DeW. Horrocks, Jr. and W. E. Collier, *J. Am. Chem. Soc., 103,* 2856 (1981).
12 J. Sowadski, G. Cornick and R. H. Kretsinger, *J. Mol. Biol., 124,* 123 (1978).
13 M.-C. Kilhoffer, J. D. DeMaille and D. Gerard, *FEBS Lett., 116,* 269 (1980).
14 M.-C. Kilhoffer, D. Gerard and J. G. DeMaille, *FEBS Lett., 120,* 99 (1980).
15 C.-L. A. Wang, R. R. Aquaron, P. C. Leavis and J. Gergely, *Eur. J. Biochem., 124,* 7 (1982).

B25

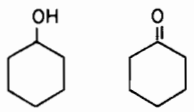
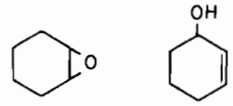
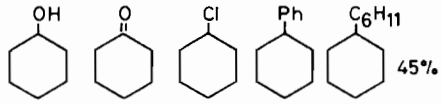
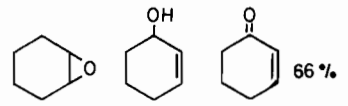
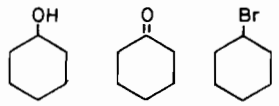
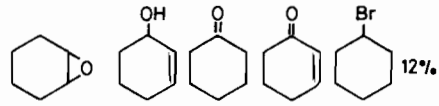
Model and Enzymatic Studies with Cytochrome P-450

DAVID DOLPHIN*, BRIAN R. JAMES and TAK LEUNG

Department of Chemistry, University of British Columbia, Vancouver, B.C., Canada V6T 1Y6

The cytochromes P-450 which have only been recognized for the past two decades are now the most

TABLE I.

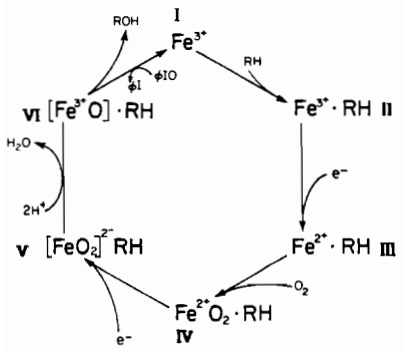
	[a] + iodosylbenzene	[a] + iodosylbenzene
FeTPPCl[c]	CH_2Cl_2  8,1 %[b]	CH_2Cl_2[c]  70 %
	7.6 : 0.5	55 : 15
MnTPPCl[d]	C_6H_6  45%	CrTPP[f] CH_2Cl_2  66 %
	26 : 5 : 11 : 2 : 0.5	4 : 75 : 21
RuOEP(PPh$_3$)Br[e]	CH_2Cl_2  3.5 %	CH_3CN  12%
	1 : 8 : 9	1 : 1.5 : 0.3 : 0.3 : 0.3

[a]Used in excess. [b]Yield based on iodosylbenzene. [c]J. T. Groves, T. E. Nemo, R. S. Myers, *J. Am. Chem. Soc., 101*, 1032 (1978), and personal communication. [d]C. L. Hill, B. C. Schardt, *J. Am. Chem. Soc., 102*, 6374 (1980). [e]This work. [f]J. T. Groves and W. J. Kruper, *J. Am. Chem. Soc., 101*, 7613 (1979).

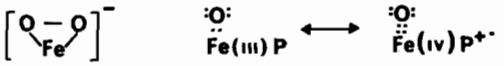
widely studied of all proteins. As is often the case with enzymes much of the detailed information concerning their mechanism of action comes principally from the chemical studies that have been carried out.

In the case of cytochrome P-450 the characteristic optical spectrum has been shown to arise from the axial coordination of a thiolate anion [1, 2] and a theoretical interpretation of the resulting 'split Soret band' in the electronic spectrum has been given [3].

The catalytic cycle for P-450 is shown in the scheme. After binding of substrate (RH) to the resting enzyme (I) the high spin ferric complex (II) is reduced to give the ferrous complex (III) which is still coordinated by the axial thiolate ligand [4]. Oxygenation then gives IV which due to the thiolate ligation has properties quite different from other oxygenated hemeproteins such as oxymyoglobin [5]. The further steps, and subsequent intermediates, in the catalytic cycle have not yet been identified in the enzymic systems. However, model studies suggest structures and mechanisms of action for the subsequent steps. Thus, the electrochemical reduction of a simple oxygenated porphyrin, aimed at mimicking the second enzymatic reduction (IV → V), gave an η^2-peroxy complex (1) which may represent the penultimate step in the enzymatic cycle. Loss of oxygen at the oxidation level of water from V (whatever its electronic configuration might be) would lead to a species which could be represented as a ferric oxene complex (2). While the chemistry that P-450 performs (alkene epoxidation and hydrocarbon hydroxylation) has been shown not to proceed via oxene-like reactions, another electronic configuration of (2), namely (3) appears as a promising candidate for the active oxidizing agent [6]. In addition, this oxo-iron (IV) porphyrin π-cation radical (3) has already been established as the key intermediate in the functioning of the catalases and peroxidases [7] and may also be a key intermediate in the cytochrome oxidase mediated for electron reduction of dioxygen to water [8].

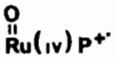


Groves and his collaborators [9] have shown that a complex having the same electronic configuration as (3) can be prepared from ferric porphyrins and iodosylbenzene. Such systems cause both epoxidation and hydroxylation with selectivity similar to those of



1 2 3

P-450 (Table I). We have extended this chemistry using ruthenium porphyrins [10] and find that a ruthenium (III) octaethylporphyrin and iodosylbenzene will catalyse epoxidation and hydroxylation in a similar fashion (Table I). In addition, a relatively stable complex, characterized as (4) has been isolated which exhibits an ESR signal at g = 2.0 confirming its porphyrin radical nature. This isolated complex performs the same oxidations as in the catalytic regime described in Table I. where the formation of,



4

inter alia, cyclohexyl bromide from cyclohexene confirms the radical nature of the oxidation processes.

1 J. P. Collman and T. N. Sorrell, *J. Am. Chem. Soc., 97,* 4133 (1975).
2 C. K. Chang and D. Dolphin, *J. Am. Chem. Soc., 97,* 5948 (1975).
3 L. K. Hanson, W. A. Eaton, S. G. Sligar, I. C. Gunsalus, M. Gouterman and C. R. Connell, *J. Am. Chem. Soc., 98,* 2672 (1976).
4 C. K. Chang and D. Dolphin, *Proc. Natl. Acad. Sci. U.S.A., 73,* 3338 (1976).
5 D. Dolphin, B. R. James and H. C. Welborn, *Biochem. Biophys. Res. Comm., 88,* 415 (1979).
6 D. Dolphin, in 'The Biological Chemistry of Iron', H. B. Dunford, D. Dolphin, K. N. Raymond and L. Sieker (eds), Reidel, Dordrecht, Holland, pp. 283–294 (1982).
7 D. Dolphin, *Isr. J. Chem., 21,* 67 (1981).
8 S. Chan, persomal communication.
9 J. T. Groves, R. C. Haushalter, M. Nakamura, T. E. Nemo and B. J. Evans, *J. Am. Chem. Soc., 103,* 2884 (1981).
10 T. Leung, B. R. James and D. Dolphin, These abstracts.

B26

The Determination of Hydration Numbers of Metal Ions in Metalloenzymes by NMR

T. KUSHNIR and G. NAVON*

Department of Chemistry, Tel Aviv University, Tel Aviv 69978, Israel

Proton relaxation enhancement (PRE) was one of the first applications of NMR to biological systems [1]. It was successfully used to monitor changes in the hydration sphere of paramagnetic metal ions embedded in the active sites of enzymes, upon binding of substrates and inhibitors [2]. However, no unambiguous quantitative interpretation of PRE in terms of hydration numbers (q), exchange lifetimes (τ_M) and correlation times (τ_C) is available [3, 4].

The evaluation of these parameters on the basis of the frequency dependencies of the proton longitudinal relaxation times cannot be made in a unique manner mainly because of the invalidity of the relaxation equations at low magnetic fields, where, at least for Mn(II), the contribution of the zero field splitting cannot be ignored. Our approach was to evaluate all the parameters at one, high magnetic field strength, using the four relaxation times, T^H_{1p}, T^H_{2p}, T^D_{1p} and T^D_{2p} of the water protons and deuterons in the same solution. As a result, in addition to the three parameters, q, τ_M and τ_C, a contribution from the outer sphere relaxation could also be characterized.

For Mn(II) bovine carboxypeptidase and Mn(II) bovine carbonic anhydrase B, a hydration number of unity was obtained, and the exchange lifetimes $\tau_M = 1.0 \times 10^{-7}$ s and 0.75×10^{-7} s were found for the two enzymes respectively.

The same q and τ_M were obtained in independent measurements at different magnetic fields, and τ_M was found to be twice as long upon reducing the temperature from 20 °C to 0 °C, as expected.

The outer sphere relaxation could not be explained either by spin diffusion from the protein protons or by dipolar interaction between the Mn(II) ion and freely diffusing water molecules [5]. A good account of this contribution could be obtained by a mechanism involving water molecules which are bound outside the first hydration sphere and have an average exchange lifetime of about 3×10^{-10} s.

1 J. Eisinger, R. G. Shulman and W. E. Blumberg, *Nature, 192,* 963 (1961).
2 A. S. Mildvan and M. Cohn, *Advances in Enzymology, 33,* 1 (1970).
3 For a recent review, see: D. R. Burton, S. Forsén, G. Karlstrom and R. A. Dwek, *Prog. NMR Spect., 13,* 1 (1979).
4 S. H. Koenig and R. D. Brown III, in 'ESR and NMR of Paramagnetic Species in Biological and Relaxed Systems', I. Bertini and R. S. Drago, Eds., D. Reidel, Dordrecht, 1979, pp. 89–115.
5 H. Pfeifer, *Ann. Phys., 8,* 1 (1961).

B27

Role of the Protein in the B_{12}-dependent Enzymes: Steric Control of a Molecular Switch

JOHN M. PRATT

Department of Chemistry, University of the Witwatersrand, Jan Smuts Avenue, Johannesburg 2001, South Africa

The initial step in the B_{12}-dependent isomerase and ribonucleotide reductase enzymes involves the reversible homolytic fission of the Co–C bond in