of the tetraol in DNA solution at high DNA concentrations. Binding by intercalation is due to a "hydrophobic" interaction between the π electrons of the pyrene moiety of BPT and those of the DNA bases. In contrast, the physiochemical properties of the second type of binding site (II) are such that the interaction between the pyrene chromophore and DNA are weaker. The fluorescence yield, decay profile, and absorption spectra appear to be the same as those of unbound tetraol and similar to the same properties displayed by the covalently bound adduct obtained by reacting *anti*-BPDE with DNA. Electric linear dichroism results are consistent with an external binding site for the covalent adduct.[16]

These results indicate that *anti*-BPDE may also bind physically to DNA in a manner similar to BPT, before reacting covalently with DNA. If this is the case, then it appears that such covalent interactions are favored at site II.

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Functional Design of Heme Proteins: Reaction with Linear Ligands

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Abstract: The reactivities of heme proteins are compared with those of an octapeptide model system derived from cytochrome *c*. Comprehensive studies of the equilibria and kinetics of ligation of linear (CN^-) and bent (N_3^-) ligands are reported for myoglobin and the model. The equilibrium constants are 2.7×10^8 M^{-1} for MbCN, 1.5×10^6 M^{-1} for OPCN, 2.5×10^4 M^{-1} for MbN_3, and 27.6 M^{-1} for OPN_3. The forward rate constants are 2.3×10^4 M^{-1} s^{-1} for MbCN and 1.4×10^6 M^{-1} s^{-1} for OPCN. Reverse rate constants, calculated from the equilibrium constants and forward rate constants, are 8.4×10^{-5} s^{-1} for MbCN and 0.95 s^{-1} for OPCN. These data provide estimates of the relative importance of protein-ligand interactions in modifying ligand binding. Estimates are made of the relative importance of electrostatic effects, steric effects, and differential M-L bond strengths. Contrary to previous suggestions, steric effects appear to be energetically small in these systems, while electrostatic effects dominate. Ligation kinetics appear to be dominated by conformational effects.

Introduction

The reactions of heme proteins with small ligands have been studied extensively to gain insight into the nature of the binding site. Nevertheless, the factors which govern the reactivity of heme proteins are not entirely understood. In the unliganded form of myoglobin the entrance to the heme pocket is too small for a ligand to have direct access to the binding site. The residues which block the opening are thought to swing out of the way so the ligand may approach the coordination site.[1] Once inside the protein, the ligand may encounter additional obstacles, such as the distal residues, depending on the size and geometry of the ligand.

One approach to understanding the functional design of heme proteins is provided by comparing the chemistry of similar model systems. By comparing the affinity of various ligands for the protein to a model system, the effect of the protein on reactivity can be analyzed. Numerous investigators have used model systems to deduce the nature of oxygen and carbon monoxide binding in myoglobin and hemoglobin, as summarized in recent reviews.[2] These studies indicate the affinity for carbon monoxide in the sterically unhindered model systems is significantly greater than in myoglobin. In MbCO, X-ray studies show the CO is bent or tilted due to steric interactions with the distal residues of the protein surrounding the binding site.[3] The model systems, which have unencumbered axial ligand binding sites, coordinate CO in the expected linear fashion.[4,5] Caughey postulated that heme proteins have lower affinities for CO due to steric effects.[6] There seems to be mounting evidence for this hypothesis from studies with a variety of heme proteins and synthetic model systems, some of which mimic these steric interactions.[6-12] However, the magnitude of these steric effects remains uncertain; some models have CO affinities which approach that of Hb.[10]

We have carried out comprehensive ligand binding studies with the isoelectronic cyanide ligand to broaden the understanding of interactions between linear ligands and heme proteins. Equilibrium and kinetic studies with cyanide are reported for myoglobin and a model system, the heme octapeptide. The equilibrium constants for the azide adducts of myoglobin and octapeptide are also reported. These studies are intended to probe as quantitatively as possible the effects of steric constraints and simple Coulombic interactions on the dynamics of ligand binding in heme proteins.

Experimental Section

Salt-free myoglobin from horse skeletal muscle was purchased from Sigma Chemical Co. Solutions were made in 0.05 M phosphate buffer, pH 7. Potassium ferricyanide was added to ensure complete oxidation of the myoglobin and then removed by desalting on a Sephadex G-25

(1) T. Takano, *J. Mol. Biol.*, **110**, 537-568 (1977).
(2) (a) J. P. Collman, *Acc. Chem. Res.*, **10**, 265-272 (1977); (b) J. P. Collman, J. I. Brauman, and K. M. Doxsee, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 6035-6039 (1979); (c) R. D. Jones, D. A. Summerville, and F. Basolo, *Chem. Rev.*, **79**, 139-179 (1979).
(3) E. J. Heidner, R. C. Ladner, and M. F. Perutz, *J. Mol. Biol.*, **104**, 707-722 (1976).
(4) S.-M. Peng and J. A. Ibers, *J. Am. Chem. Soc.*, **98**, 8032-8036 (1976).
(5) J. L. Hoard, "Porphyrins and Metalloporphyrins", K. M. Smith, Ed., Elsevier, New York, 1975, pp 356-358.
(6) W. S. Caughey, *Ann. N.Y. Acad. Sci.*, **174**, 148-153 (1970).
(7) J. O. Alben and W. S. Caughey, *Biochemistry*, **7**, 175-183 (1968).
(8) W. S. Caughey, J. O. Alben, S. McCoy, S. H. Charache, and P. Hathaway, *Biochemistry*, **8**, 59-62 (1969).
(9) J. P. Collman, J. I. Brauman, T. R. Halbert, and K. S. Suslick, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3333-3337 (1976).
(10) T. G. Traylor, D. Campbell, and S. Tsuchiya, *J. Am. Chem. Soc.*, **101**, 4748-4749 (1979).
(11) J. R. Budge, P. E. Ellis, Jr., R. D. Jones, J. E. Linard, F. Basolo, J. E. Baldwin, and R. L. Dyer, *J. Am. Chem. Soc.*, **101**, 4760-4762 (1979).
(12) J. R. Budge, P. E. Ellis, Jr., R. D. Jones, J. E. Linard, T. Szymanski, F. Basolo, J. E. Baldwin, and R. L. Dyer, *J. Am. Chem. Soc.*, **101**, 4762-4763 (1979).

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Table I. Equilibrium and Kinetic Data for Metmyoglobin and Octapeptide Reaction with CN^- and N_3^-

	$K_{eq} = k_{on}/k_{off}$, M^{-1}	k_{on}, M^{-1} s^{-1}	k_{off}, s^{-1}	ΔH^*_{on}, kcal/mol	ΔS^*_{on}, eu	ref
MbCN[a]	2.7×10^8	2.3×10^4	8.4×10^{-5}	2.5	30	16
OPCN[b]	1.5×10^6	1.4×10^6	0.9	9.2	22	this work
MbN_3[c]	2.5×10^4					this work
OPN_3[d]	27.6					this work

[a] Cyanometmyoglobin. [b] Cyano octapeptide. [c] Azidometmyoglobin. [d] Azido octapeptide.

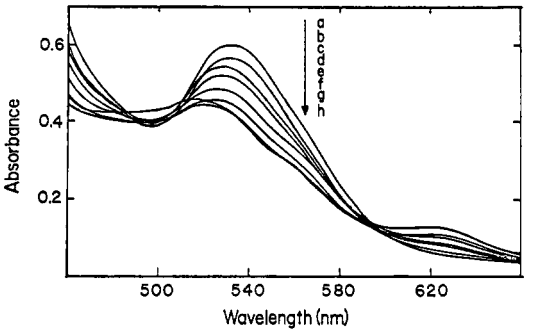

Figure 1. Spectra of the formation of OPCN at 25 °C; OP $\simeq 1 \times 10^{-4}$ M; 1-cm cuvette. Percent of OPCN formation: a, 100%; b, 83%; c, 64%; d, 53%; e, 31%; f, 16%; g, 10%; h, 0%.

column, equilibrated with the same buffer. The heme octapeptide from cytochrome *c* was prepared as reported by Harbury and Loach.[13] A Sephadex column was used in the purification steps. The purity of the octapeptide was checked by silica gel thin-layer chromatography. The TLC of the sample was compared to that of another standard sample whose amino acid analysis had been carried out previously. Potassium cyanide solutions were made in 0.05 M phosphate buffer fresh before use to minimize decomposition and evaporation. The concentration of the cyanide solutions was checked by making up identical solutions in doubly distilled water and carrying out a Leibig titration.[14] The concentrations were the same as those made up in buffer.

The equilibrium studies were carried out on a Cary 118 spectrophotometer, and the pH of the solutions was measured with a Beckman 4500 Digital pH meter. The concentration of free cyanide could then be determined from the appropriate p*K* of HCN,[15] 9.14 at 20 °C.

The kinetics of association for myoglobin and octapeptide with cyanide were carried out on a Durrum D110 stopped flow rapid kinetics system. The change in transmittance was monitored at 410 and 405 nm for the myoglobin and octapeptide systems, respectively, under pseudo-first-order conditions. The data results were analyzed with a Tektronix 4051 graphics system, using a linear least-squares program. The slope of a plot of $-\log (A_\infty - A_t)$ vs. time yielded the observed rate constants. Activation parameters were determined from rate constants at six different temperatures between 5 and 45 °C. The value of the p*K* for HCN at different temperatures was calculated from the enthalpy for the acid dissociation of −10.9 kcal/mol.[15] Myoglobin concentrations determined from the absorbance at 408 nm were 2.5–3.0 μM. Six different potassium cyanide concentrations between 5 and 25 mM were used in the kinetics with myoglobin. Octapeptide concentrations, determined from the hemochrome spectrum, were 0.7 μM. Six potassium cyanide concentrations between 15 and 40 mM were used in the octapeptide kinetic studies.

The equilibrium studies of Mb and OP with N_3^- were monitored by the change in absorbance at 410 and 396 nm, respectively. The azide solution was introduced into the sample and reference cuvettes through a rubber septum with a gas-tight Hamilton microliter syringe.

Results

Equilibrium Studies. The association constant for cyanomyoglobin, measured in 0.05 M phosphate buffer (pH 7.0), was 2.7×10^8 M^{-1}. The value is in excellent agreement with the previously determined value.[16] The association constant for the cyanide–octapeptide complex is 1.5×10^6 M^{-1} in 0.05 M phosphate buffer, pH 7.0. The visible spectra for the formation of the cyanide–octapeptide complex are shown in Figure 1. An isobestic point is clearly defined at 586 nm. The MbN_3 equilibrium constant of 2.5×10^4 M^{-1} is slightly different from a previously determined value of 1.45×10^4 M^{-1}.[16] The azide equilibrium constant is known to depend on the type and concentration of buffer used.[16] Since the equilibrium constants are being used for comparative purposes, the value in 0.05 M phosphate, pH 7, buffer determined in this study will be used. The octapeptide azide equilibrium constant in this buffer system is 27.6 M^{-1}.[17]

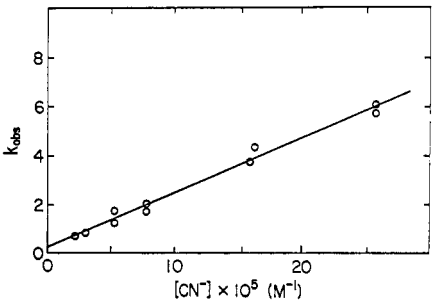

Figure 2. Observed rate constant of MbCN formation as a function of cyanide concentration at 25 °C.

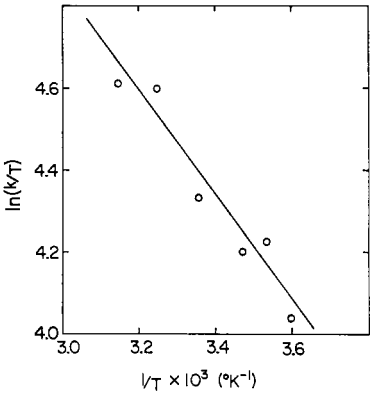

Figure 3. Eyring plot of MbCN forward rate constants.

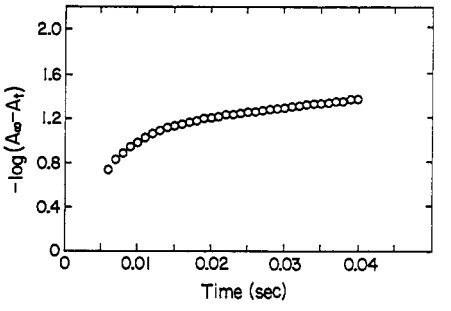

Figure 4. Pseudo-first-order treatment of kinetics of OPCN formation at 15 °C.

Kinetic Studies. The kinetics of cyanide binding to myoglobin, carried out under pseudo-first-order conditions, exhibited simple

(13) H. A. Harbury and P. A. Loach, *J. Biol. Chem.*, **235**, 3640–3645 (1960).
(14) J. G. Dick, "Analytical Chemistry", McGraw Hill, New York, 1973, p 328.
(15) R. M. Izatt, J. J. Christensen, R. T. Pack, and R. Bench, *Inorg. Chem.*, **1**, 828–831 (1962).
(16) E. Antonini and M. Brunori, "Hemoglobin and Myoglobin in Their Reactions with Ligands", Elsevier, New York, 1971.
(17) D. C. Blumenthal and R. J. Kassner, *J. Biol. Chem.*, **254**, 9617–9620 (1979). After submission of this manuscript, a limited study of MbN_3 was published. These authors reported a binding constant for azide 29.2 M^{-1} in excellent agreement with the present results.

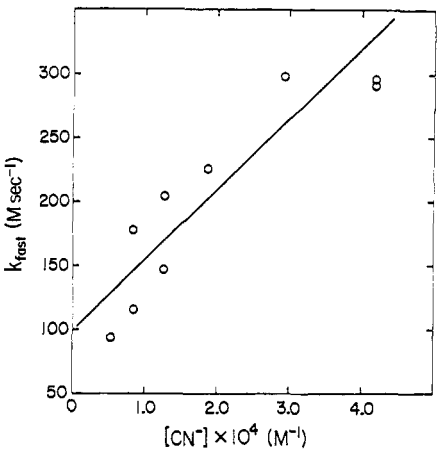

Figure 5. Fast observed rate constant of OPCN formation as a function of cyanide concentration at 15 °C.

exponential behavior. The observed rate constants varied linearly with cyanide concentration according to the equation $k_{obsd} = k[CN^-]$. A plot of k_{obsd} vs. [CN] (cyanide concentration) is shown in Figure 2. The rate constant at 25 °C for the formation of cyanomyoglobin (k_{on}) is 2.3×10^4 M^{-1} s^{-1}. Rate constants at six temperatures were used to calculate the enthalpy and entropy of activation, which are 2.5 kcal/mol and −30 eu, respectively. The Eyring plot is shown in Figure 3. The dissociation rate constant (k_{off}) was calculated from the equilibrium constant and the forward rate constant and is 8.4×10^{-5} s^{-1} at 25 °C. The equilibrium and kinetic data for the cyanomyoglobin system are tabulated in Table I.

The kinetic studies with cyanide and octapeptide were complicated. As shown in Figure 4, plots of $-\log (A_\infty - A_t)$ vs. time were not linear for this system. This type of behavior is indicative of at least two rate processes taking place. The two rate constants were obtained by analyzing the data according to a method outlined in Frost and Pearson.[18] The fast (observed) rate constant was first order in cyanide concentration, whereas the slow rate constant was zero order in cyanide. A plot of the fast observed rate constant (k_{obsd}) vs. cyanide concentration is shown in Figure 5. It should be noted that a slow rate constant was also observed by Traylor and co-workers in the formation of carbon monoxide microperoxidase.[19] This seems to be a unique feature of the heme peptides. Further study will be required to determine the exact nature of the reaction responsible for these slow rate processes. The value for the slow rate constant is 37 s^{-1} at 25 °C. For the present study though, the fast rate constant (k_{on}) is the one of interest and at 25 °C is 1.4×10^6 M^{-1} s^{-1}. This value was used to calculate the dissociation rate constant (k_{off}) from the equilibrium constant and is 0.9 s^{-1} at 25 °C.

The activation parameters for the fast and slow reactions of octapeptide were obtained from rate constants at six different temperatures. The enthalpy and entropy of activation for the fast reaction are 9.2 kcal/mol and 22 eu, respectively. The activation enthalpy for the slow, zero-order cyanide reaction is 10 kcal/mol and the entropy of activation is about 5 eu. Table I contains the relevant data.

The errors in the octapeptide kinetic studies are larger than for myoglobin due to the inherent error in separating the two rate constants in the octapeptide reaction. Another source of error is the smaller signal to noise ratio because the reaction is very fast and the time scale is at the lower limit of the capabilities of the stopped flow kinetics apparatus.

Discussion

Cyanide and carbon monoxide are isoelectronic and therefore isostructural. X-ray data for MbCN have been referred to briefly[20]

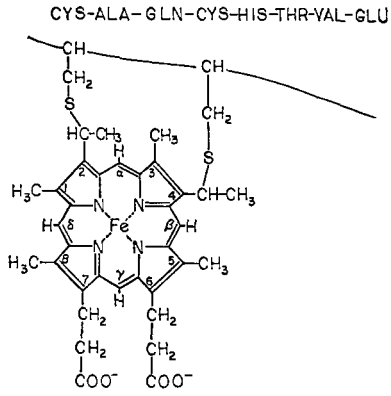

Figure 6. Schematic representation of heme octapeptide. The heme group is covalently attached to an eight-residue peptide whose sequence is also shown. The axial histidine lies below the heme plane.

Table II. Equilibrium and Kinetic Data for Carbonylmyoglobin and Carbon Monoxide Microperoxidase

	$K_{eq} = k_{on}/k_{off}$, M^{-1}	k_{on}, M^{-1} s^{-1}	k_{off}, s^{-1}	ref
MbCO[a]	3.9×10^7	5×10^5	0.017	16
MPCO[b]	2×10^9	2×10^7	0.01	19

[a] Carbonylmyoglobin. [b] Carbon monoxide microperoxidase.

but full details have not appeared. Another report of the X-ray structure of MbCN by Watson and Chance[21] does not show any movement of the protein side chains. Contact distances between cyanide and the distal residues have been calculated by others[16] (presumably from the MbCO data) which imply a similar bent or tilted geometry for cyanide in MbCN. More direct evidence for this distortion is found in the crystallographic data of HbCN where the ligand is bent or tilted in both α and β chains.[22] Since the β chain structure of Hb is very similar to that of Mb,[1] the crowded environment responsible for distorting the ligand is probably functioning in an identical manner, resulting in a bent or tilted ligand geometry in MbCN.

The equilibrium constants reported here for the cyano and azido complexes of Mb and a model system, the heme octapeptide (OP), provide additional evidence for steric interactions between linear ligands and myoglobin. The model system used in this study is a naturally occurring histidine "tailed" porphyrin derived from cytochrome *c* through proteolytic digestion. The heme group is covalently attached to an eight residue peptide through two thioether linkages. The peptide contains a histidine that serves as the fifth ligand to the iron, and at pH 7 water occupies the sixth position.[23] The heme octapeptide is shown schematically in Figure 6. The primary difference between this model system and myoglobin is the absence of protein structure which could interact with axial ligands. The cyanide in OPCN is therefore linear and normal to the heme plane. Azide, unlike cyanide, forms bent or angular bonds with metals and therefore need not bend or tilt to fit in the heme pocket of the protein. A recent analysis of equilibrium constants for Mb and model system complexes of CO, NO, and O_2 shows that the ratios of the equilibrium constants, K_{model}/K_{Mb}, for the latter two complexes, which form bent bonds naturally in both protein and models, are nearly identical and close to unity.[24] The present study shows that the ratios $K_{MbN_3}/K_{OPN_3} = 900$ and $K_{MbCN}/K_{OPCN} = 180$ are unequal and support the contention that cyanide experiences nonbonded steric interactions

(18) A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms", Wiley, New York, 1953, p 149.
(19) V. S. Sharma, H. M. Ranney, J. F. Geibel, and T. G. Traylor, *Biochem. Biophys. Res. Commun.*, **66**, 1301–1306 (1975).
(20) P. A. Bretscher, *Nature (London)*, **219**, 606–607 (1968).
(21) H. C. Watson and B. Chance, "Hemes and Hemoproteins", B. Chance, R. Estabeook, and T. Yonetani, Eds., Academic Press, New York, 1966, pp 149–153.
(22) J. F. Deatherage, R. S. Loe, C. M. Anderson, and K. Moffat, *J. Mol. Biol.*, **104**, 687 (1976).
(23) H. A. Harbury and P. A. Loach, *J. Biol. Chem.*, **235**, 3646–3653 (1960).
(24) R. W. Romberg and R. J. Kassner, *Biochemistry*, **18**, 5387–5392 (1979).

in MbCN. Myoglobin binds the linear ligand cyanide only 180 times more strongly than octapeptide, compared to the bent ligand azide which binds to the protein almost 1000 times better than octapeptide.

Comparison of the equilibrium data for the cyanide and carbon monoxide complexes shows that the affinity for cyanide is much higher in metmyoglobin than the model system. Thus, although cyanide is bent or tilted in Mb, this does not diminish its binding constant compared to the binding constant of the model system. This is in direct contrast to carbon monoxide binding to Mb and nonsterically hindered model systems, such as microperoxidase (MP), a close homologue of octapeptide. Table II lists the literature values of the thermodynamic and kinetic data for carbonylmyoglobin and microperoxidase. The implication is that some favorable interaction is present in MbCN which is absent in MbCO.

In order to evaluate the relative importance of the various interactions between linear ligands and Mb, it is helpful to consider the energetics of these effects individually. The simplest approach is to express the free energy for ligand binding to myoglobin as a sum of the following individual energy contributions: (1) the "intrinsic" free energy of the reaction ΔG_i, which depends primarily on M–L bond strength, (2) the free energy, ΔG_s, of any steric interactions between the protein and ligand, and (3) ΔG_{es}, the free energy for electrostatic and Coulombic interactions between the iron of the charged heme group in metmyoglobin and a charged ligand such as cyanide or azide. Thus, $\Delta G_{Mb\text{-}L} = \Delta G_i + \Delta G_s + \Delta G_{es}$. A similar analysis can be performed for the model system. The free energy of complex formation contains both an intrinsic free energy and an electrostatic term if a charged ligand is bound to a charged heme. The protein-specific term vanishes for the model system, $\Delta G_s = 0$, so the total change in free energy is given by $\Delta G_{OP\text{-}L} = \Delta G_i' + \Delta G_{es}'$. By taking the difference between free energies of ligation in Mb and OP, i.e., $\Delta\Delta G_L = \Delta\Delta G_i + \Delta G_s + \Delta\Delta G_{es}$, estimates of these free-energy components can be obtained. Chang and Traylor used a similar qualitative approach to compare the kinetics of oxygen and carbon monoxide binding of synthetic model systems to myoglobin and hemoglobin.[25]

Analysis of the equilibrium data for the azide complexes of Mb and OP gives $\Delta G_{MbN_3} - \Delta G_{OPN_3} = \Delta\Delta G_{N_3} = \Delta\Delta G_i + \Delta\Delta G_{es} = -4.0$ kcal/mol. Because azide forms bent bonds in the protein, the free energy due to steric interactions, ΔG_s, can be omitted or set equal to zero. This is supported by the X-ray crystal structure of MbN_3, which shows no alterations in the structure of the protein side chains.[26] Since the azide in the octapeptide complex binds with the same bent geometry as MbN_3, the intrinsic free energies of the two species are presumably the same and $\Delta\Delta G_i = 0$. The difference in the electrostatic and Coulombic interactions between the two systems is therefore responsible for the higher affinity of Mb than OP for N_3^- and $\Delta\Delta G_{N_3} = \Delta\Delta G_{es} = -4.0$ kcal/mol. Since the positively charged heme is in a hydrophobic environment in the protein, one would expect the Coulombic attraction to azide or cyanide to be greater than in the model system, where the heme can be solvated by water molecules.

Calculations were also carried out to suggest a reasonable magnitude for $\Delta\Delta G_{es}$. The theoretical model used has been proposed earlier to account for the redox potential differences between synthetic analogues and iron–sulfur proteins.[27] The change in free energy, ΔG_{es}, in both MbCN and OPCN contains an electrostatic term for charging the reactants in their respective microscopic dielectric mediums (Born equation) and a Coulombic term for the attraction between the two charged reactants. The values were −22 kcal/mol for MbCN and −9 kcal/mol for OPCN. Therefore, the predicted value for $\Delta\Delta G_{es}$ is −13 kcal/mol. It should be noted that the calculations were not refined by using a variety of different reactant radii or trying different values for the various dielectric constants. Instead, reasonable numbers for the radii were selected from crystallographic data.[4,22] Effective microscopic dielectric constants were calculated by using the Kirkwood–Westheimer equation.[28] These calculations, although rather crude, do support the more reliable value of $\Delta\Delta G_{es} = -4.0$ kcal/mol extracted from the azide equilibrium data.

The differences in free energies of ligation for the cyanide complexes, $\Delta\Delta G_{CN} = \Delta G_{MbCN} - \Delta G_{OPCN} = \Delta\Delta G_i + \Delta G_s + \Delta\Delta G_{es} = -3.0$ kcal/mol, lead to an estimate of ΔG_s by making the appropriate substitution of −4.0 kcal/mol for $\Delta\Delta G_{es}$, provided $\Delta\Delta G_i$ is small or equal to zero. The intrinsic free energies of MbCN and OPCN are probably similar even though their geometries are not, because cyanide is a poor π acceptor and its distorted bent orientation in the protein should not greatly affect the strength of the more important σ bond. This notion seems reasonable when the IR stretching frequencies of MbCN (ν_{CN} 2125 cm^{-1})[29] and that of an appropriate model system (monopyridinemonocyanohemin with ν_{CN} 2125 cm^{-1})[30] are taken into account. The free energy of these unfavorable steric interactions, ΔG_s, then is 1 kcal/mol.

The free energies of forming the CO complexes of Mb and MP, the undecapeptide precursor to octapeptide, determined by others[16,19] can be analyzed the same way. Electrostatic free energies are zero, since the reactants are neutral. Using $\Delta G_s = 1$ kcal/mol in the expression $\Delta\Delta G_{CO} = -\Delta G_{MbCO} = \Delta\Delta G_i + \Delta G_s = 2.3$ kcal/mol affords $\Delta\Delta G_i = 1.3$ kcal/mol. The intrinsic free energy in MbCO is higher than the model system. The available IR data for MbCO (ν_{CO} 1945 cm^{-1})[16,31] and other model systems (ν_{CO} 1970 cm^{-1})[32,33] suggest some difference in the metal ligand bond strengths. If CO tilting or bending in the protein reduces backbonding, the bond formed in MbCO would be weaker than the linear bonds in models, so $\Delta\Delta G_i$ would be positive. The IR data are inconsistent with this formulation unless electron-pair donation from the distal histidine into the CO π bond, as proposed by Caughey, is evoked.[6,29,33] An alternative explanation to the positive $\Delta\Delta G_i$ in this analysis is that additional free-energy terms have been overlooked and are contained in the final values of the intrinsic free energies, such as free energies of solvation in the model and the protein.

Kinetic Studies

The kinetics of ligand association and dissociation provide additional evidence for increased electrostatic attraction in MbCN. Table I contains the forward and reverse rate constants, k_{on} and k_{off}, for the Mb and OP reactions with cyanide. The difference in affinities lies in the extremely small k_{off} value for MbCN. Although the equilibrium constant is greater for MbCN than OPCN, the forward rate constant for OPCN exceeds that of MbCN. The large forward rate constant for OPCN implies that it has a more open binding site than the protein. A slow forward rate which is independent of cyanide and hydrogen cyanide concentration was also observed. Precedence exists for tailed porphyrins and other hemes reacting with linear ligands via a base elimination pathway.[34-36] Whether this mechanism is operative with the heme peptides is unclear at this point and requires additional study.

The enthalpy of activation for OPCN complex formation is reasonable compared to activation energies of substitution reactions in octahedral transition-metal complexes.[37] The positive entropy

(25) C. K. Chang and T. G. Traylor, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1166–1170 (1975).
(26) L. Stryer, J. C. Kendrew, and H. C. Watson, *J. Mol. Biol.*, **8**, 96–104 (1964).
(27) R. J. Kassner and W. Yang, *J. Am. Chem. Soc.*, **99**, 4351–4355 (1977).
(28) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506–512 (1938).
(29) S. McCoy and W. S. Caughey, *Biochemistry*, **9**, 2387–2393 (1970).
(30) W. S. Caughey, C. H. Barlow, D. H. O'Keeffe, and M. C. O'Toole, *Ann. N.Y. Acad. Sci.*, **206**, 296–309 (1973).
(31) J. O. Alben and W. S. Caughey, *Biochemistry*, **7**, 175–183 (1968).
(32) J. J. Wang, A. Nakahara, and E. E. Fleischer, *J. Am. Chem. Soc.*, **80**, 1109–1113 (1958).
(33) J. C. Maxwell and W. S. Caughey, *Biochemistry*, **15**, 388–396 (1976).
(34) J. Cannon, J. Geibel, M. Whipple, and T. G. Traylor, *J. Am. Chem. Soc.*, **98**, 3395–3396 (1976).
(35) J. Geibel, J. Cannon, D. Campbell, and T. G. Traylor, *J. Am. Chem. Soc.*, **100**, 3575–3585 (1978).
(36) D. K. White, J. B. Cannon, and T. G. Traylor, *J. Am. Chem. Soc.*, **101**, 2443–2454 (1979).

of activation is also expected, since the reaction entails two oppositely charged ions forming a neutral product. The release of tightly solvated water molecules from the ions upon forming the activated complex is the source of increased entropy and corresponds to a decrease in electrostriction.[38] The protein provides the positively charged heme with a hydrophobic environment, which increases the effective charge on the iron. This in turn is responsible for a greater attraction between the iron and cyanide and a subsequent decrease in the activation enthalpy relative to the model system. This increased Coulombic attraction is also manifested in the extremely small reverse rate constant for MbCN. Once the charge in the low dielectric hydrophobic pocket is neutralized by addition of cyanide to iron, the complex will only dissociate slowly.

The activation entropies in enzyme substrate kinetics are thought to be influenced by solvation and structural effects.[39] In enzyme substrate reactions where the substrate is an ion and subsequently neutralized, the activation entropy is usually positive. The negative activation entropy for the MbCN reaction suggests that the conformational contribution is dominant. Large negative activation entropies are common in protein substrate kinetics.[39] The conformational effects which contribute to negative activation entropies arise from the protein changing reversibly to a closed or rigid structure in the activated complex, which has fewer vibrational and rotational modes available. In the MbCN case, steric interactions of cyanide with the distal residues would produce a crowded environment in the heme pocket. The opening to the binding site must be enlarged, to release the metal-bound water and allow cyanide to enter, which would decrease the motion of the residues at the opening. A concerted mechanism of these events occurring simultaneously is unlikely. A more plausible explanation involves multiple reaction barriers, similar to those observed for MbCO.[40] The observed activation entropy in the present case could arise from a combination of negative and positive activation entropies of individual barriers. Nevertheless, a rigid conformation in the activated complex is indicated by the large negative activation entropy for MbCN formation. The exact nature of the "activated complex" cannot be determined, but the possibilities outlined above suggest a crowded environment around the binding site.

Conclusion

The increase in Coulombic attraction between cyanide and metmyoglobin accounts for the larger affinity constant of MbCN compared to the model system, OPCN. This effect is demonstrated in the equilibrium constant analysis for MbN_3 and OPN_3, in theoretical calculations, and in the kinetics of cyanide binding and dissociation (and the associated activation enthalpy). The large negative activation entropy for MbCN formation indicates that there is a large entropically unfavorable conformational change in forming the complex.

An estimate of the change in free energy due to steric interactions of linear ligands with myoglobin was obtained. This semiquantitative result implies that steric effects are smaller than previously thought.[9,2b] Analysis of MbCO and MPCO equilibrium data, using this estimate of the steric free-energy change, suggests the possibility of differences in the M-L bond strength due to these steric interactions.

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(37) F. Basolo and R. G. Pearson, "Mechanisms of Inorganic Reactions", Wiley, New York, 1967.
(38) K. J. Laidler, "Chemical Kinetics", 2nd ed., McGraw Hill, New York, 1965.
(39) K. J. Laidler and P. S. Bunter, "The Chemical Kinetics of Enzyme Action", Claredon Press, Oxford, 1973.
(40) R. H. Austin, K. W. Beeson, L. Eisentein, H. Frauenfelder, and I. C. Gunsalus, *Biochemistry*, **14**, 5355-5373 (1975).

Structural Study of the Vanadium Complex in Living Ascidian Blood Cells by X-ray Absorption Spectroscopy

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Abstract: The immediate coordination environment of vanadium in living ascidian blood cells (vanadocytes) has been studied by X-ray absorption spectroscopy. Analysis of the X-ray absorption edge data shows clearly that only a small amount (less than 10%) of the vanadium is present as the VO^{2+} ion in either living or spontaneously lysed vanadocytes. Parameters for curve-fitting analysis of V EXAFS were determined semiempirically from data on model complexes of known structure and have been tested for amplitude and phase transferability by using other structurally characterized V complexes. Analysis of the EXAFS data from the vanadocytes establishes that the vanadium in the living cells is present as V(III) ions surrounded in the first coordination sphere by a symmetric, single shell of low-atomic-weight scatterers. The data are best fit by a single shell of six oxygen atoms at an average distance of 1.99 Å. The EXAFS data show no evidence for the presence of VO^{2+}. Absence of a well-defined second shell of scatterers suggests that the vanadium complex in the living cells is simply aquovanadium(III), not associated with a ligand containing ordered, more distant shells (such as porphyrin or imidazole). Upon spontaneous lysis, little change in the vanadium coordination environment (as judged by the edge or EXAFS) is observed.

Studies of metal ions in biological systems have been successful in defining a wide variety of biochemical processes which make use of the special properties of metals.[1] However, one such system has confounded the efforts of several generations of scientists to discover its biochemical significance. This is the vanadocyte, the vanadium-sequestering cell of tunicates[2] (widely distributed, marine filter feeding animals classified within a subphylum of the Chordata[3]). Its nature is such that neither the function of this

(1) "Inorganic Biochemistry"; Eichorn, G. L., Ed.; Elsevier: New York, 1973.
(2) Biggs, W. R.; Swinehart, J. H. In "Metal Ions In Biological Systems"; Sigel, H., Ed.; Marcel Dekker: New York, 1976; Vol. 6, pp 141-196.

0002-7863/80/1502-5670$01.00/0