Mechanisms of Ligand Binding to Pentacoordinate Protoheme

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Abstract: Kinetic and equilibrium parameters for the binding of 11 alkyl isocyanides to protoheme mono-3-(1-imidazoyl)-propylamide monomethyl ester have been measured at 20 °C in benzene and in aqueous soap suspensions. The quantum yield for photodissociation of the isonitrile–heme complex is equal to 0.53 ± 0.09, is independent of solvent, and exhibits little dependence on ligand size. The rate constant for isonitrile dissociation (0.8–0.9 s^{-1}) is also independent of solvent and shows little dependence on the length of the alkyl side chain. In benzene, the association rate constant is equal to about 2×10^8 M^{-1} s^{-1} and again exhibits no dependence on ligand size. In contrast, the association rate constant measured in 2% myristyltrimethylammonium bromide in 0.1 M sodium phosphate, pH 7, increases from 1×10^7 M^{-1} s^{-1} to about 2×10^8 M^{-1} s^{-1} in going from methyl to *n*-butyl isocyanide. This effect is due to an increase in the equilibrium constant for the partitioning of the isonitrile between the micelle and aqueous phases. The observed rate of ligand binding to the pentacoordinate model heme also exhibits a dependence on soap concentration. Analysis of this dependence allows an evaluation of both the equilibrium partition constant and the bimolecular rate ($\sim 6 \times 10^6$ M^{-1} s^{-1}) within the micellar phase. Regardless of the exact interpretation, it is clear that the isonitriles react intrinsically more rapidly with the model heme than any of the smaller, gaseous ligands. This appears to be a result of the large dipole moment of the isocyano group, which facilitates interaction with the positively charged iron atom. However, when the heme is dissolved in soap solutions, this favorable effect of ligand polarity is offset by stabilization of the polar isocyano group in the outer hydrated regions of the micelle.

The high-resolution crystallographic structures of hemoglobin and myoglobin have provided the framework for examining the factors which govern the functional properties of iron atoms within these and other analogous heme proteins. Ligand binding is envisioned in terms of at least three distinct steps: (1) diffusion up to and penetration into the protein surface, (2) diffusion through the macromolecular structure until the sixth coordination position of the iron atom is reached, and (3) covalent bond formation with concomittant orbital rearrangements and changes in the coordination geometry of the proximal histidine residue. Unfortunately, quantitative assessments of the significance of these processes cannot be made from structural data alone. Several approaches have been taken to resolve this problem. Frauenfelder and co-workers have attempted to visualize the individual steps by measuring the rates of rebinding of ligand molecules which are trapped within various parts of the protein molecule after laser photolysis at low temperatures and high viscosities.[2] Traylor's, Collman's, and Basolo's groups have examined the innermost process directly by studying the ligand binding properties of synthetic pentacoordinate heme compounds.[3-5] Olson and Reisberg used a homologous series of alkyl isocyanides to assess the influence of ligand size and stereochemistry on the rates and equilibria of ligand binding to hemoglobin.[6]

The latter two approaches have been combined in this current study. Rate and equilibrium constants for carbon monoxide and alkyl isocyanide binding to protoheme mono-3-(1-imidazoyl)-propylamide monomethyl ester in benzene and in various soap suspensions have been measured directly by flash photolysis and stopped-flow rapid-mixing techniques. The parameters evaluated for binding to the model heme in benzene serve both as a test of the assumption of a homologous series for the isonitriles and as reference points for interpreting the data obtained with soap solutions and proteins. The reactions carried out with heme embedded in micelles are analogous to those observed for proteins. In both cases, preferential partitioning of the ligand molecules from an external aqueous phase into nonpolar, hydrocarbon regions must be considered when interpreting the observed rate and equilibrium data. The work presented for the model heme compounds in soap solutions has allowed us to quantitate this "hydrophobic" effect experimentally in the absence of any specific, protein steric hindrance effects.

Experimental Section

Hemes. Protoheme mono-3-(1-imidazoyl)propylamide monomethyl ester was prepared as described by Traylor et al.[3] A small amount of dried material was dissolved in 0.05–0.1 mL of methanol to give a final concentration of about 2 mM. Then microliter quantities of this stock solution were added to cuvettes or larger syringes containing anaerobic soap suspensions or benzene. Samples for flash photolysis experiments were prepared as follows. A 1-cm fluorescence cuvette was sealed with a serum stopper, flushed with nitrogen, and then filled with about 4 mL of anaerobic solvent so that there was little or no gas phase. For the experiments in soap solutions, approximately 1 mg of dry sodium dithionite was added to the cuvette prior to sealing it and adding the liquid. When the hemin was added to these cuvettes, it was reduced immediately, and the spectrum of the pentacoordinate protoheme was recorded in the Soret wavelength region (λ_{max} = 430 nm, ϵ = 114 mM^{-1} cm^{-1}).[3] The addition of isonitrile produced a sharpening of the Soret peak with λ_{max} = 428 nm and ϵ = 167 mM^{-1} cm^{-1}. The binding of all the isonitriles was extremely tight so that simple titrations could not be used to estimtae equilibrium constants (Table I). Below the equivalence point, all the isonitrile added was bound by the heme compound. Reduction of the heme in benzene was more difficult and precluded titration experiments. Two complexes of dithionite were tried as nonaqueous reducing agents. Initially we used the 18-crown-6 ether (Aldrich) complex prepared in methanol as described by Mincey and Traylor.[7] In later experiments, the Kryptofix 2,2,2 (MCB) complex in methanol was used.

Ligands. Isonitriles were synthesized and stock solutions prepared as described by Reisberg and Olson.[6a,c] The solubilities of the compounds were determined by the following procedure. About 0.5 mL of 0.1 M phosphate buffer at pH 7.0 was added to an equal volume of pure isonitrile and then placed in a glass vial which was sealed with a Teflon washer and a screw cap. The sample was shaken vigorously for 5–10 min and then allowed to stand for 1–2 h at 20 °C. Aliquots were withdrawn

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Table I. Rates of Ligand Binding to Protoheme Mono-3-(1-imidazoyl)propylamide Monomethyl Ester in 2% Myristyltrimethylammonium Bromide, 0.1 M NaP_i, pH 7.0, and in Benzene, both at 20 °C, and Solubilities of the Ligands in 0.1 M Sodium Phosphate, pH 7.0[a]

	2% C_{14}-$N(CH_3)_3^+$			benzene			solubility data		fitted micelle parameters		
ligand	k', $\times 10^{-8}$ M^{-1} s^{-1}	k, s^{-1}	K, $\times 10^{-8}$ M^{-1}	k', $\times 10^{-8}$ M^{-1} s^{-1}	k, s^{-1}	K, $\times 10^{-8}$ M^{-1}	S, M	$K_{partition}$	K_p	$k_{m'}$, $\times 10^{-8}$ M^{-1} s^{-1}	$K_{m'}$, $\times 10^{-8}$ M^{-1}
				A. Isonitriles							
methyl	0.11	0.91	0.12	2.0	0.95	2.1	2.4	7.6	1.8	0.056	0.062
ethyl	0.25	0.79	0.32	1.9	1.2	1.6	0.78	17	4.1	0.066	0.087
n propyl	0.59	1.0	0.59	2.1	0.89	2.4	0.21	51	11.8	0.063	0.063
n-butyl	1.5	0.76	2.0	2.0 (2.2)[b]	0.78 (0.5)[b]	2.6 (4.4)[b]	0.056	160	31.8	0.068	0.089
n-amyl	1.8	0.74	2.4	2.0	0.70	2.9	0.030	260	89.2	0.051	0.069
n-hexyl	1.5	0.64	2.3	1.7	0.72	2.4	0.0049	1410	26.1	0.037	0.058
iso-propyl	0.60	1.5	0.40	1.8	1.2	1.5	0.207	53	9.1	0.076	0.051
tert-butyl	1.1	1.8	0.61	1.4	1.6	0.9 (1.7)[b]	0.088	98	14.7	0.095	0.053
isobutyl	1.1	0.88	1.25				0.066	140	31.8	0.056	0.064
± *sec*-butyl	1.2	1.5	0.80				0.070	135	24.6	0.072	0.048
c-hexyl	1.9	0.90	2.11						68.9	0.065	0.072
benzyl	2.1	0.22	9.55						55.6	0.079	0.360
				B. Gases							
CO	0.027 (0.036)[c]	0.009[c]	3.0	0.082 (0.11)[b]	(0.025)[b]	3.3	0.00096	12.2[e]	3.9	0.009	1.0
O_2	0.26[c] (0.14)[d]	47[c] (150)[d]	0.0055 (0.0009)[d]	(0.35)[d]	(2500)[d]	(0.00014)[d]	0.00125				

[a] Association rate constants were obtained from the slopes of plots of k_{obsd} vs. ligand concentration as described in Figure 2. Dissociation rate constants were obtained from the analysis of CO replacement time courses. The equilibrium constants were computed from the ratio of the association and dissociation rates. The solubility, S, represents the concentration of isonitrile present in the aqueous phase when pure liquid is equilibrated with 0.1 sodium phosphate buffer. $K_{partition}$ was calculated from S as described in the text. K_p represents the partition constant between the micelle and aqueous phase taken from Figure 4; $k_{m'}$ is the fitted (CO through nHNC) or calculated (iPNC through BzNC) bimolecular rate constant inside the micelle, using eq 6, and $K_{m'}$ is the association equilibrium constant within the micelle phase which was calculated from $k_{m'}/k$. [b] Data in parentheses from ref 11. [c] Traylor, T. G.; Berzinis, A. *Proc. Natl. Acad. Sci. U.S.A.* 1980, *77*, 3161-3175. [d] Data for mesoheme derivative in 2% cetyltrimethylammonium bromide and toluene: Chang, C. K.; Traylor, T. G. *Ibid.* 1975, *72*, 1166-1170. [e] Computed from the ratio of the solubilities of 1 atm of CO in *n*-heptane (0.0117 M) and in water (0.0096 M): Gjaldbaek, J. C. *Acta Chem. Scand.* 1952, *6*, 623-633.

from the lower aqueous phase and assayed directly or diluted further into more phosphate buffer, depending on the solubility of the compound being examined. The concentration of isonitrile (S in Table I) was measured by titrating a sample of the reduced model heme in soap with aliquots or dilutions taken from the aqueous phase in the partitioning vial.

Kinetic Experiments. Rapid-mixing experiments were carried out with a Gibson-Durrum stopped-flow apparatus equipped with an On-Line Instrument Systems Model 3820 data collection system. The flash photolysis apparatus has been previously described.[6b,8] For the quantum yield experiments, a 0.5-ms pulse width was used to achieve maximum photolysis. In all cases, time courses from 4 to 8 separate flashes were averaged and then stored on diskettes for further analysis.

Soaps. Sodium dodecyl sulfate was purchased from Sigma, and the three trimethylammonium bromide soaps, dodecyl, myristyl, and cetyl, were obtained from Aldrich.

Results

Quantum Yield Determinations. Gibson and Ainsworth[9] measured a quantum yield of 0.05 for the ethyl isocyanide complex of hemoglobin which is only slightly greater than the value for oxyhemoglobin. In a more recent paper, Brunori et al.[10] reported that the quantum yields for myoglobin isonitrile complexes depend markedly on the nature of the alkyl side chain. The measured yields for ethyl, *n*-propyl, isopropyl, and *n*-butyl isocyanide sperm whale myoglobin were 0.04, 0.18, 0.08, and 0.32, respectively.[10] Traylor and Stynes suggested that the quantum yield for the model pentacoordinate protoheme compound in benzene was too low to allow direct photolysis of the *n*-butyl isocyanide complex.[11] In view of these previous results, we felt that a systematic quantum yield study would be appropriate.

The pulse method described by Brunori and co-workers was employed using the photolysis apparatus described in the Experimental Section.[10,12] The rate of change of the heme ligand complex, HX, during the flash is given by eq 1, where X and H

$$d(HX)/dt = k'(X)(H) - (k + \epsilon Iq)(HX) \quad (1)$$

represent free ligand and pentacoordinate heme, and k' and k, the association and dissociation rate constants. ϵIq is the rate of

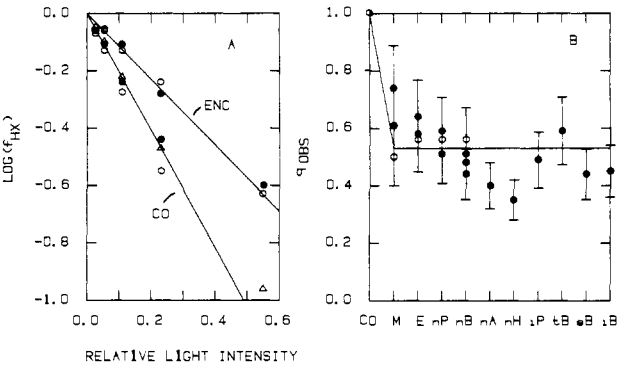

Figure 1. Quantum yield determinations for isonitrile–protoheme mono-3-(1-imidazoyl)propylamide monomethyl ester complexes in benzene and 2% myristyltrimethylammonium bromide in 0.1 M Na phosphate, pH 7, 20 °C. A, dependence of the fraction of complex remaining after the flash on the relative light intensity of the pulse: (●) data for the ethyl isocyanide (ENC) and CO complexes in 2% soap; (○) data for the complexes in benzene; (△) data for CO–myoglobin (sperm whale) in 0.1 M Na phosphate pH 7. B, dependence of the quantum yield, q, on ligand size. Ligand abbreviations are M, methyl; E, ethyl; nP, *n*-propyl; nB, *n*-butyl; *n*A, *n*-amyl (pentyl); nH, *n*-hexyl; iP, isopropyl; tB, *tert*-butyl; sB, *sec*-butyl; and iB, isobutyl isocyanide. The error bars represent estimates of the uncertainties of the slopes obtained from plots analogous to those shown in A: (○) data measured in benzene; (●) data measured in 2% soap.

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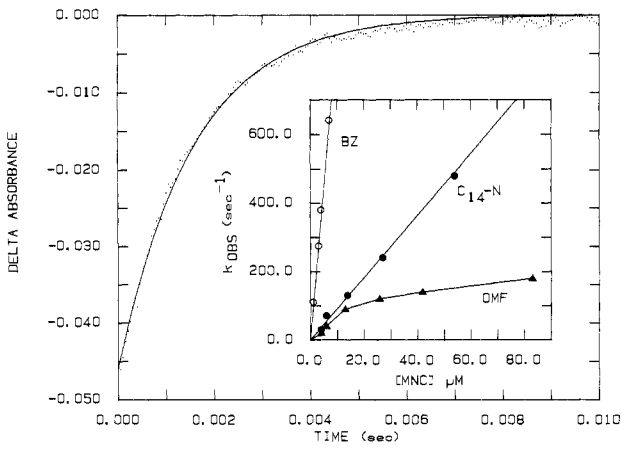

Figure 2. Time course for methyl isocyanide binding to pentacoordinate protoheme in benzene, 20 °C. The reaction was followed at 428 nm after flashing a solution containing 1.5 μM heme and 5.0 μM isonitrile. The trace represents the average of six separate flashes. The absorbance changes are given as dots (200 points) and the solid line represents a fit to a single exponential with $k_{obsd} = 640\ s^{-1}$. As shown, the fitted curve is superimposable on the real data. The inset shows the dependence of the observed, pseudo-first-order association rate, k_{obsd}, on free methyl isocyanide concentration (μM) for three different solvents: DMF, dimethylformamide; C_{14}–N, 2% myristyltrimethylammonium bromide in 0.1 M phosphate pH 7; and Bz, benzene.

photodissociation, where ϵ is the extinction coefficient, I the light intensity, and q the observed quantum yield. If I is large, the k term can be neglected, and if (X) is kept small, recombination during the flash pulse can be neglected. Under these conditions, eq 1 is readily integrated and the fractional amount of complex remaining after the flash, f_{HX}, is given by eq 2, where t is the time

$$\ln f_{HX} = -\epsilon I q t \tag{2}$$

of the flash pulse. In these experiments it is difficult to evaluate the absolute value of ϵI. Instead $\ln f_{HX}$ is plotted vs. the relative intensity of the flash, and the slope of the line for the unknown complex is compared with that for CO–myoglobin which exhibits a known quantum yield of 1.0.[13] The intensity of the flashing light was attenuated by the use of suitable neutral density filters. A set of results for CO and ethyl isocyanide heme complexes is shown in Figure 1A.

In the experiments with model heme compounds very dilute solutions were required in order to prevent rebinding during the flash pulse. Thus, the absolute values of the absorbance changes were small to begin with and decreased further as the flashing light intensity was reduced. As a result, the quantum yield computed from the relative slopes (see Figure 1B) exhibit an error of about ±20%. As shown in Figure 1, the value of q is independent of solvent condition and exhibits little dependence on ligand size, particularly when compared to the myoglobin results of Brunori et al.[10] The average value for all the isonitriles tested, regardless of solvent, was 0.53 ± 0.09.

Association and Dissociation Rate Constants. A typical time course for methyl isocyanide binding to pentacoordinate protoheme in benzene is shown in Figure 2. The dependence of the observed rate on ligand concentration is shown in the inset. The bimolecular rate in benzene is very large, $2 \times 10^8\ M^{-1}\ s^{-1}$ (Table I), whereas that in 2% myristyltrimethylammonium bromide is 20-fold lower, $1 \times 10^7\ M^{-1}\ s^{-1}$. Dimethylformamide also causes a reduction in the reaction velocity, but the observed rates do not exhibit a linear dependence on ligand concentration. In this case, ligand binding appears to involve the dissociation of weakly bound solvent molecules followed by the bimolecular combination of the isonitrile with the heme iron atom. Benzene and toluene appear to be the only suitable organic solvents for these kinetic studies. The more commonly used solvents such as dimethyl sulfoxide and dimethylformamide are weak ligands, and heme is insoluble in more inert aliphatic liquids.

A summary of the measured association rate constants is presented in Table I. As shown, the rates measured in benzene exhibit little or no dependence on ligand size and are 10–100 times greater than the rates of O_2 and CO binding. This is in marked contrast to the situation with proteins where the isonitrile association rates are always significantly less than those of the diatomic gases.[6,14] In 2% myristyltrimethylammonium bromide, the situation is more complex. The association rate increases monotonically from 1×10^7 to $\sim 2 \times 10^8\ M^{-1}\ s^{-1}$ in going from methyl to *n*-amyl isocyanide.

Dissociation rate constants were measured by mixing the isonitrile heme complex with a high concentration of carbon monoxide in the stopped-flow apparatus. The observed rate of replacement, r, is given by eq 3, where k' and k represent the

$$r = \frac{kl'(\mathrm{CO}) + lk'(\mathrm{RNC}) + kl}{l'(\mathrm{CO}) + k'(\mathrm{RNC}) + k} \tag{3}$$

isonitrile association and dissociation rate constants, respectively, and l' and l, the corresponding carbon monoxide rate constants.[15] In most of our experiments the CO concentration was very high (0.48 mM in the soap solutions, 3.8 mM in benzene after mixing), and the free concentration of isonitrile was kept as small as possible (equal to the heme concentration, ≤0.002 mM). Under these conditions r equals k, the isonitrile dissociation constant. The validity of eq 3 was tested for the case of *n*-butyl isocyanide binding by increasing the free isonitrile concentration. As expected, the replacement rate decreased markedly with increasing (RNC)/(CO), and the dependence corresponded quantitatively with that predicted by eq 3.

A summary of the dissociation rate constants is also presented in Table I. In contrast to association constants, the dissociation rates are independent of solvent conditions, invariant with soap concentration (from 2 to 8% myristyltrimethylammonium bromide), and only weakly dependent on the structure of the alkyl side chain. The *n* series of compounds exhibit an average rate of about 0.8–0.9 s^{-1}; α substitution causes a monotonic increase to about 1.7 s^{-1} in going from ethyl to *tert*-butyl isocyanide; and benzyl isocyanide exhibits an abnormally low value, 0.22 s^{-1}, indicating some type of specific effect due to the aromatic side chain.

Dependence of the Association Rate on Soap Concentration. As shown in Table I, there is an almost linear dependence of log (k') on side chain length when the rates are measured in 2% myristyltrimethylammonium bromide. This is most readily explained in terms of partitioning of the ligand molecules into the micellar phase of the suspension. The larger ligands exhibit a greater partition constant so that their local concentration in the micelle is significantly greater than in the bulk solution. The net result is a larger measured association rate constant. This "hydrophobic" effect can be quantitated if the rates of partitioning between the aqueous and hydrocarbon phases are much greater than the bimolecular processes within the micelle. The mechanism is described by the following scheme:

$$X_w \overset{K_p}{\rightleftharpoons} X_m + H_m \xrightarrow{k_m'} HX_m \tag{4}$$

(13) This method assumes that the amount of exciting light absorbed by the unknown sample is the same as that absorbed by CO–myoglobin (ref 12). In our experiments only light at wavelengths greater than 500 nm was used for photolysis. In this region the spectrum of the model protoheme–CO complex is very similar to that of myoglobin. Comparisons between isonitrile and carbon monoxide derivatives are also readily made. Even though the α and β band peaks for the isonitrile complexes are blue shifted by about 8 nm, the ratio of the integrated absorbances, $\int \epsilon(\lambda) d\lambda$ for isonitrile vs. CO derivatives, is 1.03 ± 0.05 in the 500–700-nm region. Thus, in Figure 1B we have presented simply the ratio of the slopes obtained from plots of $\ln f_{HX}$ vs. relative light intensity. In the original work of Bücher and Kaspers (Bücher, T.; Kaspers, J. *Biochim. Biophys. Acta* **1947**, *1*, 21–34), the quantum yield of CO–myoglobin was reported to be 0.91 ± 0.03 for exciting light at 546 nm and 1.02 ± 0.03 for light below 400 nm. Most recent workers have assumed that the quantum yield for MbCO is unity and independent of wavelength, but the possibility that the model protoheme–CO complexes could exhibit q values slightly greater than that for MbCO should be kept in mind. The ±20% relative error in our work precludes any distinctions between these derivatives.

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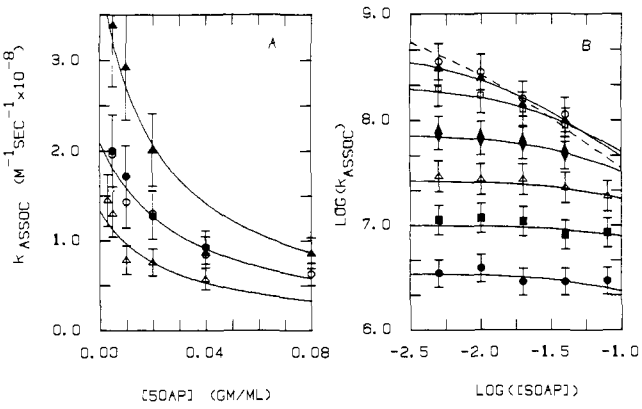

Figure 3. Dependence of the observed association rate constants on soap concentration. A, rate constants for the binding of *n*-butyl isocyanide to pentacoordinate protoheme in sodium dodecyl sulfate (▲) and three trimethylammonium soaps: dodecyl (○), myristyl (●), and cetyl (△). The solutions were made in 0.1 M sodium phosphate, pH 7. The solid lines represent fits to eq 6 as described in the text. The fitted parameters: $K_p = 37$, $k_m' = 3.6 \times 10^6$ M^{-1} s^{-1} for △; $K_p = 32$, $k_m' = 6.6 \times 10^6$ M^{-1} s^{-1} for ○ and ●; and $K_p = 43$, $k_m' = 9.1 \times 10^6$ M^{-1} s^{-1} for ▲. B, rate constants for CO and isonitrile binding in myristyltrimethylammonium bromide solutions: (●) CO; (■) MNC; (△) ENC; (♦) nPNC; (□) nBNC; (▲) nANC; and (○) nHNC. The dashed line was added for clarity and represents the theoretical curve for nHNC. All the lines represent fits to eq 6 as described in the text. The resultant parameters are listed in Table I. The error bars in both panels represent 20% uncertainty in the values of the rate constants.

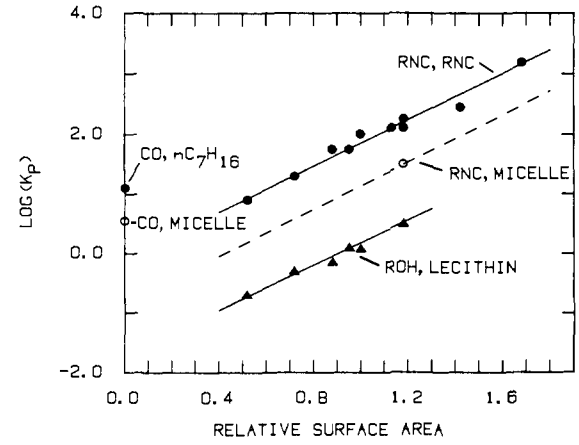

Figure 4. Dependence of the equilibrium partition constant on the relative surface area of the alkyl side chain. The upper curve (●, RNC, RNC) represents K_p values computed from the solubility data in Table I as described in the text. The slope of the fitted line is 1.87 which corresponds to a free energy change at 20 °C of −2.50 kcal/mol per unit increase in surface area. The lower curve (▲, ROH, lecithin) represents K_p values reported by Katz and Diamond[19] for the partitioning of alcohols into dimyristylphosphatidylcholine at 20 °C. The slope of this curve is 1.73 which corresponds to −2.31 kcal/mol per unit surface area. The open circles represent K_p values obtained by fitting *n*-butyl isocyanide and CO binding data to eq 6 (Figure 3 and Table I). The dashed line represents the assumed dependence of K_p on alkyl surface area for partitioning of the isonitrile ligands between aqueous and myristyltrimethylammonium micelle phases. The relative surface areas for the various ligand side chains were taken from Reisberg and Olson.[6a]

where X represents the ligand molecule, H the model heme, the subscripts w and m the water and micelle phases, K_p the partition constant defined as $(X)_m/(X)_w$, and k_m' the apparent association rate constant within the micelle. The rate of change of free heme concentration is given by $d(H)_m/dt = -k_m'(H)_m(X)_m$. Since all of the heme is inside the micelles, the subscripts for $(H)_m$ can be dropped and the overall, observed rate constant evaluated from $k_m'(X)_m$.

The total ligand concentration is equal to $f_w(X)_w + f_m(X)_m$, where f_w and f_m represent the fractional volumes of the aqueous and soap phases. The fractional volume of the soap phase is given by V_mC_m, where V_m is the partial specific volume of the soap and C_m is its concentration in g/mL; f_w is then given as $1 - V_mC_m$. Granath[16] measured the partial specific volumes for a variety of soaps including sodium lauryl sulfate and cetyltrimethylammonium bromide. The former exhibited $V_m = 0.89$ mL/g in 0.2 M NaBr and the latter 1.01 mL/g under the same conditions. We have assumed $V_m = 1.0$ mL/g for all of the soaps used in this study. The concentration of ligand in the aqueous phase is given by $(X)_w = (X)_m/K_p$. Rearranging these expressions yields

$$(X)_m = \frac{K_p(X)_t}{1 + (K_p - 1)V_mC_m} \quad (5)$$

where $(X)_t$ is the total solution concentration of ligand. The expression for the observed second-order rate constant is obtained by substituting eq 5 into the differential equation for the rate of change of free pentacoordinate heme.

The rate of *n*-butyl isocyanide binding was examined as a function of soap concentration in several surfactant solutions, and the results were analyzed in terms of eq 6 and are shown in Figure

$$k_{obsd}' = \frac{k_m'K_p}{1 + (K_p - 1)V_mC_m} \quad (6)$$

3A. The agreement between the observed and predicted dependence on soap concentration is reasonably good. The concentration of soap which can be used is limited to values greater than the critical micelle concentration and less than the concentration at which insoluble lamellar structures are formed. For myristyltrimethylammonium bromide in 0.1 M NaP_i pH 7.0, this corresponds to a concentration range of about 0.002–0.10 g/mL.[17] The fitted values of K_p are 30–40 so that within the available range only a limited, but certainly measurable, dependence on soap concentration is observed. Thus, although the product $k_m'K_p$ is well-defined, the individual values of the constants exhibit some uncertainty.

As shown in Figure 3A, there is some dependence of the rate of *n*-butyl isocyanide binding on the structure of the surfactant molecule. For the quaternary amines, the K_p and k_m' values are unchanged when the alkyl chain is increased from 12 to 14 carbon atoms. The addition of two more carbons does cause k_m' to decrease from 6.8×10^6 M^{-1} s^{-1} to 3.6×10^6 M^{-1} s^{-1}. The internal bimolecular rate increases by 30–40% when sulfate is substituted for the trimethylammonium group. However, none of these differences are as great as the effect of increasing the length of the alkyl side chain of the ligand molecule (Figure 3B).

Assignment of Partition Constants. The soap concentration dependence for CO binding and the binding of the *n* series of isonitriles was also examined and analyzed in terms of eq 6. For the smaller compounds which exhibit low K_p values, very little dependence on C_m is observed and it is difficult to define the individual constants since $k_{obsd}' \simeq k_m'K_p$. In order to circumvent this problem, we have assigned values for K_p based on other experimental data and the fitted results for *n*-butyl isocyanide binding.

The solubilities of the isonitriles in 0.1 M phosphate pH 7 were measured as described in the Experimental Section. These values were used to compute partition constants between the neat liquid and the aqueous phase (Table I). The concentration of ligand in the organic phase was calculated from the molecular weight of the compound and its density.[6c] As shown in Figure 4, there is a linear correspondence between log $K_{partition}$ and the relative surface area of the alkyl side chain. We have used the areas computed by Harris et al.,[18] which assign a value of 1.0 to the *tert*-butyl group.[6a] The lower curve in Figure 4 represents data

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for the partitioning of short chain alcohols into dimyristylphosphatidylcholine.[19] Although the absolute values of the partition constants for the alcohols are about 2 orders of magnitude lower, the slope of the line fitted to these data is nearly identical with that obtained from the isonitrile solubility data. The partition constant obtained from fitting the *n*-butyl isocyanide–heme binding data to eq 6 is about 5 times lower than that for partitioning between buffer and the pure liquid. This difference is not unexpected; Katz and Diamond[19] also observed a decrease in K_p when partitioning between water and simple hydrocarbon phases was compared to that between water and lecithin. The micelles and membranes contain hydrated, charged outer layers and more solid inner phases which partially inhibit the uptake of simple organic molecules.

Although the absolute value of K_p may vary, its dependence on the surface area of the side chain appears to remain the same. Consequently, we used the slope of the upper curve in Figure 4, the relative surface areas of the side chains,[6a,18] and the fitted value for *n*-butyl isocyanide binding to compute K_p for all the isonitriles examined (dashed line in Figure 4 and Table I). Then the kinetic data in Figure 3B were fitted to eq 6 to obtain values of k_m', the bimolecular rate constant within the micelle, for the *n* series of ligands. The value of k_m' is (6–7) × 10^6 M^{-1} s^{-1} and independent of size for the smaller ligand molecules. There is a significant decrease in going from *n*-butyl to *n*-hexyl isocyanide which accounts for the fact that the observed rates for the *n*-amyl and *n*-hexyl compounds are about the same even though their partition constants differ by a factor of 2.9. The k_m' values for the substituted isonitriles were computed from eq 6 using the observed second-order rate constants in 2% myristyltrimethylammonium bromide and the K_p values listed in Table I. Again, little difference between the reactivities in the micellar phase is observed with changing size or shape.

The parameters for CO binding are also shown in Table I and Figure 4. The equilibrium constant for the partitioning of carbon monoxide between *n*-heptane and water is 12.2 ($K_{partition}$, Table I) which is about 1.6 times greater than the corresponding value for methyl isocyanide. Thus, it is clear that the isonitrile group is intrinsically much more polar than CO. If their polarities were the same, the hydrocarbon partition constant for carbon monoxide would be expected to be about 5 times less than that for methyl isocyanide since the surface area of the diatomic gas is roughly half that of methyl isocyanide. The fitted value of K_p for carbon monoxide binding to heme dissolved in micelles is also greater than that for methyl isocyanide. The absolute value, 3.9, is quite close to the value of 4.1 reported for the partitioning of CO between water and olive oil.[20] As expected from the lower observed rates, k_m' for CO binding is about 6 times smaller than k_m' for isonitrile binding. Thus, in both benzene and the micelles the association rates for CO binding are considerably smaller than those for the isonitriles (Table I).

Discussion

Interpretation of Equilibrium Constants. Equilibrium constants for isonitrile binding to the protoheme compound in benzene, K_{Bz}, were calculated from the observed association and dissociation rate constants. The value of K_{Bz} shows little dependence on ligand size and reflects primarily the strength of the iron–isocyano bond (Figure 5). In this solvent, the affinity of the heme group for isonitriles is nearly equal to that for carbon monoxide (Table I).

The situation in soap solutions is more complex. The observed association rate constant depends on both soap concentration and the size of the ligand molecule. In contrast, the dissociation rate constant is invariant. The variation in the observed association rate constant has been interpreted in terms of the two-step binding process described by eq 5 and 6. These equations apply in principle to the situation for ligand binding to proteins. The major difference is that the fractional volume of the protein phase in most

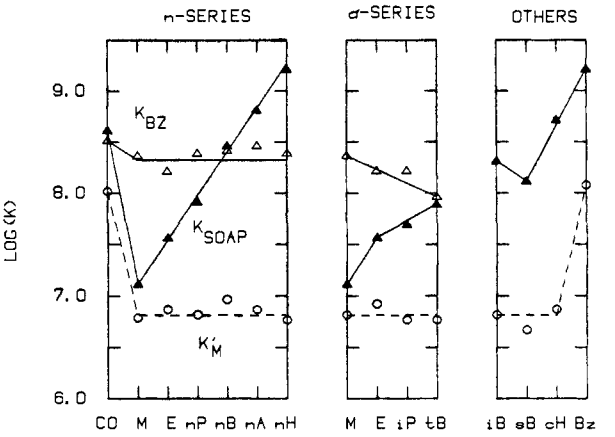

Figure 5. Dependence of equilibrium association constants on ligand size and stereochemistry: (△) K_{Bz}, constants for ligand binding to pentacoordinate protoheme in benzene; (▲) K_{soap}, constants for ligand binding in myristyltrimethylammonium micelles extrapolated to infinitely dilute soap concentration ($K_pK_M' = K_{soap}$, Table I); (○) K_M', constants for ligand binding within the soap micelle phase. Ligand abbreviations are listed in Figure 1.

experiments is quite small. In the case of soap solutions, the concentration of surfactant must be kept above the critical micelle concentration so that V_mC_m is always greater than about 0.02. In order to compare the data obtained for soap solutions with those measured for proteins, the values of the apparent association rate constants at infinitely small C_m were computed from $k_m'K_p$. K_{soap} is defined as the equilibrium association constant under this condition and is given by $k_m'K_p$ divided by k, the observed dissociation rate constant which is independent of solvent composition. The equilibrium association constant for isonitrile binding within the micelle phase, K_m', is defined as k_m'/k.

As shown in Figure 5, the linear dependence of K_{soap} on ligand size is entirely a result of the dependence of the micelle–water partition constant on the surface area of the ligand side chain. K_m' remains constant for all of the isonitriles except the benzyl compound. The latter ligand exhibits a large observed equilibrium constant due to an abnormally low dissociation rate (Table I). Reisberg and Olson also observed abnormally high affinity constants for benzyl isocyanide binding to hemoglobin and its subunits.[6] In benzene, there is a small decrease in the affinity constant with increasing α substitution on the alkyl side chain. This result is due to a 2-fold increase in the dissociation rate in going from ethyl to *tert*-butyl isocyanide. It is not clear whether this small effect is due to steric interactions or to inductive effects arising from the additional carbon–carbon bonds near the isonitrile group. In soap solutions, an opposite result is observed; the apparent affinity constant, K_{soap}, increases with increasing substitution due to the "hydrophobic" effect.

The most surprising result in Figure 5 is the 30-fold decrease in affinity for isonitrile which is observed when the model heme is taken out of benzene and put into the soap micelle (K_M' vs. K_{Bz}). Only a 3-fold decrease is observed for CO binding so that it is improbable that the effect is due to a large change in the intrinsic affinity of the heme group. The markedly higher affinity of pentacoordinate heme in micelles for CO as compared to that for the isonitriles is analogous to the situation observed for proteins.[6,14] The similarity of the affinity constants for CO and the isonitriles in benzene argues against any major intrinsic differences in the stability of the two types of iron–ligand bonds. The most likely explanation of the reduced isonitrile affinity in soap solutions is that these ligands behave as weak surfactants and interact preferentially with the outer hydrated layers of the micelle structure. The dipole moments of the isonitriles are about 3.85 D,[21] which makes them as a group vary polar molecules. The smaller gaseous ligands such as O_2, NO, and CO are essentially

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nonpolar, exhibiting dipole moments of 0, 0.15, and 0.11, respectively.[21] Stephany et al. have reported that the ^{13}C chemical shift of the isonitrile carbon atom is altered in the presence of water and have attributed this change to hydrogen bonding.[22] This also suggests that alkyl isocyanides will be oriented in the micelles with the isonitrile group adjacent to the surface and the alkyl side chain interacting with the hydrocarbon interior. Thus the apparent micellar affinity, K_m', will be attenuated by a factor representing the extent of partitioning of the isocyano group between the outer hydrated regions of the micelle and the inner hydrocarbon core. Again, it should be emphasized that similar phenomena occur in the case of ligand binding to proteins. The polar isonitriles behave as weak amphipaths and are stabilized near the surface whereas the apolar diatomic ligands exhibit a more uniform concentration gradient throughout the protein structure.

Conclusion

Regardless of the exact mechanistic details, it is clear that the isonitriles react intrinsically more rapidly with heme iron than any of the smaller gaseous ligands (Table I, benzene results). The most plausible explanation of this result is that the polar nature of the isocyano group facilitates the bimolecular binding process. The magnitude of the dipole moment of these ligand molecules indicates a partial negative charge of about −0.7 e on the terminal carbon atom. This value coupled with the partial positive charge on the iron atom suggests that electrostatic considerations alone could easily account for the 30-fold increase in the observed association rate in going from carbon monoxide to methyl isocyanide. This is particularly true when the reactions are carried out in benzene, which has a very low dielectric constant. In the case of heme dissolved in soap solutions, this favorable effect of the dipole moment is attenuated by stabilization of the polar isocyano group in the outer hydrated regions of the micelle structure. As a result, the bimolecular rate constant for isonitrile binding within the micellar phase, k_m' in eq 6, is more nearly equal to that observed for carbon monoxide binding (Table I).

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Registry No. Protoheme mono-3-(1-imidazoyl)propylamide monomethyl ester, 72177-42-5; sodium dodecyl sulfate, 151-21-3; dodecyltrimethylammonium bromide, 1119-94-4; myristyltrimethylammonium bromide, 1119-97-7; cetyltrimethylammonium bromide, 57-09-0; methyl isocyanide, 593-75-9; ethyl isocyanide, 624-79-3; *n*-propyl isocyanide, 627-36-1; *n*-butyl isocyanide, 2769-64-4; *n*-amyl isocyanide, 18971-59-0; *n*-hexyl isocyanide, 15586-23-9; isopropyl isocyanide, 598-45-8; *tert*-butyl isocyanide, 7188-38-7; isobutyl isocyanide, 590-94-3; *sec*-butyl isocyanide, 14069-89-7; cyclohexyl isocyanide, 931-53-3; benzyl isocyanide, 10340-91-7; CO, 630-08-0.

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Applications of Molybdenum-95 NMR Spectroscopy. 7.† Studies of Metal–Metal Bonded Systems Including Aqueous Molybdenum(IV) and Molybdenum(V). Crystal and Molecular Structure of $Na_2[Mo_3O_4((O_2CCH_2)_2NCH_3)_3]\cdot 7H_2O$

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Abstract: Solution ^{95}Mo NMR studies are reported on spin-coupled polynuclear systems of Mo(V), Mo(IV), and Mo(II). Resonances occur at low fields compared to mononuclear species. The chemical shifts of the Mo(IV)–aquo ion in 4 M *p*-toluenesulfonic and methanesulfonic acid media and those of the Mo(IV) complexes containing oxalate, EDTA, and methyliminodiacetate ligands (whose solid-state structures are based on the $[Mo_3O_4]^{4+}$ cluster) fall in the narrow range of 172 ppm spanning 990–1162 ppm. As the known chemical shift scale for the ^{95}Mo nucleus covers 7000 ppm, this observation indicates that the ^{95}Mo nucleus is in a similar chemical environment in each of the species examined and, taken with published evidence, confirms formulation of the Mo(IV)–aquo ion as $[Mo_3O_4(H_2O)_9]^{4+}$. Two resonances are detected in the above range for $Mo(IV)_{aq}$ in 4 M hydrochloric acid and for $[(Mo_3O_4)_2(PDTA)_3]^{4-}$. Additional resonances appear at 539–608 ppm in the methanesulfonic acid, hydrochloric acid, and EDTA systems when stored in air. These are assigned to $[Mo^V_2O_4]^{2+}$-based species by comparison with the observed resonances of the Mo(V)–aquo ion, $[Mo^V_2O_4(H_2O)_6]^{2+}$, in the relevant acid media and with $[Mo^V_2O_4(EDTA)]^{2-}$ in H_2O. The $[Mo^V_2O_4(PDTA)]^{2-}$ anion exhibits two resonances associated with inequivalent molybdenum sites. Resonances for $[Mo^{II}_2(O_2CR)_4]$ (R = CF_3, *n*-Pr), which contain formal quadruple bonds, have been observed for the first time and are the most deshielded ^{95}Mo NMR signals detected to date. The methyliminodiacetate complex, $Na_2[Mo_3O_4((O_2CCH_2)_2NCH_3)_3]\cdot 7H_2O$, was isolated. Its crystal structure contains a discrete trinuclear $[Mo^{IV}_3O_4((O_2CCH_2)_2NCH_3)_3]^{2-}$ anion whose symmetry approaches C_{3v} and which is closely related to the equivalent trinuclear units connected by EDTA groups that occur in $Na_4[(Mo_3O_4)_2(EDTA)_3]\cdot 14H_2O$. Crystal data: monoclinic space group $P2_1/n$; a = 14.712 (9) Å, B = 13.919 (2) Å, c = 15.458 (3) Å, β = 99.80 (3)°, V = 3119 Å^3, Z = 4.

The nature of the Mo(IV)–aquo ion, $Mo(IV)_{aq}$, has excited a lively interest since the first demonstrations[2] of its stability in acid solution. On the basis of indirect physical and chemical techniques, the structure of $Mo(IV)_{aq}$ was suggested variously to be mono-

† For parts 6 and 8, see ref 21i and 21r.

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