and azido hemoprotein derivatives it is clear that all protoheme proteins studied have $\Delta H°$ and $\Delta S°$ values which are significantly different from the corresponding mesoheme proteins[16] in which two ethyl groups replace two vinyl groups of protoheme. Perutz et al.[65] and Kendrew[66] demonstrated that the two vinyl groups of the protoheme in hemoglobin (and myoglobin) have several contacts with neighboring residues. It has earlier been suggested[16] that substitution of these vinyl groups with ethyl groups is likely to change the interaction between porphyrin and apoprotein, thereby affecting the spin state of the heme iron through a change in the position of the iron with respect to the porphyrin plane. Alternatively, it has been suggested[28] that a change in the interaction between porphyrin and protein may affect the spin state through a core-expansion mechanism. While the difference between the spin-equilibrium properties of proto- and mesoheme proteins may suggest the importance of interactions between apoprotein and the porphyrin, the much greater difference between thermodynamic data for the spin equilibria of a protein coordinated to different axial ligands at the sixth position indicates the importance of the axial ligation. Among the different ligands included in the current studies, water is regarded to have a stronger ligand field than OH^- such that in aquoheme compounds the difference in energy between low-spin and high-spin states should be greater than the corresponding hydroxo compounds. One would, therefore, expect that a low-spin ground state should be more likely in aquohemoproteins than in hydroxohemoproteins. Reported data indicate that the ground state is low spin in hydroxohemoproteins and high-spin in most aquohemoproteins. In contrast, the ground state is low spin for both aquo and hydroxo ferric heme octapeptide complexes. It is evident therefore that the interplay between axial ligand and apoprotein-porphyrin interactions determines the spin state of a particular heme compound. The present data indicate the extent to which the equilibrium thermodynamics depend on the interaction between protein and the particular heme axial ligand complex involved.

Enthalpy-Entropy Compensation. There is an interesting relation between each pair of $\Delta H°$ and $\Delta S°$ values for the spin equilibria of various hemoproteins. It can be seen that as $\Delta H°$ gets more negative or positive, $\Delta S°$ always changes in the same direction. This behavior is referenced to as enthalpy-entropy compensation. The ratio of $\Delta H°/\Delta S°$ is defined as T_c. Tasaki[67] explained the observed enthalpy-entropy relation in protein spin equilibria in terms of a linear temperature dependence of the energy barrier between high- and low-spin states and a small enthalpy difference at temperature T_c. He attributed the temperature-dependent energy barrier as due to the weak interaction between the porphyrin and its nearest polypeptide chain. As temperature increases, the number of interactions change. Therefore, the energy difference between high- and low-spin states would change with temperature accordingly and result in a linear relationship between $\Delta H°$ and $\Delta S°$. Tamura[53] suggested that the temperature T_c is characteristic of the protein and relatively independent of the axial ligand involved. However, calculated T_c values are not identical for all ligands of a particular hemoprotein. The thermodynamic data for the aquo-, hydroxo- and azidoheme octapeptide complexes observed in this and the previously reported[28] study also indicates that $\Delta H°$ changes with $\Delta S°$ although the T_c values are not equal for each complex. The results suggest then that the compensation phenomenon may be primarily associated with interactions between the porphyrin and solvent or protein environment and that the deviations in the T_c value may be associated with particular ligand-environment interactions.

Conclusion

The present and previously observed magnetic properties of the heme octapeptide complexes indicate that the spin-state equilibria are characteristic of unconstrained model complexes which have axial ligands analogous to hemoproteins and their derivatives. The equilibrium constants and thermodynamic values for these complexes provide references for assessing the affects of protein structure on the spin-state equilibria. Further studies will be required to determine what parameters contribute to the differences between model and hemoprotein complexes.

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Electronic Effects on the Binding of Dioxygen and Carbon Monoxide to Hemes

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Abstract: Chelated protoheme (protoheme having a proximal imidazole covalently attached) is compared with two analogues in which the two vinyl groups are replaced by either two electron-donating ethyl groups or two electron-withdrawing acetyl groups. In this series neither the kinetic nor equilibrium constants for CO binding vary appreciably. By contrast, the dioxygen dissociation rate increases along the series ethyl < vinyl < acetyl, with a consequent lowering of the equilibrium constant for dioxygen binding. These results are discussed in terms of the dipolar nature of the $Fe^{\delta+}-OO^{\delta-}$ bond.

The relationship between the structure of a heme and its ligand binding properties has been studied in a wide variety of systems. Altered ligand binding to hemoproteins reconstituted with hemes bearing differing 2- and/or 4-substituents has been interpreted as reflecting electronic factors on these affinities. However, because protein conformation is also sensitive to the nature of the heme, alternative steric explanations have been offered. The binding of ligands and the interplay of steric and electronic effects have been discussed in detail in three recent reviews.[2,3]

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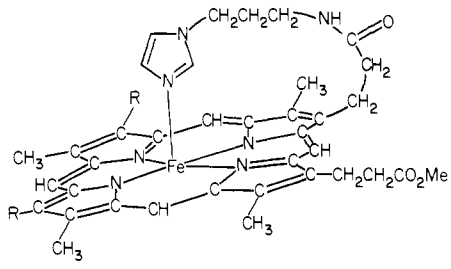
Table I. Reaction of Chelated Hemes with Carbon Monoxide and Oxygen[a,b]

compd	substituent	pK_3[c]	$10^{-6}k^{CO}$, M^{-1} s^{-1}	k^{-CO}, s^{-1}	$10^{-8}K^{CO}$, M^{-1}	$10^{-7}k^{O_2}$, M^{-1} s^{-1}	k^{-O_2}, s^{-1}	$10^5K^{O_2}$, M^{-1}	M[d]
1	ethyl	5.8	8.2	0.014	5.8	3.5	22	16	270
2	vinyl	4.8	3.6	0.005	7.2	2.6	47	5.5	980
3	acetyl	3.3	5.6	0.0085	7.0	3.4	400	0.85	6200

[a] In 2% MTAB solution, 0.05–0.10 potassium phosphate buffer, pH 7.3, 20 °C. [b] See Experimental Section for error analysis and the conversion of rates and equilibria from M^{-1} s^{-1} to $torr^{-1}$ s^{-1}. [c] Reference 10. [d] Calculated using the values for K^{CO} and K^{O_2} in $torr^{-1}$.

We have been interested in probing the electronic effects on ligand binding in the absence of steric effects. To that end we have synthesized ethyl (meso), vinyl (proto), and acetyl (diacetyldeutero) hemes **1**, **2**, and **3**, respectively. In this paper we



1, chelated mesoheme, R = CH_2CH_3
2, chelated protoheme, R = $CH{=}CH_2$
3, chelated diacetyldeuteroheme, R = $COCH_3$

report the association and dissociation rates of CO and O_2 with the chelated hemes and compare these rates with those found in heme proteins.

Experimental Section

The syntheses of **1**,[4] **2**,[4] and **3**[5] have been described previously.

Myristyltrimethylammonium (MTAB) solutions were made by using twice recrystallized MTAB (Aldrich) and glass-distilled water buffered with phosphate to the desired pH. It was necessary to use MTAB rather than CTAB (cetyltrimethylammonium bromide) solutions because 2% solutions of the latter tend to crystallize on standing below 22 °C.[4] The effect of changing from CTAB to MTAB was to increase k^{CO} and K^{O_2} by factors of <10%. Samples were prepared with 5 mL of 2% MTAB in tonometers of ~140 mL volume. Enough aqueous $Na_2S_2O_4$ was added to produce an absorbance of 0.03 at 350 nm. The hemin was added in methanol to produce a solution of $5–10 \times 10^{-6}$ M. It is not necessary that all the hemin be reduced (the hemin is kinetically inert), but it is necessary that there be no dithionite in solution when the O_2 is added. The reaction of $Na_2S_2O_4$ with O_2 in the presence of the heme destroys some of the heme and produces solutions that are kinetically inhomogeneous.

The solubilities of CO (1.035×10^{-3} M atm^{-1}, 1.362×10^{-6} M $torr^{-1}$) and O_2 (1.385×10^{-3} M atm^{-1}, 1.822×10^{-6} M $torr^{-1}$) in water at 20 °C were calculated from the Landolt–Bornstein tables.[6] The solubility of CO in benzene at 20 °C was taken as 7.50×10^{-3} M atm^{-1} (9.87×10^{-6} M $torr^{-1}$).[7]

Kinetic measurements were performed by monitoring the transmittance change of a solution after a photolyzing light pulse derived from a Phase-R DL2100D tunable dye laser with Rhodamine 6G in absolute ethanol (585 nm) or from a Braun 2000 40 VCR flashgun. The apparatus has been described previously.[4,7] In all cases [CO], $[O_2] \gg$ [heme] such that the reactions were pseudo first order. The rate constants were derived from plots of ln $(A - A_\infty)$ vs. time for 2 half-lives. Each rate was determined on at least two separate samples, with a minimum of five different concentrations of gaseous ligand in each sample. A single kinetic point consisted of an accumulation of five to ten runs and afforded pseudo-first-order rate constants with standard deviations <±1% within a series of flashes on one sample and ≤10% between samples.[8] Correction was made for the CO flashed off the heme. The temperature was maintained at 20.0 ± 0.1 °C with a circulating bath.

Stopped-flow measurements were determined on an Aminco-Morro 4-8409 stopped-flow instrument modified for accurate temperature control (±0.2 °C) as previously described.[9] Within a single run or between shots in a single pair of solutions, deviations in first-order rate constants were ±1%. Between complete runs, the rate variations were about ±10%.

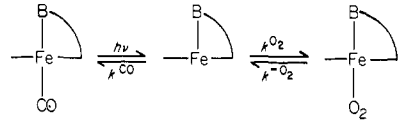
Results

Carbon Monoxide Association. The CO association rates of the chelated hemes were measured in both aqueous MTAB and in benzene (Table I). The observed rates of recombination with CO after the flash were independent of wavelength and linearly dependent on the concentration of CO. The rate constants were calculated from the slopes of the ln $(A - A_\infty)$ vs. time plots divided by [CO]. In aqueous MTAB the CO association rates increase in the order proto < diacetyl < meso chelated heme, but the change is less than a factor of 2. In benzene these three rate constants are within experimental error of 1.1×10^7 M^{-1} s^{-1}.

Carbon Monoxide Dissociation. The dissociation rates of carbon monoxide were measured by flowing one chelated heme–CO complex against another chelated heme having a different spectrum (meso-CO with diacetyl, proto-CO with diacetyl, and diacetyl-CO with meso). In aqueous solvents this technique has been shown to give the same results as the standard hemoprotein technique of flowing the heme–CO complex against a ferricyanide solution.[9] In micelles, however, ferricyanide tends to precipitate, and the trap method gives more reliable results. The off rates for the three hemes (meso, 0.014; proto, 0.0050; and diacetyl, 0.0085 s^{-1}) show little variation with structure and do not follow the electron-withdrawing properties of the substituents. The off rates are, in fact, more sensitive to environment than substituent. In methanol–water (80:20, v/v, 20 °C) the rates are meso 0.059, proto 0.029, and diacetyl 0.016, while in CTAB (2% in 0.15 M phosphate buffer, pH 7.3, 20 °C) the rates are meso 0.019, proto 0.0052, and diacetyl 0.0063.

Carbon Monoxide Equilibria. The small variations in the association and dissociation rates of carbon monoxide are in the same direction, with the result that the equilibrium constants vary little with structure. The K^{CO} for **1**, **2**, and **3** are almost within experimental error of one another in MTAB micelles. The variations in off rate with solvent indicate that it is possible that the diacetyl heme will bind CO more strongly than the mesoheme in some solvents; it is unlikely that the opposite is true.

Oxygen Association, Dissociation, and Equilibria. The rates of oxygen addition were measured by flash photolysis of the carbonmonoxy heme in the presence of both CO and O_2. The



deoxy heme formed initially may either add oxygen ($k^{O_2} \simeq 3 \times 10^7$ M^{-1} s^{-1}) or carbon monoxide ($k^{CO} \simeq 6 \times 10^6$ M^{-1} s^{-1}). If the concentration of oxygen is greater than that of carbon monoxide (e.g., $[O_2] \geq 2[CO]$), then the majority of the heme will

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add oxygen. This fast rate will be followed by a slower relaxation of HBO_2 to HBCO, governed by the rate constant for loss of O_2 and the rates of addition of CO and O_2. A plot of $(k_{slow})^{-1}$ vs. $[O_2]/[CO]$ gives a slope K^{O_2}/k^{CO} and an intercept $(k^{-O_2})^{-1}$ (ref 11).

$$K_{fast} = k^{O_2}[O_2]$$

$$k_{slow} = k^{-O_2}\frac{k^{CO}[CO]}{k^{CO}[CO] + k^{O_2}[O_2]}$$

$$(k_{slow})^{-1} = (k^{-O_2})^{-1} + \frac{k^{O_2}}{k^{-O_2}}\frac{1}{k^{CO}}\frac{[O_2]}{[CO]}$$

Flash photolysis of the heme–CO under these conditions gave k^{O_2} from the initial fast portion of the curve, measured at an isosbestic point for the HB ⇌ HBCO reaction. The slow relaxation was followed at HBO_2 ⇌ HBCO isosbestic point, and the oxygen off rate was either derived directly from the intercept of the k_{obsd}^{-1} vs. $[O_2]/[CO]$ plot or from the CO on rate, O_2 on rate, and O_2 equilibrium constant. For values of k^{-O_2} smaller than 50 s^{-1}, both calculations gave the same k^{-O_2}, providing an independent check of the method. Faster off rates, however, gave an intercept near zero and were more accurately determined from the other three constants. For chelated hemes, this technique is preferable to the oxygen pulse stopped-flow measurement[12] of k^{-O_2} both because k^{-O_2} is somewhat fast to measure by stopped flow and because the heme–O_2 species is not stable for extended periods.

The values determined for k^{O2} and k^{-O_2} are given in Table I. The oxygen on rates in chelated hemes are not particularly sensitive to the groups at the heme periphery. The off rates, however, do show the expected increase in rate with increasing electron-withdrawing capability with a consequent decrease in K^{O_2} in this order.

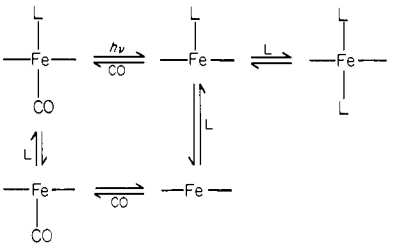
Discussion

The kinetics and equilibria reported here are not subject to steric or other environmental influences that are generally present in heme proteins. The results reported in Table I therefore reflect only the influence of electron donation to the heme on the CO and O_2 ligation dynamics.

Carbon Monoxide Binding. The first, somewhat surprising, finding is the insensitivity of CO association, dissociation, and equilibrium constants to electronic effects over the series meso to acetyl. These results seem to be at variance with other model studies on kinetics and equilibria of CO binding, and some rationalization of these discrepancies is in order.

Alben and Caughey investigated the effect of ring substituent on the equilibrium constant for CO binding by measuring the conversion of dipyridine heme to carbonmonoxy pyridine heme.[13] In benzene at 20 °C with 3.5×10^{-5} M total heme, 1.3 M pyridine, and 4.5×10^{-4} M CO, the conversion from heme(py)$_2$ to heme (py)CO was 77, 60, 56, and 17% for meso-, deutero-, and proto-, and diacetyldeuterohemes, respectively. If the equilibrium constants for binding CO were all essentially the same, then the observed differences in conversion would be due to the variations in binding of the second pyridine. The equilibrium constants for binding of pyridine to heme(py) are not available, but the binding of pyridine to substituted Co(II)[14] and Zn[15] tetraphenylporphyrins exhibits ρ values of 0.168 and 0.188, respectively. Similar results have been found for the binding of piperidine to Ni(II)[16] and Co(II)[14] tetraphenylporphyrin derivatives. It is therefore possible that the majority of the effect seen in the heme(py)$_2$ ⇌ heme(py) ⇌ heme(py)CO equilibrium may be due to the pyridine rather than to the CO equilibrium constants.

Scheme I



Studies of the kinetics of CO binding to substituted free hemes by Smith[17] and Sono et al.[18] have shown an increasing combination rate with decreasing electron-withdrawing power of the porphyrin substitutents. The situation for the free heme is, however, complicated by the fact that liganded hemes can add carbon monoxide in two ways—by direct association or by base elimination.[19] Flash photolysis of HLCO results in HL which may add CO, add L, or lose L (Scheme I). The observed rate of return of HL to HLCO after the flash in a solution containing external ligand is therefore a function of the 10 rate constants for the processes indicated. This scheme has been studied for 1-methylimidazole and mesoheme dimethyl ester in 2% aqueous CTAB and in benzene.[7] The rate constants for addition of CO to four-coordinated mesoheme are $\sim 3 \times 10^8$ and 5×10^8 M^{-1} s^{-1}, respectively, while that of addition to the five-coordinated imidazole mesoheme is $\sim 10^7$ M^{-1} s^{-1} in both solvents. Thus even a few percent of the HL → H → HCO → HLCO base elimination is enough to increase the expected HL → HLCO rate measureably. Sono et al.[18] noted that the kinetic difference spectrum obtained by flash photolysis was not the same as the equilibrium difference spectrum between the reduced and carbonmonoxy hemes. This is consistent with Scheme I, with heme$(H_2O)_2$ being the equilibrium species and heme(H_2O) the major kinetic species. It is probable that some or all of the difference in rates as a function of heme substituent reflects the effect of substituent on K^{H_2O} rather than the k^{CO} for either four- or five-coordinated heme. Free heme reacts much faster in benzene (5.7×10^8 M^{-1} s^{-1}, deuteroheme dimethyl ester; 1.9×10^8 M^{-1} s^{-1}, mesoheme dimethyl ester).[7] It is therefore difficult to compare the results obtained for the free hemes in ligating solvents with either the chelated models or the reconstituted proteins. These uncertainties do not apply to the chelated heme studies reported here because reaction does not proceed through the four-coordinated species. Our previous studies of the pH dependence of rates of CO association with **1** indicated that, at pH 7.3 in micellar suspension, **1** reacts over 90% by direct association with the five-coordinated form.[19] The rate and the percent reaction through four-coordinated heme increase as the pH is lowered. However, reaction with dioxygen proceeds at the same rate at pH 7.3 and 5.[4] These results provide strong evidence that both CO and O_2 react by direct association (like heme proteins) under the present conditions. Both protoheme and diacetyldeuteroheme are more electron deficient than mesoheme and therefore bind imidazoles with higher affinity,[14] assuring direct association mechanisms for CO and O_2 ligation with **2** and **3**.

The rate constants for the addition of CO to reconstituted hemoglobin, myoglobin, and horseradish peroxidase have been reported; representative data are shown in Figure 1. For hemoglobin both the first (k_1^{CO}, addition to T state) and fourth (k_4^{CO},

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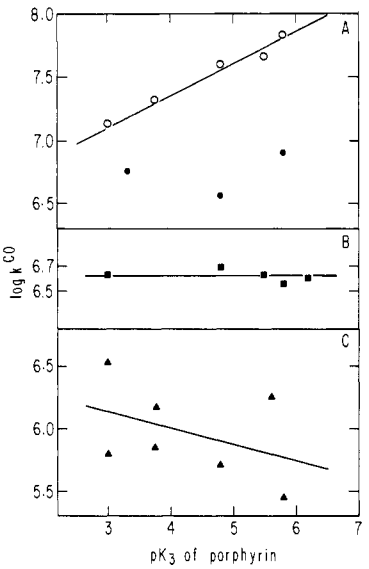

Figure 1. The log of the CO association rate (k^{CO}) as a function of the pK_3 of the porphyrin.[10] A: Free heme in ethylene glycol (○), ref 18; chelated heme in MTAB (●), this work. B: Hemoglobin, binding of the fourth molecule of CO (■), ref 20. C: Myoglobin (▲). In some cases different hemes have the same pK_a values but different values of k^{CO}, ref 2.

addition to R state) rates are of interest. Both k_1^{CO} and k_4^{CO} are independent of the electron-withdrawing properties of the heme; the k_1^{CO} (not shown) show more scatter than the k_4^{CO}.[20] Myoglobin association rates increase as the electron density at the heme iron decreases.[2a] There is a good deal of scatter in the data, presumably indicating a substantial steric interaction of the heme with the globin. The horseradish peroxidase rates[21] (proto 5.7×10^3, meso 6.0×10^3, deutero 2.3×10^5 M^{-1} s^{-1}) show no correlation between the rate and pK_3 of the porphyrin.

We conclude that binding of carbon monoxide is without appreciable side chain electronic effect. This conclusion is consistent with the theoretical calculations of Case et al.,[22] which indicate little or no charge separation in the Fe–CO bond. Since both σ electron donation and π back-bonding are important in transition-metal carbonyl bonding and both should respond to electron donation to the transition metal,[23] our results suggest that these two kinds of bonding contribute approximately equally to the Fe–CO bond strength in these particular complexes. Certain physical properties of the Fe–CO system such as the CO stretching frequency or ^{13}CO chemical shift might respond more to changes in the π back-bonding than to electron density in the Fe–CO σ bond. This would explain our finding[5] and that of others[24,25] that these properties do not correlate with the CO affinities.

Oxygen Binding. The details of oxygen binding to ferrous hemes have been a matter of debate since the first structures were proposed by Pauling,[26] Griffith,[27] and Weiss.[28] The basic heme–oxygen structure is that of a covalent Fe(II)–O_2 bond with some transfer of electron density from the iron to the oxygen. Binding of O_2 to the heme is expected, therefore, to be sensitive

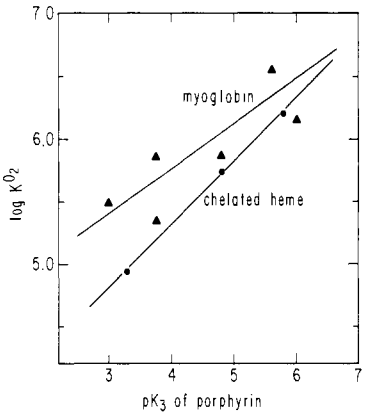

Figure 2. The log of the oxygen equilibrium constant (K^{O_2}) as a function of the pK_3 of the porphyrin[10] for myoglobin (▲), ref 2a, and chelated heme (●), this work.

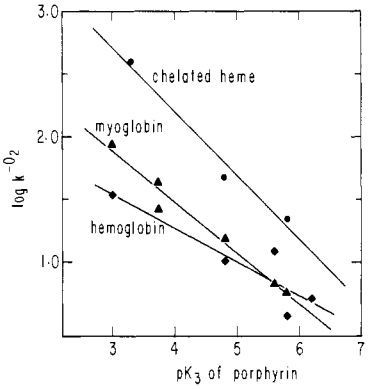

Figure 3. The log of the oxygen dissociation rate (k^{-O_2}) as a function of the pK_3 of the porphyrin[10] for hemoglobin (♦), ref 20, myoglobin (▲), ref 2, and chelated heme (●), this work.

to the electron density at the iron and thus to the substituents at the heme periphery. This is seen to be the case for both models and myoglobin (Figure 2). For the chelated hemes, K^{O_2} decreases by 1 order of magnitude as the two substituents are changed from ethyl to acetyl groups. Myoglobin experiences a similar effect, although the difference is not quite as large. The myoglobin data show more scatter than the chelated heme data, indicating a steric interaction of the heme with the pocket. This is seen most clearly in the two isomeric monoformylmonovinyl myoglobins, which show substantial differences in their K^{O_2} (3.5×10^5 and 7.2×10^5 M^{-1} for 2-formyl-4-vinyl and 4-formyl-2-vinyl, respectively).[29]

The situation for hemoglobin is even more complicated, because the overall K^{O_2} represents not only the intrinsic affinity of each chain for oxygen but also the change in cooperativity with changes in the substituents at the heme periphery. This change in cooperativity is expressed by the Hill coefficients, which are 2.2, 1.3, 2.6, and 2.4 for dimethyldeutero-, meso-, proto-, and dibromodeuterohemes, respectively.[20] Mesoheme-reconstituted hemoglobin has the highest equilibrium constant for oxygen binding of the four but is the least cooperative of the examples studied, probably indicating a substantial steric effect on protein conformation. Proteins reconstituted with deuteroheme usually behave anomalously, again indicating a steric effect. Binding of the fourth oxygen to hemoglobin ($K_4^{O_2}$) should be free of differences in the Hill coefficient. The available data[30] (proto, 1.72×10^6; 2-formyl-4-vinyl, 1.89×10^5; 4-formyl-2-vinyl, 3.66×10^5; and 2,4-diformyl, 1.25×10^5 M^{-1}) show a dependence on ring substituent similar to that of myoglobin and chelated heme.

A more detailed look at oxygen binding reveals that the changes in the equilibrium constants are dominated by the off rates. As

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seen in Figure 3, the logarithms of the oxygen dissociation rates are inversely proportional to pK_3, with the chelated hemes, myoglobin, and hemoglobin all showing about the same dependence of K^{O_2} on pK_3. The oxygen on rates are less sensitive than the off rates. In myoglobin the on rates are about half as sensitive as the off rates, while the chelated heme on rates show no regular dependence on substituent. Appropriate data are not available for the hemoglobin tetramer. Although there is a substantial conformational or steric effect seen in the binding of oxygen to various reconstituted heme proteins, it is nonetheless the case that both the models and the proteins show the same general overall dependence of oxygen binding on substituent.

These results and the decreasing oxygen dissociation rates with increasing solvent polarity[31] or proximal base electron donation[32] agree with the conclusion that the $Fe^{\delta+}-O-O^{\delta-}$ has considerable charge separation, placing more electron density on the oxygen atoms. Theoretical calculations indicate that, although there is only a small negative charge on the oxygen ligand, the oxy complex has a larger amount of electron transfer into the oxygen than does the carbonmonoxy complex into the carbon monoxide, in qualitative agreement with our findings.

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Conclusions

Side chain electron donation in chelated hemes decreases oxygen dissociation rate constants but has little or no effect on association rates of oxygen or carbon monoxide or dissociation rates of carbon monoxide. Although changes in the side chain heme substituents in heme proteins sometimes cause steric effects, the general trends of such substituent effects in heme proteins are similar to those in model compounds.

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Fluorescence Detected Circular Dichroism of Proteins with Single Fluorescent Tryptophans

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Abstract: The first fluorescence detected circular dichroism (FDCD) measurements on proteins are reported. The proteins studied were adrenocorticotropic hormone, glucagon, human serum albumin, monellin, and ribonuclease T1. All contain a single, fluorescent tryptophan. FDCD, transmission circular dichroism, and absorption spectra are combined to calculate the FDCD equivalent of the Kuhn dissymmetry factor, g_F. Values obtained for g_F range from -1×10^{-3} for human serum albumin to 1.65×10^{-3} for monellin compared with 4×10^{-4} for free tryptophan. The N-F pH-dependent transition of human serum albumin is monitored by this technique. The data demonstrate the sensitivity of FDCD to the tryptophan environment.

The first fluorescence detected circular dichroism[2-5] (FDCD) measurements on proteins are reported. FDCD is combined with transmission circular dichroism (CD) and absorption measurements to give an FDCD equivalent of the Kuhn dissymmetry factor, g_F.[6] Due to radiationless energy transfer, this is a measure of the chirality around the fluorescent tryptophan and any chromophores that transfer energy to it. The proteins studied were adrenocorticotropic hormone, glucagon, human serum albumin, monellin, and ribonuclease T1. All contain a single, fluorescent tryptophan. These proteins have been previously studied by fluorescence quenching techniques.[7-9] The g_F spectra measured are consistent with expectations based on the reported fluorescence quenching parameters. The results demonstrate that FDCD is a sensitive, selective probe of local conformation around tryptophan in proteins.

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Experimental Section

Methods. FDCD Spectra. The quantity measured in FDCD is $\theta_F^\circ = -28.65\ (F_L - F_R)/(F_L + F_R)$, where F_L and F_R are the fluorescence intensities excited by left and right circularly polarized light (LCPL and RCPL), respectively. Spectra were measured on a Cary 60 spectropolarimeter with Model 6001 CD accessory, modified for fluorescence detection (see Figure 1). Light from a xenon arc lamp is dispersed through a double-prism monochromator. The slits were fixed at 2.2 mm, resulting in a spectral bandwidth ranging from 5.9 nm at 290 nm to 1.6 nm at 210 nm. The monochromatic light is rendered plane polarized by a Glan-Thompson crystal polarizer and then made alternately left and right circularly polarized by a Pockels cell operated as a variable-wavelength quarter wave retarder. The Pockels cell modulation voltage is controlled by a linear Helipot, driven by the wavelength scanning mechanism. This control voltage is also used for computer digitization as a signal proportional to wavelength. Experimentally, perfect circular polarization is never achieved. To minimize the linearly polarized component of the light, the Pockels cell is on a laser mirror mount for precise angular adjustment. The alternating LCPL and RCPL is then absorbed by the sample which is contained in a 1-cm, cylindrical CD cell. For the experiments reported here, the resulting fluorescence was collected by two Hamamatsu R375 photomultiplier tubes (PMTs) perpendicular to the excitation beam and to each other. Scattered excitation light was blocked by Wratten 18-A filters (see Figure 1). The fluorescence emission from

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