Influence of Trans Weak or Strong Field Ligands upon the Affinity of Deuteroheme for Carbon Monoxide. Monoimidazoleheme as a Reference for Unconstrained Five-Coordinate Hemoproteins†

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ABSTRACT: The binding of carbon monoxide and weak or strong field ligands to deuteroheme has been studied over the widest possible ranges of carbon monoxide and ligand concentrations. The variations of the affinity constants of the monoliganded hemes for carbon monoxide (and of monocarbonylheme for a sixth ligand) have been related to the relative σ-donor-π-acceptor character of the ligands. With respect to the embedded heme group of the O_2-carrying hemoproteins, monoimidazoleheme appears in the most favorable situation to bind carbon monoxide. Comparison with published data shows that the isolated α^{SH} and β^{SH} chains of human hemoglobin, the single-chain hemoglobin of *Chironomus thummi thummi*, and the relaxed (R) form of human hemoglobin bind carbon monoxide with affinity constants as high as that of monoimidazoleheme ($K = 4.8 \times 10^8\ M^{-1}$). We conclude that the structure of these hemoproteins is as relaxed [in the terminology of Perutz, M. F., Ladner, J. E., Simon, S. R., and Ho, C. (1974), *Biochemistry 13*, 2163] as that of free, unconstrained monoimidazoleheme in solution.

The hydrophobic environment of the heme group in hemoproteins has led us and others (see, for instance, Caughey et al., 1965; Kassner, 1972, 1973) to think that a nonaqueous solution may be a more suitable medium for iron-porphyrin studies. We have previously discussed the preparation of bare, i.e. free of axial ligands, deuteroheme in benzene, and its coordination by weak or strong field ligands (Brault and Rougee, 1974a-c) and by carbon monoxide (Rougee and Brault, 1973). We present here a more complete study of the coordinating properties of deuteroheme in the presence of both a weak or strong field ligand (L) and carbon monoxide. The purpose of this study is to determine to what extent the fixation of a given ligand (or CO) alters the reactivity of heme toward CO (or a ligand). Our results give information on the unusual behavior of CO among other ligands and the very high affinity of five-coordinate hemoproteins such as myoglobin and hemoglobin toward CO. We conclude that hemoproteins which bind CO with the maximum affinity hitherto reported ($3\text{-}5 \times 10^8\ M^{-1}$) are as relaxed, according to the concept of Perutz et al. (1974a,b), as free, unconstrained monoimidazoleheme in solution.

Experimental Section

Solvents and chemicals were of the purest available grade. Chlorodeuterohemin dimethyl ester was synthesized according to routine procedure. The reduced form will be hereafter called heme.

Argon, grade U, was purchased from Air-Liquide, and pure carbon monoxide (CO) was from Matheson. Diluted CO in nitrogen, grade U (respectively, 1.08×10^{-4}, 0.98×10^{-3}, and 1.01×10^{-2} in volume), was specially supplied by Air-Liquide.

Ultraviolet (uv), visible, and near-infrared (ir) (up to 3000 nm) spectra were recorded using a Beckman DKU spectrophotometer. The temperature was regulated at 25 ± 0.1°.

The reduction of chlorodeuterohemin dimethyl ester in benzene by aqueous sodium dithionite has been previously described (Brault et al., 1971), as well as the procedure used to transfer under inert atmosphere the heme solution into the optical cells for spectrophotometric titrations (path lengths 1 and 10 mm for the Soret and visible regions, respectively) (Brault and Rougee, 1974b).

Drying of Heme Solutions. The presence of water in benzene solutions of heme ([water] = $3 \times 10^{-2}\ M$) (Riddick and Bunger, 1970) gives rise to characteristic absorption in the ir difference spectrum of water-saturated against dry benzene at 2800 and 2640 nm ($\Delta OD = 0.21$ and 0.12, respectively, for a pathlength of 10 mm). Preliminary studies have shown that under bubbling with carbon monoxide, a mixture of monocarbonylheme and monocarbonylmonoaquoheme is first obtained, as characterized by their well-distinct sharp Soret bands at 393 and 404 nm (Rougee and Brault, 1973). The latter complex can be preponderant if the benzene phase is transferred to the optical cell before complete separation of the two phases, some water being carried away with the benzene solution. Upon further bubbling, water is removed within a few minutes, as evidenced by both the progressive transformation of monocarbonylmonoaquoheme to monocarbonylheme, and the concomitant disappearance of the ir absorption at 2800 and 2640 nm. Calibrating the ir difference spectra with mixtures of water-saturated and dry benzene, containing known amounts of water below saturation, allows us to estimate that the water concentration after drying is less than $10^{-3}\ M$, the lowest concentration of the weak field ligands used in our study (see below).

Spectrophotometric Titrations. The competitive binding of CO in the presence of a constant concentration of other ligands was monitored as follows: a known amount of deoxygenated ligand was first introduced in the cell containing about $5 \times 10^{-5}\ M$ heme in dry benzene. This solution was

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then bubbled with a mixture of argon U and pure or diluted CO. Saturation of the gaseous phase with dry benzene avoids heme concentration variations. CO partial pressure, determined by measuring gas flows with standard flowmeters, was stepwise increased, and the optical spectrum recorded after equilibration of the solution with the surrounding atmosphere. Attainment of equilibrium is rapid, and checked by reading absorbances at different time intervals.

Complexation of ligands under constant CO partial pressure was carried out by stepwise addition of deoxygenated ligands using graduated, air-protected micro-syringe (Hamilton G.F.). The optical spectrum was recorded after solution equilibration.

Accidental air contamination during the experiment (transfer, drying, titration) is easily detected by the final optical spectrum (Brault and Rougee, 1974b). Any experiment showing more than 5% oxidized form was disregarded.

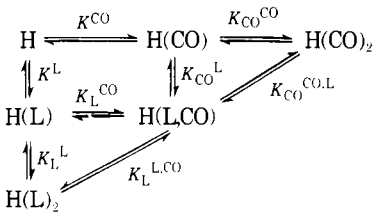
CO partial pressure ranging from 2×10^{-6} to 1 atm can be obtained with our experimental set-up. Assuming, as others implicitly do, that CO solubility in benzene obeys Henry's law, with the proportionality factor $[CO] = 6.7 \times 10^{-3}\ M\ atm^{-1}$ (Alben et al., 1968), the free CO concentration varies from 1.3×10^{-8} to $6.7 \times 10^{-3}\ M$. This experimental procedure avoids correction for bound CO. Therefore, it is possible to perform spectrophotometric titrations on heme solutions whose concentration is much higher than the free [CO] range necessary to observe the full transformation of the initial, nonliganded form to the final, CO-liganded one. Affinity constants for CO as high as $1 \times 10^7\ M^{-1}$ can thus be measured with accuracy (±10%) and values up to $2\text{-}3 \times 10^8\ M^{-1}$ can be estimated from the upper end of the saturation curve.

We have checked our experimental set-up by measuring directly the very high overall affinity constant of human hemoglobin for CO. Assuming that the solubility of CO in water is $[CO] = 1.0 \times 10^{-3}\ M\ atm^{-1}$ (Anderson and Antonini, 1968), the saturation curve, for a hemoglobin concentration of $3.0 \times 10^{-5}\ M$ in phosphate buffer (0.1 M) at pH 7.1, and 25°, is described, between 5 and 95% saturation, by a Hill coefficient $n = 2.8 \pm 0.1$ and $[CO]_{1/2} = (5.6 \pm 0.9) \times 10^{-8}\ M$. These values agree very well with those deduced from the oxygen equilibrium constant K and the partition constant M (Antonini and Brunori, 1971, p 276). The Hill coefficient is slightly but significantly higher than the value (2.3) obtained by Anderson and Antonini (1968) in their first reported direct spectrophotometric determination on very dilute hemoglobin solutions (4.5×10^{-8} and $2.34 \times 10^{-7}\ M$).

Treatment of Experimental Data

It is convenient to first present the mathematical analysis of the spectrophotometric titration of heme by a ligand L (or CO) in the presence of a constant CO (or ligand L) concentration. As shown in a previous paper (Brault and Rougee, 1974b), heme exists as a ligand-free monomer in benzene. It can bind one or two CO molecules, each species being well identified by its sharp Soret bands at 393 and 409 nm (Rougee and Brault, 1973), or one molecule of weak field ligands, such as alcohols, *p*-dioxane, tetrahydrofuran, and dimethylformamide (Brault and Rougee, 1974b), or two molecules of strong field ligands, such as imidazole, in two overlapping steps (Brault and Rougee, 1974a). In addition, mixed complexes of heme (L,CO) are well known (Hill, 1926).

The most general binding scheme is therefore:



where H stands for bare heme, i.e. free of axial ligand.

The concentration of each species is related to the concentration of any one of them ([H] for instance) via the affinity constants K^{CO}, K_{CO}^{CO}, K^{L}, K_{L}^{L}, K_{L}^{CO}, and K_{CO}^{L} (expressed as M^{-1}), and the free ligand concentrations [L] and [CO].

The dimensionless constants $K_{L}^{L,CO}$ and $K_{CO}^{CO,L}$ are defined by:

$$K_{CO}^{CO,L} = ([H(CO,L)]/[H(CO)_2]([CO]/[L])$$

$$K_{L}^{L,CO} = ([H(CO,L)]/[H(L)_2])([L]/[CO])$$

The eight constants of the above scheme are not independent and are related by the obvious relationships:

$$K^{CO}K_{CO}^{L} = K^{L}K_{L}^{CO} \quad (1a)$$

$$K_{L}^{CO} = K_{L}^{L}K_{L}^{L,CO} \quad (1b)$$

$$K_{CO}^{L} = K_{CO}^{CO}K_{CO}^{CO,L} \quad (1c)$$

or any combination between them.

Titration of Heme by a Ligand L in the Presence of a Constant CO Concentration. In order to simplify the mathematical formulation, we define the following parameters (eq 2a–c).

$$\alpha = 1 + K^{CO}[CO] + K^{CO}K_{CO}^{CO}[CO]^2 \quad (2a)$$

$$\beta = K^{L} + K^{L}K_{L}^{CO}[CO] \quad (2b)$$

$$\gamma = K^{L}K_{L}^{L} \quad (2c)$$

It is easily shown that $\alpha[H]$, $\beta[L][H]$, and $\gamma[L]^2[H]$ represent the total concentration of the species with zero, one, and two ligands (L), respectively. Therefore, the total heme concentration $[H_0]$ is given by:

$$[H_0] = (\alpha + \beta[L] + \gamma[L]^2)[H] \quad (3)$$

In the same way, let us define:

$$\alpha' = \epsilon + \epsilon_{CO}K^{CO}[CO] + \epsilon_{(CO)_2}K^{CO}K_{CO}^{CO}[CO]^2 \quad (4a')$$

$$\beta' = \epsilon_{L}K^{L} + \epsilon_{CO,L}K^{L}K_{L}^{CO}[CO] \quad (4b')$$

$$\gamma' = \epsilon_{(L)_2}K^{L}K_{L}^{L} \quad (4c')$$

where ϵ, ϵ_{CO}, $\epsilon_{CO,L}$, etc. are the molar extinction coefficients of H, H(CO), H(CO,L), etc.

The optical density (OD) of any mixture of these species is then given by:

$$OD = (\alpha' + \beta'[L] + \gamma'[L]^2)[H] \quad (5)$$

When [CO] is constant, so are α, β, α', and β'. The analysis of the variations of OD as a function of [L] is thus subjected to the same limitations as those encountered for the spectrophotometric titration of heme by the ligand L in the absence of CO (Brault and Rougee, 1974a). However, simplification occurs if $H(L)_2$ can be neglected, either because it does not form (weak field case) or because it does not appear at the end of the titration. In the strong field case, the presence of symmetrical hemochrome is easily detected by its very sharp α band around 545 nm.

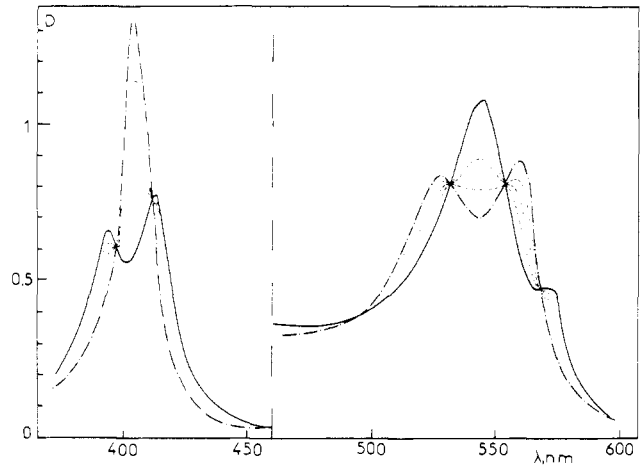


FIGURE 1: Spectrophotometric titration of 7.0×10^{-5} *M* deuteroheme in benzene by ethanol, in the presence of constant [CO] = 6.7×10^{-3} *M*. Optical paths, 1 mm (Soret) and 1 cm (visible): (—) initial spectrum, showing the contribution of the characteristic Soret bands of H(CO) at 393 nm and $H(CO)_2$ at 409 nm; (-·-) final H(L,CO) spectrum; (- - -) intermediate spectra.

In this simpler case, introducing OD_0, the optical density of the initial equilibrium mixture of H, H(CO), and $H(CO)_2$, and OD_∞, the limiting value corresponding to the final [L]-independent equilibrium mixture of H(L) and H(L,CO), one obtains:

$$OD_0 = (\alpha'/\alpha)[H_0]$$

$$OD_\infty = (\beta'/\beta)[H_0]$$

and, by a straightforward calculation:

$$OD_0 - OD_\infty = [(\alpha'\beta - \alpha\beta')/\alpha\beta][H_0] \quad (6a)$$

$$OD_0 - OD = \frac{\alpha'\beta - \alpha\beta'}{\alpha(\alpha + \beta[L])}[L][H_0] \quad (6b)$$

$$OD - OD_\infty = \frac{\alpha'\beta - \alpha\beta'}{\beta(\alpha + \beta[L])}[H_0] \quad (6c)$$

from which one deduces:

$$(OD_0 - OD)/(OD - OD_\infty) = (\beta/\alpha)[L] = K_{app}^{L}[L] \quad (7)$$

with

$$K_{app}^{L} = K^{L}\frac{1 + K_L^{CO}[CO]}{1 + K^{CO}[CO] + K^{CO}K_{CO}^{CO}[CO]^2} \quad (8)$$

On examination of the above relationships, several conclusions can be drawn. (i) From eq 6a-c, the intersection of any intermediate spectrum ([L] $\neq$ 0) with the initial one must give an isosbestic point, with $\alpha'\beta - \alpha\beta' \equiv 0$, that is $OD = OD_0 = OD_\infty$ = constant. Such a system behaves like an absorbing two species system, with regard to isosbestic points. However, their position depends on [CO].

(ii) The expected variation of OD as a function of [L] can be checked by plotting either log $(OD_0 - OD)/(OD - OD_\infty)$ vs. log [L] according to eq 7 (the slope of the straight line must be 1), or $1/(OD_0 - OD)$ vs. $1/[L]$ according to the following modified eq 7:

$$\frac{1}{OD_0 - OD} = \frac{1}{OD_0 - OD_\infty} + \frac{1}{OD_0 - OD_\infty}\frac{1}{K_{app}^{L}}\frac{1}{[L]} \quad (9)$$

The latter procedure does not require the knowledge of OD_∞, and this parameter is obtained by extrapolation.

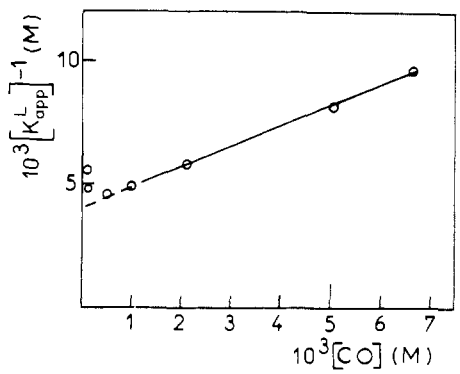


FIGURE 2: Variations of $[K_{app}^{L}]^{-1}$ vs. [CO] according to eq 13; [CO] > 5×10^{-6} *M*.

In practice, the linearity of these plots has been checked at two or more wavelengths providing maximum variations of optical density. Of course, K_{app}^{L} must be wavelength independent.

(iii) The bound ligand concentration $[L]_b$, deduced from the following relationships:

$$[L]_b = [H(L)] + [H(L,CO)] = \frac{\beta[L]}{\alpha + \beta[L]}[H_0] = \frac{OD - OD_0}{OD_\infty - OD_0}[H_0] \quad (10)$$

is, if necessary, subtracted from the known total concentration, $[L]_t$. As discussed in details by Antonini and Brunori (1971, p 167) this correction, negligible if $K_{app}^{L}[H_0] \ll 1$, becomes meaningless when $K_{app}^{L}[H_0] \gg 1$. In this case the titration appears quantitative. It is easily shown from eq 10, with $[L] \ll [L]_t$, that:

$$(OD - OD_0)/(OD_\infty - OD_0) = [L]_t/[H_0] \text{ when } [L]_t < [H_0] \quad (11a)$$

and

$$OD \rightarrow OD_\infty \text{ when } [L]_t > [H_0] \quad (11b)$$

Titration of Heme by CO in the Presence of Constant Ligand L Concentration. Provided that $[H(CO)_2]$ is negligible (the validity of this approximation will be discussed in the Results section), the same calculation as above leads to:

$$K_{app}^{CO} = K^{CO}\frac{1 + K_{CO}^{L}[L]}{1 + K^{L}[L] + K^{L}K_L^{L}[L]^2} \quad (12)$$

Results

Action of Carbon Monoxide and Weak-Field Ligands on Deuteroheme. (1) Titrations of Deuteroheme by Weak-Field Ligands in the Presence of a Constant [CO]. Whatever the relative amount of H, H(CO), and $H(CO)_2$ in the initial equilibrium mixture, the spectrum obtained at the end of the titration is [CO] independent in the range 5×10^{-6}–6.7×10^{-3} *M*. A typical spectrophotometric titration is presented in Figure 1. Well-defined isosbestic points are obtained, and the variations of OD as a function of [L] obey eq 7 or 9. The final spectrum is therefore characteristic of pure H(L,CO) species. Then, $K_L^{CO}[CO] \gg 1$, and eq 8 can be rewritten as:

$$\frac{1}{K_{app}^{L}} = \frac{1}{K^{L}K_L^{CO}}\frac{1}{[CO]} + \frac{K^{CO}}{K^{L}K_L^{CO}} + \frac{K^{CO}K_{CO}^{CO}}{K^{L}K_L^{CO}}[CO] \quad (13)$$

Table I: Affinity Constants of Deuteroheme–Carbon Monoxide–Weak Field Ligand Systems.[a,b]

Ligand	K^L (M^{-1})	$K_{CO}{}^L$(M^{-1})	$K_{CO}{}^{CO,L}$	$K_L{}^{CO}$ (M^{-1})
Ethanol	1.5	250	1.2	8×10^6
Isoamyl alcohol	1.6	230	1.1	7×10^6
Tetrahydrofuran	5.2	290	1.4	2.8×10^6
p-Dioxane	2.5	190	0.9	3.8×10^6
Dimethylformamide	3.4	290	1.4	4.3×10^6
Water	~0.1	~10	~0.05	$\sim 5 \times 10^6$
Ethyl acetate		1.2	0.006	
2-Methylimidazole	1.3×10^4	6.5×10^5	3×10^3	2.5×10^6

[a] $K^{CO} = (5 \pm 0.5) \times 10^4\ M^{-1}$; $K_{CO}{}^{CO} = (2.1 \pm 0.2) \times 10^2\ M^{-1}$. [b] Estimated error ± 20%.

In agreement with eq 13, plots of $1/K_{app}{}^L$ vs. [CO] and $1/[CO]$ tend to be linear when, respectively, the first and the last terms of the right member become negligible (Figures 2 and 3). From the linear part of these plots, $K^L K_L{}^{CO}$, K^{CO}, and $K_{CO}{}^{CO}$ are obtained. The last two constants do not depend on the particular ligand investigated, and their values agree with those obtained by direct titration of deuteroheme by CO in the absence of ligand: $K^{CO} = (5 \pm 0.5) \times 10^4\ M^{-1}$, $K_{CO}{}^{CO} = (2.1 \pm 0.2) \times 10^2\ M^{-1}$.[1]

Up to $[CO] = 2 \times 10^{-6}\ M$, the initial concentrations of H(CO) and $H(CO)_2$ are negligible, and the titration leads to the [L]-independent equilibrium mixture of H(L) and H(L,CO). Accordingly, $K^{CO}K_{CO}{}^{CO}[CO]^2 \ll K^{CO}[CO] \ll 1$, and eq 8 simply gives: $K_{app}{}^L = K^L + K^L K_L{}^{CO}[CO]$.

The slope of the linear $K_{app}{}^L$ vs. [CO] plot gives again $K^L K_L{}^{CO}$, and its intercept with the ordinate axis, K^L, agrees with the value obtained by direct titration of heme by ligand L. The individual constants $K_L{}^{CO}$, $K_{CO}{}^L$, and $K_{CO}{}^{CO,L}$ characterizing several weak field ligands are reported in Table I.

(2) Titration of Deuteroheme by CO in the Presence of a Constant Ligand L Concentration. Two concentration ranges are distinguished. For $[L] > \sim 5 \times 10^{-2}\ M$, the titration by CO occurs in a single step and leads to pure H(L,CO). Increasing [CO] up to $6.7 \times 10^{-3}\ M$ does not modify the final spectrum and particularly does not give rise to even minute amounts of $H(CO)_2$, a prerequisite for the validity of eq 12. Possible variations of the Henry's law coefficient may slightly affect our measured $K_{app}{}^{CO}$ values in the presence of a very high ligand concentration (up to pure ligand), but no significant decrease of $K_{app}{}^{CO}$ is observed, as it would occur if, according to eq 12, $K_L{}^L$ has a nonnegligible value. This result confirms the very low concentration, if any, of symmetrical $H(L)_2$ species in pure ligand (Brault and Rougee, 1974b).

For [L] ranging from 10^{-3} to $5 \times 10^{-2}\ M$, two steps are observed. The first one gives the [CO]-independent equilibrium mixture of H(CO) and H(L,CO), but at high [CO], $H(CO)_2$ appears in spectrophotometrically detectable amounts. However, the two steps are sufficiently distinct to allow the analysis of the first one according to the analog of eq 9:

[1] A more accurate determination of the affinity of deuteroheme for CO shows that our previous K^{CO} value (Rougee and Brault, 1973) was underestimated.

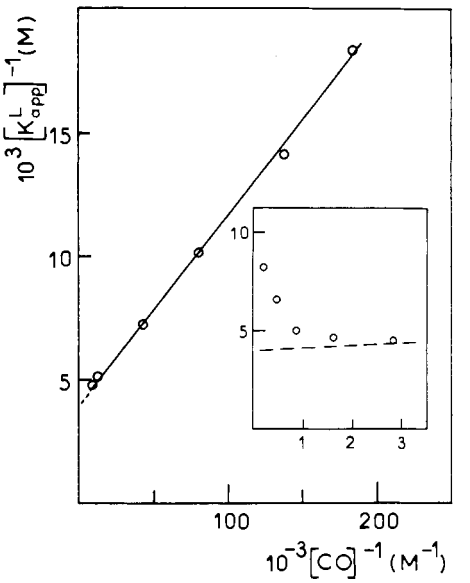


FIGURE 3: Variations of $[K_{app}{}^L]^{-1}$ vs. $[CO]^{-1}$ according to eq 13; $[CO] > 5 \times 10^{-6}\ M$.

$$\frac{1}{OD_0 - OD} = \frac{1}{OD_0 - OD_\infty} + \frac{1}{OD_0 - OD_\infty}\frac{1}{K_{app}{}^{CO}}\frac{1}{[CO]}$$

For the sake of clarity, the experimental curves corresponding to these titrations have not been represented. The measured $K_{app}{}^{CO}$ values are in complete agreement with those calculated from eq 12 (with $K_L{}^L = 0$) and the individual constants previously determined. $K_{CO}{}^L$ for water has been estimated from the $H(H_2O,CO)/H(CO)$ ratio in water-saturated benzene, assuming the same molar extinction coefficient for the mixed species $H(H_2O,CO)$ as the approximate common value observed for the other weak-field ligands. The $K_{CO}{}^L/K^L$ ratio is of the same order as that of these weak field ligands ($\simeq 10^2$). The low, but easily measurable $K_{CO}{}^L$ value for ethyl acetate agrees with the very weak coordinating properties of this pure solvent which cannot be detected in the absence of CO.

Action of Carbon Monoxide and Strong Field Ligands on Deuteroheme. (1) Titration of Deuteroheme by CO in the Presence of a Constant Imidazole (L) Concentration. Imidazole gives the well-known symmetrical bisimidazoleheme with the successive high affinity constants $K^L = 4.5 \times 10^3\ M^{-1}$ and $K_L{}^L = 6.8 \times 10^4\ M^{-1}$ (Brault and Rougee, 1974a). Accordingly, bisimidazoleheme is the major species when $[L] > 5 \times 10^{-4}\ M$. In this case, titration by CO gives pure monocarbonylmonoimidazoleheme (Figure 4). As predicted by eq 12, with $K_{CO}{}^L[L] \gg 1$ and $K^L K_L{}^L[L]^2 \gg 1 + K^L[L]$, $K_{app}{}^{CO}$ is proportional to $[L]^{-1}$, up to saturation ($2 \times 10^{-2}\ M$), which leads to $K_{app}{}^{CO}[L] = K^{CO}K_{CO}{}^L/K^L K_L{}^L = K_L{}^{L,CO} = 7 \pm 0.7) \times 10^3$. From this constant, a straightforward calculation, using eq 1, gives $K_{CO}{}^L$, $K_L{}^{CO}$, and $K_{CO}{}^{CO,L}$ (Table II).

(2) Titration of Deuteroheme by Imidazole in the Presence of a Constant [CO]. For $[CO] > 10^{-6}\ M$, the titration of any initial equilibrium mixture of H, H(CO), and $H(CO)_2$ by imidazole gives pure H(L,CO) and appears quantitative, as expected when $K_{app}{}^L[H_0] \gg 1$ (eq 11). For $[CO] < 10^{-6}\ M$, in a well-defined subsequent step, excess imidazole gives some amounts of bisimidazoleheme, detected by its sharp visible α band.

(3) 4-Cyanopyridine. For the sake of comparison, the competition between CO and the weaker base 4-cyanopyri-

Table II: Affinity Constants of Deuteroheme–Carbon Monoxide–Strong Field Ligand Systems.[a]

Ligand	K^L (M^{-1})	$K_L{}^L$ (M^{-1})	$K_{CO}{}^L$ (M^{-1})	$K_L{}^{CO}$ (M^{-1})	$K_{CO}{}^{CO,L}$	$K_L{}^{L,CO}$
Imidazole	4.5×10^3	6.8×10^4	4.3×10^7	4.8×10^8	2×10^5	7×10^3
4-Cyanopyridine	4.7×10^3	7.1×10^4	5.3×10^6	5.6×10^7	2.5×10^4	8×10^2

[a] Estimated error ± 30% (except for $K_L{}^{L,CO}$, ±10%), arising mainly from the uncertainty on K^L and $K_L{}^L$ (Brault and Rougee, 1974a).

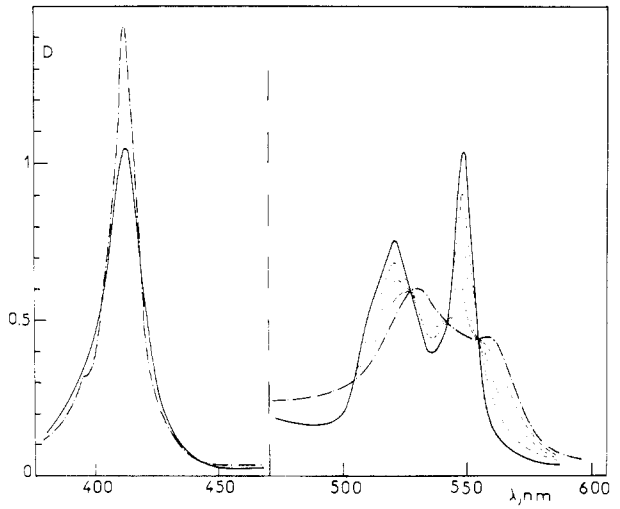


FIGURE 4: Spectrophotometric titration of 5.1×10^{-5} M bisimidazoledeuteroheme in benzene by carbon monoxide; [imidazole] = 2.1×10^{-3} M. Optical paths, 1 mm (Soret region) and 1 cm (visible region): (—) initial spectrum (bisimidazoledeuteroheme); (-·-) final spectrum (monocarbonylmonoimidazoledeuteroheme); (- - -) intermediate spectra.

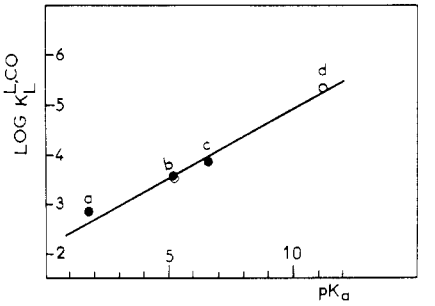


FIGURE 5: Variations of $K_L{}^{L,CO}$ vs. ligand L pK_a: (●) Deuteroheme; (○) protoheme; (a) L = 4-cyanopyridine (this work); (b) L = pyridine (from Alben and Caughey, 1968); (c) L = imidazole (this work); (d) L = piperidine (from Stynes and James, 1974).

dine has also been investigated. The successive constants K^L and $K_L{}^L$ have been determined according to the method used for imidazole. $K_{app}{}^{CO}$[L] is constant in the wide concentration range 5×10^{-4}–1.2 M (saturation). The individual constants characterizing both imidazole and 4-cyanopyridine are given in Table II.

(4) 2-Methylimidazole. Special attention has been devoted to 2-methylimidazole. The constants characterizing this ligand are given in Table I. It behaves like other weak field ligands; in particular, no decrease of $K_{app}{}^{CO}$ is observed when [L] is increased from $10/K^L = 8 \times 10^{-4}$ M up to saturation (3×10^{-2} M), as it would occur if, according to eq 12, $K_L{}^L$ has a nonnegligible value. The apparent ambiguous behavior of this ligand, with respect to the weak field–strong field concept, will be discussed later.

Discussion

The above results provide the first complete thermodynamic description of the heme–CO weak or strong field ligands, and shed some light on the unusual behavior of CO. Data summarized in Tables I and II, including our earlier results on the coordination of deuteroheme by weak or strong field ligands (Brault and Rougee, 1974a–c), undoubtedly prove the validity of the binding scheme in the widest possible concentration range.

Biscarbonylheme is the first example of low-spin, six-coordinate complex having the second affinity constant smaller than the first one: $K_{CO}{}^{CO}/K^{CO} \simeq 4 \times 10^{-3}$. An explanation based only on spin state or steric considerations (Hoard, 1971) can be ruled out, owing to the small size of CO, and the expected diamagnetic state of monocarbonylheme, as deduced from the measurements of Wang et al. (1958) on monocarbonyl monoaquoheme, and from theoretical calculation of Zerner et al. (1966). Interpretation of this unusual behavior may involve the specific nature of the CO binding to iron. Carbon monoxide is a rather weak σ donor and a strong π acceptor, as illustrated by its ability to stabilize low oxidation states of transition ions (this property may be related to the inability of CO to react with hemins). As pointed out by Cotton and Wilkinson (1966) and discussed by Caughey (1970), the binding mechanism in carbonyl complexes is synergic, that is, the σ-bond formation strengthens the π bonding and vice versa, as illustrated by the very high first affinity constant, $K^{CO} = (5 \pm 0.5) \times 10^4$ M^{-1}. However, the withdrawal of π electron density from iron to carbon monoxide tends to decrease the affinity of iron for a second strong π acceptor ($K_{CO}{}^{CO}/K^{CO} = 4 \times 10^{-3}$), but increases the affinity of iron for the σ-donor, weak field ligands ($K_{CO}{}^L/K^L \simeq 10^2$).

The balance between the relative σ-donor–π-acceptor character of ligands is well illustrated by the competition between CO and strong field ligands. Alben and Caughey (1968) have emphasized the great importance of the "trans effect" in 4-substituted pyridines and of the "cis effect" of substituted porphyrins upon the carbonyl stretching frequency ν_{CO} of monopyridinemonocarbonylhemes. As can be seen in Figure 5, and in agreement with the above predictions, the greater the basicity, i.e. the σ-donor strength of the ligand, the easier is the replacement of the sixth ligand by the strong π-acceptor CO. In addition, our data emphasize the much greater affinity $K_{CO}{}^L$ of monocarbonylheme for strong σ donors such as imidazole and 4-cyanopyridine than for weak field poor σ donor ligands.

The observation that K^L for imidazole and 2-methylimidazole are of the same order (a base strengthening effect has even been observed) led us to conclude that in 2-methylimidazoleheme, repulsive interactions between the methyl group and the electron cloud of the porphyrin plane are negligible (Brault and Rougee, 1974a). This result is corroborated by the recently measured large displacement (0.42–0.55 Å) of the iron atom out of the porphyrin plane in 2-methylimidazoletetraphenylheme (unpublished results of L. J. Radonovich and J. L. Hoard quoted by Hoard and

Scheidt, 1973). However, these repulsive interactions become important when iron is drawn back to the porphyrin plane upon CO binding. The resulting destabilization is illustrated by a 200-fold decrease of the affinity constant K_L^{CO} with respect to that of monoimidazoleheme.

Alternatively, the exceptional stability of the various mixed heme–CO–weak- or strong-field ligand complexes is shown by their overall stability constants $K^{CO}K_{CO}^{L} = K^{L}K_{L}^{CO}$, ranging from about 1×10^7 to 2.2×10^{12} M^{-2}. Moreover, their spectra appear very similar, with a very narrow Soret band at 402–412 nm and two more or less resolved visible bands centered around 530 and 560 nm. This analogy confirms the preponderant influence of CO on the electronic structure of these mixed complexes.

Comparison with Hemoproteins

With respect to the embedded heme group of the high-spin, O_2-carrying hemoproteins, we may expect monoimidazoleheme to be in the most favorable situation in reacting with CO, and it may be inferred that its affinity for CO is among the highest that can be conceivably observed. As shown by the measurements of Alben and Caughey (1968) reported in Figure 5 the replacement of the hydrogens in deuteroheme by the vinyl group in protoheme (the prosthetic group of the O_2-carrying hemoproteins) does not markedly affect their affinity for CO.

Caughey et al. (1969) and Caughey (1970) have discussed in detail the influence of various factors which are expected to affect the strength of the iron–CO bond in hemoproteins. Among them is the bending of this bond, as a consequence of interactions, in the distal face of the heme, between CO and the β^{63} residue. This bending is strongly suggested, in hemoglobin and myoglobin, by ir measurements, and has been recently proven by X-ray structure determination by Huber et al. (1970) in the case of *Chironomus thummi thummi* hemoglobin (Fe–C–O = 145 ± 15°). However, no direct correlation between the strength of the iron–CO bond per se (as related to ν_{CO}) and the overall affinity for CO was found.

On the other hand, Perutz (1972) and Perutz et al. (1974a,b) have recently pointed out that in hemoglobin, the observed increase of the O_2 affinity upon increasing saturation can be ascribed to a transition between the deoxy or T (tense) form to the oxy or R (relaxed) form of the protein. This transition results in both a shortening of the iron–porphyrin plane distance, and a decrease of the tension exercised by the protein chain on the heme iron. The partition constant M being nearly independent of the saturation degree (Antonini and Brunori, 1971, p 263) this conclusion also holds for the CO binding. On the basis of X-ray determinations, the isolated α^{SH} and β^{SH} chains of human hemoglobin and the *Chironomus thummi thummi* single chain hemoglobin are also expected to be, according to Perutz, in a relaxed R form. It is noteworthy that their affinity for CO is the highest hitherto reported. The corresponding affinity constants are 3.0, 4.8, and 3.1×10^8 M^{-1}, respectively (Brunori et al., 1966; Amiconi et al., 1972). A similar value can be deduced for the R form of hemoglobin, because both its O_2 affinity (Perutz et al., 1974b) and its partition constant M (Brunori et al., 1966) are similar to those of the isolated α and β chains. It is still more significant that these values are of the same order as that we have measured for monoimidazoleheme. Thus, we can conclude that the structure of these hemoproteins and of the R form of hemoglobin is as relaxed as free, unconstrained monoimidazoleheme in solution. In other words, the latter provides a valuable reference, with respect to the reactivity toward CO, for unconstrained hemoproteins.

This conclusion is further supported by preliminary experiments on a five-coordinate heme compound having one histidine (covalently linked, via a peptide bond, to one of the two propionic acid side chains of deuteroheme) liganded to iron (M. Momenteau, personal communication). Its affinity for CO, which reaches the maximum value that can be determined with our experimental set-up ($K \simeq 3$–4×10^8 M^{-1}), is comparable to that of unconstrained five-coordinate hemoproteins, and much higher than the surprisingly small value reported by Chang and Traylor (1973a,b) for their five-coordinate monopyridinemesoheme (Py-mesoheme, $K = 4.4 \times 10^5$ M^{-1})

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Studies on Self-Association of Proteins. Self-Association of α-Chymotrypsin at Its Isoelectric Point in Buffer Solutions of Ionic Strength 0.1†

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ABSTRACT: The self-association of α-chymotrypsin at its isoelectric point has been studied in two buffer solutions of μ (ionic strength) = 0.1: phosphate buffer (pH 6.9) and Tris buffer (pH 8.3). The weight-average molecular weight (by the Archibald method) and sedimentation coefficient were determined as a function of protein concentration. The molecular weights measured were the same in both the buffers. In sedimentation velocity experiments unimodal peaks were obtained at all the protein concentrations. The molecular weight data could be fitted to a nonideal indefinite self-association equilibrium or a hexamerization equilibrium with all the intermediate species coexisting. The sedimentation data could be fitted to an octamerization equilibrium.

The self-association of α-chymotrypsin (EC 3.4.4.5) has been studied extensively (see Pandit and Rao, 1974a, for earlier references). In buffer solutions of μ = 0.2[1] and above association proceeds essentially to dimerization or trimerization (Steiner, 1954; Egan et al., 1957; Rao and Kegeles, 1958; Winzor and Scheraga, 1964). On the other hand, at its isoelectric point in buffer solutions of μ = 0.05, extensive association occurs (Massey et al., 1955; Nichol and Bethune, 1963; Pandit and Rao, 1974a). Sedimentation velocity data under these conditions fit a monomer–hexamer equilibrium (Gilbert, 1955, 1959; Pandit and Rao, 1974a). However, the molecular weight data by the Archibald method can be best described by an indefinite self-association equilibrium (Pandit and Rao, 1974a).

The self-association of α-chymotrypsin at acid pH values and μ = 0.1 has been studied by the light scattering method (Steiner, 1954). Essentially dimerization was observed. A decrease in pH or increase in ionic strength favored association. However, no measurements have been reported at μ = 0.10 at the isoelectric point of the protein. In this investigation the self-association of α-chymotrypsin has been studied at μ = 0.1 at its isoelectric point, by measuring the weight average molecular weight and the sedimentation coefficient as a function of protein concentration.

This enzyme has an isoelectric point of pH 8.3 in uni-univalent buffers (Anderson and Alberty, 1948; Rao and Kegeles, 1958). However, its isoelectric point in phosphate buffer of μ = 0.20 is pH 6.2 and this increases to pH 6.9 in phosphate buffer of μ = 0.10 (Rao and Kegeles, 1958).

In this investigation two buffer solutions were used, phosphate buffer of pH 6.9 and μ = 0.10 and Tris buffer of pH 8.3 and μ = 0.10. It has been reported that in Tris buffer of pH 8.3 and μ = 0.05, considerable autolysis of the protein occurs (Pandit and Rao, 1974a). However, no such autolysis was observed in Tris buffer solution of μ = 0.10.

Materials and Methods

α-Chymotrypsin. Worthington α-chymotrypsin, 3× crystalline, CDI 7-JC, was used without further purification.

Chemicals. The chemicals used were guaranteed reagent grade or chemically pure grade.

Archibald Molecular Weight. The molecular weight measurements were made with a Spinco Model E ultracentrifuge equipped with schlieren optics and a RTIC unit. Solutions prepared in phosphate buffer were dialyzed in the cold for 12 hr. However, for measurements in Tris buffer the protein was directly dissolved in the buffer solution and used. For the false bottom, 0.1 ml of fluorocarbon oil (FC 43) was used. The temperature was maintained at 25 ± 1° with the RTIC unit. The experimental details were the same as described earlier (Pandit and Rao, 1974a).

Sedimentation Velocity. The measurements were made at 25 ± 1°. From the pictures taken at different intervals of centrifugation, the sedimentation coefficient was calculated by determining the movement of the second moment of the

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[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; μ, ionic strength.