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PROTON ACTIVITIES FOR THREE STATES OF CYTOCHROME P-450cam

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SUMMARY: *Changes in proton concentration during the binding of dioxygen, carbon monoxide, and for the exchange of dioxygen by carbon monoxide, at ferrous-cytochrome P-450cam were measured by direct titration. Insufficient proton release was observed to support protonation-deprotonation of an axial cysteinyl sulfur donor as a mechanism for generation of hyper spectra in only the carbonylated ferrous state. Measurement of the $p_{\frac{1}{2}}$ value for CO binding as a function of pH (the carbon monoxide Bohr effect) confirms the direct titration data.*

INTRODUCTION:

Overwhelming evidence has been presented for cysteine thiolate ligation to the ferric and ferrous-CO forms of cytochrome P-450 (1-5); however, the reduced and oxygenated forms of the cytochrome have been less well characterized in this respect. Axial thiolate coordination in these has been assumed as an extension of its presence in the other states, but there is little evidence that all states of the reduced cytochrome maintain such ligation. The ferrous-CO complex shows a strong Soret band at 450 nm, and this absorption led to the discovery and naming of the enzyme (6). Such a strong absorption at 450 nm is atypical for a heme protein, and can only be reproduced *in vitro* when a ferrous-heme-CO complex (protein-free) is coordinated by a thiolate (RS^-) but not a thiol (RSH) ligand (7).

The model studies of Chang and Dolphin (7) on $[(^nBuS)Fe(porphyrin)(CO)]^-$ systems revealed not only the 450 nm band but also a second less intense Soret band at ~370 nm. Hanson *et al.*(8) have defined such two peak

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electronic spectra of metalloporphyrins as hyper-spectra, and have shown by molecular orbital calculations that the two bands result from an interaction between a porphyrin $\pi \rightarrow \pi^*$ transition (the normal Soret band) and a ligand (or metal)$\rightarrow\pi^*$ charge transfer transition. For cytochrome P-450cam hyper-Soret absorptions could result from a $p \rightarrow \pi^*$ charge transfer from a lone pair of thiolate p-electrons, and the porphyrin $\pi \rightarrow \pi^*$ transition. Indeed, a reexamination (8) of the optical spectrum of the CO complex of ferrous cytochrome P-450 revealed the second band at higher energy, comparable to that found in the model studies.

The MO calculations (8) also predict that the oxygenated ferrous cytochrome should exhibit a hyper spectrum if there is an axial thiolate ligand, and this is supported by model studies on $[(^{n}BuS)Fe(porphyrin)(O_2)]^-$ models (9). Similar hyper spectra are *not* observed for the ferrous or the oxygenated ferrous forms of P-450cam, and it is possible that either the coordinated sulfur ligand in these two states is protonated and coordinated as a thiol (at near neutral pH), or the thiol is locally displaced and a non-sulfur ligand occupies the proximal coordination site. Oxidation to the ferric state, or coordination of CO at the ferrous center, would result in an increased electron demand at the metal, and this could then polarize the proximal cysteine thiol and induce it to become coordinated as thiolate. For this hypothetical case, the cysteine thiol would experience a shift in its dissociation constant sufficient to release about one proton per heme in going from **1** to **3**, or from **2** to **3**:

$$\begin{array}{ccc} & \text{P-450}(Fe^{2+}),\ \text{high spin}\ (\mathbf{1}) & \\ {\scriptstyle O_2}\swarrow & & \searrow{\scriptstyle CO} \\ \text{P-450}(Fe^{2+})O_2,\ \text{low spin} & \underset{O_2}{\overset{CO}{\rightleftharpoons}} & \text{P-450}(Fe^{2+})CO,\ \text{low spin} \\ (\mathbf{2}) & & (\mathbf{3}) \end{array}$$

We have measured changes in proton concentration during reactions **1**→**2**, **1**→**3**, and **2**→**3**, to test the hypothesis.

Proton balance can be measured in protein solutions of low buffer capacity by direct titration, and this method has been applied widely to O_2-carrying proteins to investigate the Bohr effect for oxygenation. Visible spectroscopic techniques are also useful for such measurements, and an accurate method for the quantification of reversible dissociation of protons induced by the binding of gaseous ligands is to determine the variation of ligand affinity with pH. For example, monitoring the absorption band of the ferrous-CO form of the cytochrome provides a way to measure ligand affinity by means of spectral $p_{\frac{1}{2}}$ values, where $p_{\frac{1}{2}}$ is the partial pressure of CO required to convert half of the cytochrome to the CO-bound form. The situation is entirely analogous to the classical Bohr effect (10) where the number of protons bound or released by the protons (ΔH^+) is given by the expression $\Delta H^+ = \delta \log p_{\frac{1}{2}}/\delta pH$. This variation of $p_{\frac{1}{2}}$ with pH also gives a direct measure of proton activity.

MATERIALS AND METHODS:

Cytochrome P-450cam in the ferric form was prepared from *Pseudomonas putida* (PpG 786) by the method of Gunsalus (11), and was stored at liquid N_2 temperature at a concentration of 0.2 mM in 10 mM phosphate buffer, pH 7.0. The cytochrome concentration was determined from extinction coefficient data (11): ferrous form, λ_{max}408 nm, ε=86.5 $mM^{-1}cm^{-1}$; ferrous-CO form, λ_{max} 446 nm, ε=120 $mM^{-1}cm^{-1}$. Aliquots for direct titration were ultrafiltered continuously in an Amicon device (UM-10) with 10 volumes of 50 mM KCl, 100 μM in camphor, to reduce the buffer capacity. Solutions for the spectroscopic measurements were prepared by a 50-fold dilution of the cytochrome into standard buffers (50 mM acetate, phosphate, and borate solutions, prepared by mixing appropriate amounts of the acid and the potassium salt). An overall $[K^+]$ of 100 mM was maintained in each buffer, by KCl addition if necessary, although no specific ion effects were observed in control experiments. NADH was purchased from Sigma Chemical Co. Visible spectra were recorded on a Cary 17D equipped with a thermostated cell compartment.

Gas mixtures for the determination of spectral $p_{\frac{1}{2}}$ values were prepared by allowing standard volumes of prepurified argon and carbon monoxide to mix in a closed system. The major component gas (argon) was scrubbed through the buffer solution to compensate for the water vapor pressure within a tonometer. The absolute pressure in the system was measured with a difference mercury manometer relative to barometric pressure.

Direct Titration Procedure. The method described by Benesch and Benesch (12) was used, the apparatus being equipped with a magnetic stirrer and a combination electrode. Gas equilibrations and titrations were performed with the apparatus immersed in an ice-water bath in a 5°C coldroom. Unbuffered cytochrome solution was brought to the desired pH in the ultra-

filtration procedure by adding 10 mM HCl or KOH from a microsyringe. The solution was equilibrated with Ar or O_2 by passing the gas over the surface of the stirred solution for 10 min. Two equivalents of NADH in 50 mM KCl were added at this point, and the pH was adjusted by reagent additions until stable (2-5 min). The formation of the stable ferrous or oxygenated ferrous cytochrome within this period was verified spectrally. The cytochrome solution was then equilibrated with O_2 or CO, the pH being maintained constant by additions of 10 mM HCl or KOH. When no further change occurred, the solution was titrated using a microsyringe at the equilibration pH to determine the extent to which the residual buffer capacity of the protein limited the detection of small pH changes. The changes in proton activity were determined by dividing the moles of titrant added by the total moles of cytochrome present. Tabulation of these values over a pH range yields a plot of the Bohr effect for the process observed (Figure, upper part).

Spectrophotometric Ligand Affinity Method. Cytochrome solutions ($\sim$4 μM) were prepared by dilution of the stock solution with buffer, 100 μM in camphor. Acetate, phosphate, and borate buffers spanned the desired pH range and provided adequate buffer capacity. A tonometer was made to which a quartz optical cell was attached (10). The solution in the cell was transferred by tilting into the tonometer cavity for equilibration with the gas mixture, which took $\sim$2 min with gentle swirling.

A typical determination of $p_{\frac{1}{2}}$ consisted of deaerating the ferric cytochrome solution in the tonometer by alternately evacuating and filling with argon. Two equivalents of NADH, dissolved in buffer, were injected through a serum stopcock under Ar, and the spectrum recorded after 5 min. The tonometer was then attached to the gas-handling apparatus, twice evacuated and then filled with a CO/Ar mixture at a known pressure; after equilibration the spectrum was recorded at 23°C. The tonometer was then flushed with pure CO, and the spectrum of the fully carbonylated cytochrome recorded; this was independent of pH over the range 5.0-8.5. The fraction Y of carbonylated species formed at a pressure p_{CO} was readily determined using the extinction coefficient data; plots of log Y/(1-Y) *vs.* log p_{CO} (13,14) were linear and yielded the reproducible values of $p_{\frac{1}{2}}$ shown in the Figure (lower part). Our $p_{\frac{1}{2}}$ values are in good agreement with a reported binding constant of about $10^5 M^{-1}$ (15); for example, at pH 7.00, $p_{\frac{1}{2}}$=5.55 mm Hg (Figure), and since the solubility of CO under our conditions is $1.36 \times 10^{-6} M$ mm^{-1} (16), this corresponds to a binding constant of $1.3 \times 10^5 M^{-1}$.

Spectral $p_{\frac{1}{2}}$ values for O_2-binding could not be determined accurately because of decomposition of the oxygenated species to the ferric form at 23°C.

RESULTS AND DISCUSSION:

The differential titration and ligand affinity methods are applicable to any change of state in the cytochrome for which an equilibrium can be established and for which the proton release directly involves only the protein. In this respect the cytochrome reduction step cannot easily be studied since protons are released upon oxidation of NADH and possibly upon association of the reducing agent with the protein. The interaction of the reduced cytochrome with gaseous ligands, however, is a simpler

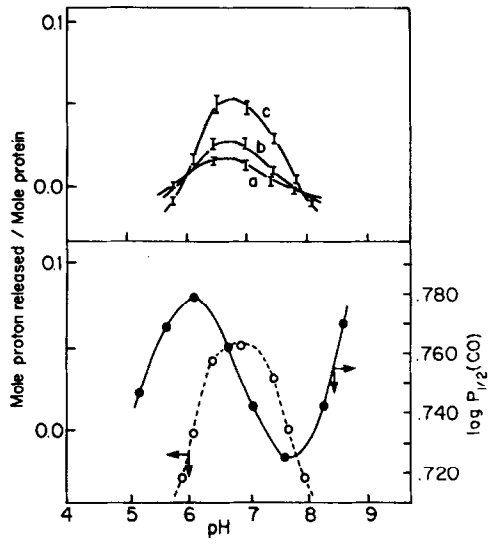

Figure. *Upper part: direct titration data at 0°C for (a) O_2-binding, (b) replacement of O_2 by CO, (c) CO-binding, at a ferrous cytochrome P-450cam center.*
Lower part: ●, spectrophotometric determination of $p_{\frac{1}{2}}$ for CO-binding as a function of pH at 23°C; ○, first derivative curve.

process. Since the cytochrome is a monomeric protein, there are no cooperative effects and all proton balances for O_2- and CO-binding can be associated with changes at the interaction site.

The direct titration data for binding of O_2 and CO, and for the exchange of O_2 by CO (Figure) show very small changes in proton concentration, indicating that only minor changes in acid dissociation constants accompany binding of the gaseous ligands. A maximum of about 0.05 H^+ per cytochrome are liberated during carbonylation at pH 6.8, while at pH 6.0 and 7.9 there is essentially no change in $[H^+]$. The oxygenation and O_2/CO replacement reactions show very similar, but less marked behavior. The Bohr effect for CO binding measured spectrophotometrically is in excellent agreement with the titration data as seen from the first derivative curve (Figure, lower part, open circles) which corresponds to curve *c* (Figure, upper part).

Clearly, protonation and deprotonation of a coordinated cysteinyl sulfur does not appear to be an important process during carbonylation or oxygenation and cannot be responsible for the change from a normal to

hyper spectrum. The indication is that the cysteine sulfur ligand of the heme in P-450cam is bound deprotonated in all states (ferric, ferrous, carbonylated ferrous, and oxygenated ferrous). The acidity of ethanethiol on coordination to a $Ru(NH_3)_5^{2+}$ center increases by $\sim$3 log units (17); the pK_a of the -SH group of free cysteine is $\sim$8.5(18), and these data are consistent with our conclusions that at pH $\geq$ 5.5 this group is likely to be deprotonated in all states of P-450.

Considering our analysis, it is difficult to see how any proton released could be masked by concomitant changes in dissociation constants of amino acid residues, or by other complexities resulting from the properties of the protein. For example, during carbonylation, for the liberation of a proton from the coordinated thiol to become accompanied by concomitant association with some free protein base would require the latter to modify its pK_a value from below 5.5 to above 8.0, and this would also have to be linked to formation of the hyper spectrum.

With the model systems in dimethylsulfoxide solution, the hyper spectra have been observed only with coordinated thiolate (RS^-), while use of neutral RSH or R_2S (R=alkyl or aryl) leads to normal spectra for carbonylated ferrous species (7). It is noteworthy that microsomal P-450 with ethyl isocyanide coordinated does undergo a reversible pH-dependent change from a hyper to normal spectrum around pH 6.0 (19), and this could result from protonation of the thiolate ligand. The absence of hyper spectra for the ferrous and the oxygenated ferrous forms of P-450 still requires an explanation.

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