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Electronic Control of Ferroporphyrin Ligand-Binding Kinetics

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Abstract: Measurements of the rates of CO binding to ferrous porphyrins have been used to examine two different mechanisms which have been proposed to explain protein control of heme reactivity. The results indicate that electronic control through π-donor/acceptor interactions with the macrocyclic porphyrin ring is *not* important in controlling the heme reactivity of hemoglobin or of other hemoproteins. However, hydrogen bonding to the metal-bound imidazole can have a powerful influence on heme reactivity.

Introduction

The primary control of heme reactivity in proteins is achieved by the axial ligand(s) arrived at through molecular evolution. However, powerful secondary control mechanisms also exist. As the best-known example, cooperative ligation of hemoglobin reflects a conformational equilibrium between one protein form (T)[1] with low ligand affinity and a second form (R) with $\sim 10^2$-fold higher affinity, yet the single endogenous heme-ligand is the same in both forms.[2-4] This influence of conformation on reactivity is correspondingly expressed in the modulation of ligand-binding kinetics: for example, the CO on-rate for the T state is ~20- to 60-fold less than for the R state.[5] This paper discusses two different control mechanisms involving purely electronic effects local to the heme, one associated with protein-induced perturbations of the proximal histidine which we shall call a "proximal" effect, the other with perturbations of the porphyrin ring which we call a "peripheral" effect.

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Peisach and collaborators[5] have proposed that changes in protein conformation lead to proximal control of reactivity through alteration in hydrogen bonding to the proton on the amino nitrogen of a metal-coordinated imidazole. The stronger the hydrogen bond formed by the proton, the more electron rich is imidazole; the limit of full proton transfer is the imidazolate anion. Differences in the imidazole "protonation state" would alter the electron-donating power of the imido nitrogen, thus modifying the electronic structure of the metal to which it is coordinated. This proposal has been considered in several recent publications.[6-9]

An alternate mechanism for peripheral electronic control of heme reactivity, originally noted by Abbott and co-workers,[10] involves π-donor/acceptor (D/A) interactions between the heme macrocycle as acceptor and an aromatic amino acid (e.g., phenylalanine, tyrosine, tryptophan, histidine) as donor.[9,11,12] If these interactions significantly alter the electron density on the ring, they could also indirectly influence the electron density at the metal and thus its reactivity. Considering hemoglobin in particular, two aromatic residues in contact with the heme, the phenyl rings of Phe CD1 and G5, have altered orientations in the T and R structures.[13] Enhanced D/A interactions in the R form could be proposed to account for the increased ligand affinity; the results of high-precision difference resonance Raman studies have been interpreted in this fashion.[12] Note that the proposal of control through electronic interactions at the heme periphery can be compared usefully with the suggestion that, upon transition from T → R, changes in van der Waals contacts with the porphyrin are important in control of reactivity,[14] and with more recent, related proposals.[15,16] In a continuation of our studies of ferroporphyrin reactivity,[6,9] this report tests the applicability of these two mechanisms. We examine the rates of ligand binding to ferroporphyrin models and use the modulation of these rates by diverse "effector" compounds as the indicator of heme reactivity.

Experimental Section

General Procedures. All reactions were carried out under an atmosphere of dry N_2 at room temperature unless otherwise noted. Toluene was purified by distilling from sodium and stored with molecular sieves under nitrogen. Imidazole was recrystallized from benzene. Pyridine, piperidine, and 1-methylimidazole were distilled from KOH. All glassware used for hydrogen-bonding studies was flamed to drive off water. Solute π donors and π acceptors were recrystallized from appropriate solvents or sublimed. KIm was prepared and purified as described by Nappa and Valentine, and the crown ethers (Aldrich) were purified as described.[8] Carbon monoxide (Matheson) was pretreated with Drierite, Ascarite, and aminated silica gel[17] to remove H_2O and CO_2. Free-base porphyrins were purchased (Alfa), and the iron was inserted by literature methods.[18] Fe(DPD)(Pip)$_2$, Fe(TPP)(Pip)$_2$, and Fe(TPP)(Im)$_2$ were synthesized by literature methods.[19]

Fe(TPP)(B)(CO) Samples. Samples containing Fe(TPP) in the presence of neutral nitrogenous bases were all prepared in an identical manner. Solutions of Fe(TPP)(B)(CO) were prepared by first placing solid Fe(TPP)(Pip)$_2$ into a cuvette which could be sealed with a serum cap, and then flushing with N_2 for 10 min. Three milliliters of deoxygenated solvent (toluene, dichloromethane, or cyclohexane) was then transferred with a gas-tight syringe into the cuvette, followed by the addition of a known amount of deoxygenated base or base solution. Both the solvent and base solution had been deoxygenated by bubbling N_2 through the liquids for at least 10 min. CO was then bubbled through the solution for 15 min. The concentration of CO was calculated from the solubility of CO at 20 °C (toluene, 7.5 mM/atm; CH_2Cl_2, 8.5 mM/atm; C_6H_{12}, 11.0 mM/atm).

Final concentrations of Fe(TPP) were typically 5×10^{-6} M. After measurement of the recombination rate of a given sample, the addition of base and CO was repeated, but only until the sample volume changed by ~10%. Base concentration ranges employed were 0.015 to 0.90 M piperidine, 0.005 to 1.1 M pyridine, 0.0001 to 0.01 M imidazole, and 0.002 to 0.20 M 1-methylimidazole.

Samples with Added Effectors. The influence of added effectors was examined by σ-base titrations in the presence of a constant effector concentration and/or by an effector titration done at constant [base]. These experiments employed a cuvette with a side arm that could be charged with a known amount of the solid effector. The Fe(Por)(B)(CO) solution (Por = TPP or DPD) was prepared in the cuvette and the CO recombination rate measured before and after addition of the effector. In measurements using B = Im, to make sure that the stoichiometric piperidine present from the starting material was not affecting the experiments at low [Im], Fe(Por)(Im)$_2$ was prepared and used as a starting material. The same results were obtained.

Fe(Por)(Im$^-$)(CO) Samples. Toluene solutions of Fe(TPP)(Im$^-$)(CO) and Fe(DPD)(Im$^-$)(CO) were prepared under N_2 by addition of a large excess ($\geq 10^3$) of KIm, solubilized by a crown ether, to Fe(Por)(Pip)$_2$. The CO pressure was then established as described above.

Myoglobin Samples. Samples of whale skeletal muscle Mb (Sigma) in pH 7.0 potassium phosphate buffer (μ = 0.1) were deaerated with a gently flowing stream of N_2 gas, reduced by the addition of a minimum of potassium dithionite (Fisher), and converted to the CO adduct with the addition of 1 atm of CO gas. Ethylene glycol was added to buffered samples for low-temperature studies.

Flash Photolysis Apparatus. Kinetic measurements were made by monitoring absorbance changes after flash photolysis of a Fe(TPP)(B)(CO) solution using apparatus of conventional design. Photolysis employed a Xenon Corp. flash lamp assembly Model No. 457 Micro-Pulser (flash duration ~20 μs). The flash was screened from the sample by a yellow filter (Corning No. 3-71; 50% transmittance at 480 nm), and by neutral density filters when needed, and then focused on the sample. The analyzing beam was monochromatized with a Jobin Yvon H-20UV-V grating monochrometer and monitored with an RCA No. 1P28 phototube which was preceded by a second JYH-20UV-V monochrometer to reject stray light. The reaction trace was captured by a Biomation 610 B transient recorder and processed in a Nova 3 computer.

Optical Spectra. Optical spectra were recorded at ambient temperature on a Beckman Acta III spectrophotometer. The static difference spectra [Fe(B)$_2$ − Fe(B)(CO)] were obtained by subtracting absorbance readings for a given sample before and after addition of CO. Readings were taken from the digital display at 1-2-nm intervals. Kinetic difference spectra were directly measured point-by-point (1-2-nm intervals) from the zero-time absorbance change, ΔA_0, immediately after photolysis. Since complete photolysis typically was not achieved, ΔA_0 was dependent on flash intensity. This could be held constant or corrected for by monitoring the relative photon flux of a flash by integrating the photocurrent generated in an SD-40A photodiode located next to the sample. The diode was calibrated over 3 decades with neutral density filters (Melles-Griot).

Quantum Yields. Photorelease quantum yields, Φ, were determined relative to Mb(CO) at 20 °C (Φ = 1). As shown in ref 20, for fixed temperature:

$$-\ln\left(1 - \frac{\Delta A_0(I)}{\Delta A_\infty}\right) = \omega I \qquad (1)$$

where ΔA_∞ is the zero-time absorbance change upon full photolysis, $\Delta A_0(I)$ is the observed change for a flash of intensity I, and ω is a constant proportional to the photorelease quantum yield at the wavelength of flash excitation. ΔA_∞ is obtained either from direct difference-spectrum measurement or from a least-squares fit to eq 1, with the constraint that the theoretical line extrapolates back to an intercept of zero at I = 0. Since Φ is wavelength independent for CO photorelease in these systems,[21] eq 1 also holds for the broad-band flash excitation, except that $\omega = \gamma\Phi$, where γ involves a convolution of the flash lamp profile, the blue-cutoff screening filter, and the absorbance spectrum of the particular sample. For the present purposes, it is adequate to ignore differences in γ among the different Fe(Por)(B)(CO) and MbCO since these are small. A plot of the left-hand side of eq 1 vs. I gives a straight line whose slope, normalized to that of MbCO (Φ = 1), is taken to be the quantum yield. Overall, this procedure appears to give a good measure of the temperature dependence of Φ for a given system and reasonable absolute numbers in comparison with MbCO.

Activation Energies. Variable-temperature studies employed a liquid nitrogen boil-off Dewar fitted with optical windows. This low-temperature cell was designed to maintain a constant CO concentration so that no correction in measured activation energies was

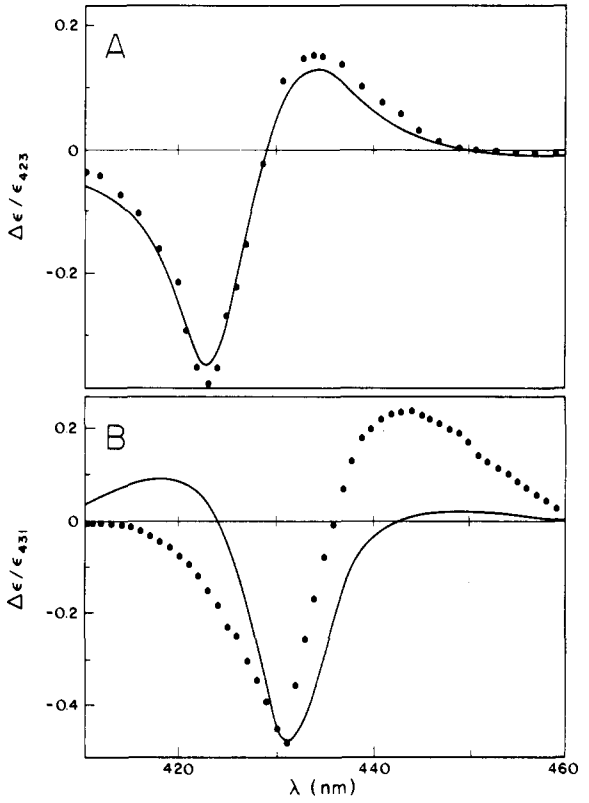

Figure 1. (A) Static difference spectrum, $Fe(TPP)(Im)_2$ − Fe(TPP)(Im)(CO) (—); kinetic difference spectrum after flash photolysis of Fe(TPP)(Im)(CO) (···); (B) static difference spectrum, $Fe(TPP)(Im^-)_2$ − Fe(TPP)(Im^-)(CO) (—); kinetic difference spectrum after flash photolysis of Fe(TPP)(Im^-)(CO) (···).

required for the enthalpy of solution of CO in toluene. The temperature (+40 °C to −60 °C) was monitored with a copper-constantan thermocouple, which was in thermal contact with the optical cell.

Results and Analysis

Fe(TPP)(B)(CO). The Fe(TPP)(B)(CO) (B = Im, 1-MeIm, Py, or Pip) complexes are all highly photolabile, and the photoproduct rebinds CO with a pseudo-first-order rate, $k_{obsd} \propto [CO]$. The product obtained immediately after the flash (~20 μs) does not correspond to the formation of Fe(TPP)(B), which should have a Soret maximum at ~435 nm (Figure 1A). Rather, the kinetic difference spectrum is the same as the static difference spectrum, [Fe(TPP)$(B)_2$] − Fe(TPP)(B)(CO)], also presented in Figure 1A, which is obtained by direct subtraction of the absorbance spectra of the appropriate complexes. This indicates that a second B is bound within the flash lifetime. Therefore, the overall stoichiometry of the CO rebinding reaction as observed in the regeneration of Fe(TPP)(B)(CO) after the photolysis flash is ligand replacement, $Fe(TPP)(B)_2 + CO \rightarrow Fe(TPP)(B)(CO) + B$, not the ligand addition reaction,

$$Fe(TPP)(B) + CO \xrightarrow{k_5} Fe(TPP)(B)(CO)$$

which would correspond to the CO binding reaction by Hb.

This observation is expected from previous studies, in particular those of Traylor.[22a-d] Binding of CO to an iron(II) porphyrin in the presence of excess base (B) can usually be described in terms of preequilibrium between the $Fe(B)_n$ (n = 0, 1, 2),[22a,b,23] where we have suppressed the porphyrin abbreviation (Scheme I). Here K_1 and K_2 are the measured static binding constants, and k_4 and k_5 are the second-order CO binding rates, of which k_5 is the quantity of interest; the reverse reaction, loss of CO, can be neglected on our time scale.

Scheme I

$$\begin{array}{ccccc} & & \overset{K_1}{\underset{-B}{\overset{B}{\rightleftharpoons}}} & & \overset{K_2}{\underset{-B}{\overset{B}{\rightleftharpoons}}} \\ Fe & & Fe(B) & & Fe(B)_2 \\ & k_4 \downarrow +CO & & k_5 \downarrow +CO & \\ Fe(CO) & \xrightarrow[+B]{k'} & Fe(B)(CO) & & \end{array}$$

When comparing parameters for different bases, we will write, for example, $k_5(B)$.

Under the conditions of our experiments, the results of White et al.[22b] show that CO addition is rate limiting, and that the ligand rebinding rate obeys an equation which may be written:

$$\kappa \equiv \frac{k_{obsd}}{[CO]} = \frac{k_4}{\Sigma} + k_5 \frac{K_1[B]}{\Sigma} \qquad (2)$$

where $\Sigma = 1 + K_1[B] + K_1K_2[B]^2$. With this equation, if K_1 and K_2 are known for a given base, B, k_4 and k_5 can be obtained directly. The equilibrium constants for binding of Im and Py to Fe(TPP) in toluene are known:[24] (K_1(Im) = 8.8 × 10^3 M^{-1}; K_2(Im) = 7.9 × 10^4 M^{-1}; K_1(Py) = 1.5 × 10^3 M^{-1}; K_2(Py) = 1.9 × 10^4 M^{-1}). The CO binding rates have been measured as a function of the concentration of these two bases, and from the plot of:

$$\kappa\Sigma = k_4 + k_5K_1[B] \qquad (3)$$

vs. [B], (for example, Figure 2) k_4 was obtained from the intercept and k_5 from the slope (Table I). The values of k_4 so obtained agree ($k_4 \simeq 5 \times 10^7$ M^{-1} s^{-1}), and compare favorably with a value reported recently by Traylor et al.[22b] for Fe(DPD) + CO in benzene: $k_4 = 5.7 \times 10^8$ M^{-1} s^{-1}.

It is generally easy to achieve nitrogenous base concentrations which satisfy the condition $K_1K_2[B]^2 \gg K_1[B] \gg 1$. In such a case,[9] $\Sigma \simeq K_1K_2[B]^2$ and a plot of:

$$\kappa[B]^2 \simeq \frac{k_4}{K_1K_2} + \frac{k_5}{K_2}[B] \qquad (4)$$

vs. [B] is linear (Figure 3). When K_1 and K_2 are known (Im, Py in toluene), values of k_4 and k_5 can be calculated from the intercept and slope, respectively; these rates agree to within 10% with those in Table I. For bases whose binding constants are not known, the product K_1K_2 may be obtained from the intercept, k_4/K_1K_2, by adopting the uniform value, $k_4 = 5.0 \times 10^7$ M^{-1} s^{-1}. This has been done for 1-MeIm and Pip in toluene and for Py in C_6H_{12} and CH_2Cl_2. If K_2 can be approximated, an estimate of k_5 may also be obtained from the slope of eq 4, k_5/k_2. Typically, $K_2/K_1 \simeq 10$ for neutral nitrogenous bases,[24] and this assumption has been used with the experimental values of K_1K_2 and k_5/K_2 to obtain the k_5 listed in Table I. The ratio k_5/K_2 decreases by a factor of ~45 in the order Pip ≫ Py > Im > 1-MeIm. The unusual behavior for Pip undoubtedly reflects a low value for K_2, which arises from steric hindrance within the low-spin [Fe(TPP)][Pip]$_2$ complex. The approximate values of k_5 change by only a factor of ~5, although decreasing in approximately the same order. The values of K_1K_2 obtained from eq 4 decrease in the order Im > 1-MeIm > Py > Pip, which agrees with the observation made by Balch et al. for Im and 1-MeIm.

Peripheral Effects. (a) Solvent Effects. The CO recombination rates for Fe(TPP)(Py)(CO) have been measured using toluene, dichloromethane, and cyclohexane as solvents. Analysis of the data with eq 4 gives the ratios listed in Table I. Considering the major differences in the physical properties of the three solvents and the large solvent effects seen in some chemical reactions, the changes in CO binding kinetics with solvent are small. The ratio k_5/K_2 changes by a factor of less than 3 among the three solvents, and there is only a 15% dif-

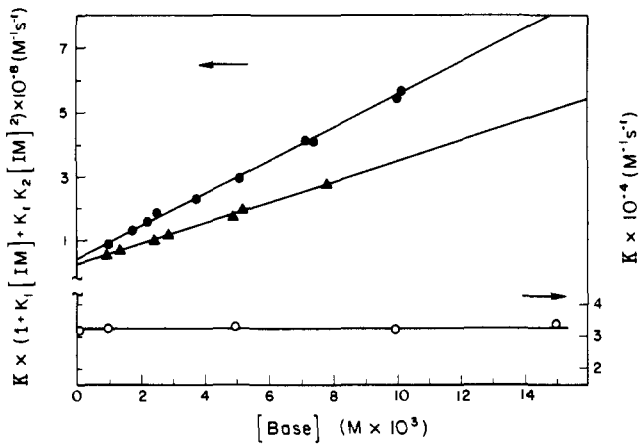

Figure 2. Plot of the CO binding rate function $\kappa\Sigma$ (eq 3) for Fe(TPP) vs. nitrogenous base concentration, [B]: (●) B = Im; (▲) B = Im in the presence of [phen] = 5×10^{-6} M; (○) B = Im$^-$ with [DC-18-C-6] = 0.2 M. (See Table III.) Conditions: toluene, 21 °C, [Fe(TPP)] = 6×10^{-6} M, [CO] = 7.5×10^{-3} M.

Table I. Rate Constants and Experimentally Measured Ratios for CO Binding[a]

base	k_4/K_1K_2, M s^{-1}	k_5/K_2, s^{-1}	k_4, M^{-1} s^{-1}	k_5, M^{-1} s^{-1}	K_1K_2, M^{-2}
Im[b]	0.070	71.9	4.9×10^7	5.7×10^6	
Py[b] (toluene)	1.9	156	5.4×10^7	3.0×10^6	
Py[c] (dichloromethane)	15	182	4.3×10^8	3.5×10^6	
Py[c] (cyclohexane)	0.44	65	1.3×10^7	1.2×10^6	
1-MeIm[d]	0.15	31.8		1.8×10^6	3.33×10^8
Pip[d]	10.8	1420		9.7×10^6	4.63×10^6

[a] Ratios listed in the first two columns were obtained through use of eq 4. [b] Values of k_4 and k_5 obtained from eq 3; equivalent values of k_4 and k_5 are obtained from the ratios in columns 1 and 2. The values of K_1 and K_2 (in toluene) were obtained from ref 24a for Im and ref 24b for Py. [c] Rate constants were obtained from ratios in columns 1 and 2, assuming K_1 and K_2 values measured in toluene. [d] K_1K_2 obtained from k_4/K_1K_2, using $k_4 = 5 \times 10^7$ M^{-1} s^{-1}; k_5 calculated by further assuming $K_2 = 10K_1$.

ference between toluene and dichloromethane. These differences undoubtedly reflect changes in both k_5 and K_2, but an upper bound for the influence of solvent upon k_5 may be obtained by assuming that K_2(Py) is the same for all three solvents ($K_2 = 1.9 \times 10^4$ M^{-1}); this forces the modest solvent effects on k_5/K_2 to appear as changes in rate constants and gives rise to the k_5 values listed in Table I.The ratio k_4/K_1K_2 changes by only a factor of 4 between cyclohexane and toluene, but is much larger in CH_2Cl_2. A reasonable conclusion is that this ratio is more sensitive to the solvent dielectric constant.

(b) Solute π-Donor/Acceptors. We employed Fe(TPP)(Py)(CO) and Fe(DPD)(Py)(CO) to examine the influence of solute π-donor/acceptors on the CO rebinding rate after photolysis. Pyridine was chosen as the base because it is convenient to handle, it cannot H-bond as does Im, and it does not react with other reagents. Measurements were made in solvents which do not participate in π interactions, methylene chloride and cyclohexane. The donors employed and their maximum concentrations were: *N,N,N,N*-tetramethyl-*p*-phenylenediamine, 1.0 M; 1,10-phenanthroline, 0.18 M; hexamethylbenzene, 0.8 M; pyrene, 0.02 M; perylene, 2.0×10^{-4} M; anthracene, 3.7×10^{-3} M. Recall that Abbott and co-workers first discussed this mechanism because of observations made using 1,10-phenanthroline.[10]

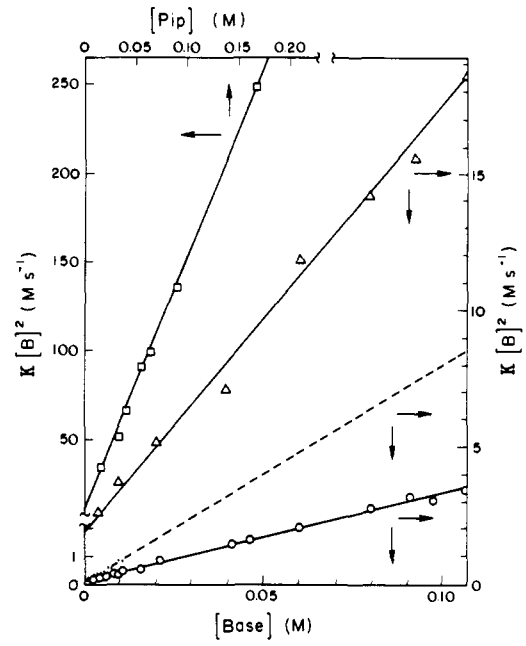

Figure 3. Plot of the CO binding rate function $\kappa[B]^2$ (eq 4) for Fe(TPP) vs. nitrogenous base concentration, [B]: (□) B = Pip; (△) B = Py; (○) B = 1-MeIm. Straight lines are calculated from the use of eq 3 and the parameters in Table I. Dashed line is the calculated behavior for imidazole over this concentration range, based upon the measurements made at lower concentrations (Figure 2). Conditions: toluene, 21 °C, [Fe(TPP)] = 6×10^{-6} M, [CO] = 7.5×10^{-3} M.

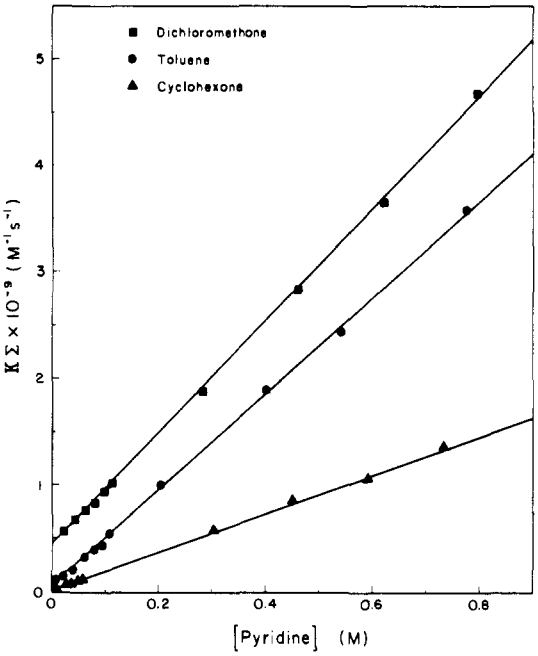

Figure 4. Plot of the CO binding rate function $\kappa\Sigma$ (eq 3) for Fe(TPP) vs. pyridine concentration in three solvents: (■) dichloromethane, [CO] = 8.5×10^{-3} M; (●) toluene, [CO] = 7.5×10^{-3} M; (▲) cyclohexane, [CO] = 11.0×10^{-3} M. (See Table I.) Conditions: 21 °C, [Fe(TPP)] = 6×10^{-6} M.

No effects of *any* donor on CO binding rates could be detected either by performing a base titration with Py over the range 0.02 to 0.79 M in the presence of a fixed high donor concentration, or by varying the donor concentration at a fixed pyridine concentration. It may be noted further that the linear behavior of $\kappa\Sigma$ vs. [B] (Figure 4) plotted according to eq 3

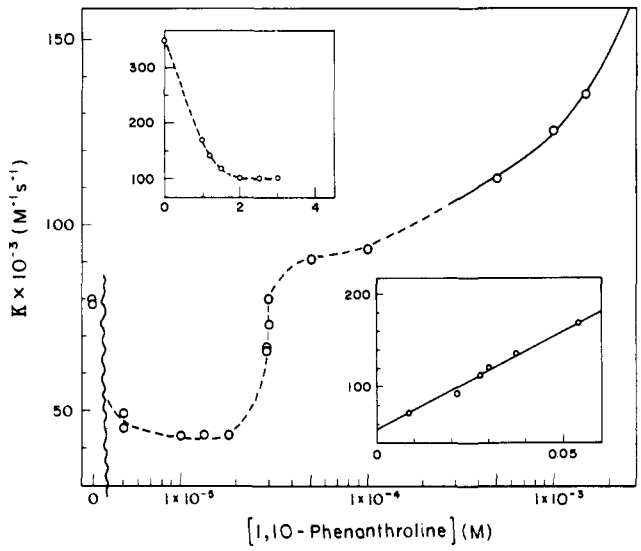

Figure 5. Observed CO binding rates, κ, for Fe(TPP) at [Im] = 1.5×10^{-3} M vs. log [phen]. Solid line represents the linear relation between κ and [phen] which exists at high [phen] as shown in the inset (right) plot of κ vs. [phen] ([Im] = 2.2×10^{-3} M). Broken line has no theoretical significance. The rapid drop in κ with initial phen additions is shown in the plot of κ vs. [phen] inset (left) with abscissa in units of 10^{-5} M. The concentration of Im is 5.6×10^{-4} M. Conditions: toluene, 21 °C, [Fe(TPP)] = 5×10^{-6} M, [CO] = 7.5×10^{-3} M.

means that pyridine itself does not act as a π donor which can influence the binding rate through "peripheral" interaction.

The acceptors employed and their maximum concentrations were: 1,2,3,5-tetracyanobenzene, 0.8 M; 1,3,5-trinitrobenzene, 0.2 M; 2,4,7-trinitro-9-fluorenone, 0.8 M. Again, no effects on CO binding rates were detected through either procedure.

Proximal Effects. (a) Fe(Por)(Im$^-$)(CO). The Fe(TPP)(Im$^-$)(CO) complex is also photolabile, and the photoproduct also returns to the starting material with a rate proportional to [CO]. However, the kinetic difference spectrum upon photolyzing Fe(TPP)(Im$^-$)(CO) does not correspond to the static [Fe(TPP)(Im$^-$)$_2$] − [Fe(TPP)(Im$^-$)(CO)] difference obtained by subtraction of the appropriate absorbance spectra (Figure 1B), nor does it agree with what would be observed if the four-coordinate Fe(TPP) were formed.[24a] Instead, the red-shifted peak with a maximum at 444 nm corresponds to the formation of the high-spin five-coordinate Fe(TPP)(Im$^-$).[22c] In addition, the isosbestic point observed at 436 nm is independent of [Im$^-$], and the rebinding of CO takes place with pseudo-first-order kinetics. We conclude that the five-coordinate Fe(TPP)(Im$^-$) produced upon photolysis directly adds CO to regenerate the Fe(TPP)(Im$^-$)(CO) without observable formation of Fe(TPP)(Im$^-$)$_2$ on the time scale of the experiment.

In a series of measurements, the observed second-order CO binding rate, κ, was independent of [Im$^-$] (Figure 2). Examination of the above observations in light of the general Scheme I indicates that in this case the observed and intrinsic rate constants are the same: $\kappa = k_5$(Im$^-$).[26] The same analysis holds for Fe(DPD)(Im$^-$)(CO) data. The second-order rates, k_5(Im$^-$), for both Fe(TPP) and Fe(DPD) are listed in Table III. Traylor and co-workers have measured the kinetics of CO binding to Fe(DPD)(Im),[22b] and the reported value of k_5 will be used as a reference (Table III).

All results presented for Fe(TPP)(Im$^-$) and Fe(DPD)(Im$^-$) are for samples in which DC-18-C-6 (~0.1 M) has been used to solubilize the KIm. The results of Nappa and Valentine[8] make it likely that there is minimal interaction between this crown ether and the porphyrin, and we find that varying the crown ether concentration between 0.005 and 0.15 M causes no change in measured properties. The smaller ether, 18-C-6, interacts with anion-bound porphyrins, and we find that the rate constant for CO binding vs. Fe(Im$^-$) is influenced to a small degree by the presence of 18-C-6, although no effect is seen in static and kinetic difference spectra or in quantum yields. Addition of a small amount of 18-C-6 (~0.003 M) to a sample prepared with DC-18-C-6 (~0.1 M) reduces k_5(Im$^-$) by a factor of only approximately two. More 18-C-6 does not further reduce k_5(Im$^-$).

(b) 1,10-Phenanthroline as Effector. 1,10-Phenanthroline was chosen to study the effects of partial hydrogen bonding with an iron-coordinated imidazole because it is known to hydrogen-bond with a metal-coordinated imidazole[25] and stoichiometrically form a 1:1 complex with Fe^{III}(DPD)(Im)$_2$(Cl).[10] Phen also is unable to compete with Im for an axial base site,[10,25] and the results presented above show that it does not affect CO binding rates through π-donor interaction with Fe(TPP)(Py). Thus, changes in CO binding rates for Fe(TPP)(Im) caused by small additions of phen may be attributed solely to hydrogen bonding.

The optical spectra of Fe(TPP)(Im)(CO) in the presence of high [phen], [phen]/[Im] $\simeq 10^2$, exhibits only a 1-2-nm red shift in the Soret maximum. Also, the kinetic difference spectrum for Fe(TPP)(Im)(CO) shown in Figure 1A changes only slightly. Both the maximum and minimum are unchanged, but the isosbestic point shifts from 429.5 nm in the absence of phen to 427.5 nm at the 100:1 ratio. Thus, the optical data support the premise that phen does not compete with Im for an axial base site.

The presence of phen markedly affects the CO rebinding rates after photolysis of Fe(TPP)(Im)(CO). Figure 5 plots κ as a function of [phen] in the presence of constant [Im]. When [phen] $\gg$ [Fe] (see Figure 5, right inset), the rate, κ, is linear in [phen]. We interpret this behavior as a "trivial" phenomenon reflecting an H-bonding equilibrium between phen and Im which reduces the effective concentration of Im free in solution.[25,27] Taking such an equilibrium into account in eq 2 leads to a linear increase in κ. A crude measurement of the H-bonding equilibrium constant gives a value of ~100 M^{-1}.

In contrast, added low concentrations of phen cause a nontrivial reduction in κ. As shown in Figure 5 (left inset), a precipitous drop in rate occurs upon phen addition, and continues until phenanthroline is approximately stoichiometric with the porphyrin (~5×10^{-6} M). The measured values of k_5/K_2 from imidazole titrations in the presence of fixed [phen] also decrease as [phen] is increased: $k_5/K_2 = 71.9$ for [phen] = 0; $k_5/K_2 = 41.1$ for [phen] = 5.0×10^{-6} M; $k_5/K_2 = 25.2$ for [phen] = 1.0×10^{-5} M. At these concentrations the interaction between free phen and Im should have no effect, and we interpret this decrease as resulting from H bonding between phen and Im bound to the metal; binding of Im to a metal is well known to increase the acidity of the imido proton.[27] A transition between the two different H-bonding regimes occurs in the intermediate [phen] region.

If K_1 and K_2 for Im are assumed to be unaffected by H bonding to phen, the ca. threefold decrease in k_5/K_2 with [Fe(TPP)] $\approx$ [phen] is wholly assigned to a decrease in k_5. As an alternate method of estimating k_5, K_1 might be approximated from the intercept k_4/K_1K_2, using k_4 (5.0×10^7 M^{-1} s^{-1}) and an assumed $K_2 = 10K_1$. The two estimates of k_5 are comparable (see Table III). These considerations, if anything, minimize the calculated effects of added phen. Both k_5 and K_2 may be expected to change upon interaction with phen. However, keeping in mind that K_2 for Im is 7.9×10^4 M^{-1} and K_2 for Im$^-$ is $\lesssim 10^2$ M^{-1}, it is reasonable to assume that any influence on the Im binding constant, K_2, caused by the presence of phen would decrease K_2 and thus *increase* k_5/K_2. Therefore, k_5 must decrease even more than does the ratio k_5/K_2. Thus, these experiments provide direct evidence that

Table II. Temperature-Dependent Quantum Yields for Photorelease of CO[a]

	21 °C	0 °C	−20 °C	−30 °C	−40 °C
MbCO[b]	1	0.94	0.92	0.91	
Fe(TPP)(Im)(CO)[c]	1.0	1.0	0.66		0.10
Fe(TPP)(Im⁻)(CO)[d]	0.92	0.66	0.53		0.35

[a] Estimated uncertainty, ±0.03. [b] Conditions: 0.05 M Tris buffer, 50% ethylene glycol, pH 7. [c] Toluene solution. [d] Toluene solution, ~0.2 M DC-18-C-6 and KIm.

Table III. Rate Constants for CO Binding

	k_5, M^{-1} s^{-1}
Fe(TPP)(Im)(CO)	5.7×10^6 [a]
Fe(TPP)(Im)(CO) + [phen]	2.3×10^6 [b]
Fe(TPP)(Im⁻)(CO)	3.4×10^4 [c]
Fe(DPD)(Im)(CO)	1.2×10^7 [d]
Fe(DPD)(Im⁻)(CO)	1.0×10^5 [c]
R-Hb(CO)	1.1×10^7 [e]
T-Hb(CO)	5.0×10^5 [e]
Mb(CO)	5.0×10^5 [f]

[a] Toluene, 21 °C. [b] Toluene, 21 °C, calculated as described in text, [phen] = 1×10^{-5} M ≃ [Fe(TPP)]. [c] Toluene solution, ~0.2 M DC-18-C-6 and KIm, 21 °C. [d] Benzene solution, 20 °C, ref 22b. [e] Conditions: 0.05 M sodium borate buffer, pH 9.2, 20 °C, ref 6. [f] Conditions: 0.05 M Tris buffer, 50% ethylene glycol, pH 7, 21 °C.

hydrogen bonding to the Im proton substantially decreases k_5.

Temperature-Dependent Phenomena. In order to compare both the photorelease and ligand recombination processes for the model compounds and hemoproteins, quantum yields were measured at various temperatures and activation energies were determined where possible. The results are presented in Table II. No temperature dependence was observed for the CO photorelease quantum yield of MbCO over the range studied ($\Phi \simeq 1$ from 21 to −30 °C), in agreement with previous results over a smaller temperature range.[21] The Fe(TPP)(Im)(CO) quantum yield is ~1 between ~0 and 25 °C, but decreases as the temperature is reduced, while the Fe(TPP)(Im⁻)(CO) quantum yield was relatively low and temperature dependent over the whole temperature range studied (21 to −40 °C). On the basis of these results, the Im complex more closely resembles myoglobin than does the Im⁻ adduct.

The kinetic difference spectrum upon photolysis of Fe(Im⁻)(CO) does not change qualitatively down to −40 °C, the lowest temperature employed, and the pseudo-first-order rate constants remain independent of [Im⁻]. Thus, over this temperature range the product of photolysis is Fe(Im⁻) and the second-order rebinding rate is k_5. Plots of ln k_{obsd} vs. T^{-1} give the Arrhenius activation energy for CO binding of ΔE = 21.2 kcal/mol for Fe(TPP)(Im⁻) and 20.0 kcal/mol for Fe(DPD)(Im⁻) (see Figure 6). The activation energies for CO + Fe(Por)(Im⁻) are considerably larger than the 4.3 kcal/mol activation energy measured for Mb + CO. This difference in observed activation energies is consistent with the conclusion that Fe(Por)(Im⁻) is a poor model for Mb.

Discussion

We have examined the possibility that H bonding to an imidazole axial base can modify the CO binding rates of Fe(Por), and have also examined changes in rates caused by solvents and π-donor/acceptor interactions. These latter might be called "peripheral effects", since they do not act directly through bonding to the metal. This section uses the results to draw conclusions about the mechanism of protein control of heme reactivity.

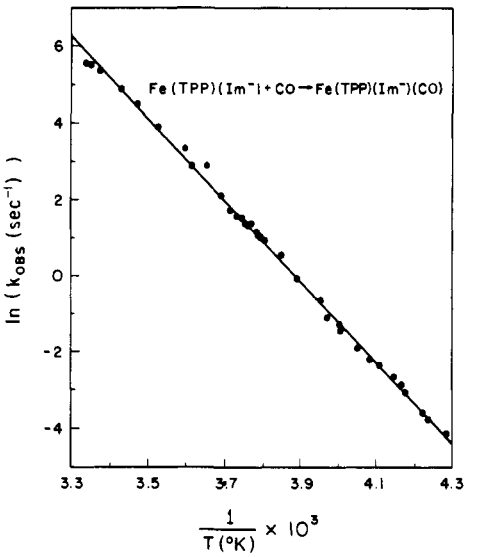

Figure 6. Arrhenius plot for CO binding to Fe(TPP)(Im⁻), toluene, [CO] = 7.5×10^{-3} M, [Fe(TPP)] = 6×10^{-6} M, [DC-18-C-6] ≃ 0.2 M.

Solvents as Donors. The similarity of CO binding rates to Fe(TPP)(Py) in dichloromethane, cyclohexane, and toluene strongly argues against the importance of π interactions between the heme and a phenyl side chain in modulating heme reactivity (Table I). Since the change from a nonaromatic solvent (C_6H_{12}, CH_2Cl_2) to one in which the heme is surrounded by phenyl rings (toluene) leaves the binding rates unchanged to within a factor of 3, it seems quite unrealistic to attribute a 20- to 60-fold rate increase from T-R conversion to a mere change in orientation of nearby phenyl groups. The small solvent effects observed could easily be explained by differences in viscosity and dielectric constants[27,28] (1.02 cP, 17 °C, C_6H_{12}; >0.59 cP, 20 °C, C_7H_8; ≳0.45 cP, 15 °C, CH_2Cl_2) (ϵ = 2.023, 20 °C, C_6H_{12}; ≲ϵ = 2.379, 25 °C, C_7H_8; <ϵ = 9.08, 20 °C, CH_2Cl_2) because experimentally measured ratios follow the same progression.

Solute Donors and Acceptors. The experiments in which donors and acceptors were added to solutions of Fe(Por)(Py)(CO) argue even more strongly against any appreciable influence of π interactions on heme reactivity. Since the binding rates, which are a function of all the kinetic and equilibrium constants (eq 2, 3, and 4), are totally unaffected by added donors or acceptors, π interactions thus give no measurable effects on any of these constants. It may be noted further that the linear behavior of the observed rate vs. [Py] in the absence of added D/A, when plotted according to eq 3, means that pyridine itself does not act as a π donor.

A more detailed analysis of these experiments is possible. In the Appendix, equations are derived for the binding kinetics expected for a solution in which a ferroporphyrin is in equilibrium with a ferroporphyrin-donor π complex. In these equations we insert π-complex formation constants measured for similar metalloporphyrin systems,[29] and utilize our observation that the CO binding rates are unchanged by donor addition to within an uncertainty of ±5%. The result is that the CO rebinding rate for a ferroporphyrin-TMPD complex differs from that of the free porphyrin by less than ~±6%. Similar analysis indicates that a ferroporphyrin complexed to the π acceptor, TNB, has CO rebinding rates within ~30% of those for the uncomplexed porphyrin. Even if the effective association constants have been overestimated by more than an order of magnitude, the equations in the Appendix show that π-complex formation with TMPD or TNB alters the CO rebinding rate of Fe(TPP)(Py)(CO) by less than 54 and 65%, respectively. We emphasize that the donor chosen for study in detail at high concentrations, TMPD, is one of the strongest

donors known;[30] it has been extensively used in the study of D/A complexes and is a far better donor than any amino acid residue. Analogous statements are true for TNB. Thus, it is clear that meaningful changes in heme reactivity cannot be produced by any change in the weak D/A π interactions possible for a porphyrin and an adjacent amino acid residue. We interpret these results as strong evidence against peripheral D/A electronic control of heme reactivity in Hb, or in any other protein.

Interactions with Bound Imidazole. The deprotonation of an iron-coordinated imidazole results in a large reduction in CO binding rate constants; for Fe(TPP), $k_5(\mathrm{Im})/k_5(\mathrm{Im}^-) = 168$, and for Fe(DPD), $k_5(\mathrm{Im})/k_5(\mathrm{Im}^-) = 120$. This is of significance because Im^- can be imagined as the product of a strong H-bond acceptor with Im. The magnitude of rate change seen upon deprotonation is more than enough to account for a 20- to 60-fold rate change seen in Hb T-R switch, and the rate constants for CO binding to T- and R-state Hb (Table III) both fall within the range spanned by the models studied here: $k_5(\mathrm{Im}^-) < k_5(\mathrm{T}) < k_5(\mathrm{R}) \simeq k_5(\mathrm{Im})$, with DPD = Por rates most closely resembling those of Hb. Note, however, that the changes are inverse to a naive expectation: deprotonation causes the rate constant to decrease, whereas if increased electron donation by the fifth ligand were the critical factor, the rate constant should increase. Instead, we interpret the rate decrease caused by H bonding to result from stabilization of Fe(Por)(B) relative to the transition state on the CO-binding reaction pathway. This is supported by the high activation energy for CO binding to Fe(Por)(Im^-).

The likelihood of total deprotonation of an Im in a protein is slight. Furthermore, the reduction in k_5 might be argued to relate more to the binding of an anion. However, a reduction in k_5 by a factor of 2 to 3 is also observed for the partial H bonding which occurs between phen and iron-bound Im in solution (this is an excess of any possible influence of H-bonding by solution IM), and one might expect a much larger reduction in k_5 if a H-bond acceptor in a protein were held in a favorable orientation for bonding with the Im proton. Thus, the general possibility of proximal control of heme reactivity through hydrogen bonding[5] is supported by the present results. Its actual occurrence in Hb is dependent on associating the T state with the structure in which the proximal imidazole is H bonded, contrary to published suggestions.[5,7]

Summary

Measurements of CO binding rates to ferroporphyrin model compounds indicate that electronic control through π-donor/acceptor interactions is not an important mechanism for controlling the heme reactivity of hemoglobin or of other hemoproteins. However, control through changes in the electronic properties of the metal-bound imidazole can have a powerful influence on heme reactivity. This latter mechanism must be included in discussions of the reactivity of nonheme metalloproteins as well, since in many such cases a histidine imidazole functions as a metal ligand.

Appendix

The estimate of π-D/A influence on CO binding rates can be obtained by considering the ferroporphyrin with m axial ligands, $\mathrm{Fe(B)}_m$, which in the absence of donor binds CO with rate constant k_n (i.e., $(m, n) = (0,4)\ (1,5)$). In the presence of donor, the $\mathrm{Fe(B)}_m$ will be in equilibrium with a π complex, $[(\mathrm{D})\cdot\mathrm{Fe(B)}_m]$, which binds CO with a possible altered rate constant k_n^{D}. CO binding will occur according to Scheme II, where K_m^{D} is the formation constant for the π complex. Considering the slow rate of CO binding and the lability of the D/A complexes,[29,31] it is clear that $\mathrm{Fe(B)}_m$ and $(\mathrm{D})\cdot\mathrm{Fe(B)}_m$ will be in rapid equilibrium, with CO binding the rate-limiting step.

Scheme II

$$\begin{array}{ccc} & \overset{K_m^{\mathrm{D}}}{\rightleftharpoons} & \\ \mathrm{Fe(B)}_m + \mathrm{D} & & (\mathrm{D})\cdot\mathrm{Fe(B)}_m \\ k_n \downarrow +\mathrm{CO} & & k_n^{\mathrm{D}} \downarrow +\mathrm{CO} \\ \mathrm{Fe(B)}_m(\mathrm{CO}) & & (\mathrm{D})\cdot\mathrm{Fe(B)}_m(\mathrm{CO}) \end{array}$$

If the donor-associated porphyrin binds CO with a rate different from that of the free $\mathrm{Fe(B)}_m$, then since the CO binding is slow, the observed rate in the presence of D will be the weighted average of the rate constants for free and associated porphyrin. Under this condition, the observed rate constants for CO binding, $\bar{k}_n$, will be a function of [D], and will be given by the equation:

$$\bar{k}_n = k_n(1 - f_m^{\mathrm{D}}) + k_n^{\mathrm{D}} f_m^{\mathrm{D}} \qquad \text{(A5)}$$

where f_m^{D} is the fraction of m-coordinate heme ($m = 0, 1$) which is complexed with D, and $(1 - f_m^{\mathrm{D}})$ is the fraction remaining uncomplexed:

$$f_m^{\mathrm{D}} \equiv \frac{K_m^{\mathrm{D}}[\mathrm{D}]}{1 + K_m^{\mathrm{D}}[\mathrm{D}]} \qquad \text{(A6)}$$

From the observed fractional change in rate induced by a donor at concentration [D], $\Delta\bar{k}_n/k_n \equiv [\bar{k}_n - k_n]/k_n$, one obtains the fractional change in the intrinsic rate constants for the $(\mathrm{D})\cdot\mathrm{Fe(B)}_m$ complex:

$$\frac{\Delta\bar{k}_n}{k_n} = \frac{(k_n^{\mathrm{D}} - k_n)}{k_n} f_m^{\mathrm{D}} \equiv \frac{\Delta k_n^{\mathrm{D}}}{k_n} f_m^{\mathrm{D}} \qquad \text{(A7)}$$

The formation of D/A complexes with $\mathrm{Co^{II}}$(p-$\mathrm{CH_3}$-TPP) has been studied in detail by La Mar.[29] Equilibrium constants for complex formation are $K^{\mathrm{D}} = 10\ \mathrm{M}^{-1}$ for TMPD, one of the strongest π donors known, and $K^{\mathrm{A}} = 17\ \mathrm{M}^{-1}$ ($\Delta H^\circ = 5.7$ kcal/mol) for TNB, one of the strongest π acceptors known. It seems reasonable to take the behavior of the Fe(TPP) system as comparable to that of the Co(p-$\mathrm{CH_3}$-TPP) system, in which case eq A6 would give $f_m^{\mathrm{D}} = 0.91$ for [TMPD] = 1.0 M, the highest concentration used. A tenfold reduction of the effective K_m^{D}, say because of orientation effects, would yield $f_m^{\mathrm{D}} = 0.5$.

References and Notes

(1) Abbreviations: porphyrin, Por; hemoglobin, Hb; myoglobin, Mb; $\alpha,\beta,\gamma,\delta$-tetraphenylporphyrin, TPP; deuteroporphyrin IX dimethyl ester, DPD; imidazole, Im; imidazolate, Im^-; piperidine, Pip; pyridine, Py; *N*-methylimidazole, 1-MeIm; 18-crown-6, 18-C-6; 1,2,7,8-dicyclohexyl-18-crown-6, DC-18-C-6; 1,10-phenanthroline, phen; *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TMPD, π-donor molecule, D; π-acceptor molecule, A.
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Structure of Arogenate (Pretyrosine), an Amino Acid Intermediate of Aromatic Biosynthesis

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Abstract: L-(8*S*)-Arogenate (previously named pretyrosine), a newly recognized precursor of L-phenylalanine and L-tyrosine biosynthesis, is widely distributed in nature. Proof of structure for arogenate, β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine, was established through the application of spectroscopic techniques (ultraviolet, ^{1}H NMR, ^{13}C NMR, and mass spectrometry) following the application of an improved procedure for isolation of L-arogenate from the culture supernatant of a mutant strain of *Neurospora crassa*. The (*S*) configuration of the chiral amino acid carbon at C-8 of L-arogenate was established by circular dichroism.

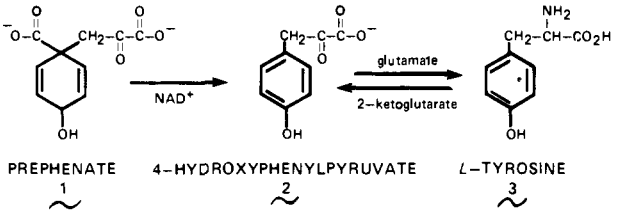
Introduction

Until 1974, the only biochemical route known for the biosynthesis of L-tyrosine in nature was as illustrated in Scheme I. Prephenate is the last nonaromatic intermediate in this 4-hydroxyphenylpyruvate sequence. A second sequence, the arogenate branchlet of L-tyrosine biosynthesis (Scheme II), has been identified as the sole route of L-tyrosine biosynthesis in species of cyanobacteria,[2,3] coryneform bacteria,[4–6] and in at least one yeast organism.[7] In the latter cases arogenate is the last nonaromatic intermediate. Pseudomonad bacteria[8–10] and plants[11] possess both enzymatic sequences simultaneously.

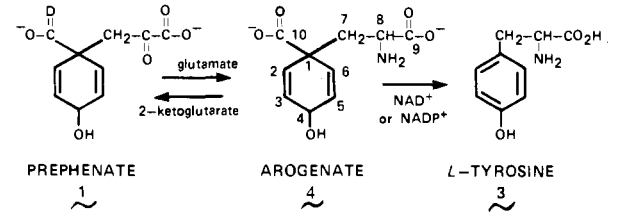
The structure depicted in Scheme II for arogenate was deduced[2] on the basis of enzymological results. Transamination of prephenate via a partially purified aminotransferase from *Agmenellum quadruplicatum* in the presence of L-leucine as the amino donor produced a ninhydrin-positive product that was easily distinguished from L-leucine by thin-layer chromatography. A dehydrogenase was partially purified which was able to oxidize the unknown compound to tyrosine in the presence of either NAD^+ or $NADP^+$. Synonymy of tyrosine and the product formed by the dehydrogenase was established by thin-layer chromatography, fluorescence profile, and amino acid analysis. Furthermore, acid treatment of arogenate yielded phenylalanine in a reaction analogous with the nonenzymatic conversion[12] of prephenate to phenylpyruvate at acidic pH. Since in some organisms[13] arogenate is a substrate for arogenate dehydratase, an enzyme which forms phenylalanine, the previous name (pretyrosine) is now abandoned in favor of the more appropriate designation.[14]

Rigorous chemical proof of structure for arogenate has not been offered prior to this study. In particular, the existence of the carboxyl group at C_1, the positioning of the double bonds in the cyclohexadiene moiety, and the stereochemistry of the C_8 chiral carbon (*R* or *S*) were not established. The ubiquity in nature of this newly found amino acid intermediate highlights the importance of structural data. The purpose of this work is (i) to establish the structure of arogenate and (ii) to assign the configuration of the asymmetric carbon at C-8.

Scheme I. 4-Hydroxyphenylpyruvate Pathway to L-Tyrosine



Scheme II. Arogenate Pathway to L-Tyrosine



Results and Discussion

Isolation of Arogenate. Small amounts of arogenate were originally made by using prephenate as substrate for a partially

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