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Garfield P. Royer,* G. M. Anantharmaiah
Department of Biochemistry, The Ohio State University
Columbus, Ohio 43210
Received June 12, 1978

Kinetics of CO Binding to Fe(TPP)(Im) and Fe(TPP)(Im$^-$): Evidence Regarding Protein Control of Heme Reactivity

Sir:

Protein structure modulation of the reactivity of a heme prosthetic group has been a focus for numerous investigations.[1] For example, ligand binding kinetics of hemoglobin are affected by protein conformation.[1a,2] The CO on-rate for the low-affinity T state is ~20-60-fold less than that for the high-affinity R state.[3] One plausible mechanism of this modulation has been advanced by Peisach and collaborators.[4] It is generally proposed that a protein can induce changes in heme reactivity through structural changes at the proximal imidazole. For hemoglobin, in the low-affinity state the proximal histidine is thought to be in its neutral form with a proton on N-1. The high-affinity form is considered to have a strong hydrogen bond to that proton, which in the limit might be thought of as corresponding to a deprotonated imidazole as the sixth ligand. Such a mechanism also has been invoked to discuss the electronic structure of cytochromes *c* from different organisms,[5] and the crystal structures of a number of hemoproteins.[6]

Prompted by our previous work on the mechanisms of heme-protein interactions,[5,7] we now have tested the relation of heme reactivity and the imidazole protonation state by the direct comparison of the CO binding rate, for a five-coordinate ferrous porphyrin model with neutral imidazole as fifth ligand, Fe(P)(Im),[8] with that for the model with deprotonated imidazole as the fifth ligand, Fe(P)(Im$^-$). Deprotonation can indeed alter the CO binding rates to a degree in excess of the difference in binding rates to the T and R states of Hb. However, the difference is in the opposite sense to that which would normally be expected: deprotonation *decreases* the rate of CO binding.

Kinetic measurements were made by monitoring absorbance changes after flash photolysis of an Fe(TPP)(B)(CO), B = Im, Im$^-$, solution,[9-12] using a computer-interfaced apparatus of conventional design.[13] The Fe(TPP)(Im)(CO) complex is highly photolabile and the photoproduct rebinds CO with a rate constant, $k_{obsd} \propto [CO]$. However, the difference spectrum of Fe(TPP)(Im)(CO) and the product obtained immediately after the flash (~20 μs) does not correspond to the formation of Fe(TPP)(Im), which should have a Soret maximum at ~435 nm.[11] Rather, the kinetic difference spectrum is the same as the static difference spectrum [Fe(TPP)(Im)$_2$] − [Fe(TPP)(Im)(CO)], obtained by direct subtraction of the absorbance spectra of the appropriate complexes, which is also presented in Figure 1A. This indicates that a second Im is bound within the flash lifetime. Therefore the overall stoichiometry of the CO rebinding reaction as observed in the regeneration of Fe(TPP)(Im)(CO) after the photolysis flash is ligand replacement

$$\text{Fe(TPP)(Im)}_2 + \text{CO} \rightarrow \text{Fe(TPP)(Im)(CO)} + \text{Im}$$

and not the ligand addition reaction

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

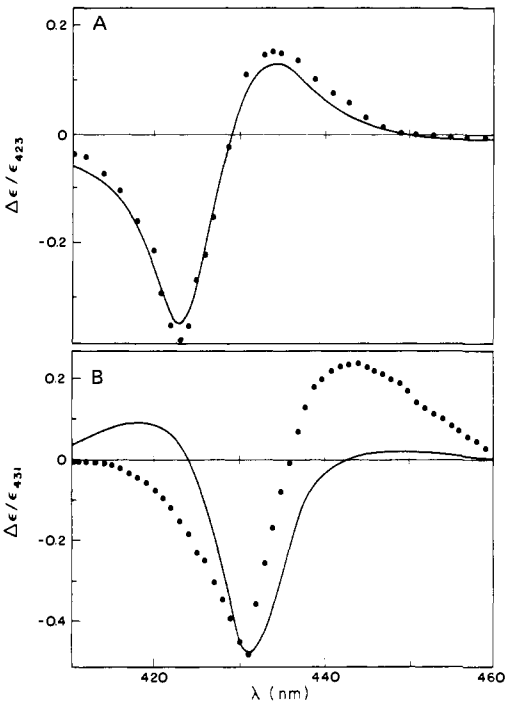
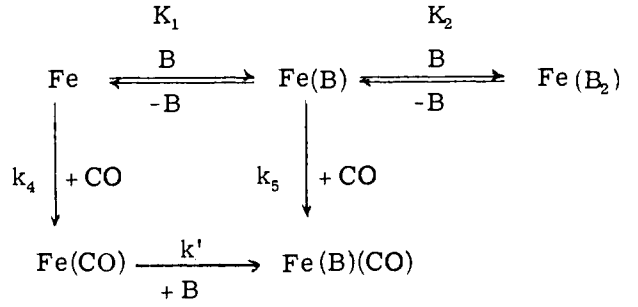


Figure 1. (A) Static difference spectrum, Fe(TPP)(Im)$_2$–Fe(TPP)(Im)(CO), —; kinetic difference spectrum after flash photolysis of Fe(TPP)(Im)(CO), ···. (B) Static difference spectrum, Fe(TPP)(Im$^-$)$_2$–Fe(TPP)(Im$^-$)(CO), —; kinetic difference spectrum after flash photolysis of Fe(TPP)(Im$^-$)(CO),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

Scheme I



which would correspond to the CO binding reaction by Hb.

This observation is expected from previous studies, such as those of Traylor;[14] binding of CO to an iron(II) porphyrin in the presence of excess base (B) can usually be described in terms of a preequilibrium between the Fe(B)$_n$ (n = 0, 1, 2),[11,14] 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. When comparing rates for different bases, we will keep track by writing k_5(B).

Under the conditions of our experiments, the results of White et al.[14c] show that CO addition is rate limiting, and that the ligand rebinding rate obeys an equation

$$k_{obsd}/[\text{CO}] = k_4/\Sigma + k_5(K_1[\text{B}]/\Sigma) \qquad (1)$$

where $\Sigma = 1 + K_1[\text{B}] + K_1K_2[\text{B}]^2$. The equilibrium constants for binding Im by Fe(TPP) are known: $K_1 = 8.8 \times 10^3$ M^{-1} and $K_2 = 7.9 \times 10^4$ M^{-1}.[11] We performed a series of rate measurements at constant [CO] and with varying [Im], and, from the plot of $k_{obsd}\Sigma/[\text{CO}] = k_4 + k_5K_1[\text{B}]$ vs. [B] (Figure 2), we obtain k_4 from the interecept and k_5 from the slope (Table I).

The Fe(TPP)(Im$^-$)(CO) complex is also photolabile, and

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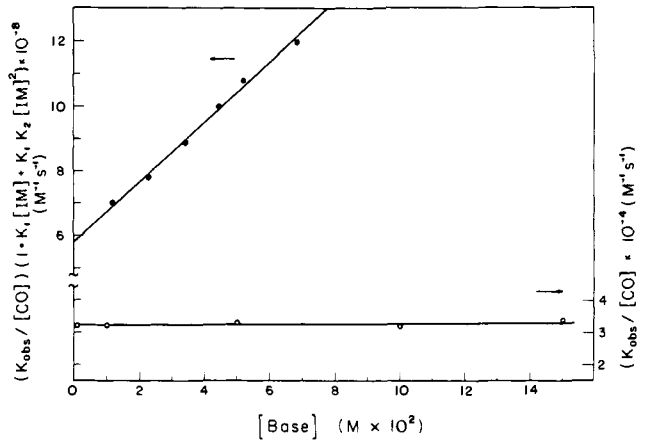

Figure 2. CO binding kinetics for Fe(TPP) as a function of nitrogenous base concentration, [B]: closed circles represent $k_{obsd} \times [1 + K_1[B] + K_1K_2[B]^2]/[CO]$ (ordinate to left) for B = Im, [CO] = 5×10^{-4} M, [Fe(TPP)] = 6×10^{-6} M; open circles represent $k_{obsd}/[CO]$ (ordinate to right) for B = Im^-, [DC-18-C-6] = 0.2 M, [CO] = 7.5×10^{-3}, [Fe(TPP)] = 6×10^{-6} M.

Table I. Rate Constants for CO Binding

	k,[a] M^{-1} s^{-1}	source
FeTPP	5.8×10^8	this work[b]
Fe(TPP)(Im)	1.1×10^7	this work[b]
Fe(TPP)(Im^-)	3.4×10^4	this work[c]
Fe(DPD)(Im^-)	1.0×10^5	this work[c]
Hb(R)	1.1×10^7	ref 3[d]
Hb(T)	5×10^5	ref 3[d]
Mb	5×10^5	ref 2a[e]

[a] For Fe(TPP) $k = k_4$; for Fe(TPP)(B), $k = k_5$ (Scheme I in text); k for the proteins is equivalent to k_5. [b] Toluene, 21 °C. [c] Toluene solution, ~0.2 M DC-18-C-6 and KIm, 21 °C. [d] Conditions: 0.05 M sodium borate buffer, pH 9.2, 20 °C. [e] Conditions: 0.1 M P_i buffer, pH 7, 20 °C.

the photoproduct also returns to the starting material with a rate ∝ [CO]. The kinetic difference spectrum upon photolyzing Fe(TPP)(Im^-)(CO) surprisingly 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.[11] Instead, the red-shifted peak with maximum at 444 nm corresponds to the formation of the high-spin five-coordinate Fe(TPP)(Im^-).[15] 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. Thus, although under equilibrium conditions Fe(TPP)(Im^-) can bind a second imidazolate, with a low binding constant ($K_2 \sim 10^2$ M^{-1}),[13] the above observations further indicate that, for purposes of the analysis of kinetic data by eq 1, we may ignore the term in Σ which is proportional to [B^2], which is equivalent to setting $K_2 = 0$. In a series of measurements at constant [CO] and with 10^{-2} M ≤ [Im^-] < 0.2 M the observed CO binding rate was independent of [Im^-] (Figure 2). Examination of eq 1 shows that such behavior validates our method of analysis and implies that the binding constant of Im^- and Fe(TPP) is $K_1 \gg 10^2$ M^{-1} and that the observed pseudo-first-order binding rate, k_{obsd}, is just k_5[CO]. The second-order rates, $k_5(Im^-)$, for Fe(TPP)(Im^-) and Fe(DPD)(Im^-) are listed in Table I.[16]

The deprotonation of a coordinated imidazole results in a large reduction in CO binding rate constants: for Fe(TPP), $k_5(Im)/k_5(Im^-) = 320$ (Table I). However, note that the changes are inverse to 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. It is probable that Im^- as an axial ligand stabilizes the five-coordinate ferroporphyrin, and that this effect far overbalances any influence of increased charge donation by Im^- in the transition state on the reaction path by which the six-coordinate Fe(**P**)(Im^-)(CO) is formed.

The rate constants for CO binding to T and R state of Hb, although sensitive to solution conditions, differ only by ~X20–X60 (Table I), and both fall within the range spanned by the models studied here: $k_5(Im^-) \ll k_5(T) < k_5(R) \approx k_5(Im)$. Thus, the Peisach mechanism, if inverted, is in principle more than adequate to explain the protein modulation of the CO binding rates, and only a relatively minor change in "protonation state" upon the T ↔ R conversion need be invoked. However, the kinetic difference spectra for both the TPP and DPD complexes show that the Soret band of the five-coordinate imidazolate adduct is appreciably red shifted from that of the imidazole adduct, and the same is expected for heme itself. In contrast, the Soret band of T state of Hb is blue shifted from that of the R state.[3] Moreover, the analysis of hemoprotein crystal structures suggested stronger hydrogen bonding in the R state.[6a]

Protein modulation of heme reactivity can be extremely subtle. For example, myoglobin can bind ligands with an equilibrium constant ($P_{1/2}$) comparable with that of the R state of Hb,[2a] but the CO on-rate is equal to that of the T state (Table I). The present results as well as others[17] would appear to make it unlikely that the Peisach mechanism applies to hemoglobin function. However, the large change in ligation rates brought about by altering the properties of the fifth heme ligand is itself of intrinsic interest and importance. Moreover, it enhances the plausibility of applying this mechanism to other proteins.[5]

Acknowledgments. We thank Dr. Terry E. Phillips for his technical assistance with the electronics and computer interfacing of the flash apparatus. This work was supported by the National Institutes of Health (HL-13531 (B.M.H.) and GM-24759 (J.S.V.) and the National Science Foundation (PCM76-81304 (B.M.H.)). James C. Swartz is a National Institutes of Health postdoctoral fellow (F32-HL05787-0).

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(8) Abbreviations: porphyrin (P); hemoglobin (Hb); myoglobin (Mb); α,β,γ,δ-tetraphenylporphyrin (TPP); deuteroporphyrin IX dimethyl ester (DPD); imidazole (Im); imidazolate (Im^-); piperidine (pip); 18-crown-6 (18-C-6); 1,2,7,8-dicyclohexyl-18-crown-6 (DC-18-C-6).
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James C. Swartz, Marlene A. Stanford
James N. Moy, Brian M. Hoffman*
Department of Chemistry, Northwestern University
Evanston, Illinois 60201

Joan S. Valentine
Department of Chemistry, Busch Campus
Rutgers, The State University
New Brunswick, New Jersey 08903
Received February 2, 1979

Matrix Isolation of a Triplet Biradical from a Carbene Precursor

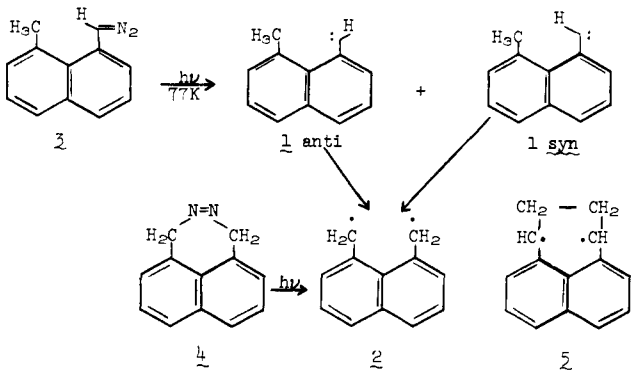
Sir:

Low temperature ESR spectroscopy has been used to determine the geometry,[1] spin distribution,[2] ground-state multiplicity,[3] and dynamical properties[4] of thermally accessible triplet biradicals immobilized in solid solution. The biradical is usually prepared by direct photofragmentation of a cyclic azo or carbonyl compound. The success of the method is dependent upon the synthetic availability of the precursor and a finite quantum yield for formation of the triplet biradical. Even if the triplet is the ground state of the biradical, and it lies in a potential minimum, there may be no pathway for populating and observing it by ESR.

It appeared possible that intramolecular hydrogen abstraction of a carbene might provide an alternate method of biradical matrix isolation. In some cases the acyclic carbene precursor may be more accessible than the requisite cyclic compound. Inter- and intramolecular hydrogen abstractions are well-known reactions of singlet and triplet carbenes.[5,6] The intermolecular process has been detected spectroscopically by NMR (CIDNP)[7] and ESR.[8]

Carbene **1** was chosen to test the feasibility of this method of biradical matrix isolation as α-naphthylcarbene[9] and 1,8-naphthoquinodimethane (**2**)[10] have stable, thermally accessible triplet states at liquid nitrogen temperature.

Mercuric oxide oxidation of the hydrazone[11] of 8-methyl-1-naphthaldehyde[12] yields the diazo compound **3**.[16] Photolysis



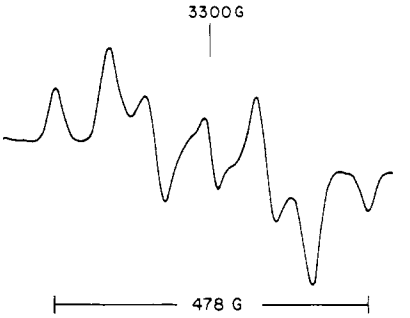


Figure 1. The triplet ESR spectrum of 1,8-naphthoquinodimethane in 2-methyltetrahydrofuran at 77 K.

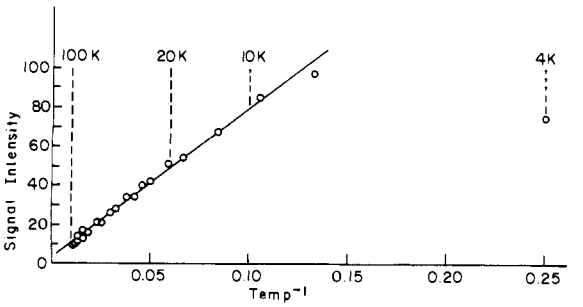
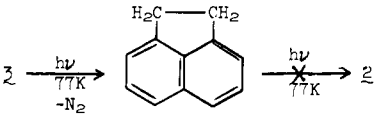


Figure 2. The Curie-Weiss Law analysis of 1,8-naphthoquinodimethane in 2-methyltetrahydrofuran.

of **3** ($350 < \lambda < 700$ nm) in hexafluorobenzene or 2-methyltetrahydrofuran at 77 K produces the spectrum of a randomly oriented triplet state (Figure 1).[17] This spectrum ($|d/hc| = 0.024 = 0.002\ cm^{-1}$, $|E/hc| = 0.001\ cm^{-1}$) is virtually identical with that reported for 1,8-naphthoquinodimethane obtained from azo compound **4**.[10b] Control experiments with acenaphthene demonstrate that triplet **2** does not arise from a secondary process.[18]



Trozzolo, Wasserman, and Yager have shown that triplet α-naphthylcarbene exists in two conformations, syn and anti.[9] The two analogous geometric forms of triplet **1** will show markedly different reactivity toward hydrogen abstraction, provided that their rate of interconversion is slower than the rate of chemical reaction. The photolysis of **3** at 77 or 4 K ($\lambda > 350$ and > 470 nm, respectively) produces no resonances which can be attributed to either form of **1**. However, it remains to be shown whether the free carbene is indeed a direct precursor of the biradical. If this proves to be the case, the carbene must be extremely short lived owing to the proximity of the reactive moieties.

A Curie-Weiss Law[19] analysis of **2** over the temperature range 4–98 K is in agreement with recent work of Wirz on the ethano-bridged biradical **5**.[20] The straight-line plot obtained between 10 and 98 K indicates that 1,8-naphthoquinodimethane is most probably a ground-state triplet biradical (see Figure 2).[21] Although very low microwave power (0.01 mW) is employed in the analysis, the triplet resonance absorptions are still saturated below 10 K, producing apparent nonlinear Curie-Weiss Law behavior in the lower limit of the temperature range.

Further work with carbenes and nitrenes to prepare new heteroatomic biradicals is in progress.

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