Kinetics of CO Binding to Cytochrome *c* Peroxidase: pH and CO Concentration Effects

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The role of the protein moiety in modulating the ligand-binding activity of a heme group is of considerable importance in the functioning of heme proteins. For example, both oxygen-carrying proteins and peroxidases contain a protoheme group bonded to the protein via an iron–histidine bond but only the former reversibly bind oxygen. Since the crystal structure of cytochrome *c* peroxidase (CCP; EC 1.11.1.5) has recently been refined at high resolution,[1,2] it is now possible to compare structure–function relationships in both classes of proteins. The kinetics of CO combination have been used extensively to probe the reactivity of myoglobin (Mb);[3a] thus, the availability of similar data for CO binding to CCP would provide an experimental assessment of protein effects. One of us recently reported CO recombination data for ferroCCP following photodissociation of carboxyCCP.[4] Further investigations of this reaction revealed a number of novel features which are presented here.

FerroCCP–CO recombination can be measured by monitoring absorbance changes due to the growth of carboxyCCP or the decay of ferroCCP after a short photolyzing flash.[4] Closer examination of the decay of the absorbance at 440 nm at room temperature[5] indicates that the recombination process is biphasic, (Figure 1A). At pH 7.0, 30% of the protein recombines with CO within 10 ms after the flash, whereas the process reported previously occurs on a 0.1-s time scale. Both rates are independent of CO concentration (Table I) in contrast to the rates observed following rapid mixing of ferroCCP and CO, which show definite first-order dependence on CO concentration.[6] This suggests that photodissociated CO is "trapped" either in the heme pocket or the protein matrix and fails to reach the solvent prior to recombination. Geminate recombination has been observed in the photolysis of carboxylMb at low temperature[3a] and in the room temperature photolysis of carboxyhemoglobin.[3b] However, in the latter, geminate recombination occurs within 100 ns[3b] unlike the slow, biphasic geminate recombination of CCP.

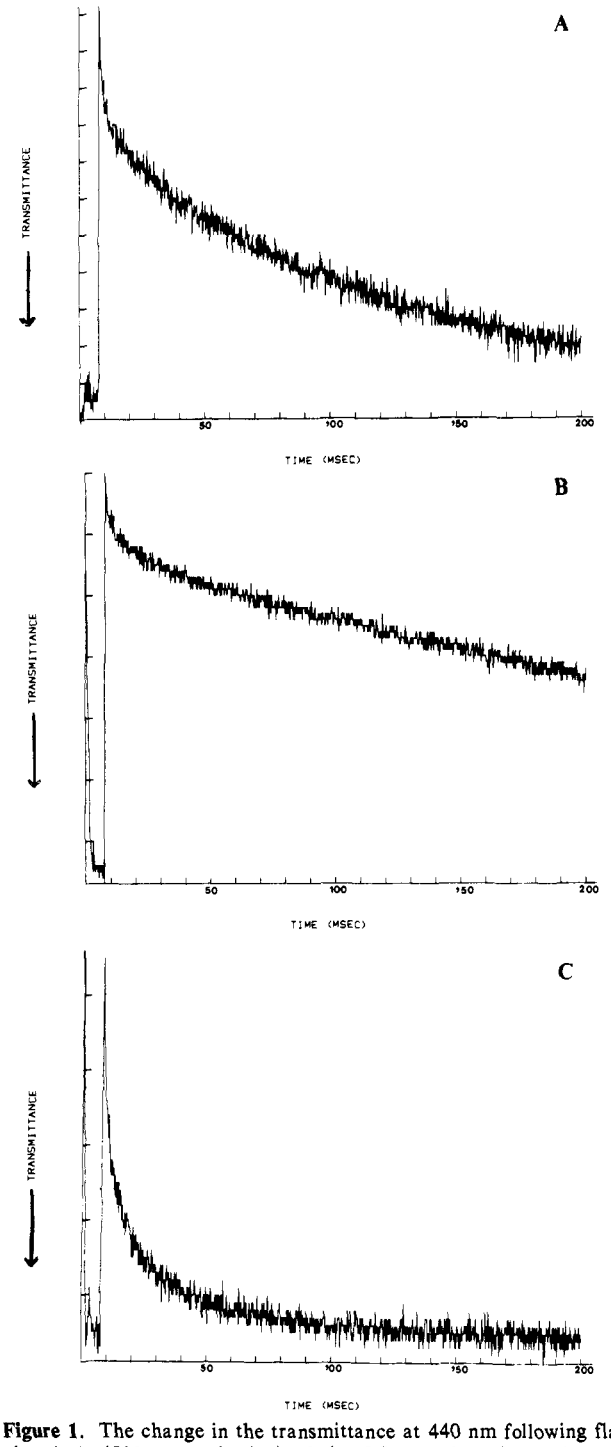

Figure 1. The change in the transmittance at 440 nm following flash photolysis (532-nm excitation) of the CO complex of cytochrome *c* peroxidase (20 μM) in 10 mM phosphate buffer: (A) pH 7.0, (B) pH 6.0, (C) pH 8.0.

Table I. FerroCCP and CO Recombination Rate Constants after Flash Photolysis at Room Temperature and pH 7.0[a]

P_{CO}, atm	k_f, s^{-1}	k_s, s^{-1}
1	400	5
0.1	600	4
0.04	300	6

[a] FerroCCP denotes ligand-free, Fe(II) cytochrome *c* peroxidase. The experimental conditions are given in ref 4. Approximately 100%, 70%, and 20% of the recombination occurs via the slow component (k_s) at pH 6.0, 7.0, and 8.0, respectively.

The biphasic kinetics of trapped CO could arise from a number of factors. Although a single heme CO-binding site is expected, the bound ligand could have different geometries or environments

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(1) CCP denotes the ligand-free, Fe(III) form of the peroxidase, which is its resting form. Only the ferrous enzyme binds CO; therefore, in the text ferroCCP and carboxyCCP refer to ligand-free and carboxy forms of the ferrous enzyme, respectively.

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(4) English, A. M.; Sharma, D.; Walsh, M. *Inorg. Chim. Acta* **1984**, *91*, L27.

(5) CCP (20 μM) in 10 mM phosphate buffer (pH 6–8) containing 0.008% acetophenone and 2% isopropyl alcohol was sealed in a 1-cm cuvette under CO. CarboxyCCP was formed in situ by photoreduction of CCP to ferroCCP following the procedure of: Ward, B.; Chang, C. K. *Photochem. Photobiol.* **1982**, *35*, 757. The absorption spectra of carboxy- and ferroCCP have Soret maxima at 423 and 438 nm, respectively. The decrease in the absorbance at 440 nm was found to be a convenient wavelength to monitor the recombination under our experimental conditions. Laser flash photolysis was carried out using a Quanta Ray Model DCR2 Nd:YAG laser (532-nm excitation) and a 150-W tungsten probe beam. The transients were recorded with a RCA 1P28 PM and a Biomation Model 6500 transient recorder. CCP was isolated from baker's yeast by the published procedure (Nelson, C. E.; Sitzman, E. V.; Kang, C. H.; Margoliash, E. *Anal. Biochem.* **1977**, *83*, 622).

(6) At pH 7.0, CCP (5 μM) and CO combine with pseudo-first-order rate constants of 0.955 and 0.425 s^{-1} at CO pressures of 1 and 0.5 atm, respectively; thus, the bimolecular on rate for CO is 1×10^3 M^{-1} s^{-1} (Olson, J. S., private communication. Mims, M. P.; Porras, A. G.; Olson, J. S.; Noble, R. W.; Peterson, J. A. *J. Biol. Chem.* **1983**, *258*, 14219). These observations have been checked and confirmed in our own lab, obviating any sample differences as the source of the discrepancy.

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with different on rates, or CO could be trapped in different sites, in which case the slow on rate could conceivably be a rate-limiting protein conformational change preceeding recombination.

Optical studies indicate that ferroCCP undergoes a heme-linked, cooperative two-proton ionization with a pK = 7.7.[7] To determine if the kinetics were also pH dependent, we examined the recombination at pH 6–8. At pH 6.0 only the slow phase is observed; the fast phase predominantes at pH 8.0 with the decay being ~80% monophasic. In addition, a cooperative two-proton ionization (7 < pK < 8) appears to control the rate transition.[8] Since both the spectral and rate transitions exhibit similar pH dependencies, it is plausible that deprotonation of the same group or groups is involved in each case. This in turn would imply that the CO association rate is largely controlled by the properties of the heme pocket rather than the protein matrix.

However, the protein matrix must play a role in preventing the escape of CO to the solvent following photodissociation, since the crystal structure of CCP reveals that its ligation pocket lies ~10 Å below the protein surface.[2] In Mb the pocket is exposed allowing rapid CO escape at room temperature.[3a] To further probe the kinetic barriers to ligand binding in ferroCCP, we are currently investigating the temperature dependence of CO recombination. Also, to ascertain whether similar barriers are present in the ferric form of the enzyme which binds H_2O_2, flash studies on the NO complex of CCP are under way.

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(8) The rate transition between pH 7 and 8 is too sharp to arise from the ionization of a single proton. Kinetic titration in this pH region suggests that a cooperative 2.0 ± 0.3 proton ionization is involved (Taylor, K., unpublished results).

Magnetic Behavior of Nonet Tetracarbene, *m*-Phenylenebis((diphenylmethylen-3-yl)methylene)

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Recently we presented the ground-state nonet molecule *m*-phenylenebis((diphenylmethylen-3-yl)methylene) (**1**)[1] as a model for organic ferromagnets[2] and discussed the unique molecular structure which makes the high-spin ground-state accessible. Characterization of **1** was based on the ESR fine structures. We have now studied the magnetic behavior of this highly important tetracarbene **1** as a molecular ferromagnet and found that the high-spin multiplicity of polycarbenes could be effectively determined by magnetic susceptibility measurements. An interesting behavior relevant to the magnetic interaction of the tetracarbene molecules has also been found.

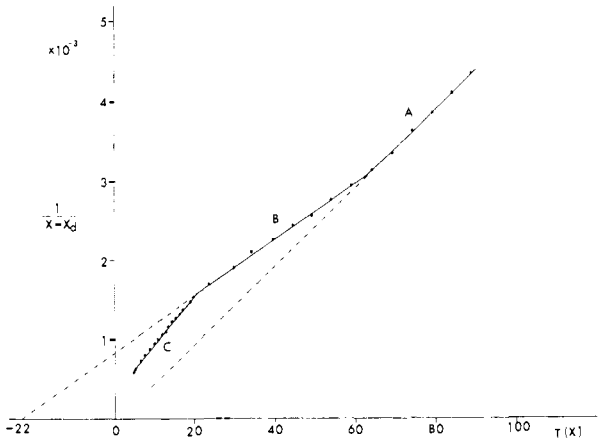
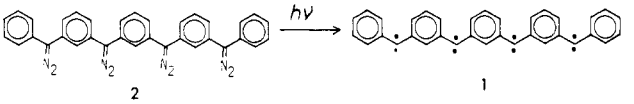


Figure 1. The temperature dependence of paramagnetic susceptibility of tetracarbene **1** in a glassy matrix of 2-methyltetrahydrofuran (8.7 × 10^{-4} M).

A single crystal of benzophenone doped with tetrakis(diazo) compound (**2**) (5.0 × 10^{-4} M) in a cylindrical quartz cell (10 mm in diameter and 12 mm depth) was placed in a cryostat of an Oxford magnetic balance system. After evacuation to remove oxygen, no ferromagnetic impurities were detected at 4.2 K by the field dependence of magnetization of the sample. Irradiation of the diazo compound was performed through a quartz window at the bottom of the cryostat with an ultra-high-pressure mercury lamp (Philips SP 500W).[3] Magnetization of the photolyzed sample was recorded at various temperatures, the field gradient being fixed at 5 T/m by separate coils from a main coil (0.5 T).



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The amount of **1** generated in the cell was determined by the decrease of a UV absorption at 520 nm due to the diazo compound after the magnetic measurement. The diamagnetic susceptibility (χ_d) of the photolyzed sample was determined by the χ vs. $1/T$ plot and the value −0.598 × 10^{-6} g^{-1} was subtracted to obtain paramagnetic susceptibilities ($\chi_p = \chi - \chi_d$). The $1/(\chi - \chi_d)$ vs. T plots in this system gave a straight line in the whole temperature range (2–100 K). The slope of the line gave μ_{eff} = 9.08 μ_B and the spin number n of 8.1 in good agreement with the theoretical values. These results clearly show that **1** has the nonet spin multiplicity in the ground state and is homogeneously dispersed in the crystal, as previously shown by the EPR spectra.[1]

The magnetic susceptibility of **1** was then measured in a glassy matrix of 2-methyltetrahydrofuran (2-MTHF) (3.77 × 10^{-3} M). Plots of $1/\chi_p$ vs. temperature are not represented by a simple line (Figure 1), suggesting that the magnetic interaction between the tetracarbene molecules is now important. The straight line in region A passes through the origin when extrapolated. The individual magnetic moment is considered not to suffer from the molecular field made by the other paramagnetic species presum-

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(3) The ESR signals due to the nonet spin multiplicity come out first when irradiation is started in matrices and single crystals at cryogenic temperatures, and the initial rates of the formation of the nonet species are linearly dependent on the incident light intensity. The formation of **1** from **2** is therefore concluded to be a one-photon process under these conditions. This excludes the presence of carbene species carrying the unreacted diazo groups and therefore of lower spin states. The conversion of **1** from **2** was 23% after irradiation for 30 min. See: (a) Itoh, K.; Takui, T.; Teki, T. "Abstracts of Papers", 46th Annual Meeting of the Chemical Society of Japan, Niigata, Japan, Oct 1982; Chemical Society of Japan: Tokyo, 1982; p 17. (b) Sugawara, T.; Inada, M.; Iwamura, H. *Tetrahedron Lett.* **1983**, *24*, 1723. (c) Sugawara, T.; Bethell, D.; Iwamura, H. *Ibid.* **1984**, *25*, 2375.

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