Sol−Gel-Encapsulated Heme Proteins. Evidence for CO_2 Adducts

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Heme proteins have been the subjects of numerous studies of photoinduced ligand dissociation and recombination (e.g., O_2, CO, and NO). Much of this work[1] is aimed at unraveling the dynamical behavior of myoglobin (Mb), which lacks a crystallographic channel connecting the iron center with the solvent. Despite its importance as a conspicuous metabolic breakdown product, CO_2 interactions with metalloproteins are not well understood. CO_2 is required for the assembly of the active sites of some multinuclear metalloproteins (e.g., the Ni_2 center of urease[2]). In carbonic anhydrase, zinc-coordinated hydroxide is believed[3] to attack CO_2, forming a bicarbonate product complex. Deoxyhemoglobin forms carbaminohemoglobin[4] in red blood cells upon reaction of CO_2 with the amino termini more readily than oxyhemoglobin does; oxygenation in the lungs causes CO_2 release. More importantly, there is no direct evidence for the existence of a metal-ligated CO_2 adduct of any metalloprotein.

In addition to transporting gases, hemoglobin and myoglobin possess enzymatic activity: reactions of the met forms with peroxides generate intermediates[5,6] containing ferryl (Fe^{IV}═O) and protein radical species that are capable of oxidizing small substrates. In the case of linoleic acid, it has been established[7] that substrate oxidation by Mb occurs by direct ferryl oxygen atom transfer. In this paper, we demonstrate that ferrylMb oxidizes carbon monoxide to produce CO_2. In addition, we present the first spectroscopic evidence for $Fe^{III}-CO_2$ coordination in heme proteins.

The reaction[7] of metMb with excess hydrogen peroxide generates a metastable intermediate, containing ferryl iron, that decays within minutes to produce degraded forms of myoglobin. However, in a saturated CO solution, Mb is observed[8] to produce a gaseous product. Table 1 presents the results of mass spectrometric analyses[9] of the gases in sealed pear flasks containing combinations of metMb, H_2O_2, and CO in phosphate buffer (50 mM, pH 7.0). The $m/e = 44$ signal clearly indicates that the product of the catalyzed reaction is CO_2. However, the low solubility of CO_2 precluded attempts to obtain the electronic spectrum of the product complex under these conditions.

Table 1. Summary[a] of GC-MS Analyses of Myoglobin-Catalyzed CO_2 Formation from CO and H_2O_2

reaction mixture	$m/e = 44$ (CO_2) (%)	$m/e = 28$ (CO) (%)
metMb + CO + H_2O_2	25[b]	2
CO + H_2O_2	<0.05	12

[a] Reaction conditions: 7.5 mL of 50 mM phosphate buffer (pH 7.0), 200 μL of 30% H_2O_2 (Fisher); 1.2 mM metMb was added to the first mixture; solutions were exhaustively degassed prior to addition of 1 atm of 99.99% CO (Matheson, purified over Ascarite II); samples were incubated (22 °C) for 30 min prior to analysis[9] of the head gases. [b] Percentages are reported relative to the $m/e = 16$ fragment peak.

Following the suggestion of Ibers[10] that liquid CO_2 be used in searching for new CO_2 complexes, we reasoned that supercritical CO_2 would be an even better choice, owing to its gaslike viscosity. Furthermore, supercritical fluids constitute reaction media that are of emerging biotechnological interest.[11]

MetMb, in buffered solutions or in lyophilized form, denatures when exposed[12] to liquid or supercritical CO_2. We therefore explored sol−gel immobilization[13] as a means of attenuating the denaturation of Mb. Immobilization solutions containing ca. 1 mM metMb were prepared by the hydrolysis of tetra(methoxy)-silane (TMOS) in 10 mM phosphate buffer (pH 6.0), following recent reports.[14] After gelation, aging (22 °C) for 2 weeks, and slow solvent evaporation, sol−gel monoliths, suitable for optical spectroscopy in the visible region, were produced. Figure 1 displays[15] electronic absorption spectra (Soret band) of an immobilized metMb sample before, during, and after exposure to supercritical CO_2. The 10 nm blue shift in the metMb Soret spectrum, to 398 nm, upon formation of the CO_2 adduct[16] is in contrast to observations[17] of red shifts for low-spin anionic adducts (e.g., N_3^-, CN^-) of metMb. The blue shift of the Soret band is,

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(8) From horse heart (Sigma). MetMb was purified by gel filtration chromatography (Sephacryl S-100 HR) prior to use.

(9) The 75 μL head gas samples were analyzed using a computer-interfaced Hewlett-Packard 5971A mass-selective detector with a 5890 Series II gas chromatograph (70 eV, 30 m DB column, 20:1 split).

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(12) MetMb (and cytochrome *c*) samples were placed in an optical cell designed for flash photolysis of solutes in supercritical fluids, as described in the following: Ji, Q.; Eyring, E. M.; van Eldik, R.; Reddy, K. B.; Goates, S. R.; Lee, M. L. *Rev. Sci. Instrum.* **1995**, *66*, 222−226. Approximate dimensions of the sol−gel monoliths were 1 × 1 × 2 cm.

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(15) No significant spectral changes are seen in the Soret band when metmyoglobin or cytochrome *c* solutions are subjected to high pressures (1−1000 atm) alone. The inequivalence of spectra A and C is likely due to formation of small cracks in the sol−gel monolith after return to the laboratory atmosphere and immersion in water.

(16) Bicarbonate adduct formation is ruled out on the basis of our observation that xerogels soaked in buffered solutions containing bicarbonate rapidly become opaque.

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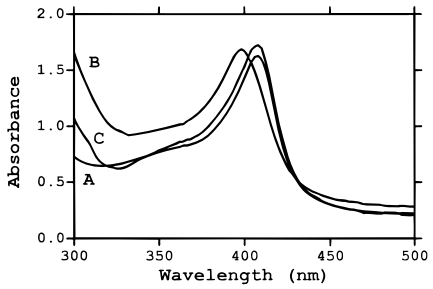

Figure 1. Soret absorption spectra of sol−gel-immobilized metmyoglobin (pH 6.0). (A) Before immersion in supercritical CO_2 (21.5 °C, 1 atm). (B) Formation of the CO_2 adduct under supercritical conditions (42 °C, 114 atm). (C) Return to the laboratory atmosphere.

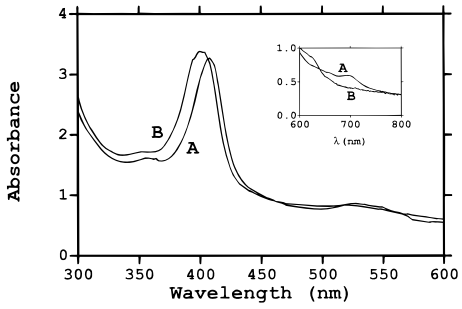

Figure 2. Absorption spectra of sol−gel-immobilized cytochrome *c* (pH 6.0). (A) Before immersion in supercritical CO_2 (21.5 °C, 1 atm). (B) Formation of the CO_2 adduct under supercritical conditions (42 °C, 128 atm). The spectrum after return to the laboratory atmosphere is identical with spectrum A.

however, reminiscent of blue-shifted spectra[18,19] of NO adducts of guanylate cyclase and deoxyMb (at pH < 5). In these cases, the ferrous heme is 5-coordinate (i.e., the Fe^{II}−His bond is broken). Further spectroscopic studies are underway to clarify the Fe coordination number of the Mb CO_2 adduct. We additionally note (data not shown) that supercritical CO_2 displaces other bound ligands from metMb, such as cyanide.

Horse heart cytochrome *c*, in which the heme iron is ligated by histidine and methionine, was also studied[20] in a similar sol−gel matrix. As indicated in Figure 2, the Soret band is also blue-shifted (8 nm) when the monolith is exposed to supercritical CO_2. The 695 nm band, assigned[21] to a methionine S → Fe^{III} charge-transfer transition, disappears under these conditions. The (reversible) loss of this signature demonstrates that the axial methionine is displaced. Other heme proteins (e.g., horseradish peroxidase) display similar Soret shifts to lower energy when exposed to supercritical CO_2.

Laser flash photolysis (532 nm, 6 ns pulse) was used to probe the stability of the CO_2 adducts. Figure 3 displays transient metMb absorbance changes at 398 nm following the laser pulse. Surprisingly,[22] both increases in absorbance are exponential, yielding observed rates of $(2.4 \pm 0.2) \times 10^4$ and 321 ± 11 s^{-1}. Work[23] on deoxyMb using other ligands (O_2, CO, NO) indicates that these geminate recombinations occur at much faster time

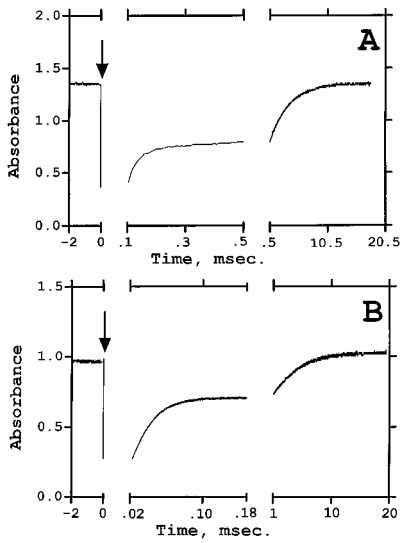

Figure 3. (A) Absorbance changes (398 nm) for the recombination of CO_2 with metMb after laser photolysis (532 nm, 6 ns pulse indicated by the arrow). The data were fitted to two single-exponential rises, yielding observed rates of $(2.4 \pm 0.2) \times 10^4$ and 321 ± 11 s^{-1}. (B) Absorbance changes (400 nm) for the recombination of CO_2 with cytochrome *c* after laser photolysis as in A. Rate constants of $(3.9 \pm 0.1) \times 10^4$ and 270 ± 10 s^{-1} were observed for fitting the data to two single-exponential rises.

scales. A similar (Figure 3) biphasic recombination is observed for the CO_2 adduct of cytochrome *c*: $k_{obsd} = (3.9 \pm 0.1) \times 10^4$ and 270 ± 10 s^{-1}. These photolysis results demonstrate that the blue-shifted Soret spectra cannot simply be explained by dehydration (i.e., loss of axial water). Carbon dioxide must be coordinated to the ferric centers in these proteins; these kinetic traces are not observed in the absence of supercritical CO_2. An additional experiment, involving the photolysis of MbCO in a TMOS sol−gel, only resulted in one observed recombination rate ($k_{obsd} = (2.1 \pm 0.3) \times 10^4$ s^{-1}) on this time scale. We interpret this different result (vis-à-vis $MbCO_2$) to indicate that there is an additional barrier to CO_2 re-entry, possibly involving CO_2 binding to lysine[24] side chains.

In addition to myoglobin (and hemoglobin, results not shown), cytochrome *c* oxidase,[25] cytochrome cd_1,[26] methane monooxygenase,[27] and carbon monoxide dehydrogenase[28] have been reported to oxidize CO to CO_2. However, product (i.e., CO_2) complexes do not accumulate and have resisted direct preparation in aqueous solution. This obstacle has been overcome in this study by the use of sol−gel enzyme immobilization, which facilitated the use of supercritical CO_2 as a reactive solvent. Studies of CO_2 interactions with other metal-containing cofactors, particularly vitamin B_{12} (cobalamine), should prove rewarding. Sol−gel methodology clearly offers great potential in the spectroscopic characterization of unusual protein adducts.

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