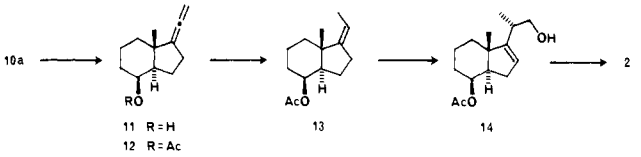
in 48% overall yield from **5**.

The best conditions found for the cyclization **9** → **10a** were as follows: titanium tetrachloride (0.24 mL, 2.18 mmol) was added to a stirred solution of 0.45 g (1.19 mmol) of acetal **9** in anhydrous dichloromethane (25 mL), containing 2,4,6-trimethylpyridine (0.16 mL, 1.21 mmol) at −78 °C under argon. After 5 min the mixture was treated with methanol (1 mL) then warmed to room temperature, and finally excess 1 M hydrochloric acid was added. The crude product contained **10a**:**10b** in the ratio 87:13 by GC.[21] Column chromatography[19b] easily separated the mixture to give **10a**[20] (82% yield) and **10b**[22] (9% yield).

The chiral auxiliary was removed from **10a** by oxidation[23] to the corresponding ketone[19a,20] followed by base-catalyzed β-elimination[24] to give alcohol **11**[19b,20] in 93% overall yield from **10a**. The optical purity of **11**, $[\alpha]^{25}_D$ −13° (*c* 0.4, CCl_4), was determined to be 92% by conversion to the (+)-MTPA ester.[25] The alcohol **11** was acetylated (Ac_2O/pyridine/10% DMAP, 70 °C, 3 h) to give **12**[19a,20] in 96% yield. The compound **12** was then subjected to semihydrogenation[26] over Lindlar catalyst.[27] As anticipated the addition occurred from the more exposed face of the terminal double bond of the allene, to give **13**[20] (89% yield) with no de-



tectable amount of the *E* double-bond isomer (^{1}H NMR spectroscopy and GC).[28] Olefin **13** is the C-8 epimer of an intermediate known to be convertible to the C/D unit of calcitriol.[4] Completion of the synthesis of the Inhoffen–Lythgoe diol **2** followed known methodology.[29] Reaction of **13** with paraformaldehyde catalyzed by $BF_3{\cdot}Et_2O$ gave **14**[19b,20] stereospecifically (72% yield).[30] Finally stereospecific hydrogenation of **14** (H_2/PtO_2) followed by deacetylation (THF/MeOH/2 M aqueous KOH, 1:1:1, 48 h, 25 °C) gave **2** in 84% overall yield from **14** (39% overall yield from **9**). ^{1}H NMR, IR, and mass spectroscopy as well as GC coinjection established the identity of **2** with an authentic sample prepared by degradation of vitamin D_2,[9] $[\alpha]^{25}_D$ +40.5 (*c* 0.38, MeOH).[31] The $[\alpha]^{25}_D$ +37.1° (*c* 0.38, MeOH) found for the synthetic material was in excellent agreement with the value calculated for a sample of 92% optical purity. A single crystallization of synthetic **2** from hexane gave optically pure material as colorless needles, $[\alpha]^{25}_D$ +40.6, mp 110–111 °C (mixture mp with authentic **2**, 110–111 °C) (lit. 113–114,[6] 109–110 °C[9]).

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Supplementary Material Available: Spectroscopic (^{1}H NMR, IR, MS) and analytical data (8 pages). Ordering information is given on any current masthead page.

(21) GC (15 m SE-54 capillary column) separation of **10a** and its 8α,13α,14β isomer was not possible though under less favorable cyclization conditions (i.e., in which a larger proportion of the latter isomer was formed, e.g., $SnCl_4$/fluorobenzene −40 °C, 4 h) the minor diastereomer appeared as a shoulder on the major GC peak.

(22) The ^{1}H NMR spectrum was consistent with the structural assignment. The predominant absolute configuration at C-8, C-13, and C-14 was assumed on the basis of earlier work (see ref 10a).

(23) Pyridinium chlorochromate (1.5 mol equiv), CH_2Cl_2, 25 °C, 18 h. Corey, E. J.; Suggs, J. W. *Tetrahedron Lett.* **1975**, 2647–2650.

(24) THF/MeOH/7.5 M aqueous KOH (4:2:1), 24–48 h, 25 °C; see ref 10b.

(25) Determined by GC analysis (base-line separation on a 15-m SE-54 capillary column) of (*R*)-(+)-MTPA esters. See: Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543–2549.

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(30) ^{1}H NMR and GC (15-m SE-54 capillary column) indicated that **14** was diastereomerically pure at C-20.

(31) The reported rotation is +36.5° (*c* 1, MeOH).[6]

Deuterium Isotope Effect on the Heme-Coordinated CO Vibration Band of Ferrous Cytochrome *c* Peroxidase–CO

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The question of distal effects as mediators of ligand binding in heme proteins has resulted in efforts to discern the role of the distal histidine at position E-7 in the primary amino acid sequence.[2-15] This amino acid is situated closest to the ligand binding site in heme globins,[3,7] but a similarly positioned "distal histidine" is present in cytochrome *c* peroxidase (EC 1.11.1.5; CcP), a native ferriheme enzyme.[15] For CcP this histidine has been implicated in the peroxidase catalytic mechanism.[16]

Ferrous heme globins bind both molecular oxygen and carbon monoxide, and evidence has been presented which suggests that nature takes advantage of the potential multifunctionality of the distal histidine.[2-5] For example, distal histidine hydrogen bonding has been reported for heme-coordinated dioxygen,[3,4,15] whereas the absence of neutron density between histidine E-7 and heme-bound CO was noted for carbonylmyoglobin.[7] In CO-ligated heme proteins, it has been suggested that the role of the distal histidine is primarily steric,[2] although evidence has been presented in support of a strong hydrogen bond between heme-coordinated CO and protein in the heme enzyme carbonylhorseradish peroxidase (EC 1.11.1.7; HRP).

In addition to structural evidence,[3,7] one of the primary arguments against hydrogen bonding involving heme-coordinated CO in hemoglobins and myoglobins has been the absence of a spectroscopically detectable deuterium isotope effect. The reasoning

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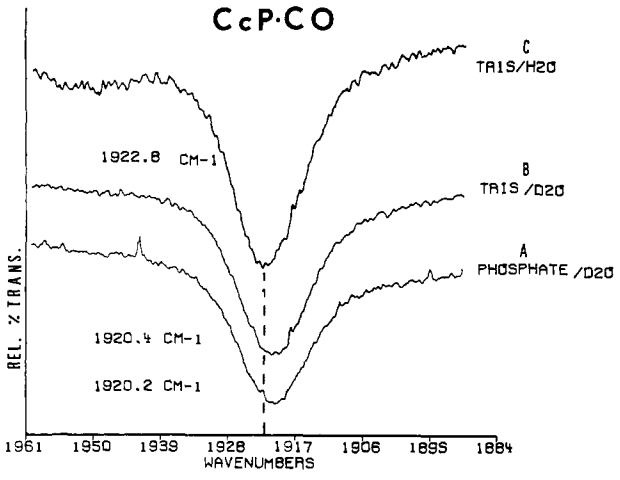

Figure 1.

is that changing the buffered solvent from H_2O to ^2H_2O should result in a shift in the CO infrared frequency because the hydrogen-bonding proton should be exchange labile. This has not been observed even for hemoglobins in which the heme prosthetic group was removed and the apoprotein allowed to extensively dialyze against ^2H_2O.[10] In addition, nuclear magnetic resonance relaxation data for ^{13}CO–hemoglobin A has recently been interpreted without invoking a specific hydrogen bond.[18] Further, a ^{13}C–^1H nuclear Overhauser effect is absent in ^{13}CO–myoglobin (sperm whale) when the experiments were carried out under conditions of protein concentration where a maximum enhancement of 0.17 could have been observed if a proton were coupled to the heme-coordinated ^{13}CO.[19]

For normal heme globins the absence of data in support of distal hydrogen bonding to heme-ligated CO seems to obviate this concept. Until now, the absence of a protein in which heme-coordinated CO displayed any of the anticipated experimental effects that are characteristic of hydrogen bonding (isotope shift in the infrared spectrum, nuclear Overhauser effect in the NMR spectrum) has even led to concern whether such effects were, in fact, detectable. As indicated above, evidence for HRP suggested that hydrogen bonding in HRP–CO is significant and that a deuterium isotope effect can be observed in the ultraviolet–visible spectrum[12] and by infrared spectroscopy.[14] Here we report that the infrared spectrum of CcP-CO shows a reproducible deuterium isotope effect in the CO stretching region that is comparable in magnitude to that measured for HRP–CO.[14]

Cytochrome *c* peroxidase was isolated from Baker's yeast as previously described.[20] As usual for this isolation procedure the enzyme was shown to be greater than 95% pure by polyacrylamide gel isoelectric focusing (LKB). In order to obtain the infrared spectrum, we dialyzed the ferric native enzyme in the desired buffer then concentrated it by pressure dialysis (Amicon). Two buffers were used: Tris (tris(hydroxymethyl)aminomethane; Sigma) and potassium phosphate (Fisher). Spectra were taken at pH 4.5, 6.25, and 7.5 in both H_2O and ^2H_2O solution. After concentrating, the protein solution was degassed with prepurified nitrogen (Matheson), saturated with ^{12}CO (Matheson), reduced with sodium dithionite (Aldrich), and transferred anaerobically to a sealed CaF_2 infrared cell. All of the manipulations just described were carried out in an inert atmosphere glovebox. The pH was measured before and after dithionite ion reduction with a Beckman pH meter and a Fisher glass pH electrode. Buffer concentrations of 0.1–0.2 M were employed.

A large amount of work[2,6,8,11,14] has shown that heme-coordinated CO vibrations occur between 1970 and 1900 cm^{-1}. Figure 1 indicates that for CcP–CO a single major CO infrared band occurs at 1920.4 ± 0.5 cm^{-1}, with a line width at half-maximum height of 17 ± 1 cm^{-1}, in ^2H_2O buffer. The absorption position is identical, within experimental error, regardless of whether Tris or phosphate buffer is used. This band exhibits a reproducible deuterium isotope effect of 2.0 ± 0.5 cm^{-1}. These results have been reproduced at the three pHs mentioned above with at least two different samples at each pH. Although not shown in this figure, the major band varies by only 1.0 cm^{-1} over the pH range 4.0–8.0, and the deuterium isotope effect is identical at each pH. Above pH 8.0 a second, broader band centered at 1944 ± 2 cm^{-1} appears, as the 1920 cm^{-1} band disappears. A reproducible deuterium isotope effect for this band has so far not been observed, probably due to this band's broadness, which causes larger uncertainty in identifying the peak maximum. All of the spectra and infrared data reported here were recorded on a Nicolet 6000/7000 FTIR spectrometer, were ratioed against the appropriate buffer solution, are difference spectra referenced to the ferric form of the enzyme, and were run at 0.5-cm^{-1} resolution.

There are distinct differences between the ferrous peroxidase–CO bands and the ferrous heme globin–CO spectrum.[8] As previously reported[8,14] HRP–CO exhibits three infrared bands over the pH range 4–9, whereas the heme globins may exhibit up to four bands in the CO stretching region.[9] In the pH range where CcP is stable (pH 4–8) only one major band has so far been detected.

The observation of a deuterium isotope effect for a second member of the peroxidase class of heme enzymes could be regarded as evidence of hydrogen bonding between a distal amino acid side chain and heme-coordinated carbon monoxide. This is plausible in view of the reported crystal structure for cytochrome *c* peroxidase, which shows the heme to be buried within the protein matrix and not directly solvent exposed.[16,17,20,21] On the distal side of the heme, three amino acid residues that are positioned close to the ligand binding site form a catalytic triad (tryptophan-51, histidine-52, arginine-48). A consequence of this triad is that it provides a more polarizing environment for heme ligands than do hemoglobin and myoglobin.[16,17,21] This results in a larger line width at half-maximum height for CcP–CO compared to CO–hemoglobin and CO–myoglobin.[9,14] In the postulated mechanism of CcP catalysis the three distal triad residues play integral roles in the hydrogen peroxide decomposition step.[16,17,21] Two of these (his-52 and arg-48) are capable of providing a deuterium-exchangeable proton for hydrogen bonding to the heme-coordinated CO. However, structural studies to date indicate that the his-52 nitrogen that carries the proton at neutral pHs is positioned away from the heme ligand, points toward the protein surface, and participates in a hydrogen bond with asparagine-82. Therefore, unless this positioning is different in ferrous CcP–CO, the distal histidine is not expected to be a hydrogen-bond donor to the heme-coordinated carbon monoxide. This participation by one (or both) of the distal amino acids is sufficient to account for our results. However, we wish to point out that the crystal structure of CcP also indicates a rather ordered network of hydrogen bonding including arginine-48 and two water molecules (Wat-648, Wat-348), which apparently form a link to the protein surface.[21] One of these water molecules that is imbedded in the protein matrix lies close enough to hydrogen bond to the axial ligand site in the native enzyme (Wat-648). Therefore, if this ordered water structure is retained in CcP–CO it could also be the source of the deuterium isotope effect on the CO stretching vibration. We are in the process of attempting to identify the source of this hydrogen bond using NMR spectroscopy.

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