Infrared spectroscopic evidence of hydrogen bonding between carbon monoxide and protein in carbonylhorseradish peroxidase C

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Carbonylhorseradish peroxidase isoenzyme C_2 (EC 1.11.1.7) exhibits two bands in the infrared spectrum attributable to the ligand CO at 1933.5 and 1905 cm^{-1}. Replacement of H_2O by D_2O results in shifts to both bands to new positions at 1932.5 and 1902.5 cm^{-1}. The results indicate strong hydrogen bonding to the terminal oxygen of CO, of strength comparable to that recently observed for oxyhemoglobin and oxymyoglobin.

Peroxidase *Infrared* *Carbon monoxide* *Hydrogen bond* *Isotope effect*

1. INTRODUCTION

Evidence has recently been presented for hydrogen bonding between the terminal oxygen of bound dioxygen and distal histidine in the α subunit of oxyhemoglobin [1], in the α and β subunits of oxycobalt hemoglobin [2], and in oxymyoglobin [3]. Reports of O_2 and CO ligation with 'basket handle' hemes conclude that hydrogen bonding to O_2 is important for stabilization of the oxygenated complex; hydrogen bonding to CO was not considered [4,5]. Spectrophotometric titrations suggested a strong hydrogen bond between CO and protein in carbonylhorseradish peroxidase (HRPC · CO) and a weaker one in carbonylmyoglobin (Mb · CO) [6]. However, hydrogen bonding directly to CO was ruled unlikely for Mb · CO as judged by infrared spectroscopy [7]. We report here, as the first instance concerning heme-bound CO, that the infrared stretch frequencies of a carbonylhemeprotein (HRPC · CO) differ significantly in H_2O and D_2O. The effect is most simply interpreted as being due to hydrogen bonding between the oxygen of CO and a distal residue.

Abbrevations: HRPC · CO, carbonylhorseradish peroxidase C; Mb · CO, carbonylmyoglobin; Hb · CO, carbonylhemoglobin; IR, infrared; ν CO, infrared absorbance maximum

2. MATERIALS AND METHODS

Horseradish isoperoxidase C_2 was purified from horseradish [8], dialyzed against 50 mM potassium phosphate, 1 mM EDTA (pH 7.0) and stored as small beads in liquid nitrogen. Before use the enzyme was concentrated to 3.5–4 mM by ultrafiltration (YM-10 membrane, Amicon) under nitrogen gas at 4°C. Exchange to deuterated buffer was made by 3 times repeated dilution with 10 vol. of D_2O buffer and concentration by ultrafiltration. Deuterated buffer was made with D_3PO_4 (Stohler Isotopes, MA), EDTA and NaOD at the above concentrations and 'pH 7.0' as read on the meter. Samples were stirred under a stream of scrubbed (chromic perchlorate-amalgamated zinc) CO gas for 30 min, reduced by the addition of a small quantity of fresh dithionite (BD Chemicals, England) and injected into a variable temperature IR cell fitted with CaF_2 windows (0.1 mm pathlength) and thermostatted at 20°C. The IR spectra were recorded (15 000 scans each) using a Digilab FTS-14C interferometer in a single-beam

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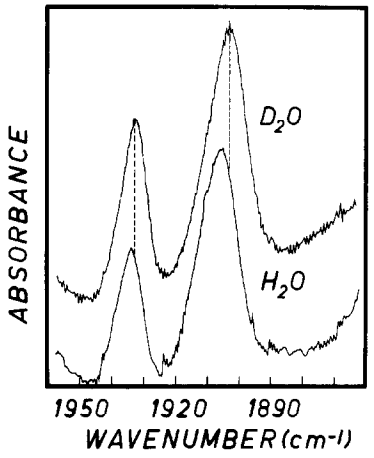

Fig. 1. Infrared spectra in carbonyl stretch region of HRPC · CO. Top, in deuterated buffer, bottom, in simple buffer.

mode at 1 cm^{-1} resolution. HRPC · CO integrity was checked after IR analysis by optical spectrophotometry directly of the IR cell using a Beckman DU-7 spectrophotometer. Separate spectra (15000 scans each) of buffer and air were recorded to permit conversion to absorbance.

3. RESULTS

In the carbonyl stretch region HRPC · CO exhibits two bands with maxima at 1933.5 (I) and 1905 (II) cm^{-1} [9,10]. In deuterium buffer both bands occupy new positions, 1932.5 and 1902.5 cm^{-1} (fig. 1). The isotopic dependence of I may not be real at the precision of the instrument (0.5 cm^{-1}), but we consider the 2.5 cm^{-1} shift of II to be significant. This shift cannot be due to the difference between 'pH' and pH because stretch maxima positions are independent of 'pH' from 4 to 11 [10].

4. DISCUSSION

The CO stretch maxima of carbonyl protoheme proteins appear within the range 1905–1970 cm^{-1}, at unique energies depending upon protein and observational conditions. The relative importance of determinants such as electronic distribution within the heme, protein steric effects, or hydrogen bonding to the ligand, upon a particular ligand stretching frequency is incompletely known. The difference in ν CO between Mb · CO (1944 cm^{-1}) and Hb · CO (1951.5 cm^{-1}) has been attributed to steric restraint in Mb · CO by the protein [11]. For a number of heme proteins the electron density in the heme, expressed as the Fe(III)/(II) reduction potential, appears to determine ν CO [10]. For the carbonyl derivatives of HRPA and HRPC [10], lactoperoxidase [12] and cytochrome *c* peroxidase [13], two strong CO stretches were observed with pH-dependent intensities.

For HRP and lactoperoxidase this dependence was attributed to hydrogen-bonding of the heme carbonyl. The direction of the proton-induced shift to a lower wave number is in accord with the weakening of the C=O bond. The magnitude of the shift is related to the strength of the hydrogen bond. The IR spectra of various α- and β-substituted anthraquinones show that the keto carbonyl group stretch frequency ($\sim$1675 cm^{-1}) is shifted by 40 to 50 cm^{-1} to lower frequency when an intramolecular hydrogen bond is formed between an α-hydroxy or amino group and the keto carbonyl [14]. The smaller, 30 cm^{-1}, shift to lower energy of the heme-bound carbonyl group may result from slightly weaker hydrogen bonding in the protein compared to substituted anthraquinones or may be a function of the lower electron density available for hydrogen bonding on a heme-bound carbonyl group compared with a keto carbonyl [15].

Our novel observation of an isotope effect on the CO stretch in HRP · CO brings conclusive evidence for the existence of a hydrogen bond to the carbonyl group. The increased mass should require a lower frequency for resonance, and we find a decrease of 2.5 cm^{-1} upon deuteration. Interestingly, in oxycobalt hemoglobin and oxymyoglobin deuteration caused an increase by 2–5 cm^{-1} [2]. This may mean that the geometry of hydrogen bonding is quite different in peroxidases and oxygen carriers or it could arise from the unusual electronic properties of bound dioxygen, which suffers Fermi resonance [16].

The amino acid sequence of HRP distal to heme includes the two histidyl residues 40 and 42 and arginyl 38 [17], all candidates for hydrogen bonding to CO. Optical [6] and IR [10] spectroscopies gave p*K* values of 8.25 and 8.8, respectively for a heme-linked proton dissociation in HRPC · CO. These values are quite high for a macroscopic

dissociation constant for a histidyl residue but seem excessively low for an arginyl group. Chemical modification of HRPC·CO by *p*-chlorobenzoyl chloride, a histidine specific reagent, completely abolishes II [18]. This is further evidence that a histidyl group is the proton donor.

We conclude, on the basis of the present and previous studies, that a hydrogen bond FeCO···H-protein exists in carbonyl HRP at neutral and acid conditions, probably to a histidyl residue. This is inconsistent with the proposal that a hydrogen bond is formed between the carbon atom of CO and a distal residue [6]. Our conclusion is consistent with two proposed peroxidase mechanisms involving proton transfer at the active site [19,20].

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