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## BBA Report

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### INFRARED STRETCHING FREQUENCIES OF CO IN CARBOMONOXY-HEMOGLOBIN FROM TROUT

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#### Summary

The infrared spectra of the carbomonoxy derivatives of the hemoglobin components I and IV from trout have been measured in the CO stretching frequency region using a high resolution infrared spectrometer. The CO stretching frequency of Hb I CO is very close to that of carbomonoxy human hemoglobin and is pH-independent. In contrast, the CO stretching frequency of Hb IV CO is higher and shows a small but significant pH dependence in the range 6.2–7.8. These results point to a decreased strength of the iron-CO bond in Hb IV CO at low pH, in agreement with the conclusions drawn from the reported difference spectra of Hb IV CO as a function of pH.

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The structural and functional properties of the hemoglobin components I and IV from trout have been actively investigated in the last few years and have been correlated with the physiological demands of the fish [1,2]. The large drop in oxygen affinity of Hb trout IV observed on decreasing pH (Root effect) or on adding organic phosphates, has been described as a proton-induced stabilization of a low affinity conformational state of the protein [1,3]. This interpretation is in line with the spectral changes observed for the CO-derivative of Hb trout IV as a function of pH [3]; the bathochromic shift of the Soret band in going from pH 8 to pH 6 and the associated ultra-violet difference spectrum have been taken as evidence of a pH-dependent structural transition in the liganded form of Hb trout IV.

The infrared spectra of Hb I CO and Hb IV CO in the CO stretching frequency region have been measured at different pH values, to gain additional insight in the structural properties of these liganded hemoglobins.

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Abbreviation: Hb, hemoglobin.

Spectra in the 1920–1980  $\text{cm}^{-1}$  region were measured with a Perkin Elmer model 180 IR spectrometer equipped with a Standard Digital Interface. The carbonyl stretching frequency was calibrated against the water vapour peak at 1942.6  $\text{cm}^{-1}$ . The band width of the instrument was set at 0.5  $\text{cm}^{-1}$  and the wavelength is accurate to approx. 0.05  $\text{cm}^{-1}$ . Each spectrum was digitalized taking data points at 0.1  $\text{cm}^{-1}$  intervals. The spectra were elaborated by subtracting the spectrum of the solvent at the corresponding pH each time and filtered using a digital filter technique [4]. The concentrations of Hb I CO and Hb IV CO (obtained by exposing a solution of HbO<sub>2</sub> to pure CO) were about 14 mg/ml, at pH 6.2 and 7.8 in 0.2 M phosphate buffer. BaF<sub>2</sub> cells with an optical path of 0.075 mm were used; the reference cell contained water.

The infrared spectra of the two hemoglobins at two pH values are reported in Fig. 1. The presence of a single narrow absorption band, which can be attributed to the CO stretching, indicates the existence of a single type of bonding for the ligand [5]; the measured half bandwidths of 9.1–8.5  $\text{cm}^{-1}$  for Hb I CO and 9.2–9.0  $\text{cm}^{-1}$  for Hb IV CO, at pH 7.8 and 6.2, respectively, are similar to that found for HbA [5]. The narrowness of the band points to a non-polar, polarizable environment around the ligand [6]. The  $\nu_{\text{CO}}$  for Hb I CO is at 1952.0  $\text{cm}^{-1}$ , independent of pH (see Table I). A very similar value has been reported for the CO derivative of human HbA, HbH ( $\beta_4$ ) and for the  $\alpha$  chains in HbM<sub>Emory</sub>, and Hb Zürich [7,8]. As shown in Table I, the position of the band for Hb IV CO is significantly higher than that of all the other hemoglobins so far examined, with the exception of the  $\beta$  chains of Hb Zürich [8]; moreover a small but significant pH dependence of the center of the band is observed.

The  $\nu_{\text{CO}}$  of various CO-hemes having different substituents in positions 2 and 4 of the porphyrin ring and the CO stretching frequency values of carbomonoxy hemoglobins and myoglobins have been recently correlated with the strength of the CO bonding to the porphyrin-iron and to the intramolecular interactions occurring in the heme pocket [6]. In the case of 2,4-substituted deuterio hemoglobins the absolute frequency values have been taken as an evidence of the decreased  $\pi$ -donor capacity of the porphyrin-iron in the order ethyl > hydrogen > vinyl [5,6,9]. The shifts observed for these reconstituted proteins and for other hemoproteins [7] have been interpreted as a decreased basicity of the tetrapyrrole nitrogens, due to the increased electron withdrawing power of the 2,4-substituents (ethyl < hydrogen < vinyl). In Table I, some of these values [5] are compared with those found for trout hemoglobins.

On this basis, the values of  $\nu_{\text{CO}}$  of Hb I CO and HbA CO are consistent with an equal strength of the CO bonding to the iron in these two heme proteins. On the other hand, the increased values observed for Hb IV CO would indicate a decrease in the strength of the iron-CO bond.

In view of the identity of the 2,4-substituents in trout hemoglobins and in HbA, other factors must be taken into account to explain the shift in the CO stretching frequency of Hb IV CO, such as interactions of the 2,4-vinyl groups with the amino acid residues of the polypeptide chains, or any other interactions that can decrease the  $\pi$ -donor capacity of the porphyrin iron.

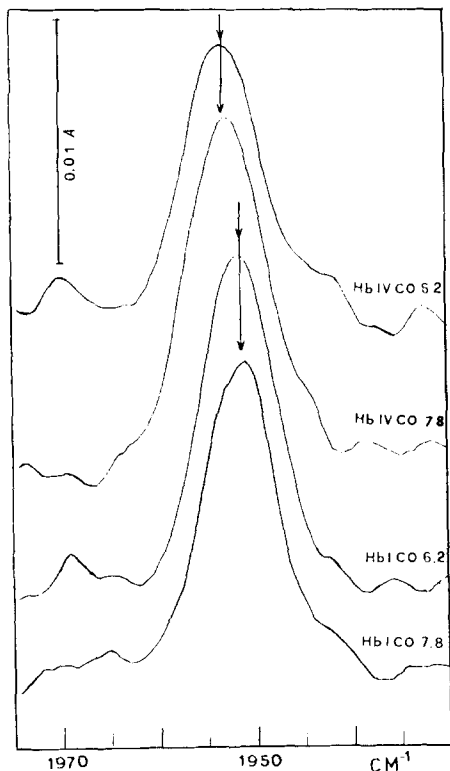


Fig. 1. Infrared spectra of trout Hb I CO and Hb IV CO in the region of the carbon-oxygen stretching vibration. Protein solutions were made in 0.2 M phosphate buffer at pH 6.2 and 7.8.

TABLE I

CO STRETCHING FREQUENCIES OF VARIOUS CARBOMONOXYHEMOGLOBINS

Hemoglobin	$\nu_{\text{CO}}$ ( $\text{cm}^{-1}$ )	Reference
Meso HbA	1946	5
Deutero HbA	1948.5	5
HbA	1951–1951.9	5
Hb Trout I pH 7.8	1951.96	This work
pH 6.2	1952.02	This work
Hb Trout IV pH 7.8	1953.06	This work
pH 6.2	1953.46	This work

Knowledge of the sequence of Hb IV, which is being completed [2], is a necessary prerequisite for the structural interpretation of these differences.

This difference, however, cannot be directly correlated with the overall ligand affinity in view of the previously reported findings by Caughey and coworkers [7] on the CO stretching of hemoglobins, characterized by different affinities. Moreover, these results are significant in relation to the understanding of the Root effect, especially in connection with the information which has been recently obtained by  $^{13}\text{C}$  NMR spectroscopy of  $^{13}\text{C}$ -Hb IV [10]. The pH dependence of the resonance of the ligand bound to the  $\alpha$  and the  $\beta$  chains in Hb trout IV shows unequivocally a structural change involving the

immediate environment of the ligand binding site. The small change of the CO stretching frequency shown in this paper, excludes a direct correlation between the proton-induced structural change observed in Hb IV CO [1,3] and a protonation of the distal imidazole. This would in fact modify substantially the electron distribution in the Fe-C-O bonds and thus shift the CO stretching frequency more drastically than observed [11]. This suggests that the structural origin of the Root effect resides somewhere else in the molecule.

Finally, we point out that the small but significant pH dependence of the  $\nu_{\text{CO}}$  in the Hb IV CO is in agreement with both the decreased strength of the iron-CO bond at low pH and the previously reported difference spectra of Hb IV CO as a function of pH [3]. In fact the red shift observed in the Soret peak at low pH indicates a decrease in the electron donating power of the porphyrin ring to the iron and a consequent decrease of the metal electron density which gives rise to a poorer  $\pi$  transfer to the ligand. These results are in line with the decreased overall CO affinity of this hemoglobin that drops by a factor of approx. 100 going from high to low pH [12], although, as commented above, a direct correlation between the value of  $\nu_{\text{CO}}$  and the ligand affinity at present is not tenable.

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