OBSERVATIONS ON CO TROUT HEMOGLOBINS BY ^{13}C NMR

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1. Introduction

The functional properties of the two major hemoglobin components from trout's blood (Hb trout IV and I) have been characterized in a number of recent papers (see [1] for review). The ligand binding equilibrium curve of Hb trout I shows strong homotropic interactions (n=2.3–2.5), but is completely insensitive to changes in proton concentration. On the other hand, Hb trout IV is characterized by marked heterotropic interactions: lowering of pH produces a large decrease both in the overall affinity for ligands such as oxygen and carbon monoxide, and in the heme–heme interactions (the value for Hill's coefficient n drops from 2.3 at pH = 7.5 to about 1 or less at pH = 6). In the case of oxygen, the affinity of the molecule for the ligand at acid pH values is so dramatically reduced that a large proportion of the oxygen binding sites are deoxygenated even at oxygen pressures above one atomsphere ('Root' effect).

With the aim of gaining a better insight in the molecular mechanism operative in the hemoglobin components from trout, we have determined the ^{13}C n.m.r. spectra of the ^{13}CO bound to both Hb trout I and Hb trout IV.

2. Materials and methods

Preparation of hemoglobin from trout and separation of the components were achieved as previously reported [2].

The sample solutions were concentrated by ultrafiltration (Amicon, UM-10 membrane) to a concentration 3–5 mM in heme. The pH was adjusted by addition of weighed amounts of sodium phosphate salts to a final 0.2 M concentration, and measured in each sample by a Radiometer microelectrode pH-meter. The ^{13}CO derivatives were prepared by addition of 90% enriched ^{13}CO (Prochem. England) to the previously deoxygenated samples. Small excess of sodium dithionite was, in some cases, added to the sample.

^{13}CO nuclear magnetic resonance spectra were obtained using the pulse fourier transform technique on a Bruker 22.63 MHz spectrometer. Data acquisition periods between 6 and 20 h were used to achieve adequate signal-to-noise ratio. D_2O (about 50% of the solvent) was used for the field frequency lock. The free ^{13}CO resonance was used as internal standard. The chemical shifts were transformed to p.p.m. from TMS using the value of 184.60 for the free CO [3].

3. Results and discussion

The ^{13}C spectra of the ^{13}CO derivatives of Hb trout I and IV under various conditions are reported in fig.1.

The spectra of Hb trout IV show two well

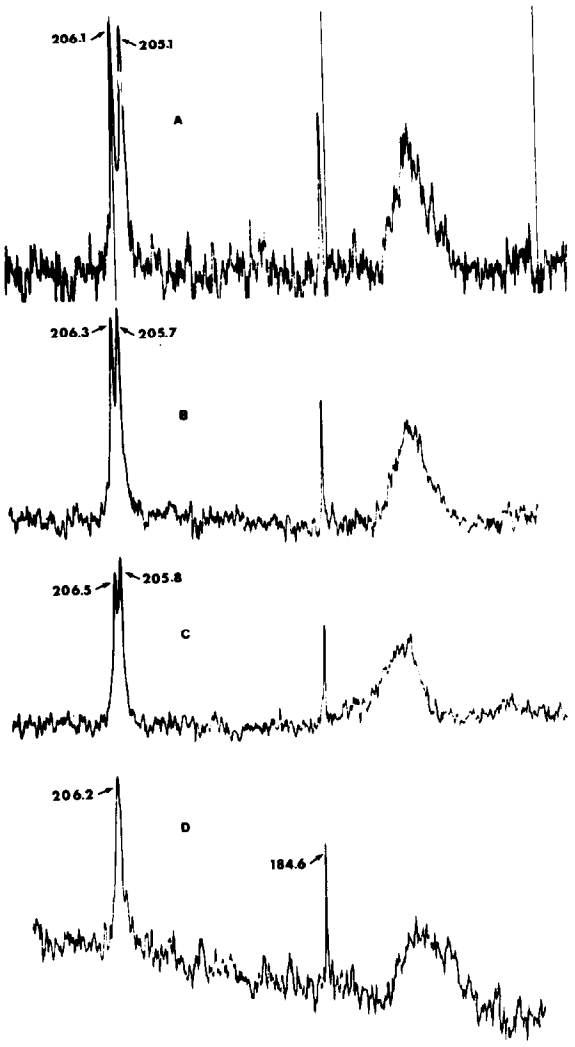

Fig.1. ^{13}C n.m.r. spectra of trout hemoglobins. (A) Hb Trout IV, pH 6; (B) same, pH 6.8; (C) same, pH 7.8; (D) Hb Trout I, pH 6. The narrow high field signal is due to free CO. Hb Trout IV conc. = 5.2×10^{-3} M, Hb Trout I = 3.8×10^{-3} M, free $^{13}CO = 9 \times 10^{-4}$ M. Temp. = 27°C.

resolved resonances of about the same intensity which can be attributed, as in the case of other hemoglobins, to the two different chains in the tetramer (α-like and β-like chains) [4,5].

Contrary to what observed for all other hemoglobins studied to date, the chemical shift of the ^{13}CO lines in the Hb trout IV is sensibly pH dependent for both the chains (see figs.1 and 2). However the effect is not equally distributed on the two chains: lowering the pH from 7.8 to 6, the up-field resonance is shifted towards higher field by 0.73 p.p.m., whereas the low field line is shifted in the same direction by 0.39 p.p.m. This finding clearly supports the idea that in Hb trout IV the intrinsic differences between the chains are strongly enhanced at low pH.

A spectrum of the Hb trout IV at pH 6 and under conditions of partial saturation ($Y \sim 0.3$) with the ligand is reported in fig.3. As in the case of human and rabbit hemoglobins [3] a difference in the affinity for carbon monoxide between the two chains is clearly evident. It is suggestive that the chain which experiences the more marked pH shift is the one with lower ligand affintiy.

For Hb trout IV no direct experiments are available to decide which resonance corresponds to which chain, and any assignment made by analogy with human hemoglobin is at best tentative. Experiments along this line are in progress.

Although, as previously suggested [1,6], the molecular interpretation of the Root effect could be envisaged as a progressive stabilization of the low affinity (T) quaternary state by protons, it was not

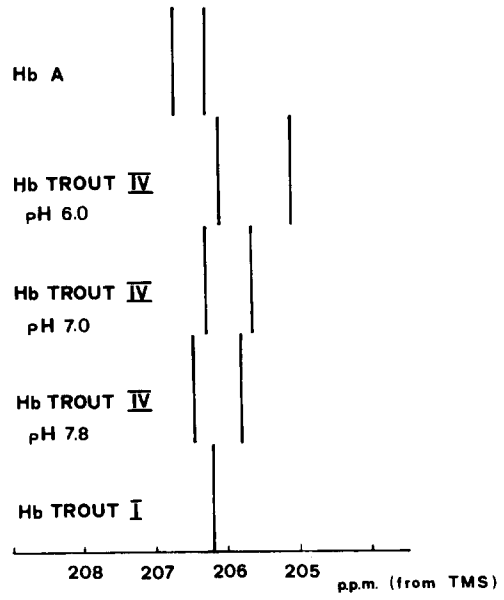

Fig.2. Comparison of bound ^{13}CO n.m.r. frequencies in human and trout hemoglobins.

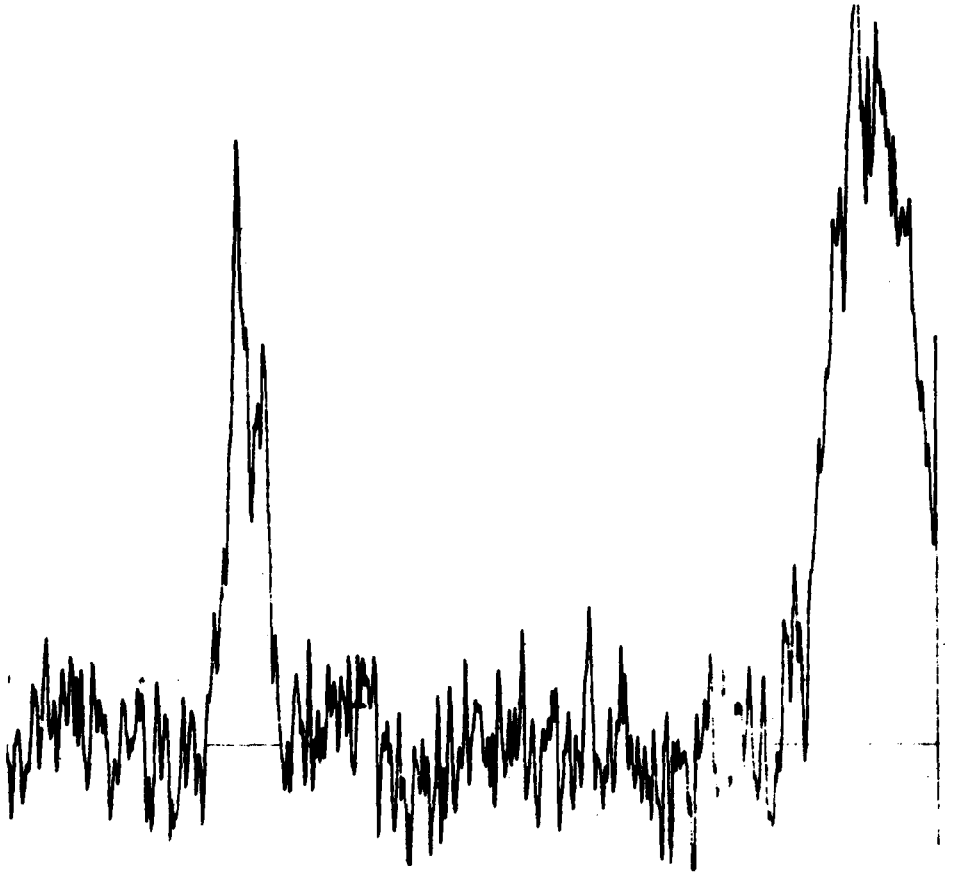

Fig.3. ^{13}C n.m.r. spectrum of Hb trout IV (pH = 6) partially saturated with ^{13}CO (Y ~ 0.3, estimated by comparison with the protein carbonyl absorption region).

possible, on the basis of the available data, to exclude on alternative mechanism involving widely different Bohr effects for the α and β chains. If this was the case, the enhancement of the functional heterogeneity induced by protons should produce an apparent decrease in the overall cooperativity. In principle the two models are not mutually inconsistent, and both of them are probably operative. The n.m.r. results on Hb Trout IV yield clear evidence for the existence of intramolecular pH dependent heterogeneity which must play a significant role in the molecular mechanism underlying the Root effect.

^{13}CO bound to Hb trout I shows, within the resolution, a single resonance at 206.20 ppm (fig.1), strictly pH independent over the range from 6 to 8. As in the case of pigeon Hb [3] the interactions between the ligand and the neighbouring groups must be very similar for the two different polypeptide chains.

In view of the extreme pH dependence of the functional properties of Hb trout IV as compared to other hemoglobins, notably to Hb trout I, it is not surprising that the ^{13}CO resonance(s) are pH independent in the latter cases and pH dependent in the former one. This comparison indicates that the immediate interactions of CO with nearby protein residues are not significantly modified by pH even for those hemoglobins which display a Bohr effect (like human HbA) [3]. On the other hand in Hb trout IV, proton dependent structural changes may involve some of the residues in the vicinity of the ligand in the heme pocket. pH-dependent structural changes in the ligand bound form were also suggested on the basis of e.p.r. spectra of the nitric oxide derivative of Hb trout IV [7]. A possibility which appears at present appealing, though still

speculative, is that the interactions between side chains on the distal side (e.g. His E7 and Val E11) and the ligand are significantly modified by pH in Hb trout IV. This possibility is presently being investigated.

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References

[1] Brunori, M. (1975) Current Topics Cell. Reg. 9, 1–39.
[2] Binotti, I., Giovenco, S., Giardina, B., Antonini, E., Brunori, M. and Wyman, J. (1971) Arch. Biochem. Biophys. 142, 274–280.
[3] Moon, R. B. and Richards, J. M. (1974) Biochem. 13, 3437–3443.
[4] Antonini, E., Brunori, M., Conti, F. and Geraci, G. (1973) FEBS Lett. 34, 69–70.
[5] Moon, R. B. and Richards, J. M. (1972) J. Amer. Chem. Soc. 94, 5093–5095.
[6] Giardina, B., Ascoli, F. and Brunori, M. (1975) Nature 256, 761–762.
[7] Brunori, M., Falcioni, G. and Rotilio, G. (1974) Proc. Nat. Acad. Sci. USA 71, 2470–2472.