PROPERTIES OF TROUT HbI IN WATER AND LIGAND LINKED BINDING OF Na^+

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1. Introduction

The structural and functional properties of trout Hb I (one of the components isolated from the hemolysate of trout, *Salmo irideus*) have been investigated in considerable detail in the last few years [1–4]. Its properties are exceptional in sofar as ligand binding is characterized by strong heme–heme interactions but lacks completely effects of protons and organic phosphates.

We have investigated the functional properties of trout Hb I in water and in various salts. From the experiments in pure water it is clear that the equilibrium and kinetics of the isoionic protein are very similar to those obtained in buffered solutions at different pH values [1–4]. This leads to the conclusion that the characteristic thermodynamic properties of trout Hb I [3] are intrinsic features of the system, and are not dominated by heterotropic effects involving small ions.

In addition the oxygen equilibrium of trout Hb I investigated as a function of NaCl, sodium citrate and sodium dextran sulfate shows two distinct effects, one related to the differential binding of Cl^- [5] and the other to the specific binding of Na^+, which is proven to stabilize the low affinity conformational state of hemoglobin. This effect is discussed in the light of the known structural properties of trout Hb I.

2. Materials and methods

Isolation of the component I from trout hemolysate (trout Hb I) was done as in [1]. Stripped isoionic trout Hb I was prepared by dialysing the sample against deionized distilled water and then passing the solution 3 times through a column filled with a mixed-bed ionexchange resin (Amberlite MB-50). Deionization of the isoionic solution was checked by conductimetric measurements (mod. CDM 3 Radiometer, Copenhagen). The isoionic pH at 20°C is 7.72, as obtained from the average of 5 independent determinations.

Oxygen equilibria were determined spectrophotometrically in [6]. The oxygen-binding curves were determined either in deionized distilled water or in 0.05 M bis–Tris buffer (pH 7.2) in the presence of known amounts of various salts (notably NaCl, sodium citrate or sodium dextran sulfate).

Spectroscopic measurements were performed with a Cary 219 spectrophotometer. Flash-photolysis experiments were carried out either with the protein in deionized distilled water or in 0.1 M phosphate buffer (pH 7.7) using an apparatus similar to that employed in [7]. Analysis of the results was carried out with a Hewlett-Packard 9330 A desk computer. Dextran sulfate (M_r 8000) was obtained from Gallard Schlesinger Chemical Corp. (Carle Place, NY); the amount of sodium/mg dextran sulfate was determined by atomic absorption in a Perkin-Elmer 603 flame spectrometer. Carbon monoxide was obtained from SIO (Rome); all other reagents were Analytical grade products.

3. Results and discussion

3.1. *Oxygen equilibria in water*

Since it is proven that trout Hb I shows no Bohr effect [1–4], oxygen equilibria can be carried out in

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water without pH changes along the binding isotherm (as controlled by independent potentiometric measurements). The intrinsic binding properties of the isoionic protein can therefore be compared with those in buffered solutions and in the presence of various salts. The oxygen equilibria, performed in deionized distilled water at 3 different temperatures (10,20 and 30°C) show that trout Hb I displays cooperative oxygen binding curves, similarly to the results obtained in buffered solution [3,4]. As shown in fig.1 an increase in temperature is associated to a relatively small increase in $p(O_2)_{1/2}$ and to a clear-cut decrease in the value of the Hill coefficient, which drops from 2.2–1.8 (as the temperature is increased from 10–30°C. The dependence of n on temperature may be interpreted in terms of a temperature-stabilization of the R-state, as shown in [8]. Since ΔH is saturation dependent, as shown by the superimposition of the oxygen binding curves at low ligand saturations, we have calculated the overall enthalpy change from the values at 50% of saturation (fig.1, inset). This type of behaviour is very similar to that obtained in buffered solutions [1,3]. Therefore, the dependence of cooperativity on temperature cannot be attributed to enthalpic contributions related to binding of small ions, but reflects intrinsic features and specific thermodynamic properties of this hemoglobin.

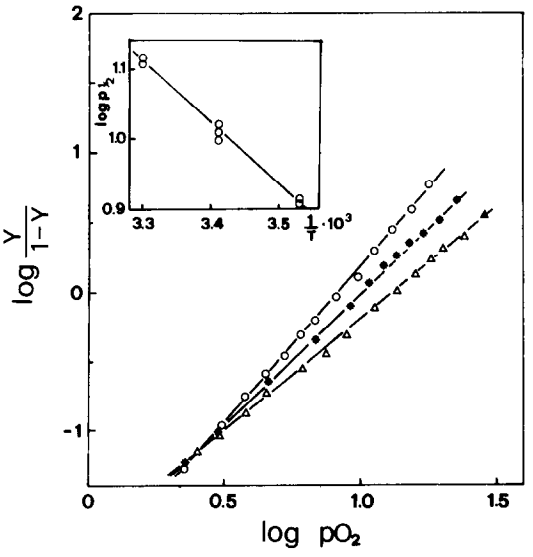

Fig.1. Hill plot of the oxygen binding curves of trout Hb I at 3 different temperatures and at the isoionic pH (7.72) in water. (○) T = 10°C; (*) T = 20°C; (△) T = 30°C. From the inset (Van 't Hoff plot) an overall $\Delta H \simeq -3.8$ kcal/mol may be calculated.

3.2. *Effect of anions and cations on oxygen equilibria*

The effect of salts was studied in the presence of increasing concentrations of NaCl, sodium citrate or sodium dextran sulfate. It was shown [5] that trout Hb I is not affected by organic phosphates (like pyridoxal phosphate, ATP or IHP) probably due to steric inaccessibility; on the other hand, chloride, ortho- and pyrophosphates decrease significantly the oxygen affinity of trout Hb I. It should be remarked that the effect of small ions on the O_2 affinity of trout Hb I is very much smaller than that characteristically observed for human HbA [9]. Addition of NaCl chloride to trout Hb I up to ~1 M (fig.2) leads to a decrease in affinity, in agreement with [5]. However, the new data indicate that the effect of NaCl is the result of 2 distinct contributions, due respectively to the anion and to the cation. Using citrate or dextran sulfate sodium salts, we could dissect out the effect of Na^+ on the oxygen affinity of trout Hb I, since the bulky anions do not affect the O_2 affinity of trout Hb I. As shown in fig.2, using these salts, the oxygen affinity decreases as the sodium concentration is raised up to 0.3 M and then levels off, indicating that the O_2-linked sodium binding sites are saturated in both quaternary states.

The shift towards lower affinity indicates that the T state of the molecule is stabilized by Na^+. Moreover,

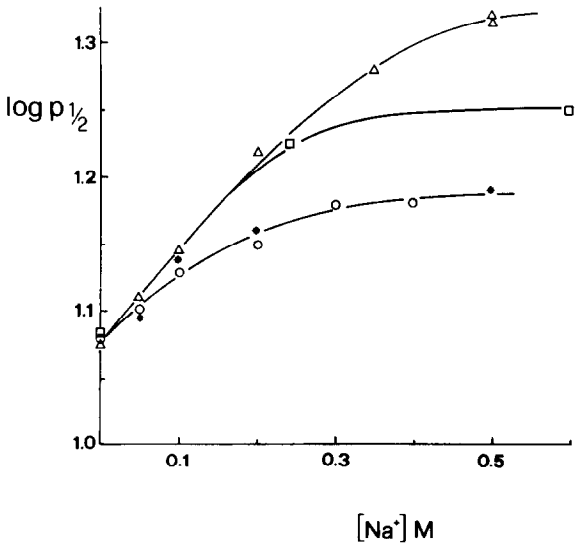

Fig.2. Effect of NaCl (△), sodium orthophosphate (□), sodium dextran sulfate (○) and sodium citrate (*), on the oxygen affinity of trout Hb I. Results in water are reported at $[Na^+] \leq 10^{-6}$ M $\simeq 0$; T = 20°C; hemoglobin $\simeq$ 4 mg/ml.

over the concentration range studied, cooperativity in O_2 binding is significantly affected, the difference in the values of n between the stripped deionized sample ($n_{1/2}$ = 2.0) and that in the presence of different salts ($n_{1/2}$ = 2.3–2.5) being outside experimental errors.

Previous data [5] on the effect of orthophosphate on the oxygen affinity of trout Hb I were normalized for the corresponding sodium concentration and are plotted with the new data in fig.2. This composite graph allows us to subtract from the overall effect of NaCl and sodium orthophosphate the contribution due to the differential binding of the common cation, i.e., sodium. The overall binding constant for sodium seems higher than that of either anions, being saturated at a concentration at which the two anions still affect the O_2 binding.

3.3. *Kinetics of CO binding*

Stripped trout Hb I deoxygenated and equilibrated with 1 atm CO at 20°C, was conveniently diluted either with 0.1 M phosphate buffer (pH 7.7) or deionized distilled water, both equilibrated with CO at the same pressure. The final protein concentration was 4 μM and final CO 1 mM. A crystal of sodium dithionite was added to ensure complete removal of oxygen; the maximum drop in pH (6.8) observed with the unbuffered solution has no effect on the time course of CO binding (see also [2]). Analysis of the time course of binding observed under conditions of complete photolysis shows that the reaction is autocatalytic both in water and in buffer (see also [2]). In no case the full photolysis data show the presence of quickly reacting components, indicating that at μM levels trout Hb I in water is fully tetrameric even in the liganded form (similarly to what clearly demonstrated in buffered solution) [2]. The shape of the time course is very similar for the two conditions (fig.3). The absolute value of the second order rate constant(s) is slightly higher (~15%) for the experiments performed in water as compared to those in 0.1 M phosphate buffer. The time course of CO recombination depends on the fractional photodissociation [10] and as the flash intensity is decreased the presence of a quickly reacting component is easily detectable. These experiments in the absence of buffer show that all the main features of trout Hb I kinetics are maintained, since the overall results are very similar to those reported for the same protein in buffered solutions [2,8,10]. This implies that the kinetic contribution to cooperativity is an intrinsic property of

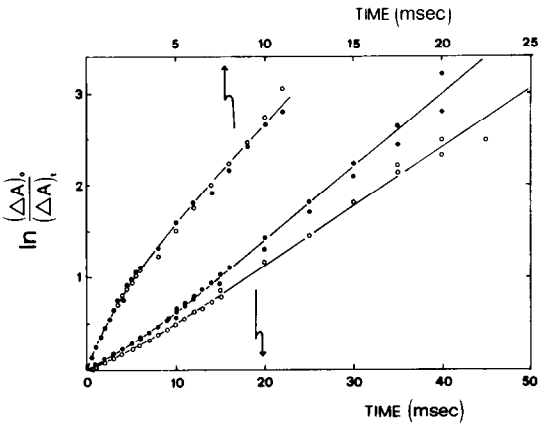

Fig.3. Kinetics of CO combination of trout Hb I after complete photolysis (lower time scale) and partial (~20%) photolysis (upper time scale). Results in water (○) and in phosphate buffer 0.1 M (○); [CO] = 1 mM; [Hb] = 4 μM; T = 20°C.

the molecule and is not determined to any substantial extent by heterotropic effects, at least for CO.

4. Conclusions

The complete absence of Bohr effect in trout Hb I is well proven, and is substantiated by the amino-acid substitutions involving the specific residues responsible for the Bohr effect in Hb A [11,12]. Thus experiments with the isoionic protein can be carried out at constant pH (i.e., the isoionic pH) in the absence of ions other than H^+ and OH^- (and of course the protein itself). This type of experiment has never been performed because with most other hemoglobins the pH cannot be kept constant along the binding curve in the absence of buffer, due to the Bohr effect. The isoionic trout Hb I is still cooperative in the binding of O_2 (as well as CO, as inferred by the kinetic experiments), proving that homotropic interactions are fully operative even in the absence of heterotropic effects. The experiments at different temperatures yield additional information relevant to the interpretation of the properties of trout Hb I. The temperature dependence of cooperative CO and O_2 binding [4] may be interpreted in terms of a large endothermic value for the enthalpy change associated to the quaternary structural change (ΔH_{L_0} = +30 kcal/tetramer), as well as the peculiar enthalpic contribution related to the binding of a ligand to a site in a T quaternary state

(ΔH_T = +6 kcal/site for CO) [4]. The experiments reported above show that these thermodynamic properties are intrinsic features of this hemoglobin, and cannot be attributed to heterotropic contributions.

A second interesting finding is the original result that Na^+ binding is O_2-linked. The experiments reported in fig.2 show that: (i) Na^+ binding is more favourable to the T quaternary state, since the affinity is lowered by Na^+; (ii) the ligand-linked effect is saturated at 0.2–0.3 M Na^+.

The structural interpretation of the Na^+ effect is at present only tentative, but it may be attempted on the assumption that ligand linked Na^+ binding in trout Hb I occurs at the DPG binding site [13]. It may be recalled that the positively charged residues coating the crevice in between the two β chains are all substituted in trout Hb I, i.e.: β(143) His → Ser, β(2) His → Glu and β (82) Lys → Leu ([12,15,16], unpublished). It is therefore possible that β (2) Glu may be involved in the differential binding of Na^+ in trout Hb I, although this proposal is at present speculative. These new results also establish that a real O_2-linked Cl^- effect is operative in trout Hb I, as seen from the results in fig.2 where the net Cl^- effect may be obtained by subtracting the specific Na^+ effect. It should be noticed that the magnitude of the Cl^- effect is very small compared to Hb A ($\Delta \log p_{1/2}$ of 0.1–0.2 against $\Delta \log p_{1/2} \sim 1.0$ for Hb A [5,9]. This seems consistent with the fact that some of the Cl^- binding sites identified in Hb A are strongly perturbed or essentially abolished in trout Hb I (e.g., the one between α_1 (1) Val and α_1 (131) Ser, which in trout Hb I are, respectively, α_1 (1) acetyl Ser and α_1 (131) Ala) [17,18]. It is possible, however, that the site identified between α(126) Asp, α(127) Lys and α(141) Arg [19], which are all conserved in trout Hb I, is the site responsible for the Cl^- effect characterized here.

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