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Thermodynamic characterization of the allosteric transition in trout hemoglobin

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Abstract. The pH induced spectral changes in the Soret region occurring in the carbomonoxy derivative of trout HbIV have been measured under carefully controlled conditions of temperature and organic phosphate concentration. Parallel experiments on the kinetics of carbon monoxide dissociation by NO replacement have been performed over the same pH range. Both sets of results agree satisfactorily with a thermodynamic scheme independently drawn on the basis of functional data previously analyzed within the framework of a two state allosteric model. Thus, the whole body of data strongly supports the idea that the spectral changes are themselves an indication of the pH induced structural transition, thereby reflecting the stabilization of the liganded *T*-state of the molecule at low pH. This allows us to definitely conclude that the functional changes induced in trout HbIV-CO by protons are associated with a red shift of the Soret absorption band.

Key words: Allosteric transition in trout HbIV, spectral changes in liganded HbIV, fish hemoglobins

Introduction

Hemoglobins from teleost fish, and occasionally amphibians, display a very marked Bohr effect (called Root effect), associated with a large change in the position and the shape of the oxygen binding curve, which becomes less and less cooperative as the pH is lowered (at pH $\leq$ 6, Hill coefficient of 1 or even less). Leaving aside the role of functional heterogeneity, whose extent has been shown to be protein and ligand dependent (Brunori et al. 1978; Giardina et al. 1978), the essential feature of this phenomenon appears to be a proton induced stabilization of the low affinity state of the molecule (Brunori 1975). At the structural level, the Root effect has been correlated (Perutz and Brunori 1982) with two additional H-bonds involving the protonated form of His HC3 (146β) and a serine at position F9 (93β) that, in Root effect hemoglobins, substitutes for the cysteinyl residue normally found in mammals. We have previously shown (Giardina et al. 1975) that the Soret absorption band of trout HbIV (that component from trout blood which displays the Root effect) fully saturated with carbon monoxide is red-shifted by about 1 nm on going from pH 8 to 6 and/or upon addition of P_6-inositol, which is known to greatly stabilize the low affinity states of hemoglobin. The red shift of the absorption spectrum has been found to be accompanied by a small increase (0.46 cm^{-1}) in the CO stretching frequency (Ascoli et al. 1978) at low pH and by changes in the near ultraviolet and visible circular dichroism spectra (Ascoli et al. 1983). The origin of all these spectroscopic changes was associated with the allosteric transition, which is induced by protons in the liganded form of the molecule. In contrast to this hypothesis, Greenwood and Gibson (1983) have concluded that the allosteric functional parameters obtained from kinetic studies on hemoglobins from bluefin tuna (*Tunnus thynnus*) and menhaden (*Brevoortia tyrannus*) are not correlated with CD spectral changes, which therefore are not monitoring quaternary structural changes.

Since the functional properties of trout HbIV have been studied in detail, the system is well suited for verifying if specific spectral changes may be directly linked to the populations of different quaternary forms. In order to test the validity of our previous proposal, which correlated spectroscopic observations and the quaternary structure transition in trout HbIV (see also Perutz et al. 1978), we have reinvestigated the pH induced spectral changes in

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the Soret region at different temperatures, and added observations on the kinetics of carbon monoxide dissociation. The new results agree satisfactorily with a thermodynamic scheme independently drawn on the basis of functional data previously analyzed within the framework of a two-state allosteric model (Wyman et al. 1978).

Materials and methods

Trout HbIV was prepared as previously described (Binotti et al. 1971) and its CO-derivative was obtained by exposing the protein to pure CO. The protein concentration of the carbomonoxy derivative was determined spectrophotometrically at pH 7.8 using an extinction coefficient $\varepsilon = 12.9\ mM^{-1}\ cm^{-1}$ at 566 nm (Falcioni et al. 1978). Buffers used in the kinetic and spectrophotometric experiments were 0.05 *M Bis-Tris*/HCl, *Tris*/HCl and sodium phosphate buffer.

The pH values were measured with a Radiometer pH M64 pH meter, at the temperature of the experiment.

In the difference spectra the reference and sample cells (1 cm optical path) contained a solution of HbIV-CO at the same concentration (say 6 μ*M*) but different pH; the reference was kept at pH 8.1 and the sample changed systematically. The two compartments of the spectrophotometer were maintained at constant temperature with thermostatted circulating water. In the experiments as a function of temperature the reference cell contained the protein solution at pH 8.1, as above, and the sample cell contained the same solution but at constant pH value selected between 6.0 and 7.0. Both compartments were thermostatted at the same temperature, which was increased (or decreased) stepwise, in the range 10–30 °C. Spectra were taken after equilibration (usually after 15 min) at the desired temperature.

Difference spectra were recorded in a Cary 219 spectrophotometer between 370 and 450 nm; base lines were taken before and after each set of the experiments. From the total ΔOD at 424 nm, obtainable at low pH, the data were analyzed in terms of a two-state equilibrium constant and plotted as a function of pH or temperature.

The kinetics of CO dissociation from HbIV-CO was measured by displacing CO with an excess of NO. Using a stopped flow apparatus, HbIV-CO solutions (protein concentration, 7 μ*M*; CO concentration 0.1–0.4 m*M*) were mixed with a 1 m*M* solution of NO in the desired buffer, as described by Antonini and Brunori (1971). The time course of the reaction was followed at 419 nm and the rate constant for dissociation of CO was obtained by applying the equations developed and used by Gibson and Roughton (see Gibson 1959).

Results

Difference absorption spectra

Difference spectra of the CO derivative of trout HbIV were measured as a function of both temperature and pH, in the absence and in the presence of inorganic or organic phosphates. A typical experiment concerning the pH dependence of the difference spectra at 20 °C is shown in Fig. 1. As is evident from Fig. 2, the temperature dependence of the spectral transition (determined from the difference spectra) is almost negligible within the range 10–30 °C; the van't Hoff plot of the data at a constant pH of 6.1 (shown as an inset) gives $\Delta H = +1.2 \pm 1$ Kcal/tetramer, thus resulting in a negligible enthalpy contribution to the observed transition, which, at the apparent pK value, has a slope characterized by a value higher than 1.

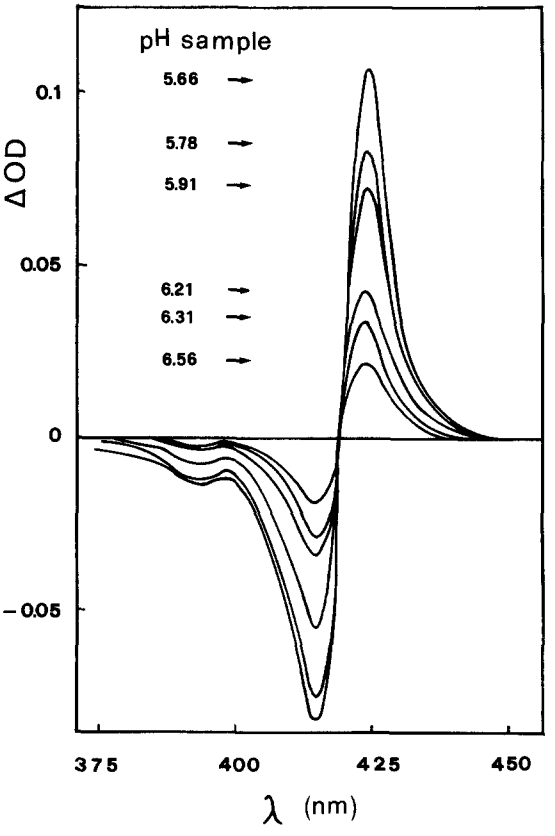

Fig. 1. pH dependence of the Soret absorption spectrum of HbIV-CO. Protein concentration, 6 μ*M*; temperature, 20 °C. Sample cell, at various pH values as indicated; reference cell, at pH 8.1. Buffers: 0.05 *M Tris*/HCl and *Bis-Tris*/HCl

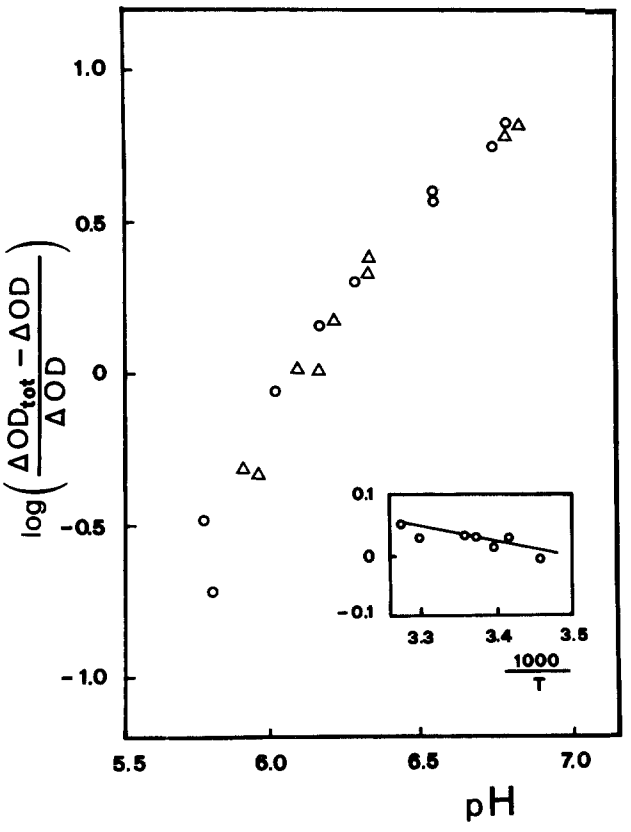

Fig. 2. pH dependence of the absorption changes at 424 nm in trout HbIV-CO. Experiments performed at 20 °C (△) and 30 °C (○). Buffers as in Fig. 1. *Inset:* van't Hoff plot at pH 6.1. For other details, see text

As expected on the basis of previous knowledge on the effect of organic polyphosphate (Brunori et al. 1975), addition of P_6-inositol (1 m*M* concentration) further increases the fraction of liganded molecules in the low affinity *T*-state (by 15% within the pH range 6.0–7.0). Moreover, binding of P_6-inositol induces an increase of the temperature dependence of the allosteric transition (apparent ΔH is $\sim +6$ Kcal/tetramer). Similar results were obtained in 0.05 *M* phosphate buffer. The binding constant of P_6-inositol, measured at various pH's by spectrophotometric titration of HbIV-CO with increasing quantities of this polyphosphate, has been shown to increase linearly upon lowering the pH. In particular, at 20 °C, the overall affinity constant was found to increase by a factor of 9 in going from pH 6.8 to 6.0 ($0.6 \times 10^2\ M^{-1}$ vs $5.5 \times 10^4\ M^{-1}$).

Kinetics of carbon monoxide dissociation

The pH dependence of the kinetics of CO dissociation was measured by replacement of carbon monoxide by NO. This method allows one to maintain, at every pH, the protein in a fully liganded form and hence to follow a functional parameter which relates to the completely saturated molecule. The quantitative analysis of the results proved to be difficult because of two complexities: (a) the time course of the replacement was complex, except at low pH (~ 5.5) where a single exponential process is observed in the presence of 5 m*M* P_6-inositol: we believe that the observed kinetic heterogeneity may reflect chain differences, but we have no independent proof for this interpretation; (b) the recovery of absorbance at the same wavelength ($\lambda = 419$ nm) was not constant at the various pH's examined, and tended to become smaller at more alkaline pH values. This was partly accounted for by the finding that the spectra of both species are pH dependent; in particular the extinction of HbIV-NO at the maximum ($\lambda = 416$ nm) was lower at pH 5.5 as compared to pH 6.5 and above.

Analysis of the pH dependence of the faster kinetic component (~ 30%) indicates that a significant increase in the rate constant of CO dissociation occurs over the pH range examined (pK ~ 6.2–6.3). The overall change both in the absence and in the presence of 5 m*M* P_6-inositol is quite small, as shown in Fig. 3. This is not unexpected since it is known that, in the case of CO, the major contribution to the Root effect comes from a large change in the combination rate constant (Giardina et al. 1973). It should be mentioned that the acid asymptotic value of the data reported in Fig. 3 is consistent with the dissociation rate constant for CO previously obtained from flash photolysis experiments (Binotti et al. 1971) at pH 6.0 in 0.1 *M* phosphate buffer ($l_4 = 0.4\ s^{-1}$).

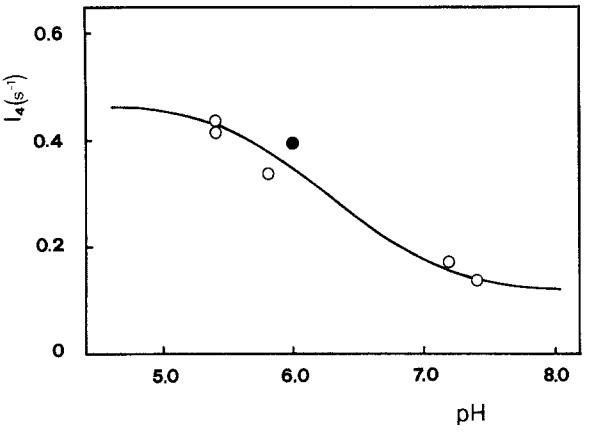

Fig. 3. pH dependence of the apparent CO dissociation rate constant (l_4) for trout HbIV-CO. *Open symbols:* values obtained by NO replacement; temperature, 20 °C; observation wavelength, 419 nm; buffers as in Fig. 1. *Full symbols:* value previously obtained from the CO binding kinetics to HbIV (Binotti et al. 1971). The continuous line is the theoretical curve for titration of one group with a pK of 6.25

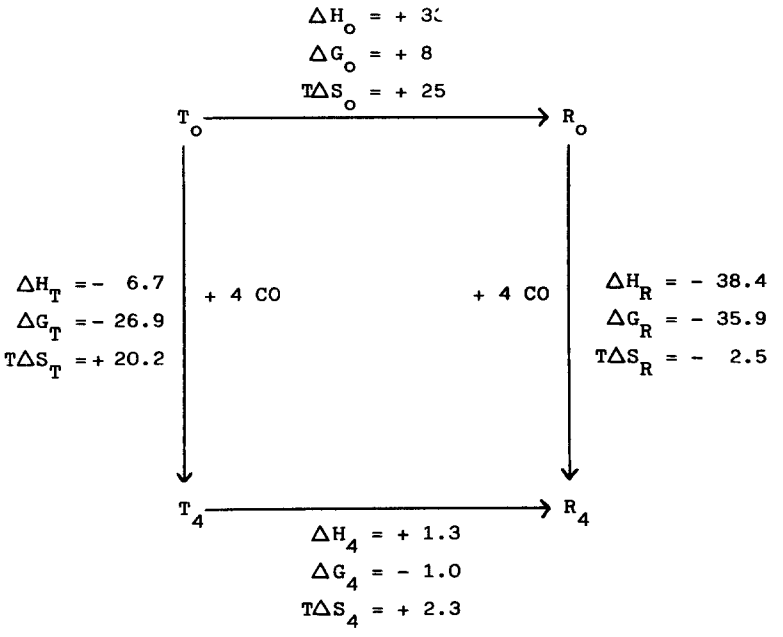

Fig. 4. Thermodynamic parameters (expressed in Kcal/mole of tetramer) for CO binding to trout HbIV and for the associated allosteric transition at pH 6.0

Discussion

The pH and temperature dependence of the reaction of trout HbIV with carbon monoxide has been extensively investigated (Wyman et al. 1978) by different and parallel experimental approaches which comprise (i) microcalorimetric measurements, (ii) determination of accurate CO binding curves and (iii) differential titrations of the deoxy- and CO-derivatives of the molecule. From a combination of these results analyzed with a two state allosteric model it was possible to obtain a complete thermodynamic characterization showing the free energy, enthalpy and entropy changes associated with the binding of carbon monoxide to trout HbIV. The thermodynamic cycle derived from those data is shown in Fig. 4 and refers to the reaction with CO and to the associated allosteric transitions in HbIV and HbIV-CO, at pH 6.0. It should be noted that the thermodynamic parameters reported for both the $T_0 \rightarrow R_0$ and $T_4 \rightarrow R_4$ transitions were not directly measured but are derived from the fit of the binding data with a two state allosteric model. Examination of the scheme shows that in the absence of the ligand, the T_0 state is stabilized over R_0 ($L_0 = [R_0]/[T_0] = 1.2 \times 10^{-6}$) by a large and positive enthalpic contribution, which does not compensate the increase in entropy of the allosteric $T_0 \rightarrow R_0$ transition ($T\Delta S = +25$ Kcal/tetramer). Moreover, binding of CO to the T state is favoured both by the negative value of the apparent enthalpy change and by the positive value of the $T\Delta S$ term (+20.2 Kcal/tetramer), the latter representing the major part of the driving force of the reaction. In contrast, CO binding to the R state is totally enthalpy driven, since the entropy change associated with this reaction is small and unfavorable ($T\Delta S = -2.5$ Kcal/tetramer).

The spectroscopic data reported above (Figs. 1 and 2) allow us to calculate the following thermodynamic parameters for the pH-induced transition in trout HbIV-CO: $\Delta G = +0.23$ Kcal/tetramer, $\Delta H = +1.2$ Kcal/tetramer, $T\Delta S = +1$ Kcal/tetramer (all at pH 6.0 and 20 °C). The ΔG value obtained is in good agreement, within the experimental error, with the free energy change associated with the conformational transition of the completely liganded molecule ($\Delta G = -1$ Kcal/tetramer, see Fig. 4). Moreover, the ΔH value obtained from the red shift of the Soret absorption band (+1.2 Kcal/tetramer, see inset of Fig. 2) is very close to the enthalpy change (+1.3 Kcal/tetramer) associated with the $T_4 \rightarrow R_4$ transition, at the same pH value, as shown in Fig. 4. The whole body of the data allows us to assign the spectral changes to the allosteric transition of liganded HbIV on the basis of: (i) the good agreement of the thermodynamic parameters obtained from the difference spectra with those derived from independent measurements of ligand binding (Wyman et al. 1978); (ii) the pH-dependent kinetics of CO displacement by NO, which shows that the increase in l_4 occurs over a pH range consistent with the pK of the spectroscopic transition. Thus, we conclude that the spectral transition and the increase of l_4 upon acidification reflect the same phenomenon, which we believe to be the pH stabilization of the liganded T-state of the molecule.

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