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A macromolecular transducer as illustrated by trout hemoglobin IV

(heme proteins/allostery/oxygen transport)

M. BRUNORI, M. COLETTA, B. GIARDINA, AND J. WYMAN

Consiglio Nazionale delle Ricerche, Centre for Molecular Biology, Institutes of Chemistry and Biochemistry, Faculty of Medicine, University of Rome, Rome, Italy

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ABSTRACT **Oxygen binding by trout Hb IV has been investigated as a function of pH up to 10 atmospheres (1 MPa) of pure O_2. The results bring out an extreme proton–oxygen linkage, which gives rise to a Root effect. They are discussed in relation to the function of the hemoglobin as an oxygen pump. The system is of special interest as providing a prototype of a macromolecule acting as a transducer by coupling two allosterically linked reactions.**

The basic function of the hemoglobins is to ensure an adequate supply of oxygen to all parts of the organisms in which they occur. In order to accomplish this task they have developed, in the course of evolution, a common molecular mechanism based on the principle of ligand-linked conformational change in a multisubunit structure (1–3). Within the framework of this common mechanism, however, different hemoglobins, sometimes in the same organism, have acquired special features to meet special needs. This is illustrated in certain of the teleost fish, such as the rainbow trout (*Salmo irideus*), in which different hemoglobins, characterized by distinct properties, have been isolated and investigated (4). One of the components of trout blood, in fact the major one (trout Hb IV), has acquired very special properties that enable it to serve as a pump for injecting O_2 into the swim bladder against a very high opposing pressure. This function, widespread among teleost fishes, was recognized long ago by Root (5) and was subsequently studied in the unfractioned hemolysate of benthic fishes and qualitatively analyzed by Scholander and van Dam (6).

The object of this communication is the presentation of the oxygen equilibria of isolated trout Hb IV, which is responsible for this phenomenon. These equilibria are of interest not only because they illustrate the variations possible within the scope of an overall allosteric mechanism, but, even more, because this hemoglobin represents a type case of how a macromolecule, operating under steady-state conditions, can serve as a transducer.

In view of the very low O_2 affinity of trout Hb IV at acid pH, which is what makes the pump effective, the experiments called for the application of high partial pressures of O_2; we developed, therefore, a special spectrophotometric cell capable of operating up to approximately 25 atmospheres (1 atm = 101 kPa), following the pioneering work of Scholander and van Dam (6). This makes it possible to undertake a complete thermodynamic characterization of O_2 binding by this Hb, in the same way as was done in the case of CO (7). In that case, the thermodynamic analysis of the data obtained by calorimetric and equilibrium experiments provided clear evidence for the strong dependence of the allosteric equilibrium constant, $L_0 = (R_0)/(T_0)$, on the concentration of allosteric effectors, such as H^+ and organic phosphates, according to prediction based on a simple two-state model (1). It may be recalled here that in going from pH 7.5 to pH 6 the value of L_0 changes by over 10^4, and it is this change that accounts quantitatively for the pH dependence of both the position and the shape of the CO equilibrium curve, which at low pH becomes essentially noncooperative (Hill coefficient $n_{\bar{Y}=0.5} \simeq 1$) (7).

This paper begins a complete thermodynamic analysis of the O_2 binding by trout Hb IV. Fig. 1 depicts the O_2 binding curves of this hemoglobin in 0.05 M Bistris at one temperature, 14°C. The extremely low O_2 affinity displayed at pH values below 6.5 is responsible for the fact that only partial saturation is achieved in air. A Hill plot of the data brings out the following characteristic features: (*i*) the very large range of binding energies covered by the system, corresponding to a shift in O_2 affinity of approximately three orders of magnitude within two pH units; (*ii*) the tremendous dependence on pH of the shape of the O_2 binding curve, which is highly cooperative at pH 7.15 ($n_{\bar{Y}=0.5} = 2.2$) and is strongly anticooperative at pH 6.1.

These results prompt a number of considerations:

(*a*) The behavior of trout Hb IV is dominated by the strong linkage between proton binding and the stability of the allosteric states, as deduced qualitatively and quantitatively from previous data. The stabilization of a low-affinity state by protons* has already been well documented not only for trout Hb IV but also for Hb from carp (7, 9, 10).

(*b*) An important point shown by the present results is the clear qualitative difference between the equilibria of O_2 and CO, the latter never showing anticooperative binding (7), while the former does. In this respect trout Hb IV, like other fish hemoglobins (10), is different from Hb A (3).

(*c*) The oxygen binding curves at low pH show that the binding sites must be functionally unequivalent, which is presumably associated with the presence of α and β chains in the tetramer.

These considerations suggest that the properties of trout Hb IV may be understood on the basis of at least two classes of allosteric phenomena: one involves a proton-linked quaternary conformational change, expressed in the pH dependence of the allosteric equilibrium constant; the other involves proton-linked tertiary structural changes, which are different in the two kinds of chain. The net result of these two types of linkage is a strong dependence of the Bohr effect on saturation, as shown by the data reported in Fig. 2. It will be seen that while at low saturation the Bohr effect reaches a plateau at pH $\leq$6.5, at high saturation it continues to rise with a constant slope $(\partial \log pO_2/\partial pH)_{\bar{Y}=0.9} \simeq 1.6$ from pH 7.7 to pH 6.1.

The properties just described bring out the significance of trout Hb IV as a prototype of a *macromolecular transducer*, built specifically to act as an oxygen pump for injecting oxygen in the swim bladder (11). Because under working conditions

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* Although here we have focused our attention on the proton–oxygen linkage, it is known that anions such as polyphosphates may act like protons as control ligands in fish hemoglobins (9, 10).

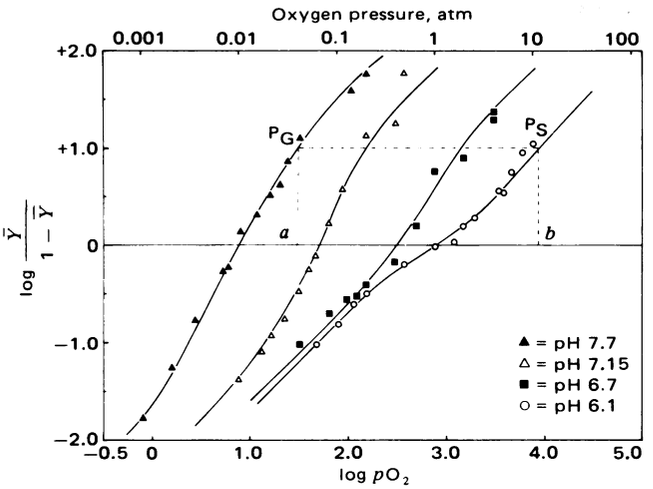

FIG. 1. Oxygen binding curves of purified stripped trout Hb IV at different pH values in 0.05 M Bistris [bis(2-hydroxyethyl) imino-tris(hydroxymethyl)methane] buffer at 14°. The binding isotherms were obtained spectrophotometrically on the basis of absorbance changes between 650 and 450 nm. In the low-affinity range the measurements were made possible by an especially built high-pressure observation cell to be described elsewhere. The achievement of equilibrium at any pressure was followed by observing spectral changes as a function of time. Values of average saturation $\bar{Y}$ at pH 6.1 were calculated taking the spectrum of trout Hb IV at this pH and 18 atm of pure O_2 as representative of the fully saturated protein. This was supported by the invariance of the spectra recorded at equilibrium from 12 to 18 atm. However, it should be pointed out that the spectrum so obtained is not identical to that of fully saturated trout oxyhemoglobin IV at pH ≥7.0. The level of methemoglobin was kept well below 5% by employing suitable amounts of a reducing enzymatic system (8). For the definition of symbols see text. The partial pressure of O_2, pO_2, is given in torr (1 torr = 133 Pa) and in atm (top scale).

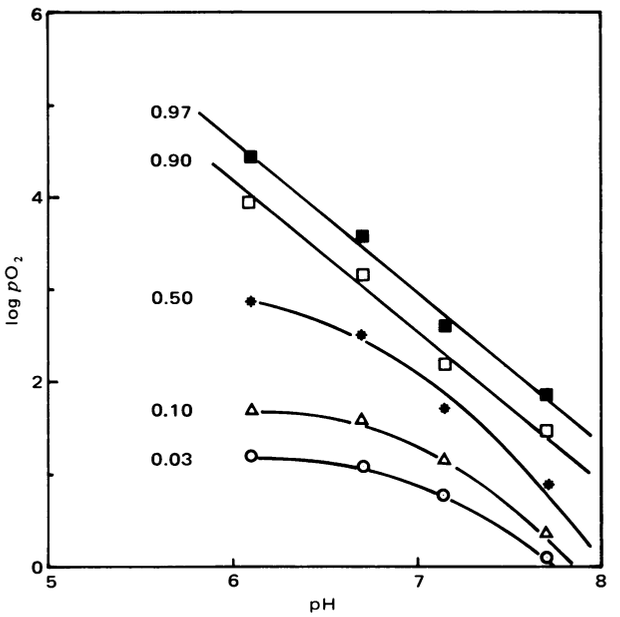

FIG. 2. Oxygen Bohr effect for trout Hb IV, obtained from the data given in Fig. 1 at different fractional saturations (as indicated). Conditions were as for Fig. 1.

the O_2 pressure in the swim bladder can be very high, in any case higher than that prevailing at the level of the gills, the operation of the pump requires a mechanism providing enough energy to transport O_2 against a concentration gradient. The operation of the pump may be understood on the basis of the following considerations, on the assumption that reaction rates are fast enough to allow equilibrium to be reached in both gills and swim bladder. If P_G represents the condition in the gills, where the oxygen pressure is p_G and pH = $-\log H^+_G$, and P_S represents the condition in the swim bladder, where the oxygen pressure is p_S and pH = $-\log H^+_S$ (see Fig. 1), then P_G and P_S will define the state of the hemoglobin as it leaves each of these regions. When an amount of hemoglobin is transferred from the gills to the swim bladder, three possibilities have to be considered: (*i*) When $p_S < a$, the hemoglobin will come to equilibrium after giving off oxygen at a partial pressure less than that in the gills, as it would have done in the absence of a pH effect. This case, which corresponds to the behavior of a cooperative pH-independent hemoglobin, such as trout Hb I (4), illustrates the situation in which the efficiency of transport depends largely on cooperativity of the binding curve ($n > 1$). (*ii*) When $a < p_S < b$, the hemoglobin comes to equilibrium only after giving off oxygen at a partial pressure greater than that in the gills. It is under these conditions that the hemoglobin acts as a *pump* for transferring oxygen from a region of low to one of higher partial pressure. The efficiency of the pump is primarily due to the strong pH dependence of the functional heterogeneity, which, as can be seen from Fig. 1, is considerably greater at the higher saturations associated with high O_2 pressures. (*iii*) When $p_S > b$, equilibrium is reached after an uptake of oxygen from a region of higher pressure, and this may be looked upon as a reversal of the pump's action.

Looked at as a whole, the blood does not change as it circulates, although it exists in two different states, one as it leaves the capillaries of the gills, the other as it leaves those of the swim bladder. In case *ii*, in which the hemoglobin is acting as a pump, the work of pumping is of course paid for by the constant return of protons from swim bladder, where H^+ is large, to gills, where H^+ is smaller. The maintenance of the pH gradient, which ensures continuing operation of the system, is due to the metabolism of the fish. It is known that the gas gland, associated to the swim bladder, produces large amounts of lactic acid even in the presence of oxygen (12).

Thus trout Hb IV can be looked upon as a *macromolecular transducer*, which operates by coupling the concentration gradients of two ligands, both of which bind to the macromolecule in a linked fashion. The downhill flow of H^+, which is maintained at high concentration in the swim bladder, provides the free energy necessary to pay for the opposite, uphill, flow of O_2, the overall thermodynamic process being of course symmetric with respect to the two ligands.

The interest of the system resides in large part in its being a prototype of a class of macromolecular machines (transducers) that includes complex enzymes, capable of converting the free energy derived from one chemical reaction into some other form of free energy. The principles involved in the operation of such systems are discussed in more general terms elsewhere (13).

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1. Wyman, J. (1968) *Quart. Rev. Biophys.* **1**, 35–80.
2. Perutz, M. F. (1970) *Nature* **228**, 726–739.

3. Antonini, E. & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands* (North-Holland, Amsterdam).
4. Brunori, M. (1975) *Curr. Top. Cell. Regul.* **9,** 1–39.
5. Root, R. W. (1931) *Biol. Bull. (Woods Hole, Mass.)* **61,** 427–456.
6. Scholander, P. F. & van Dam, L. (1954) *Biol. Bull. (Woods Hole, Mass.)* **107,** 247–259.
7. Wyman, J., Gill, S. J., Noll, L., Colosimo, A., Giardina, B., Kuiper, H. A. & Brunori, M. (1978) *J. Mol. Biol.* **123,** in press.
8. Hayashi, A., Suzuki, T. & Shin, M. (1973) *Biochim. Biophys. Acta* **310,** 309–316.
9. Giardina, B., Ascoli, F. & Brunori, M. (1975) *Nature* **256,** 761–762.
10. Noble, R. W., Parkhurst, L. J. & Gibson, Q. M. (1970) *J. Biol. Chem.* **245,** 6628–6633.
11. Fänge, R. (1966) *Physiol. Rev.* **46,** 299–322.
12. Ball, E. G., Strittmatter, C. F. & Cooper, O. (1955) *Biol. Bull. (Woods Hole, Mass.)* **108,** 1–17.
13. Wyman, J. (1978) *Atti Accad. Naz. Lincei,* in press.