A Comparative Approach to Protein- and Ligand-Dependence of the Root Effect for Fish Haemoglobins

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Ligand-binding equilibria, kinetics and ^{13}C n.m.r. spectra of bound ^{13}CO , of the haemoglobins from two fishes that are very distant on the evolutionary scale, i.e. the fourth haemoglobin component from *Salmo irideus* and the single component from *Osteoglossum bicirrhosum*, were studied. The C-terminal sequence was also determined for the haemoglobin from *Osteoglossum*. The results show that (i) the C-terminal residues of both chains are not directly responsible for the characteristic heterotropic effect known as Root effect, since for both fish haemoglobins these residues are identical with those of human haemoglobins. (ii) In all haemoglobins characterized by the Root effect a dependence of the ^{13}CO n.m.r. resonances on pH is observed. However, the extent of the shift(s) depends on the particular protein, and is probably the result of a combination of both tertiary and quaternary conformational changes. (iii) Both haemoglobins from trout and *Osteoglossum* manifest a functional heterogeneity between the two types of chains in the tetramer, which increases with proton activity. For CO, the effect is very small for trout haemoglobin IV, and very marked for *Osteoglossum* haemoglobin; for O_2 strongly heterogeneous binding curves were obtained at approx. pH 6.2 with both haemoglobins. (iv) Estimations of the relative values of the affinity constants for the α and β chains in the tetramer were obtained for both haemoglobins from ^{13}CO n.m.r. spectra at low fractional saturation. On the basis of these findings the molecular mechanism underlying the Root effect is discussed.

Haemoglobins from teleost fishes are generally characterized by possession of a special type of Bohr effect, referred to as the Root effect (Brunori, 1975). This consists of a pronounced and parallel decrease of both ligand affinity and co-operativity as the pH becomes more acid, below approx. 7.5. Thus haem-haem interactions, generally present at alkaline pH values, tend to vanish progressively as proton concentration is increased.

As to the mechanistic interpretation of this phenomenon, present data may be accounted for qualitatively on the basis of two different hypotheses, which, however, are not mutually exclusive. On one hand, the apparent decrease in co-operativity, expressed by the drop in the Hill coefficient, may be related, even in the presence of homotropic interactions, to intrinsic functional non-equivalence between the α and β subunits, which become more and more different as pH decreases; on the other hand, the same effect may be ascribed to a progressive stabilization, induced by protons (or organic phosphates), of a 'low-affinity' state of the haemoglobin molecule (T state), according to a classical two-states model (Brunori, 1975; Tan & Noble, 1973).

Expressed in terms of conformational changes, it may be stated that the latter effect is related to quaternary, and the former to tertiary pH-dependent structural transitions. Experimental data suggesting that both mechanisms may be present in fish haemoglobins were reported (Giardina *et al.*, 1976; Giacometti *et al.*, 1976).

To discriminate how far these two kinds of Bohr effect operate in proteins from different fish, we have measured, in parallel, the ^{13}CO n.m.r. spectra, as well as the ligand-binding kinetics and equilibria, of (i) one of the haemoglobin components from trout (*Salmo irideus*) notably trout Hb IV (Binotti *et al.*, 1971), and (ii) the single haemoglobin of an amazonian fish, the aruana (*Osteoglossum bicirrhosum*; Galdames-Portus *et al.*, 1978).

The CO-binding kinetics of these two proteins have been investigated. For trout Hb IV at low pH (approx. 6) the time course of CO combination is second order, without indication for either co-operative or anti-co-operative kinetic effects (Giardina *et al.*, 1973); on the other hand, kinetic investigations on haemoglobin from *Osteoglossum* have clearly indicated a very heterogeneous CO

combination time course, which has been tentatively attributed to intrinsic kinetic differences among different sites in the molecule (Galdames-Portus *et al.*, 1978).

The results reported here are of interest also from a comparative viewpoint, since trout and *Osteoglossum* are very distant on the evolutionary scale, *Salmo irideus* being a member of the superorder Protacanthopterygii and *Osteoglossum bicirrhosum* being a member of the superorder Osteoglossomorpha (Greenwood *et al.*, 1966).

Materials and Methods

Preparations of haemoglobins from trout (component IV) and aruana were made by the procedures reported (Galdames-Portus *et al.*, 1978; Binotti *et al.*, 1971).

The sample solutions for the n.m.r. experiments were concentrated by ultrafiltration or dialysis against Aquacite (Calbiochem, La Jolla, CA, U.S.A.) to a concentration of 3–5 mM in haem. The pH was adjusted by dialysis against the appropriate buffer (0.2 M-sodium phosphate or 0.2 M-sodium maleate).

The ^{13}CO derivatives were prepared by addition of 90% enriched ^{13}CO (Prochem, London SW19, U.K.) to the previously deoxygenated samples. Small excess of sodium dithionite was used to assure full deoxygenation of the samples. ^{13}CO n.m.r. spectra were obtained by pulse-Fourier-transform technique on a Bruker 22.63 MHz spectrometer. Data acquisition periods of up to 24 h were used to achieve a reasonable signal to noise ratio. $^2\text{H}_2\text{O}$ (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 tetramethylsilane by using the value of 184.60 for the free ^{13}CO (Moon & Richards, 1974).

Oxygen-dissociation curves were determined spectrophotometrically in the usual region of wavelengths. Owing to the very low affinity, oxygen-binding curves were obtained by the use of a special spectrophotometric cell which makes it possible to operate at pressures as high as 2533 kPa (25 atm) of pure O_2 (Brunori *et al.*, 1978).

The CO-dissociation curve for the aruana Hb was determined spectrophotometrically on a Cary 14 spectrophotometer in 0.1 M-Bistris (2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol) buffer at pH 6.2 by the procedure described by Wyman *et al.* (1977).

The kinetics of CO binding to trout Hb IV and aruana Hb was measured by flash photolysis as described by Bonaventura *et al.* (1973).

The determination of the C-terminal amino acids for aruana Hb was performed by carboxypeptidase A or B digestion of the protein, both with and without haem, by the procedure of Bonaventura

et al. (1974) after addition of sodium dodecyl sulphate (final concentration 3 mM). The extent of digestion was checked by amino acid analysis of the residues released.

Results

Determination of the C-terminal residues of aruana haemoglobin

The role played by the C-terminal region of the α and β chains in the regulation of the functional properties for human HbA is well documented (Perutz & Ten Eyck, 1971; Antonini & Brunori, 1971). The identity of the C-terminal sequence of the two chains of human HbA and trout Hb IV (Brunori *et al.*, 1973) suggested that the C-terminal residues are not exclusively responsible for the peculiar functional properties of this latter haemoprotein (Root effect).

As observed for the trout haemoglobins (Brunori *et al.*, 1973), attempts to digest the terminal residues of aruana Hb in its native conformation were unsuccessful, whereas positive results were obtained by performing the carboxypeptidase (A or B) digestion in 3 mM-sodium dodecyl sulphate. The analysis of the digested residues indicated that aruana Hb contains equimolar amounts of two different types of chains characterized by the very same C-terminal sequence of trout Hb IV and human HbA, i.e. -Tyr-Arg for the α chains and -Tyr-His for the β chains.

On the basis of this finding the two types of chain of fish haemoglobins have been designated as α and β .

^{13}CO n.m.r. experiments

The ^{13}C resonances of the ^{13}CO bound to both trout Hb IV and aruana Hb were measured as a function of pH and saturation. The two distinct resonances observable in the region around 206 p.p.m. from tetramethylsilane have been assigned for human HbA to the α chains (at 206.75 p.p.m.) and the β chains (at 206.19 p.p.m.) (Antonini *et al.*, 1973). For the corresponding resonances observed in trout Hb IV (Giacometti *et al.*, 1976) and in aruana Hb (see Fig. 1) we have not been able to reach unequivocal assignment since the native isolated chains of these fish haemoglobins are not available. Thus tentative assignment is based exclusively on the analogy with human HbA, the β chain resonance being taken as that at the higher field.

The chemical shift of the ^{13}CO resonance in trout Hb IV is pH-dependent (Giacometti *et al.*, 1976), and involves both resonances, but is more pronounced for the β -chains, which move up field by 0.73 p.p.m. from pH 7.8 to 5.0. This finding has been confirmed by similar observation per-

Fig. 1. ^{13}C n.m.r. spectra of aruana haemoglobin

Aruana haemoglobin partially (a) and fully (b) saturated with ^{13}CO . Sodium phosphate buffer (0.2M, pH6.0). Temperature 20°C.

formed on goldfish (*Carassius* sp.) haemoglobin (personal communication, V. Viti, K. Wütrich & K. H. Winterhalter). Although limited to the β chains, a similar effect is observed in aruana Hb ($\Delta = 0.25$ p.p.m. for β chains from pH7.3 to 6.0). This effect, which has not been reported for mammalian haemoglobins, was correlated to the Root effect, the characteristic property of teleost fish haemoglobins. The relative heights of the resonances assigned to the two types of chains have been measured as a function of the total saturation with the ligand at pH6.0. These conditions were selected because the ligand-binding curves (see below) indicated that possible functional differences among the two types of chains should be more pronounced.

Fig. 1 shows the ^{13}C n.m.r. spectra of fully saturated and partially ($\bar{Y} = 0.5$) saturated aruana Hb.

Quantitative analysis of the data was performed by plotting the ratio of saturation of the two individual chains (as measured by the ratio of the heights of the resonances of the corresponding chains) versus the overall fractional saturation (estimated by the total area under the ^{13}CO resonances).

The results for trout Hb IV and aruana Hb are compared (Fig. 2) with a set of computed curves calculated on the assumption of independent sites with different ratios of the affinity constants for CO. The data obtained on trout Hb IV allow us to conclude that, at pH6.0 and 20°C, the maximum difference between the affinity of the α and β chains is approx. 50%, i.e. that the sites can be regarded as almost equivalent for all subsequent analysis. For aruana Hb only one experiment is available at intermediate saturations; this single value indicates a much greater intrinsic affinity difference of the two chains for the ligand (a factor of 3–10).

The finding that the position of the n.m.r. lines is more pH-sensitive in trout Hb IV than in aruana Hb, although the intrinsic heterogeneity between the chains is vastly more marked in the latter one, suggests that the pH effect on the position of the CO resonances cannot be correlated only with the functional heterogeneity among the chains. It seems therefore that the dependence of the CO resonances on pH may be due to the combination of both tertiary (i.e. local effects within the individual chains) and quaternary modifications whose relative importance may be different in different haemoproteins showing a Root effect.

Oxygen equilibrium of aruana haemoglobin

The pH-dependence of the O_2 binding by aruana haemoglobin, reported by Galdames-Portus *et al.* (1978) shows that below pH7.0 the affinity drops drastically and the apparent value of h becomes smaller than 1. Fig. 3 shows the O_2 -dissociation curve of aruana haemoglobin as obtained with a high-pressure spectrophotometric cell at 10°C and pH6.2 in 0.05M-Bistris.

The presentation in the form of a Hill plot demonstrates the very marked heterogeneity observed under these conditions. The presence of a clear plateau at approximately 50% saturation indicates (i) that the two types of binding sites may be assigned to the two types of chain, α and β , and (ii) that any estimate of either $p_{\frac{1}{2}}$ or $h_{\frac{1}{2}}$ may be very misleading in the absence of a complete saturation curve.

The data were fitted with two independent binding sites, with the binding constants given in Table 1.

CO equilibria and kinetics

Fig. 4 shows CO-dissociation curves of trout Hb IV and aruana Hb determined at pH6.2 and 23°C

Fig. 2. Quantitative analysis of the ^{13}CO n.m.r. data on trout haemoglobin IV and aruana haemoglobin. Ratios of saturation with ^{13}CO of the two individual chains versus the overall fractional saturation for trout Hb IV and aruana Hb. Full lines were calculated on the assumption of independent sites with different ratios of the affinity constants as indicated. ●, Trout Hb IV in maleate buffer (0.2M, pH 6.0); ○, trout Hb IV in sodium phosphate buffer (0.2M, pH 6.0); △, aruana Hb in sodium phosphate buffer (0.2M, pH 6.0). Temperature 20°C.

Fig. 3. Hill plot of the oxygen-binding curve of aruana haemoglobin at pH 6.2 in 0.05M-Bistris buffer at 10°C. Points were obtained spectrophotometrically by recording absorbance at from A_{650} to A_{450} . The proportion of methaemoglobin was always kept well below 5% by employing suitable amounts of a reducing enzymic system (Hayashi *et al.*, 1973). The curve was fitted by assuming two sets of independent binding sites characterized by the intrinsic equilibrium constants given in Table 1. (Values of Y were calculated by taking the spectrum of aruana Hb at 18 atm of pure oxygen as representative of fully saturated protein. This was supported by the invariance of the spectra recorded from 6 to 18 atm. However, it should be pointed out that at this pH the spectrum is not identical with that obtained at alkaline pH values.)

under identical conditions. The difference in binding properties is evident. Analysis shows that for trout Hb IV the Hill coefficient (h) of the ligand dis-

sociation curve is unity up to $\bar{Y}$ approx. 0.5 and tends to increase at higher saturations, being as high as 1.5 at $\bar{Y} \geq 0.7$. This finding indicates that some residual co-operativity is still present at this pH, as shown by more extensive analysis of CO binding (Wyman *et al.*, 1978). On the other hand, the dissociation curve of aruana Hb shows a distinct plateau at $\bar{Y}$ approx. 0.5, clearly indicating the presence of two types of sites characterized by different binding constants. Quantitative analysis in terms of two sets of independent binding sites yields the equilibrium constants given in Table 1. It should be noticed that the fractional abundances (γ) of the independent sites obtained from the fit of the curve are 45% for the higher affinity sites and 55% for the lower ones. Since the amino acid analysis of the carboxypeptidase digests of aruana Hb shows that the α and β chains are present in equimolar amounts, we have tentatively interpreted this non-equivalent contribution as a spectral difference between the two chains in the Soret region. However, this point remains to be substantiated.

CO-combination kinetics were studied by flash photolysis in parallel experiments on trout Hb IV and aruana Hb in 0.1M-Bistris, pH 6.2 and approx. 25°C. The results obtained may be summarized as follows.

(a) After complete photolysis the CO combination shows that although trout Hb IV follows almost (pseudo-) first-order kinetics (the reaction being slightly autocatalytic at $\bar{Y} > 0.5$), aruana Hb displays

Table 1. *Equilibrium and kinetic constants for the reaction of aruana and trout IV haemoglobins with O₂ and CO*
 For CO: Bistris buffer, 0.1 M, pH 6.2, temperature 23°C; for O₂: Bistris, 0.05 M, pH 6.2, temperature 10°C. Attribution of the high-affinity sites to the α chains and the low-affinity sites to the β chains has been made tentatively only on the basis of the analogies of the ¹³CO n.m.r. spectra of aruana Hb with human HbA (see the text). For trout Hb, the constants reported below apply only to CO, for which the equation $\alpha \cong \beta$ is justified. *K*, Equilibrium constant for O₂ binding; *L*, equilibrium constant for CO binding; *l'* and *l*, combination and dissociation rate constants respectively for CO binding.

Haemoglobin		10 ² <i>K</i> (kPa)	10 ⁻⁵ <i>L</i> (M ⁻¹)	10 ⁻⁵ <i>l'</i> (M ⁻¹ ·s ⁻¹)	<i>l</i> (s ⁻¹)
Aruana	α chains	7.33	9.5	2.17	0.229
	β chains	0.09	0.4	0.058	0.145
Trout IV	$\alpha \cong \beta$		1.2	0.31	0.258

Fig. 4. CO-binding curves of haemoglobin aruana (●) and trout haemoglobin IV (----)

Bistris buffer, 0.1 M, pH 6.2 and temperature 23°C. Protein concentrations range from 0.5 to 1 μ M (haem). Observations were made at from 450 to 400 nm. The CO-binding curve of trout Hb IV was calculated from the thermodynamic parameters of Wyman *et al.* (1978).

Fig. 5. Kinetics of CO combination by flash photolysis ○, Trout Hb IV; ●, aruana Hb. Bistris buffer, 0.1 M, pH 6.2 and temperature approx. 25°C. Haemoglobin concentration, 6 μ M; CO concentration, 1 mM.

a very heterogeneous time course (Fig. 5). In this case the marked kinetic heterogeneity allows an easy analysis of the process in terms of two exponential decays, characterized by the second-order rate constants given in Table 1. This finding is consistent with the data of Galdames-Portus *et al.* (1978). It is worth recalling that at alkaline pH both proteins display an autocatalytic time course (Giardina *et al.*, 1973; Galdames-Portus *et al.*, 1978).

(b) Substitution of Bistris buffer by 0.2 M-maleate at the same pH has no detectable effects on the kinetics. This finding represents a necessary control because some of the ¹³CO n.m.r. experiments on trout Hb IV were performed in maleate buffer.

(c) The effect of inositol hexaphosphate (1 mM), which was not investigated before, was very minor and limited to the later stages of the reaction. For trout Hb IV addition of the compound results in the disappearance of the autocatalytic character observed at higher fractional saturation ($\bar{Y} > 0.5$), and makes the reaction identical with that observed in phosphate buffer (Giardina *et al.*, 1973), i.e. strictly

first order in protein and ligand. For aruana Hb the effect of inositol hexaphosphate consists essentially of a reduction of the rate constant for the slow kinetic component, which slows down by approx. 20%, but the faster component is not affected at all.

(d) Both proteins, at low as well as high pH, show no indication of a quickly reacting component, provided that photodissociation is complete. In view of what is known for mammalian haemoglobin (Antonini & Brunori, 1971), this proves that at the protein concentration used (1–5 μ M-haem) there is no appreciable dissociation of tetramers into dimers, information that is relevant to the analysis of the binding curves given above.

(e) In the presence of inositol hexaphosphate the kinetics of recombination after full or partial (25%) photodissociation are the same, confirming that both proteins are essentially in a low-affinity quaternary state under the conditions used, as substantiated by independent evidence for trout Hb IV (Giardina *et al.*, 1975).

The equilibrium and kinetic data collected in

Table 1 show that for CO the pH-dependent functional heterogeneity stems primarily from differences in the combination rate constant(s), the dissociation rate constant(s) having a partially compensating effect.

Discussion

Comparison of the functional properties of the haemoglobins from two fish, both characterized by a Root effect, illustrates the danger of interpreting their behaviour within one single model, and indicates that more than one experimental approach is required to describe the system completely. The partial photolysis experiments show that, under the conditions used, neither fish haemoglobin displays a rapidly reacting form, indicating that only one quaternary state is populated at low pH. However, the functional behaviour is very variable, being both protein- and ligand-dependent.

With trout Hb IV, O₂ equilibrium experiments performed at sub-zero temperatures (Giardina *et al.*, 1976) and at high oxygen pressures (Brunori *et al.*, 1978) showed a heterogeneous binding curve at a pH where CO binding is not heterogeneous. Therefore, for this protein, binding data with two different ligands (O₂ and CO) show that tertiary-linked heterotropic effects are more marked for O₂ than for CO. For aruana haemoglobin the functional heterogeneity for O₂ is three times more pronounced than for CO, as can be seen from the data in Table 1. If we limit the comparison to CO-binding data, it is evident that the Bohr effect local to each subunit is different for the α and β chains in aruana Hb, although this difference is not significant in trout Hb IV.

Thus it appears that the feature common to all fish haemoglobins having a Root effect is a 'low affinity' $\leftrightarrow$ 'high affinity' pH-dependent quaternary transition. However, the overall functional behaviour is a complex average of both quaternary and tertiary effects, which may be both ligand- and protein-dependent. The structural features underlying these various functional effects will be difficult to distinguish, although some conclusions may already be advanced, such as the relatively secondary role played by the C-terminal sequence in modulating these phenomena. Thus, although this region of the molecule is certainly necessary for maintaining basic structural features, it does not seem to be primarily responsible for differences emphasized in the present work.

Although the physiological significance of these various effects in different animals is not understood, the functional behaviour of these haemoglobins from distantly related fish represents an interesting

example of the interplay of quaternary and tertiary effects in molecular control.

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