A kinetic and equilibrium study of ligand binding to a Root-effect haemoglobin

Thomas BRITTAIN

Department of Biochemistry, University of Auckland, Auckland, New Zealand

(Received 9 November 1984/28 January 1985; accepted 25 February 1985)

The blood of the striped marlin (*Tetrapturus audax*) contains one major Root-effect haemoglobin. Titrations of this haemoglobin with CO show that at high pH the molecule is highly co-operative (Hill coefficient 2.8) whereas at low pH the titration data can best be described as the sum of contributions from non-co-operating subunits of different affinity. In terms of the two-state model the R-state affinity constant is much more sensitive to pH than is that of the T state. Flash-photolysis studies were used to characterize the kinetics of ligand binding to this haemoglobin. Both T and R states show kinetic heterogeneity in their recombination time courses, associated with the α - and β -chains of the molecule. The rate constants for ligand binding to each chain, in each quaternary state, were determined, and in conjunction with the allosteric equilibrium parameters determined at pH 8.0 were used in the two-state analysis of reaction curves, over a range of ligand concentration. The two-state model, extended to take account of chain difference, adequately fits the homotropic effects observed for this haemoglobin. The two-state model is, however, inadequate in its description of the heterotropic effects produced by protons.

Unlike the cases for human haemoglobins, the haemoglobins from various teleost fishes show the presence of extreme forms of the Bohr effect known as the Root effect (Root, 1931; Root & Irving, 1941). These haemoglobins are characterized by extreme pH-sensitivity of such magnitude that at acid pH the Hill coefficients associated with ligand binding at half saturation may reach values of <1 . Under these conditions the O₂ affinity may be so low that full saturation may not be achieved even at 10 MPa (100 atm) pressure (Scholander & Van Dam, 1954). The inability of part of the haemoglobin to bind ligands at acid pH has been assigned to functional heterogeneity in the low-pH form of the molecule (Saffran & Gibson, 1978). Within the formalism of the two-state model for co-operativity (Monod *et al.*, 1965) this observation may be expressed as functional heterogeneity within the T state. A number of Root-effect haemoglobins have been studied by both equilibrium and kinetic methods (Noble *et al.*, 1970; Brunori, 1975; Saffran & Gibson, 1978, 1979; Saito *et al.*, 1983). In most cases the physiological role of the Root-effect haemoglobins has been assigned to their function in the control of the gas volume within the swim bladder (Brunori, 1975). In at least one case, however, it has been suggested that the prime role of the Root-effect haemoglobin

may not be related to its function in control of the swim bladder but may be related to the temperature-dependence of the ligand affinity (Carey & Gibson, 1977). In the tuna, muscle mass may be raised in temperature by 15°C above ambient (Carey & Gibson, 1977; Carey & Teal, 1966) with the consequent increase in muscle efficiency. It has been proposed that the role of the Root-effect haemoglobin in this species is to compensate for what would otherwise be a dramatic loss of O₂-carrying capacity under these circumstance.

In the present paper I report equilibrium and kinetic studies on the major haemoglobin species present in the blood of the striped marlin (*Tetrapturus audax*). I have determined a variety of parameters, over a range of pH values, in a preliminary attempt to discover if the characteristic Root effect seen in tuna might be common to other active swimming teleosts.

Materials and methods

The blood used in the studies reported in this paper was obtained by heart puncture of a single striped marlin. Erythrocytes were repeatedly washed with saline and then sedimented by low-speed centrifugation. Erythrocytes were lysed by the addition of 2 vol. of distilled water, and the cell-

membrane fragments were removed by centrifugation at 10000*g* for 20 min. The resulting haemoglobin solution was reduced with a small excess of $\text{Na}_2\text{S}_2\text{O}_4$, passed down a column (40 cm $\times$ 1.5 cm) of Sephadex G-50 and finally treated with CO to produce carbonmonoxyhaemoglobin. Separation of the component haemoglobins was achieved by ion-exchange chromatography as previously described (Brittain, 1984).

Equilibrium CO-binding measurements and flash-photolysis kinetic measurements were performed by using the techniques and apparatus previously described (Brittain, 1984; Brittain *et al.*, 1982a).

Allosteric equilibrium constants were obtained from the CO-binding curves by using the equations given by Levitzki (1978). Kinetic analysis was based on the equations derived by Bateman (1910) as previously described (Brittain, 1984; Brittain *et al.*, 1982a,b). In the present work the equations originally derived by Hopfield *et al.* (1971) were modified to take account of chain differences in the reactions of the R-state and T-state conformations.

Computer simulations of the overall co-operative kinetic binding curves were obtained without recourse to any variable parameters. Equilibrium binding curves were used to obtain values of the allosteric equilibrium constant (*L*) and the affinity constants for both the T and R states. These data were then substituted directly into the appropriate equations (Hopfield *et al.*, 1971) together with the appropriate kinetic constants obtained from flash-photolysis measurements. This procedure allows the prediction of the overall co-operative kinetic curves for the binding of CO to the haemoglobin.

Results

Preliminary electrophoretic investigation of the marlin whole haemolysate indicated the presence of one major and three minor haemoglobin components. Ion-exchange chromatography of whole haemolysate on CM-cellulose, with a pH-gradient elution, allowed easy resolution of the major haemoglobin component (Fig. 1), and it was this species that was used in all further investigations.

Equilibrium binding studies showed that the major haemoglobin component of marlin blood exhibited extreme pH-sensitivity in its affinity for CO, typical of a Root-effect haemoglobin. At high pH a typical sigmoidal binding curve was obtained, whereas at low pH the haemoglobin showed a very low affinity for CO (Fig. 2). Hill plots of the binding data showed Hill coefficients at half saturation of 2.8, 2.4 and 0.6 at pH values of 8.0, 7.15 and 6.2 respectively. In all cases initial

Fig. 1. Ion-exchange chromatographic separation of marlin haemoglobins

Separation of the haemoglobin components of whole haemolysate was achieved by using a 25 cm $\times$ 1 cm column of DEAE-cellulose. The protein was applied as a carbonmonoxy form and eluted with a linear gradient (2 $\times$ 100 ml) of 10–100 mM-Tris/HCl buffer, pH 7.15 (□). O, A_{560} .

Fig. 2. Hill plots of CO-binding curves of marlin haemoglobins as a function of pH

Titration curves were obtained anaerobically by titrating 6 μM -protein (4 ml) with portions of CO-equilibrated buffer at pH 6.0 (O), pH 7.15 (□) and pH 8.0 (Δ). The temperature was maintained at 20°C. *Y* represents the fractional saturation of the protein by ligand. A line of unit slope has been included for comparison (----).

and final unitary slopes were observed from which apparent T-state and R-state binding constants were obtained with values of 0.6 μM and 0.016 μM , 5 μM and 0.2 μM , and 5 μM and 22.4 μM at pH values of 8.0, 7.15 and 6.0 respectively. The effect of pH on the binding curves was to alter the low-saturation unitary slope intercept (T-state binding constant) by a much smaller factor than the high-saturation intercept (R-state binding constant). The extreme pH-sensitivity of the binding curves allowed the estimation of the kinetic allosteric parameters for the R and T states in isolation from each other.

The binding curves of Fig. 2 indicated that, in terms of the two-state model, at the lowest pH the

haemoglobin could be expected to reside in the low-affinity or T state in the unliganded or even possibly fully liganded states. In fact even at pH 7.15 the calculated switch-over point (i_0) from the T to the R state is such that this quaternary-structural change is expected to occur only after nearly three of the four haem groups have bound CO. Thus full photolysis of the carbonmonoxy form of marlin haemoglobin at pH 6 should present recombination kinetics characteristic of the low-affinity T state. Both full and partial photolysis of the CO complex, at pH 6.0, produced identical recombination time courses. These time courses were characterized by the presence of two concentration-dependent exponential phases (Fig. 3) that contributed equally to the total absorbance change at 432 nm over the entire ligand concentration range employed. The second-order rates constants obtained for the two phases were $1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. Moreover, a similar pattern was observed for full photolysis at pH 7.15, indicating that, as calculated, even at this pH the haemoglobin resides in the T form even to

Fig. 3. Time course of CO recombination with marlin haemoglobin

Haemoglobin ($6 \mu\text{M}$) in 100 mM-Tris/HCl buffer, pH 6.0, in the presence of $100 \mu\text{M}$ -CO was subjected to full (○) and partial (approx. 14%; □) photolysis. Recombination with the ligand was monitored at 432 nm. Traces are normalized to the same total absorbance change for clarity.

extended degrees of ligation. The switch to the R state appears to occur too late on in the reaction to produce measurably co-operative recombination kinetic curves. At wavelengths other than 432 nm measurements showed that the contribution of each phase to the overall absorbance change was wavelength-dependent. The wavelength-dependence of the contribution of each phase is shown in Fig. 4. The overall difference spectrum of each phase is essentially the same but the two spectra are shifted by approx. 2 nm relative to one another. The possibility that these findings might arise from the presence of both dimers and tetramers in solution can be ruled out by the finding that the same pattern was observed if the protein sample employed was $6 \mu\text{M}$ or $70 \mu\text{M}$.

By using measurements at pH 8.0 it proved possible to obtain kinetic parameters associated with the R state or high-affinity form of the haemoglobin. From the equilibrium parameters obtained above and by using the equations given by Hopfield *et al.* (1971), assuming that all the haem groups within the haemoglobin have equivalent quantum yields, it is clear that partial photolysis (approx. 14%) should yield predominantly $\text{Hb}(\text{CO})_3$, which would be present at approx. 95% in the R state. Full photolysis at pH 8.0, on the other hand, should produce haemoglobin predominantly in the T state, with a switch-over from T to R state occurring at approx. 2.3 bound CO molecules/molecule. Full photolysis should thus yield accelerating time courses in which the binding of CO is initially to the T state and is accompanied by conversion of the T state into the more reactive R state as the reaction proceeds. Recombination with CO after partial photolysis yielded time courses that showed two exponential

Fig. 4. Kinetic difference spectra for CO recombination with marlin haemoglobin at pH 6.0

The contribution of the fast (□) and slow (○) kinetic phases observed at pH 6.0 after full photolysis are shown. The data were obtained under conditions as in Fig. 3.

Fig. 5. Effect of the extent of photolysis on the CO recombination reaction of marlin haemoglobin at pH 8.0. Haemoglobin ($6\mu\text{M}$) in 100mM -Tris/HCl buffer, pH 8.0, in the presence of $100\mu\text{M}$ -CO was subjected to full (O) and partial (approx. 14%; □) photolysis at 20°C . The time courses have been normalized to the same total absorbance change for clarity.

phases, each of which was concentration-dependent (Fig. 5). The second-order rate constants associated with these two phases were $7.5 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$ and $1.5 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$. An investigation of the wavelength-dependence of the contributions of each of the two phases to the total absorbance change showed that the two processes contributed equally at all wavelengths in the region 400–450 nm. Full photolysis at pH 8.0 produced the predicted co-operative processes, characterized by an accelerating time course of recombination with CO (Fig. 5).

Co-operative time courses of CO recombination at high pH were simulated by using the equations derived by Hopfield *et al.* (1971) and Bateman (1910). Equilibrium ligand-binding data were used to define the position of the quaternary-state equilibrium at each step of ligation by using the equations derived by Hopfield *et al.* (1971). Under the conditions of high ligand concentration employed in these studies it has been shown (Brunori *et al.*, 1980) that, if the quaternary equilibrium is established rapidly relative to ligand binding, then

Fig. 6. Comparison of the simulated and observed time courses of CO recombination with marlin haemoglobin at pH 8.0.

A comparison of the simulated (—) and observed time courses for CO recombination of $6\mu\text{M}$ -haemoglobin at 20°C after full photolysis at pH 8.0 is shown for CO concentrations of $100\mu\text{M}$ (O), $250\mu\text{M}$ (Δ), $500\mu\text{M}$ (□) and $750\mu\text{M}$ (●). Simulations were performed as described in the text.

the process of ligand binding reduces to that of a sequence of simple pseudo-first-order steps for which Bateman (1910) has derived an analytical solution. The rate constant associated with each step of the reaction is then a function of the proportion of the haemoglobin molecules present in each of the quaternary states and the second-order rate constant associated with the R state and the T state, as shown by Hopfield *et al.* (1971). The simulated time courses were then compared with those obtained experimentally over the CO concentration range 100 – $750\mu\text{M}$ (Fig. 6). In order to simplify the simulation process, two major restrictions were imposed on the system. Firstly, only processes at 432 nm were simulated. At this wavelength the contribution from each component to the overall change in absorbance is equal and thus the requirement for absorption coefficients for each type of chain can be eliminated. Secondly, a single affinity constant was used to describe the ligand binding in each quaternary state, this parameter being that derived from the unitary slope extremes of the appropriate binding curve. [A full description of the equations employed in this study has been published previously (Brittain, 1984).]

Discussion

The CO-binding properties of the major haemoglobin fraction of marlin blood clearly identify it as a Root-effect haemoglobin, similar in

character to the haemoglobins of some other teleost fishes, such as tuna (Morris & Gibson, 1982), carp (Noble *et al.*, 1970) and trout (Brunori, 1975).

Examination of the binding curves within the framework of the allosteric model proposed by Monod *et al.* (1965) shows that at high pH the binding properties of the haemoglobin are consistent with the homotropic effects described by this model. Initial binding appears to occur to a low-affinity form, yielding an initial unitary slope of the Hill plot. Subsequent binding is consistent with the proposed accompanying switch to a more reactive form at higher saturations, with a switch-over point from the predominantly low-affinity to high-affinity forms occurring when approx. 2.3 molecules of CO/molecule have bound at pH 8.0. The final stages of ligand binding again present a unitary slope, this time characteristic of the high-affinity form. When the binding curves are examined with regard to the heterotropic effects of proton concentration, at low pH a very different form of binding curve is observed. The mid-saturation Hill coefficient in this case is <1 , and the upper unitary-slope intercept is at a higher concentration than the lower. The simple Monod *et al.* (1965) model cannot account for these findings. In similar cases, as observed, for example, for tuna and carp haemoglobins, the low-pH binding curves have been interpreted in non-allosteric terms (Noble *et al.*, 1970; Morris & Gibson, 1982). In these cases it is proposed that the haemoglobin molecules reside in the low-affinity state both in the liganded and in the unliganded forms, and that the biphasic binding arises from the presence of non-identical chains with different affinities for the ligand. In the case of tuna haemoglobin this difference in affinity approaches two orders of magnitude. This interpretation also accounts for the findings for marlin haemoglobin, although in this case the difference in affinity is only a factor of approx. 2-fold. Over the pH range investigated the intercept associated with the upper portion of the Hill plot changes by a factor of approx. 100-fold whereas the lower portion shifts by a factor of approx. 4-fold. Whatever may be the molecular interpretation of these findings, clearly the two-state model is insufficient to explain both the homotropic and the heterotropic effects in this haemoglobin.

Kinetic studies appear to vindicate the proposition that, at low pH and high pH, measurements of rate data allow the characterization of T-state and R-state parameters respectively. Both full and partial photolysis at low pH indicate that under these conditions the haemoglobin is present in the low-affinity T state in both the liganded and the unliganded forms. Evidence has also been

obtained that within this state both kinetic and spectral heterogeneity exist. These findings are similar to those reported, on tuna haemoglobin, by Morris *et al.* (1981), who suggested that the α -chains within the T-state tetramer represent the high-affinity rapidly reacting species and the β -chains the low-affinity relatively slowly reacting species. This interpretation appears valid in the present case also. The possibility that heterogeneity might have arisen from dimer formation at low pH can be ruled out on two counts. Although no measurement has been made of the dissociation constant for marlin haemoglobin, values for similar fish haemoglobins show very low values, characteristically of the order of 10^{-9} M (Morris *et al.*, 1981). The fact that the same kinetic and spectral observations were made at 6 and 70 μ M would, taken together with the likely K_D value, appear to rule out the possibility of heterogeneity arising from dissociation at low pH. Kinetic heterogeneity was also observed at high pH. The fact that the proportion of each kinetic phase was equal at pH 8.0 and heterogeneity was also seen at pH 6.0 would require, in addition, that dissociation was not affected by pH between 6.0 and 8.0. I thus follow Morris *et al.* (1981) in suggesting that in this case kinetic heterogeneity arises from chain differences with a quaternary state. This behaviour is similar to that seen for tuna haemoglobin, but is in sharp contrast with that of the haemoglobins from many other species. In the case of human and porbeagle-shark haemoglobins chain differences are apparent in only the R state (Olson & Gibson, 1971; Andersen *et al.*, 1973; Dickinson & Gibson, 1981). With menhaden and carp haemoglobins the reverse pattern is observed, although the differences within the T state are small in the case of carp haemoglobin (Saffran & Gibson, 1978; Tan *et al.*, 1972, 1973).

The ability of the two-state model to explain the findings of co-operative CO recombination kinetics at pH 8.0, subsequent to full photolysis, was tested by using the equations derived by Hopfield *et al.* (1971). The homotropic effects at pH 8.0 seem to be reasonably well described by means of the two-state model extended to take account of chain heterogeneity in both the T state and the R state. In simulations using only the faster rate constants for ligand binding in each quaternary state large deviations from the observed reaction curves were found. These deviations were found to give residual sum-of-squares of deviations from the experimental data in the region of 10^{-2} absorbance units, whereas the model when extended to take account of chain heterogeneity yielded values for the residual sum-of-squares of deviations in the region of 10^{-4} absorbance units. This quality of fit may at first appear surprising considering that no

adjustable parameters were employed and only single affinity constants were used for each quaternary state. In the case of tuna haemoglobin, which shows qualitatively similar reactivity, no such fit is possible (Morris *et al.*, 1981). However, reference to the low-pH binding curve indicates that, if the two unitary slopes correspond to binding to the α - and β -chains, then in this case the difference in affinity is only a factor of approx. 3-fold. In comparison the tuna shows a difference in affinity of approx. 100-fold in the T state (Morris *et al.*, 1981). We have no measure of the differences in affinity of the α - and β -chains within the R state, but the relatively good fit of the simulations suggests that in this state the chains once again do not differ markedly in their affinity for the ligand. In this case the use of the single affinity constant probably represents a reasonable approximation to the average affinity in each state at high pH and so leads to a reasonable fit to the data. It should be stressed, however, that this simple model cannot fit the heterotropic effects observed.

The question arises as to the physiological role of this Root-effect haemoglobin. As described above, in many cases Root-effect haemoglobins appear to control swim-bladder gas volumes in many species. In the case of tuna haemoglobin the Root effect appears to counteract, at least in part, the otherwise inevitable loss in O₂-carrying capacity that would otherwise arise in circulation through a muscle mass significantly higher in temperature than ambient. Whether this haemoglobin's pH-sensitivity is controlled by a serine residue at β_{93} , as proposed for other Root-effect haemoglobins (Perutz & Brunori, 1982; Parkhurst *et al.*, 1983), must await sequence data on this protein.

Equipment was purchased from grants from the University Grants Committee (N.Z.) and the Golden Kiwi Scientific Distribution Fund. Marlin blood was provided by Dr. R. M. Wells.

References

Andersen, M. E., Olson, J. S., Gibson, Q. H. & Carey, F. G. (1973) *J. Biol. Chem.* **248**, 331–341

- Bateman, H. (1910) *Proc. Cambridge Philos. Soc.* **15**, 423–427
- Brittain, T. (1984) *Biochem. J.* **221**, 561–568
- Brittain, T., Barber, D., Greenwood, C. & Wells, R. M. (1982a) *Comp. Biochem. Physiol.* **72**, 684–695
- Brittain, T., Barber, D., Greenwood, C. & Wells, R. M. (1982b) *Comp. Biochem. Physiol.* **73**, 991–995
- Brunori, M. (1975) *Curr. Top. Cell. Regul.* **9**, 1–39
- Brunori, M., Giardina, B., Colosimo, A., Falcioni, G. & Gill, S. J. (1980) *J. Biol. Chem.* **255**, 3841–3843
- Carey, F. G. & Gibson, Q. H. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1376–1382
- Carey, F. G. & Teal, J. M. (1966) *Comp. Biochem. Physiol.* **28**, 199–204
- Dickinson, F. M. & Gibson, Q. H. (1981) *Biochem. J.* **197**, 436–446
- Hopfield, J. J., Shulman, R. G. & Ogawa, S. (1971) *J. Mol. Biol.* **61**, 425–433
- Levitzki, A. (1978) *Mol. Biol. Biochem. Biophys.* **28**, 1–106
- Monod, J., Wyman, J. & Changeaux, J. P. (1965) *J. Mol. Biol.* **12**, 88–118
- Morris, R. M. & Gibson, Q. H. (1982) *J. Biol. Chem.* **257**, 4869–4874
- Morris, R. M., Neckameyer, W. S. & Gibson, Q. H. (1981) *J. Biol. Chem.* **256**, 4598–4603
- Noble, R. W., Parkhurst, L. J. & Gibson, Q. H. (1970) *J. Biol. Chem.* **245**, 6628–6633
- Olson, J. S. & Gibson, Q. H. (1971) *J. Biol. Chem.* **246**, 5241–5253
- Parkhurst, L. J., Gass, D. J. & Perutz, M. F. (1983) *Biochemistry* **22**, 5401–5409
- Perutz, M. F. & Brunori, M. (1982) *Nature (London)* **299**, 421–426
- Root, R. W. (1931) *Biol. Bull. (Woods Hole, Mass.)* **61**, 427–456
- Root, R. W. & Irving, L. (1941) *Biol. Bull. (Woods Hole, Mass.)* **81**, 307–323
- Saffran, W. A. & Gibson, Q. H. (1978) *J. Biol. Chem.* **253**, 3171–3179
- Saffran, W. A. & Gibson, Q. H. (1979) *J. Biol. Chem.* **254**, 1666–1670
- Saito, M. I., Yonetani, T. & Gibson, Q. H. (1983) *J. Mol. Biol.* **168**, 673–686
- Scholander, P. F. & Van Dam, L. (1954) *Biol. Bull. (Woods Hole, Mass.)* **107**, 247–259
- Tan, A. L., De Young, A. & Noble, R. W. (1972) *J. Biol. Chem.* **247**, 2493–2498
- Tan, A. L., Noble, R. W. & Gibson, Q. H. (1973) *J. Biol. Chem.* **248**, 2880–2888