Studies on carbon monoxide binding by shark haemoglobin

Francis M. DICKINSON* and Quentin H. GIBSON

Department of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, U.S.A.

(Received 26 February 1981/Accepted 25 March 1981)

The kinetics of the reactions of Pacific-porbeagle haemoglobin with CO were studied by flash-photolysis and stopped-flow methods, and the equilibrium binding curves for CO were measured in spectrophotometric titrations. Measurements were made in the pH range 6–8 and in the temperature range 0–40°C. The results are discussed in terms of the allosteric model proposed by Monod, Wyman & Changeux [(1965) *J. Mol. Biol.* 12, 88–118]. Within this framework the results indicate that in the R-state the haem groups fall into two classes of different reactivity with different spectral characteristics, but that in the T-state the groups may be essentially equivalent. The physiological importance of the temperature-insensitivity of the equilibrium ligand-binding curves for porbeagle haemoglobin is discussed.

Carey & Gibson (1977) have reported that O₂ binding by the haemoglobin of the Atlantic bluefin tuna (*Thunnus thynnus*), examined in phosphate buffers in the pH range 7.8–8.0, is markedly temperature-dependent, but that the effect is reversed in sense as compared with mammalian haemoglobins. The O₂ affinity increased with increasing temperature, rather than decreasing, and the shape of the equilibrium curve was markedly altered, with much lower apparent co-operativity at lower temperatures. The molecular basis suggested for this effect was an unusually great temperature-dependence of the allosteric parameter *L*, favouring the low-affinity T-state at low temperatures, and outweighing the effect of temperature on the O₂-ligand reactions themselves. The physiological significance of the result was related to the heat-exchange mechanism, which, by conserving metabolic heat, permits the tuna to maintain a muscle temperature as much as 15°C above that of the ambient water.

If the molecular and physiological speculations of Carey & Gibson (1977) are well-founded, other warm-bodied fish may be expected to have haemoglobin with analogous properties. The most prominent warm-bodied group after the scombrid fishes is the lamnid sharks, which have a heat-exchange mechanism similar to that of the tuna (Carey & Teal, 1966). Of these sharks, which include the mako, the great white and the porbeagle sharks, the two last-mentioned show the greatest differences between muscle and environmental temperatures.

In addition to its interest in relation to muscle

temperatures and circulation, work with blood from elasmobranchs allows comparisons to be made with previous studies of teleost bloods, where the haemoglobins from the carp (*Cyprinus carpio*) and menhaden (*Brevoortia tyrannus*) were examined in relation to the two-state model (Noble *et al.*, 1970; Tan *et al.*, 1972; Pennelly *et al.*, 1975; Saffran & Gibson, 1978).

The present work was performed with a sample of blood from the Pacific porbeagle shark (*Lamna ditropus*).

Experimental

Gas solutions

Saturated CO solutions were prepared by bubbling CO gas through water or appropriate buffer solutions; deoxygenated solutions were prepared by bubbling with N₂ gas. Saturated NO solutions were made by shaking deoxygenated buffer in an NO-filled tonometer and withdrawal into a syringe.

Haemoglobin

Washed erythrocytes were prepared by three cycles of centrifugation and resuspension in 1% NaCl, being then stored in liquid N₂ until needed. Haemolysates were prepared by addition of 5–10 ml of water to 1–1.2 g of frozen cell pellets, followed by centrifugation at 5000 g for 10 min to remove insoluble material. The supernatant was then dialysed for 4 h against 3 × 250 ml of 50 mM-Tris/HCl buffer, pH 8.5, saturated with CO, and then applied to a 1.2 cm × 20 cm column of DEAE-cellulose equilibrated with the same buffer. The

* Present address: Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

column was developed with a pH gradient formed by running 500 ml of 50 mM-Tris/HCl buffer, pH 7.5, into 500 ml of 50 mM-Tris/HCl buffer, pH 8.5, all buffers being previously saturated with CO. For routine preparations, where fraction F4 (see below) was required to be as concentrated as possible, the gradient elution was interrupted as soon as fraction F3 was eluted, and the column was washed directly with 50 mM-Tris/HCl buffer, pH 7.5, saturated with CO.

For kinetic and equilibrium experiments CO was removed from the haemoglobin samples by displacement with O₂. A tonometer containing the sample was gassed with O₂ under a strong light-source, and O₂ was subsequently removed by flushing the tonometer with N₂ for several minutes.

Flash photolysis

The apparatus used was described by Parkhurst & Gibson (1967). Partial-flash measurements were made by introducing a coloured glass filter between flashlamp and sample to attenuate the photolysing light. Another glass filter was placed between the sample and the observing beam to minimize sample photolysis by the latter.

Equilibrium measurements

The equilibrium binding of CO to haemoglobin was studied by using the computer-controlled spectrophotometer of Knowles & Gibson (1976). Deoxyhaemoglobin solutions for a standard spectrum were prepared by addition of deoxyhaemoglobin to a syringe of deoxygenated buffer containing 50 μ M-Na₂S₂O₄. This solution was then transferred to a stoppered 1 cm-path-length cuvette previously flushed with N₂ gas. Carbonmonoxyhaemoglobin was prepared by the same method, but with buffer containing 92 μ M-CO. Partially liganded haemoglobin solutions were prepared as described for deoxyhaemoglobin, but with addition of a small volume (5 or 10 μ l) of a CO-containing solution to the spectrophotometer cuvette. All measurements were made in the Soret region.

Results and discussion

Haemoglobin fractions

Chromatographic treatment of porbeagle haemolysates on DEAE-cellulose resulted in the elution of four bands, with very little material retained by the column. The bands designated F1 to F4 contained 8, 5, 35 and 45% respectively of the total haem. The heterogeneity of the haemolysate is in line with the electrophoretic pattern reported by Andersen *et al.* (1973) for the Atlantic porbeagle.

The CO derivative of fraction F1 had a markedly lower value for the A_{420}/A_{260} ratio than the other three fractions, which were the same as each other in

this respect. No further work was done on fraction F1. When the absorption spectra of the CO compounds of the other three fractions were examined, fractions F3 and F4 had identical spectra in the Soret region, whereas the spectrum for fraction F2 was shifted by about 1 nm towards longer wavelengths.

In flash-photolysis experiments in 60 mM-potassium phosphate buffer, pH 6.0, with 90 μ M-CO the recombination reactions for fractions F3 and F4 were identical in both full and partial photolysis. The rates for fraction F2 were about 1.5-fold greater. Fractions F3 and F4 had identical O₂ dissociation curves at 20°C in 0.1 M-potassium phosphate buffer, pH 7.0. These experiments established that fraction F2 is different from the bulk of the haemoglobin, and showed that fractions F3 and F4 are functionally similar. No further work was done with fractions F2 and F3, and all later experiments were performed with fraction F4. As fractions F3 and F4 run close together, it is possible that some admixture may have occurred, but, in view of their functional similarity, this should not influence the results. The overall effect of the minor fractions was in any case small, since equilibrium binding curves for CO at 20°C and pH 7.0 in 0.1 M-potassium phosphate buffer for the whole haemolysate and fraction F4 agreed within experimental error. This result also establishes that the separation procedure does not alter the properties of the haemoglobin. Careful examination of fraction F4 as the deoxy, oxy and carbonmonoxy forms showed that, within experimental error, porbeagle haemolysate fraction F4 had the same spectral characteristics as human haemoglobin.

Representation of results

Some years ago Edelstein (1971) and Hopfield *et al.* (1971) showed that the two-state model proposed by Monod *et al.* (1965) could explain the broad features of the kinetics and equilibria of a range of mammalian haemoglobins, and, with some modification, it has been used successfully to describe the kinetics and equilibria for haemoglobin from menhaden (Saffran & Gibson, 1978). The two-state model has therefore now been used to describe the results with porbeagle haemoglobin. To use the model, the properties of the R- and T-states must be determined and the value of the allosteric parameter L , which prescribes the relation between the states, specified. In the following sections, evidence is presented that a transition between T- and R-states does indeed occur, and that it is rapid. The basic form of the model assumed that all sites within each conformation are equivalent in their reactions with ligand. This is not true for porbeagle haemoglobin, and data establishing functional and spectrophotometric differences between haem

groups (perhaps because of association with α - and β -chains) are given below. Kinetic and equilibrium data were used to obtain estimates of R- and T-state binding, and, finally, all of the data were used together with the model, to estimate the parameter L .

R-state–T-state transition in porbeagle haemoglobin

If the two-state model is assumed, the fact that co-operativity is observed in ligand binding to porbeagle haemoglobin (Figs. 1, 4 and 6) is itself evidence that either state can predominate under accessible conditions. In several haemoglobins the deoxy-R-state has been associated with a smaller absorption coefficient at 432 nm than that of the deoxy-T-state. In flash photolysis, the ligand may be removed in a time less than a microsecond, so that, if the carbonmonoxyhaemoglobin is in the R-state to begin with, the product of photolysis will be R-state deoxyhaemoglobin, which relaxes rapidly to the stable deoxy-T-state. The relaxation reaction may be followed by means of the increase in absorbance at 432 nm, and more easily at the deoxyhaemoglobin–carbonmonoxyhaemoglobin isosbestic point of 425 nm.

In experiments with porbeagle haemoglobin with a laser flash of 300 ns duration, a small rapid increase in absorbance at 432 and at 425 nm was seen. The amplitude and rate of this change were independent of the CO concentration, and, with a half-time of 10–15 μ s, it was very much faster than any of the ligand recombination reactions. In addition, the rapid absorbance change at 432 nm was in the reverse direction to that associated with ligand recombination, with $\Delta\epsilon = 7 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and an amplitude about one-half that reported for mammalian haemoglobin (Sawicki & Gibson, 1977).

The kinetic experiments intended to define the properties of the R-state, and that are described more fully under that heading, also confirm the existence of two states, and show that the rate of their equilibration is rapid as compared with the binding of CO for partly liganded species as well as for deoxyhaemoglobin. The basic flash experiment was extended by making a series of determinations with the same haemoglobin, but with a wide range of CO concentrations. The rate of the recombination reactions thus varied widely also. If the R-state and T-state species were effectively at equilibrium throughout the reaction, the records of the reaction with various CO concentrations should differ only by a scale factor. This was true within experimental error both at full and at small (<10%) breakdown. For unknown reasons the records at intermediate degrees of breakdown showed rather more variability, approaching the limits of probable experimental error. Unfortunately, the effect varied from

one preparation of haemoglobin to another, and was not even always in the only direction possible if the rate of transition from R-state to T-state is contributing to the observed rate of CO recombination. Whatever the cause of the problem, it does not seem to be related to the allosteric transition.

Comparisons were also made between the CO combination reaction as followed by stopped flow and by full photolysis. The time courses of the reactions were identical. As the flash experiments were performed under conditions where a transition should have occurred after photolysis, whereas the flow experiments began with deoxyhaemoglobin in the T-state, the agreement implies that full conversion of R-state deoxyhaemoglobin into T-state deoxyhaemoglobin had occurred in a time short as compared with that of CO recombination.

The conclusion drawn from these varied experiments is that there was no measurable contribution from the R-state–T-state transition to the observed rates, and that the assumption of R-state–T-state equilibrium is justified.

Properties of R-state-haemoglobin

Combination with CO. At low extents of photolysis (<15%) in the presence of excess of CO one expects the observed reaction to result from the recombination of CO with $\text{Hb}(\text{CO})_3$ (Hb representing haemoglobin) (Gibson, 1956). It cannot immediately be asserted that the $\text{Hb}(\text{CO})_3$ is predominantly in the R-state, because the value of the equilibrium constant between R- and T-states may not be small. However, for porbeagle haemoglobin at pH 8.0 in 0.1 M-potassium phosphate buffer, ligand binding is strongly co-operative. Also, we have observed that at low extents of photolysis the recombination curves are identical in 50 mM-Tris/HCl and 0.1 M-potassium phosphate buffers, though they are very different with high extents of photolysis. Both observations suggest that the recombination curves after low-extent photolysis are characteristic of the R-state. A typical reaction profile for 9% breakdown and 92 μ M-CO is shown in Fig. 1. It is evident, however, that there is a complication at small breakdown, for the reaction is biphasic and shows two widely separated rates. The same two rates, accounting for equal proportions of the reaction at 432 nm, were observed under all conditions where it might reasonably be supposed that R-state haemoglobin was reacting. The rates did not depend on the buffer used, i.e. they were not influenced by the allosteric parameters. The same rates were found for fractions F3 and F4, and for different haemoglobin preparations. They are believed to represent a property of porbeagle haemoglobin and are not an artifact. The proportions of the reaction accounted for by the two rates were roughly equal in regions of the spectrum

Fig. 1. Kinetics of CO combination with porbeagle haemoglobin

The reactions followed full (●) and partial (○) photolysis in 0.1 M-potassium phosphate buffer, pH 8.0, at 20°C (left-hand scale), and full (▲) photolysis in 50 mM-Tris/HCl buffer, pH 8.0, at 20°C (right-hand scale). Reaction mixtures contained 92 μM-CO. For full experimental details see the text.

Fig. 2. Deoxyhaemoglobin-minus-carbonmonoxyhaemoglobin Soret difference spectra for porbeagle haemoglobin

The spectra were obtained after partial (10%) photolysis of mixtures on haemoglobin (4 μM-haem) and 90 μM-CO in 0.1 M-potassium phosphate buffer, pH 8.0, at 20°C. For full experimental details see the text. The spectrum marked ● was obtained from the amplitude of the rapid phase of the recombination reaction and that marked ○ from the amplitude of the slow phase.

remote from the isosbestic points, and two distinct difference spectra were obtained by systematic repetition of the partial photolysis experiment with different wavelengths for observation. These spectra are shown in Fig. 2. It is reasonable to assign them to the haemoglobin chains and to extend the allosteric model to accommodate the difference in function. On this basis, specific rates of combination of R-state haemoglobin with CO in 0.1 M-potassium phosphate buffer, pH 8.0, were measured at temperatures in the range 0–40°C, and the values are recorded in Table 1.

Dissociation of CO. This reaction was measured spectrophotometrically at 420 nm by replacement of CO by NO (Gibson & Roughton, 1957). NO has a much higher affinity than CO for haemoglobin. The replacement of one ligand by another helps to keep the haemoglobin in the R-state, though, as elegantly demonstrated by Salhany *et al.* (1975), the allosteric effects of NO and CO are not the same. The observed reactions in 0.1 M potassium phosphate buffer, pH 8.0, with 3 μM-haem, 4 μM-CO and 120 μM-NO were precisely first-order throughout their course, and one may thus be confident that the rates of CO dissociation are characteristic of the R-state. The rates of CO replacement by NO for the R-state were measured at pH 8.0 at temperatures in the range 0–40°C, and the results are included in Table 1. It is noteworthy that the observed

rates were essentially pH-independent in the range pH 6–8.

Properties of T-state haemoglobin

These are less accessible than those of the R-state, because there is no way to keep the haemoglobin in the T-state after ligand has bound, without adding potent effectors such as inositol hexaphosphate. It seems, also, that for porbeagle haemoglobin the binding of inositol hexaphosphate may modify the properties of the T-state as well as stabilizing it.

Combination with CO. CO binding to the T-state was followed by both stopped-flow and flash-photolysis methods. Assurance that the haemoglobin is in the T-state can be provided by varying the nature and pH of the buffer, as was done for experiments with the R-state. The initial slope of the combination record is a measure of the required rate. In experiments of this kind with 92 μM-CO and 3 μM-haemoglobin just the same rate was obtained in 0.1 M-potassium phosphate buffers, pH 6–8. Fig. 3 shows that in the presence of 0.7 mM-inositol hexaphosphate the same rate is also obtained, although the later part of the progress curve is much altered. Values for the rate constant of the combination reaction in 0.1 M-potassium phosphate buffers over the temperature range 0–40°C are

Table 1. *Specific rates of the combination and dissociation reactions of CO with porbeagle haemoglobin at various temperatures*

Measurements were performed in 0.1 M-potassium phosphate buffers as described in the text. The subscripts T and R applied to the rate constants refer to T-state and R-state haemoglobin respectively. Unprimed rate constants apply to association reactions of haemoglobin and CO, and primed rate constants refer to the dissociation reactions. Values of k'_T were calculated from the dissociation constant for CO from T-state haemoglobin and from the measured values of k_T .

Temp. (°C)	k_T ($\mu\text{M}^{-1}\cdot\text{s}^{-1}$)	k'_T (s^{-1})	$k_{R,\text{slow}}$ ($\mu\text{M}^{-1}\cdot\text{s}^{-1}$)	$k_{R,\text{fast}}$ ($\mu\text{M}^{-1}\cdot\text{s}^{-1}$)	k'_R (s^{-1})
0	0.0096	0.013	0.062	0.55	0.0018
7	0.016	0.022	0.09	0.73	0.0039
14	0.036	0.05	0.125	0.94	0.0077
20	0.057	0.08	0.175	1.2	0.0145
26	0.093	0.13	0.23	1.45	0.026
32	0.17	0.24	0.31	1.75	0.045
38	0.31	0.43	0.41	2.2	0.073

included in Table 1. The Q_{10} is about 2.5. A problem remaining is to determine whether different chains react at different rates in the T-state. If the chains were to differ greatly in this respect then the slower-reacting chain might not contribute to the observed reaction. Instead, CO may bind to one chain only in the T-state with subsequent binding to the other type of chain in the R-state.

Further consideration of Fig. 3 provides additional information. In 0.1 M-potassium phosphate buffer alone the course of CO binding is autocatalytic, with a marked increase in the rate of binding as the reaction proceeds. Under these conditions the switch from T-state to R-state must take place when no less than 2 and no more than 3 molecules of CO have bound/molecule. If the transition occurred after the first molecule had been bound the remaining molecules would all be bound with the characteristic R-state velocity constant. This is so much greater than the T-state rate (between 10- and 60-fold faster, depending on the temperature) that all the vacant sites would be filled almost at once. The overall reaction would proceed with the same numerical rate as the binding of the first molecule of CO, and without showing any acceleration as the reaction proceeds. If, on the other hand, the transition occurred after the third molecule of CO had bound, numerical analysis (Gibson & Roughton, 1957) indicates that only a very modest increase in the overall rate of CO binding will follow. The large increase observed experimentally therefore gives a rough indication of the point at which the switch in conformation occurs. It is clear, too, that under the conditions of the experiment virtually all the haemoglobin must start in the T-state and end equally completely in the R-state.

Addition of 0.7 mM-inositol hexaphosphate does not change the initial rate of CO binding, but the later course of the reaction is altered. There is no

Fig. 3. *Effect of inositol hexaphosphate on the kinetics of CO combination with porbeagle haemoglobin*

The reactions followed full photolysis in 0.1 M-potassium phosphate buffer, pH 6.0, containing $90\mu\text{M}$ -CO at 20°C . Experiments were performed in the absence (O) and in the presence (●) of 0.7 mM-inositol hexaphosphate. For full experimental details see the text.

acceleration at all for the first 80% of the reaction. By the same arguments as above the T-state-R-state transition, if it occurs at all, must do so after no less than 3 molecules of CO have bound/molecule. The initial rate may be regarded as a fair measure of the rate of binding to the T-state, and the agreement

with the results in the absence of inositol hexaphosphate suggests that both are valid measurements. The constancy of the rate of CO binding in the presence of inositol hexaphosphate also answers the question of possible inequality in the rates of reaction of the chains in the T-state. The switch is so long delayed in the presence of the effector that if one chain reacted much more slowly than the other the low rate would be expressed towards the end of the reaction. It seems justifiable at this stage to take the rates of binding to the two chains in the T-state as the same. This is an interesting result, for by contrast the few Root-effect haemoglobins that have been studied in detail have all shown large differences in the rate of CO binding to the chains in the T-state. The report by Morris & Gibson (1980) on the haemoglobin of the Atlantic bluefin tuna cites a difference of no less than 100-fold for this reaction, and Saffran & Gibson (1978) found a 10-fold difference between chains in menhaden haemoglobin. The contrast between the shark and Root-effect haemoglobins is further heightened if the R-states are also considered. In the shark the chains are widely different, but in the Root-effect haemoglobins, e.g. that of menhaden (Saffran & Gibson, 1978), they are not.

Dissociation of CO. This is the least accessible of the four kinetic quantities required to define the properties of the allosteric states. The only direct kinetic experiment takes advantage of the combined effects of pH and of the effector inositol hexaphosphate on the affinity of the haemoglobin. A solution of porbeagle haemoglobin in 10 mM-Tris/HCl buffer, pH 8.0, was titrated with CO until the sample was about fully saturated. At this pH the affinity of the haemoglobin for CO is very high and there is scarcely any CO free in solution. The carbonmonoxyhaemoglobin solution was then mixed at 2°C in the stopped flow apparatus with 0.1 M-potassium phosphate buffer, pH 6.0, containing 0.7 mM-inositol hexaphosphate. The combined effects of the pH-jump and the addition of inositol hexaphosphate switch the haemoglobin to the T-state and greatly diminish its affinity for CO. The T-state carbonmonoxyhaemoglobin dissociates and the reaction can be followed in the usual way. The result is a dissociation reaction at a rate severalfold faster than that of the R-state, but one that is complicated by having two phases of about equal amplitude differing 5-fold in rate. This result could not be due to allosteric effects because, as the CO is lost, the tendency would be towards the T-state, and towards increasing rates of CO dissociation. The phases may therefore be associated with the behaviour of the chains in the T-state. Unfortunately, the experiment just described cannot be used over a wide range of conditions because the affinity of the haemoglobin is too high and in-

sufficiently sensitive to pH to give a substantial degree of CO dissociation except when inositol hexaphosphate is present.

Equilibrium binding of CO

If the deoxy form of a haemoglobin is in the T-state and the fully liganded form in the R-state, a Hill plot of the dissociation curve should have two limbs at either end with a slope of 1, which, if extended, will intercept a line at ordinate zero at points giving the dissociation constants of CO from the R- and T-states. It is well known that this principle is easier to state than to apply, but the haemoglobin from the porbeagle has favourable properties at the lower end of the dissociation curve, as shown in Fig. 4. The toe of the curve with a slope of 1 extends to about 10% saturation in 0.1 M-potassium phosphate buffer, pH 6.0, and to almost 20% when inositol hexaphosphate is included. It is therefore practicable to obtain fair estimates of the T-state dissociation constant of 1.0 μM for data relating to 0.1 M-potassium phosphate buffer, pH 6.0, and of 3.2 μM when 0.7 M-inositol hexaphosphate is included. These values were essentially temperature-independent over the range 0–40°C, and the

Fig. 4. *Equilibrium binding of CO to porbeagle haemoglobin*

The Hill plots were constructed with results obtained in 0.1 M-potassium phosphate buffer, pH 7.0, at 20°C in the presence (●) and in the absence (O) of 0.5 mM-inositol hexaphosphate. The determinations of fractional saturation, $\bar{Y}$, were made by using spectrophotometric methods as described in the text.

implication is that the temperature coefficient of the dissociation velocity constant from the T-state is just the same as that for CO binding, i.e. $Q_{10} = 2.5$. This value is considerably less than has been reported for CO dissociation from (R-state) mammalian haemoglobin (Gibson & Roughton, 1957). The shapes of the upper parts of the dissociation curves in Fig. 4 are quite different and are consistent with an earlier T-state–R-state transition in the absence of inositol hexaphosphate, as is expected. Unfortunately, however, the difference between the T-state affinities in the presence and in the absence of inositol hexaphosphate is not foreseen by the model. In its simple form effectors should act by stabilizing the allosteric states, extending the toe at the lower end of the dissociation curve, but not altering its position. The observed difference in position seen in Fig. 4 has been confirmed in several experiments and is well outside the experimental error. It seems necessary to suppose that inositol hexaphosphate not only stabilizes the T-state, but also alters its ligand-binding properties. In consequence, it seems best not to use CO-dissociation rates from the T-state haemoglobin obtained in the presence of inositol hexaphosphate in testing the fit of the allosteric model to results obtained in the absence of this agent. Instead, we obtain the required estimates by combining T-state 'on' rates with the T-state dissociation constants obtained in the absence of inositol hexaphosphate. It is not possible to deduce from these data if the chains are alike in the rates of dissociation of CO, but it is plausible to suggest that in the biphasic process in the presence of inositol hexaphosphate (see above) the quicker process is an effect produced by that ligand. On this basis the change in affinity due to inositol hexaphosphate could be explained by a 5-fold change in the rate of dissociation from one type of chain, and the chains may be treated as alike in the absence of the effector.

A number of CO-binding experiments similar to the one illustrated by Fig. 4 were performed in 0.1 M-potassium phosphate buffers, pH 6.0, 7.0 and 8.0, at temperatures in the range 0–40°C. A representative curve for pH 7.0 at 1°C is shown as an insert to Fig. 6. The equilibrium curves at pH 6.0 and 7.0 were within the error identical, whereas at pH 8.0 the affinity increased somewhat with a decrease of the half-saturation point by 2.5-fold at 20°C and 1.6-fold at 40°C. In relation to the temperature change the remarkable result was found that the CO-dissociation curve is scarcely affected by temperature over the range used and not at all between 0 and 20°C. The half-saturation point decreased by 1.3-fold at pH 6.0 and 1.9-fold at pH 8.0 on a change from 40°C to 20°C.

In the discussion of the properties of the R-state it was pointed out that the chains show different spectra. These spectra must contribute to the

spectrum of any partially saturated solution of haemoglobin, and as a result its spectrum cannot be exactly represented by any combination of the spectra of deoxy- and carbonmonoxy-haemoglobins unless the chains have equal affinities. The system used for recording the data for the dissociation curves is particularly well suited to look into this matter. Every spectrum was collected over the wavelength range 400–460 nm, and a simple programme was used to determine the best fitting weights to apply to the reference (deoxy and carbonmonoxy) spectra in order to represent the spectrum of any partially saturated solution. These weights were used to generate a calculated spectrum, which was compared with the observed one. It is convenient to call the difference between the calculated and observed spectra the residual spectrum. It was found that significant residual spectra, far outside reasonable experimental errors, appeared. Examples of the forms of these spectra are given in Fig. 5. The general shape of these residual spectra was the same at pH 8.0 in both 0.1 M-potassium phosphate and 50 mM-Tris/HCl buffers, but the maximum amplitude was reached at lower saturation and was greater in Tris/HCl. The double peak in the residual spectrum is consistent with a shift in position of an absorption maximum in a least-squares fitting process.

The data for the residual spectra in Fig. 5 may be rationalized with the equilibrium curve and the kinetic data for CO binding to the R-state, since one may expect that the high-affinity R-state species will be populated first. As the two R-state species are equally represented in the saturated solution and unequally represented in the partially saturated samples, a residual spectrum should appear as the R-state is populated, and disappear as the populations of the two R-state species (chains) approach equal concentrations. The maximum amplitude of the residual spectrum will occur at greater than 50% saturation, as was observed, because, at low saturations, particularly in phosphate buffer, there will be appreciable population of liganded T-state forms.

When the titration was performed in the presence of inositol hexaphosphate a different result was seen. The sign of the residual spectrum was reversed and the amplitude was considerably decreased. This spectrum may be associated with differences in the liganded T-state, but, in the absence of independent evidence of this, further attempts at interpretation are not warranted.

Curve fitting

The detailed fit of the allosteric model utilizing two types of R-state chain and one type of T-state chain was tested by using the procedures described by Saffran & Gibson (1978). Rate equations in terms of the various possible intermediates were

Fig. 5. Residual spectra in the Soret region for mixtures of haemoglobin and CO

CO concentrations were chosen to give approx. 50% saturation of haem groups, and mixtures contained (●) 0.1 M-potassium phosphate buffer, pH 8.0, at 20°C and (○) 0.1 M-potassium phosphate buffer, pH 6.0, plus 0.5 mM-inositol hexaphosphate at 2°C. The residual spectra were calculated from the observed spectra of the mixtures and those of deoxy- and carbonmonoxy-haemoglobins as described in the text.

solved by numerical methods with the use of a second-order Runge-Kutta process (Pennington, 1970), and the fit of the data to the model was optimized by a non-linear least-squares method. For photolysis data an equal quantum yield was assumed for all species, and the initial concentrations of intermediates were calculated from the observed fractional photolysis by assuming a binomial distribution. The experimental findings used in the tests were the equilibrium CO-binding curves obtained in 0.1 M-potassium phosphate buffers, pH 6–8, at 0–40°C described above and a new set of full- and partial-photolysis curves obtained under the same conditions. Representative samples of the data are shown in Fig. 6.

For most curve-fitting operations the 'on' and 'off'

Fig. 6. Fits of kinetic and equilibrium binding data for reactions of CO with porbeagle haemoglobin

The values (●) were observed after full photolysis and (○, ▲ and △) after partial photolysis of a mixture of haemoglobin (3.5 μM-haem) and 90 μM-CO in 0.1 M-potassium phosphate buffer, pH 7.0, at 1°C. The inset (■) shows the CO equilibrium binding curve under the same conditions. For full experimental details see the text. The lines are the fits to the observed results by using the computational methods described in the text and by using the appropriate R-state and T-state 'on' and 'off' rates given in Table 1.

rates of R-state and T-state haemoglobin used in the computations were taken from Table 1, and only the value L , the allosteric constant, was allowed to vary. In this way fits to all of the equilibrium curves were obtained that were certainly within the experimental error. The kinetic data provide a more stringent test, and the kind of fit obtained is shown in Fig. 6. The results shown for pH 7.0 at 1°C are representative of what was obtained between 0 and 20°C at all pH values. There was agreement between observed and calculated absorbance values to within 5% and the sum of the squares of the residuals was about 0.009 (for a family of curves at four extents of photolysis).

At 40°C the agreement was less good, with the sum of the squares of the residuals some 3-fold greater than at the lower temperatures. The particular problem with the kinetic data, especially the partial-photolysis curves, is that the residuals are not distributed randomly, with the computed curve therefore being slightly the wrong shape. Attempts were made to correct this discrepancy by various alterations to the input data. We tried allowing two T-state 'off' rates and hence two T-state affinities for CO. We also tried allowing different absorption coefficients for α -chain and β -chain haem groups. Both of these procedures decreased the sum of the squares of residuals slightly, but did not alter the shapes of computed curves in the right direction. We conclude that these features are not required in the data. In any case, the spectra shown in Figs. 2 and 5 indicate that the two chains have very similar absorption coefficients at 432 nm.

It will be recalled that problems were encountered in partial-flash-photolysis experiments when an attempt was made to establish that at all points of CO-recombination curves complete equilibration between R-state and T-state species was achieved. Working at different CO concentrations and application of scale factors to allow for the different CO concentrations did not give complete agreement, and there was an irreproducible element in the results. It may be that part of the difficulties encountered in the fitting process is due to this cause, whatever it is. Alternatively, the fact that inositol hexaphosphate appears to stabilize a different T-state species from that apparently predominating in the absence of this agent raises the question of multiple T-state conformations.

For trout I haemoglobin (Brunori *et al.*, 1980) the two-state model has been found to represent kinetic data accurately, and for menhaden haemoglobin (Saffran & Gibson, 1978) the model, extended to allow for two types of R-state chain, provides a detailed fit to kinetic and equilibrium data. Our results are not so impressive as those for menhaden haemoglobin, but, considering our difficulties in characterizing T-state material, the range of conditions used, the very limited number of parameters allowed to vary and the fits achieved, we conclude that the model used here provides a reasonably accurate, though not complete, description of events occurring in the ligand-binding reactions.

In terms of our model the curve-fitting procedures described above indicate that at pH 6.0 and 7.0 the allosteric constant, L , increases from 400 at 40°C to 6200 at 20°C to 560 000 at 0°C, and at pH 8.0 from 100 at 40°C to 200 at 20°C to 4000 at 0°C. At all pH values the van't Hoff plots are non-linear and concave upwards. The temperature-insensitivity of the equilibrium binding curves in the region 0–20°C arise from compensatory

changes in L and the two R-state dissociation constants. At higher temperatures outside the physiological temperature range some variation in equilibrium ligand-binding curves with temperature occurs because L does not decrease sufficiently to compensate for the decrease in affinity of R-state chains at higher temperatures.

A particularly clear effect of temperature on CO binding by porbeagle haemoglobin is seen in Fig. 7, where recombinations after full photolysis at 20°C and 40°C at pH 8.0 are shown. The results were obtained with the same sample, and the changes seen on warming to 40°C were fully reversible. Although the usual type of accelerating progress curve is seen at 20°C, the curve at 40°C is biphasic and reminiscent of the curves seen with R-state haemoglobin in partial-photolysis experiments (Fig. 1). This is the kind of transition expected from our calculation of L , because at the higher temperature the conformational equilibrium would be much more in favour of the R-state and the conformational switch will occur much earlier in the reaction. For trout I (Brunori *et al.*, 1980) and menhaden (Saffran & Gibson, 1979) haemoglobins, L is also strongly temperature-dependent and analogous changes in

Fig. 7. Effect of temperature on the kinetics of CO binding to haemoglobin after full photolysis at pH 8.0. Experiments were conducted with the same mixture, containing 0.1 M-potassium phosphate buffer, pH 8.0, and 90 μ M-CO, which was flashed first at 20°C (●), then at 40°C (○) and then at 20°C again.

the form of recombination curves after full photolysis occur.

General discussion

Andersen *et al.* (1973) observed that the O₂-binding curve for the Atlantic-porbeagle haemoglobin is insensitive to temperature change. By using laser photolysis of haemoglobin/O₂ mixtures at <60% initial saturation in the temperature range 0–35°C and taking the initial excursion as a measure of the bound O₂ concentration, we have made the same observation for Pacific-porbeagle haemoglobin. It seems that the temperature-insensitivity of ligand binding (O₂ and CO) is a notable feature of porbeagle haemoglobin and one that may be of physiological importance.

The porbeagle shark is able to maintain its muscle mass at 10°C or more above ambient. The advantage to the fish in this is not surely known, but it is reasonable to suppose that the higher temperature allows it to swim faster than would otherwise be possible and so scan a larger volume of water for prey to offset the metabolic cost of the higher activity.

Although there is little metabolic cost in retaining body heat by means of a counter-current exchanger, the movement of heat from the venous to the arterial blood is inevitably associated with a corresponding transfer of O₂ from the arterial to the venous blood, and this is a physiological debit. The absolute loss of O₂ is determined by the partial pressure of O₂ in the arterial and venous bloods, i.e. by the O₂ in physical solution. The percentage loss is smaller the higher the haemoglobin concentration, since a fixed loss is spread over the larger amount of O₂ transported. It is not therefore surprising that the tuna and the lamnid sharks have very high haemoglobin concentrations. These are quite similar to those of mammals, and are 2-fold or more greater than the haemoglobin values of cold-bodied fish. The limit is presumably reached at 45% or so on the haematocrit. Above this value the viscosity of the blood increases rapidly and the circulation cannot be effectively maintained.

The loss of O₂ in the heat-exchanger can also be diminished by decreasing the driving pressures that cause O₂ transfer. This can be done by reversing the temperature effect on the O₂ affinity. The partial pressure of O₂ in the cold arterial blood is likely to be near that of the water and, with a normal (i.e. mammalian type) dependence of affinity on temperature as the blood passes through the exchanger and warms, this pressure will rise, increasing the rate of O₂ loss. If the effect of increasing temperature were to increase the affinity of the blood for O₂, the partial pressure of O₂ in the exchanger would fall as the arterial blood warmed and less O₂ would be lost to the venous blood. The size of the effect can only

be estimated, because there are so many physiological unknowns. It seems, however, that an exchanger with 99% efficiency that will support a rise of 15°C in a large fish may lose 5–15% of the arterial O₂ in doing so. The loss may be decreased by about half if the temperature coefficient is lowered to zero, and even further if it is reversed, as in the tuna. The O₂ loss in the exchanger is proportional to the temperature difference being maintained, and it may be for this reason that no abnormality has been detected in the blood of the mako shark, where the body temperature is not more than 3–5°C above ambient.

We thank Dr. Francis G. Carey of the Woods Hole Oceanographic Institution and Dr. J. Urquhart of the University of Alaska, who supplied the Pacific-porbeagle blood. F. M. D. is grateful to the Wellcome Trust for the award of a Travel Grant. We are grateful for support by U.S. Public Health Service Grant GM 14276 and National Science Foundation Grant 79-10446.

References

- Andersen, M. E., Olson, J. S., Gibson, Q. H. & Carey, F. G. (1973) *J. Biol. Chem.* **248**, 331–341
- 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
- Edelstein, S. J. (1971) *Nature (London)* **230**, 224–227
- Gibson, Q. H. (1956) *J. Physiol. (London)* **134**, 123–124
- Gibson, Q. H. & Roughton, F. J. W. (1957) *Proc. R. Soc. London Ser. B* **146**, 206–224
- Hopfield, J. J., Shulman, R. G. & Ogawa, S. (1971) *J. Mol. Biol.* **61**, 425–443
- Knowles, F. C. & Gibson, Q. H. (1976) *Anal. Biochem.* **76**, 458–486
- Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118
- Morris, R. J. & Gibson, Q. H. (1980) *J. Biol. Chem.* **255**, 8050–8053
- Noble, R. W., Parkhurst, L. J. & Gibson, Q. H. (1970) *J. Biol. Chem.* **245**, 6628–6633
- Parkhurst, L. J. & Gibson, Q. H. (1967) *J. Biol. Chem.* **242**, 5762–5770
- Pennelly, R. R., Tan-Wilson, A. L. & Noble, R. W. (1975) *J. Biol. Chem.* **250**, 7239–7244
- Pennington, R. H. (1970) *Introductory Computer Methods and Numerical Analysis*, 2nd edn., p. 475, Macmillan, London
- 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
- Salhany, J. M., Ogawa, S. & Shulman, R. G. (1975) *Biochemistry* **14**, 2180–2190
- Sawicki, C. A. & Gibson, Q. H. (1977) *J. Biol. Chem.* **252**, 5783–5788
- Tan, A. L., De Young, A. & Noble, R. W. (1972) *J. Biol. Chem.* **247**, 2493–2498