Properties of the T State of Human Oxyhemoglobin Studied by Laser Photolysis

(Received for publication, May 31, 1977)

CHARLES A. SAWICKI AND QUENTIN H. GIBSON

From the Department of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

Using a dye laser with 1 J output at 540 nm, points on the oxygen equilibrium curve in the range 0 to 0.2 saturation were determined by observing the absorbance excursion at 436 nm following photolysis. The results compare reasonably with those in the literature (Roughton, F. J. W., and Lyster, R. L. J. (1965) Histochemie 18, 155-198). The saturation is obtained directly by this method rather than by subtraction, and contamination with CO is immediately observable as a slow relaxation.

Following the laser flash the combination of oxygen with deoxyhemoglobin may be observed, and at low per cent saturations with oxygen the greater part of this reaction must be with hemoglobin molecules which were free from oxygen immediately before the flash. The reaction has two phases of roughly equal amplitude whose rates are almost independent of initial saturation in the range 0 to 0.2, but which increase with hemoglobin concentration in the range 100 to 500 μM (heme). Analysis of the two components gives combination rates of 11.8 and 2.9 μM/s and dissociation rates of 2300 and 180/s respectively. It is suggested that these components are the hemoglobin chains. The value of L, the allosteric constant, at 20° in pH 7, 0.05 M KPi buffer with 18 U.S.C. Section 1734 solely to indicate this fact.

The two-state model of Monod et al. (1) may reasonably be regarded as dominant in the field of hemoglobin kinetics and equilibria at the present time (2-4), although objections to its use have been raised by several authors who have claimed that in its simplest form it does not represent either the kinetics or equilibria accurately (5-7).

It has been difficult to compare the model with experiment, especially for oxygen where the rates of the reactions involved are really outside the proper time scale of the stopped flow apparatus, although data for oxygen combination have appeared (8). In terms of the model, the difficulty concerns the properties of the (low affinity) T state; the reported properties of the high affinity R state are more readily accessible experimentally and have shown little change over the years (9) since values for sheep hemoglobin were suggested by Gibson and Roughton more than 20 years ago.

In practice, it has been necessary to derive kinetic information about the T state from computer modeling of stopped flow data, and from studies of mutants (10, 11). These have agreed in suggesting that the rate of dissociation of oxygen from the T state is many times larger than from the R state, and this has been confirmed experimentally by the so-called oxygen pulse experiments of Gibson (12). A rapid relaxation has also been observed in the careful T jump studies of Ilgenfritz and Schuster (13), although their results were not interpreted in terms of the two-state model.

In this paper, we describe a new experimental approach which provides both kinetic and equilibrium data for the same solutions of hemoglobin. It is especially suited to the study of the properties of the T state, and so provides a more incisive approach to the application of the two-state model than has so far been possible.

EXPERIMENTAL PROCEDURES

Methods - The principle employed is to prepare a partially saturated solution of hemoglobin in equilibrium with a known concentration of free oxygen. The bound oxygen is removed quantitatively by a laser pulse, so the amplitude of the absorbance excursion gives a measure of the saturation of the hemoglobin, and the recombination reaction after the flash reflects primarily the kinetics of the reaction of deoxyhemoglobin.

Hemoglobin was prepared from pooled hospital blood samples as previously described (14). For these experiments it is essential to remove CO as completely as possible. This should always be done when an equilibrium curve is to be determined. The average content of COHb was about 2%, and this was reduced to 0.1 to 0.2% by exposure of a thin film of a 5 mM (heme) solution in a rotary evaporator at 0° to pure oxygen. The solution was illuminated for 30 min by a 300-watt floodlight placed 5 cm from the flask. Deoxyhemoglobin was prepared by rotation of a small volume (10 to 20 ml) of a 5 mM solution in a 500-ml tonometer equipped with a serum stopper as well as the usual stopcocks. The tonometer was repeatedly flushed with purified N2 delivered through a short length of butyl rubber tubing for 15 to 20 min.

Working solutions were prepared in two ways: (a) 0.05 M (pH 7.0) phosphate buffer was placed in a large syringe (30 ml) and deoxygenated by bubbling with purified N2 for 10 min. The needle used for bubbling was then removed and the syringe closed with a small serum stopper which had its dead space filled by a stream of buffer as it was placed over the hub of the syringe, care being taken to

7538
avoid leaving a residual air bubble in the recess of the stopper. The
required amount of hemoglobin stock solution was then injected
into the large syringe using a suitable gas-tight microsyringe. A
portion of the buffer solution was also equilibrated with oxygen by
bubbling at a known temperature and pressure. The required
amount of this solution, whose oxygen content was calculated from
the standard table (15), was then injected into the hemoglobin
syringe using a microsyringe.

A cuvette of 1- or 2-mm path was prepared by blowing nitrogen
through needles passing through a serum stopper for 30 min. The
inflow needle was then removed and the cuvette rinsed with a large
excess of sample solution from the 30-ml syringe, removing bubbles
as completely as possible. When this procedure was carried out
without adding any oxygen, a 50 μM solution of hemoglobin con-
tained 0.1 to 0.2% of oxyhemoglobin. The cuvettes were stored in ice
until the experiment was to be performed (less than 40 min).

Immediately before photolysis the cells were immersed in a water
bath at 20°. (b) The second method for preparing working solutions
was to fill a tonometer of 105-ml capacity with N₂ by evacuation and
refilling. The vessel had a 2-mm path length cell fused to it, and
also a Teflon stopcock at the other end. The stopcock was then
closed, and a serum stopper placed over the end of the stem of the
stopcock and the air trapped in the stem displaced with N₂ blown
through needles. A 5-ml portion of deoxygenated buffer was intro-
duced into the tonometer by fitting a long (6 inch) needle to the
stock syringe. This passed through the serum stopper and the
stopcock into the body of the tonometer.

The required quantity of stock deoxyhemoglobin solution was
introduced similarly, and successive portions of air injected to
generate data for several kinetic and equilibrium points. After each
injection the hemoglobin was equilibrated by rotation for 5 min in a
water bath at 20° and the concentration of free oxygen was calculated
from the known partial pressure of oxygen in the tonometer.

Flash photolysis was performed using a Phase-R Co., New Dur-
ham, N.H., dye laser model 2100 B, as already described (16). Typi-
cally, photolysis was 97 to 99% complete with the energies
used. For data collection a Biomation Corp., Cupertino, Calif.,
wave form recorder model 805 was used and data were transferred
to a PDP 8/E minicomputer for averaging and conversion to absorb-
ance. At least three sets of kinetic data were collected and averaged
for each experimental run.

RESULTS

Equilibrium Data—The results of several experiments on
different samples of blood are shown in Figs. 1 and 2 covering the
range from 0 to 15 and 10 to 60% saturation. Data from the
two methods using cuvettes filled with solution, and the
tonometer agree very satisfactorily. The highest points by the
cuvette method are somewhat less reliable because the propor-
tion of free oxygen decreases with increase saturation. In
our experience with some 15 blood samples, the results have
been very consistent from sample to sample. Fig. 1 includes
data showing the greatest deviations.

As a check on the results by the photolysis methods, the
spectra of the solutions were determined in the digital spectrophotom-
er of Knowles and Gibson (17) transferring the
cuvette to one apparatus to the other. The spectrophotom-
er was used to collect data at intervals of 1 nm over the
range 400 to 460 nm and the per cent composition was
Calculated by the method of least squares,

FIG. 1. Equilibrium data points between 0 and 16% saturation
for hemoglobin in 0.05 M phosphate buffer at pH 7. The temperature
was 20° unless otherwise noted. The solid curve was generated as
described in the text using an MWC model which neglected chain
differences. The alactic parameter e₁ was taken to be 1.4 x 10⁷
while c, the ratio of the microscopic dissociation constants for the R
and T states, was 0.9025. The microscopic association constants for
R state tetramers and dimers were taken to be 3.7 μM⁻¹ and 2 μM⁻¹,
respectively. K, was taken to be 1 μM and the hemoglobin concen-
tration was 50 μM. The open circles were generated from the solid
curve by taking a new origin at the point on the curve corresponding
to 2% saturation. The dashed curve was calculated from the Adair
constants used by Imai and Yonetani (6) to fit data collected for 60
μM hemoglobin at 20° for 0.1 M phosphate buffer, pH 7. The experimen-
tal conditions were: A, 41 μM hemoglobin equilibrated in

tonometer and fractional saturation determined by photolysis as
described in the text; Δ, partially saturated 47 μM hemoglobin
solutions made up in sealed cells as discussed in the text. Saturation
was determined by photolysis; ( ), fractional saturation of the same
sealed cells determined using the computer interfaced digital spec-
photometer as described in the text; •, data of Roughton and
Lyster (18) measured at pH 7 and 19° for hemoglobin concentrations
between 0.7 and 1.4 μM. The dotted curve represents a fit to the
solid triangles of an MWC model including chain differences which
will be discussed in this paper.

would be highly desirable, but the literature affords few
suitable sets of experiments. The gasometric data of Roughton
and Lyster (18) were obtained at the same pH but with much
stronger hemoglobin solutions. Their points lie close to, but
slightly to the right of ours, especially at low per cent
saturation. Their buffer was 12 times stronger than ours, and
their buffer was 12 times stronger than ours, and
the temperature 1° lower. In view of all these factors, the
agreement is surprisingly good, perhaps because of a cancel-
nation of effects due to dimerization and the temperature
difference.

Data exactly comparable to ours by an automatic recording
spectrophotometric method have been published by Imai and
Yonetani (6) for similar buffers, temperature, and hemoglobin
concentrations. Their data were fitted to the six minimal
points, but the Adair constants given in their table generate
the dashed line in Figs. 1 and 2. This lies distinctly to the left
of our data, but Imai and Yonetani did not remove CO from
their blood and also reported appreciable MetHb formation
during their determinations (up to 4%). Their curve and ours
can be quite well reconciled by assuming about 2% of COHb
in their sample, as shown by the open circles superimposed
on the dashed line. The open circles were generated from the
solid line through our data by taking a new origin at the
point on the line corresponding to 2% saturation. With this
reasonable, but rough, correction the agreement is very satis-
factory. Our solution contained 0.4% MetHb and 0.15% COHb.
in the deoxy stock; these would have rather small effects on the position of the curve. The problem of MetHb formation during an experiment is more difficult to deal with but it appears to have been quite small since the residuals in the spectrophotometric procedure did not rise markedly nor was there evidence of an increase in absorbance at 405 nm as a tonometer experiment progressed.

There is a special problem in using flash photolysis for determining points on the equilibrium curve of oxyhemoglobin. This is the possibly different contribution of R and T species to the observed excusion following photolysis. The contributions of the deoxy forms are well known (19-21) and that difficulty may be avoided by working at 436 nm. At other wavelengths dimers and the R-T deoxy change must be expected to contribute, the R-T change becoming progressively more important as the per cent saturation is increased. For the present purpose this is mainly of importance because the excursion corresponding to 100% saturation has been determined experimentally by photolysis rather than from static optical measurements. The effect is to decrease apparent saturations measured at 442 nm, for example, and to increase them at 430 nm.

A second, and less considered, problem will arise if the extinction changes associated with binding of oxygen to give T-liganded and R-liganded forms are different. In an attempt to investigate the point we have recorded the spectrum of oxy-deoxy hemoglobin mixtures over the range 400 to 460 nm and have scaled the difference spectra (mixture-deoxy) by multiplying by the reciprocal of the fractional saturation as defined by the amplitude of the difference spectra. The results presented in Fig. 3 show little difference between the spectra at 436 nm, although somewhat larger differences are observed near the oxyhemoglobin maximum. At 436 nm the effect is less than 5% of the observed saturation (i.e., less than 0.005 at 0.1 saturation) at the most. Had the spectra of Fig. 3 shown substantial differences, unambiguous determinations of fractional saturation by any spectrophotometric method would be impossible; as it is, the uncertainties are not significant for our purpose, as R-liganded and T-liganded hemoglobins do not differ in their absorption at 437 nm.

It does seem, however, that there are significant changes in the spectrum near the oxyhemoglobin maximum with an isobestic at about 410 nm and considerable variation in the difference spectrum at about 419 nm. An inset to Fig. 3 shows the amplitude of this difference as a function of fractional saturation. Although the precision with which the differences can be measured is low, there is a clear monotonic increase at low fractional saturation.

The photolysis method is particularly suited for equilibrium points at low saturations since the deoxy standard is internal and its contamination with CO or extraneous oxygen cannot pass unnoticed. Further, the quantity to be measured is observed directly and not by comparison of different samples.

**Kinetic Experiments** — Families of curves obtained by following the return to equilibrium after photolysis are presented in Figs. 4 and 5. It is evident that the recombination reaction is markedly biphasic under all conditions examined. With both 40 \( \mu M \) and 500 \( \mu M \) hemoglobin the rate constant for the rapid component is virtually independent of initial saturation up to 0.2 or so, as shown in the semilogarithmic plots of Fig. 4. These results show that the two components of the recombination reaction are only slightly dependent upon the saturation at equilibrium in the range 1% to 20% saturation. In particular, the proportion of the rapid phase remains at about a half even when the reaction proceeds only as far as 1% saturation (although of course, as the excursion available for measurement was only 0.005 in absorbance, the noise contribution is appreciable). With differing hemoglobin concentrations, the rate increases markedly at the highest concentrations which can be used without producing an excessive rise in temperature (Fig. 5).

At very low initial (preflash) saturations, the recombination reaction must be attributed to T state hemoglobin because, whatever the nature of the photoproducts, they are present in amounts too small to influence the observed reaction. For example, with 50 \( \mu M \) hemoglobin, 1% saturation corresponds to binding of oxygen to 0.5 \( \mu M \) hemoglobin, and total oxygen will be about 1.8 \( \mu M \); then even if all of the photoproduc had R-like behavior and a rate constant of 50 \( \times 10^6 \) the apparent rate of

![Fig. 2. Equilibrium data points for hemoglobin in pH 7 phosphate buffer between 10 and 60% saturation. Experimental conditions and the calculated curves are as described in Fig. 1.](image)

![Fig. 3. Absorbance differences between oxygenated hemoglobin and deoxyhemoglobin in pH 7 phosphate buffer at 20°C in the spectral region from 400 to 450 nm. The hemoglobin concentration was 54 \( \mu M \). All spectra were run in the same 1-mm path length cell using a computer interfaced digital spectrophotometer as described in the text. Partially saturated solutions were produced by mixing oxygen and deoxyhemoglobin solutions of the same concentration. Further details of the experiment are given in the text. Absorbance data were: \( \Delta A \), absorbance difference between fully oxygenated hemoglobin and deoxyhemoglobin; \( \Delta A \), absorbance difference between hemoglobin with 4.7% bound oxygen and deoxyhemoglobin, scaled by multiplying the difference by the inverse of the fractional saturation or 21.3. The inset shows the dependence on fractional saturation of the absorbance difference at 410 nm between the oxy-deoxy difference spectrum and the absorbance difference between partially saturated hemoglobin and deoxyhemoglobin, multiplied by the inverse of the fractional saturation.](image)
recombination of oxygen would be less than 100/s. On the other hand, there will be almost 50 \( \mu M \) deoxyhemoglobin which was not combined with oxygen at all before the flash, and it is reasonable to attribute the rapid observed reaction with a half-time of less than 1 ms to this majority species. The argument may be completed by considering, in addition, the data presented for 10% initial saturation. The concentration of photoproduct must be 10 times greater than for 1% saturation, yet the rate of the recombination reaction after the flash is scarcely changed: the principal, and the invariant component, remains deoxyhemoglobin, and the rapid reaction observed must be attributed to it. The effect of increasing hemoglobin concentration above 50 \( \mu M \) (Fig. 5) is in agreement with this interpretation; only increased concentrations show much effect on the rapid component, an indication that the dissociation velocity is large.

Analogous arguments may be applied to the slow component of the overall reaction and suggest that deoxyhemoglobin should be assumed to contain two components with widely different kinetic properties. A more detailed discussion is needed to establish the point, however, since alternative possibilities exist which could give rise to a slow reaction component without requiring that deoxyhemoglobin itself contains two distinct species. The first is dimerization. It is expected that dimers will react quickly, and show a high affinity for oxygen (Appendix 1). Because their concentration will be low, the observed rate will be moderate and usually well below the rate for the fast component of Fig. 4. The second is redistribution of ligand to give an increase in the concentration of high affinity (R-ligated) forms. Such a redistribution will take place slowly even though each of the component reactions is rapid. This may be seen by considering two steps in oxygen binding.

\[
\text{Hb(O}_2\text{)}_n \xrightarrow{k'_1} \text{Hb(O}_2\text{)}_{n-1} \xrightarrow{k'_0} \text{Hb(O}_2\text{)}_{n+1}
\]

The solid arrows represent rapid reactions, the dashed arrows slower ones. The first two species are assumed to represent T states, the third an R state. If the rate of dissociation of the R state molecule is neglected, the rate of formation of \( \text{Hb(O}_2\text{)}_{n+2} \)

\[
k'_1k'_0[O_2]/(k_1 + k'_1[O_2])
\]

In words, the 2nd oxygen molecule must be bound, on the average, not once, but \((k'_1[O_2] + k_1)/k'_1[O_2]\) times, i.e. the probability that \( \text{Hb(O}_2\text{)}_{n+1} \) will revert to \( \text{Hb(O}_2\text{)}_n \) is considerably greater than the probability that it will acquire a 2nd ligand molecule and form the relatively stable \( \text{Hb(O}_2\text{)}_{n+2} \). The overall formation of \( \text{Hb(O}_2\text{)}_{n+2} \) will be correspondingly slower.

To obtain a better picture of these matters, it was assumed that the two-state model in its simplest form (2) could be used to calculate the concentrations of species at equilibrium. This assumption simplifies the computations, and the parameters required can be obtained from the legend for Fig. 1 and Appendix 1. It was measured, in addition, that the tetramer-dimer dissociation constant for fully liganded hemoglobin is of the order \( 1 \times 10^{-9} \) (cf. Mills et al. (7)). As Figs. 1 and 2 show, the equilibrium data are quite well represented with \( L = 1.0 \times 10^9, c = 0.0025 \), and the microscopic association constant for the R state \( K_R = 3.7/\mu M \). The proportion of ligand bound to T state species is shown as a function of \( pO_2 \),

![Fig. 4 (left). Semilog plot of the time-dependent absorbance changes following photolysis of partially oxygenated hemoglobin samples in pH 7 phosphate buffer observed at 436 nm for two hemoglobin concentrations. The monochromator band-pass was 3 nm. The solid lines are drawn through the experimental points. The time scale at the top of the figure refers to the 40.6 \( \mu M \) hemoglobin data (solid symbols). These data were scaled to give an initial absorbance excursion of 0.5. Fractional saturations were: A, 0.01; B, 0.10; and C, 0.19. They were collected using a 5-mm cell attached to the tonometer as described in the text. Kinetic data for 515 \( \mu M \) hemoglobin (open symbols) refer to time scale at the bottom of the figure. The data were scaled to give an initial absorbance excursion of 0.5. Fractional saturations were: [O], 0.06; L, 0.114; and C, 0.22.](http://www.jbc.org/)
in Fig. 6. If the slow component in the experiments of Fig. 4 is to be attributed to the sum of dimers binding oxygen and rearrangement of T state intermediates to R state intermediates, it follows that the dashed line labeled T in Fig. 6 represents the least proportion of the reaction which can occur at rates determined by the properties of the T state. In the limit, at very small saturation, it is obvious that the concentration of dimers and R state species must approach zero (Fig. 6) and hence the observed reaction should represent the properties of the T state only. As shown in Fig. 4, there is little or no change in the observed reaction which continues to be about half-rapid and half-slow even at 1% saturation when calculation using the MWC1 model (Fig. 6) suggests a minimum of 90% T state and 10% R state binding.

The inference is that there are two components in the reaction of deoxyhemoglobin with oxygen as followed by the laser method. Although these two components must be associated with the bulk of the deoxyhemoglobin rather than with any product of photolysis of oxyhemoglobin, the possibility remains that the deoxyhemoglobin components may be associated with a long lived photochemical effect of the intense radiation upon deoxyhemoglobin. Such a possibility appears exceedingly unlikely because (a) there is no effect of the laser flash upon the spectrum of deoxyhemoglobin in the time scale of our experiments. A major functional change such as that observed might reasonably be expected to be associated with some spectral change. (b) No analogous effect can be observed with COHb when a partially saturated solution is photolyzed with the laser. The greater photosensitivity of COHb permits the experiment to be performed with pulse energies less than 5% of those required with oxyhemoglobin but the time course of combination was independent of the amount of light used for photolysis. (c) Experiments with oxygen and myoglobin show no anomaly whatever. As illustrated in Fig. 12, the time course of oxygen recombination is accurately exponential over at least 80% of the reaction at a saturation of 0.04, and the rate of recombination is proportional to the myoglobin concentration. The same second order rate constant is obtained under these conditions as in an experiment with a substantial excess of oxygen (248 \mu M). This experiment also appears to eliminate the possibility of significant instrumental artifacts. Our experiments, which are all kinetic, cannot establish the nature of the two components responsible for the biphasic kinetics of Figs. 4 and 5, but it is at least plausible that these may be the \(\alpha\) and \(\beta\) chains.

Analysis and Simulation of Kinetic Data—The qualitative conclusions of the preceding section have been quantified and extended by comparing the data with the results of numerical integration of appropriate rate equations. As a start, the simplest MWC model was used to represent a set of kinetic data with various oxygen concentrations, setting the parameters to the values suggested by Hopfield et al. (2) for pH 7 and 20\% and adding terms for dimers. As the qualitative discussion would lead one to expect, the results were not altogether satisfactory; they are presented in Fig. 7 where the arrows join corresponding data points and calculated lines. Minimum seeking procedures, although giving a better fit than that shown in Fig. 7, still give systematic mismeasure, and multiple solutions were obtained which represented a good fit to some one portion of the data with corresponding local minima in the (residuals)\(^2\) – parameter surface. An example is presented in Fig. 8.

Three modifications to the MWC model were examined in some detail. In one, the extinction coefficient for binding of oxygen to give a T-liganded species was allowed to vary freely. A better fit was obtained by this means, using a coefficient 0.8 times that for binding to give an R-liganded species, but the distribution of residuals was not satisfactory, and, as already discussed in connection with the equilibrium curve, no large difference in the extinction coefficients for formation of R- and T-liganded species is observed at 437 nm. Kinetic experiments to check this point were performed at several wavelengths in the Soret region. Results at 442 and 430 nm differed most widely with 437 nm and 414 nm yielding virtually identical results. The difference between 442 and 430 nm decreased considerably as the initial saturation before the flash was decreased below 20%, but because of the large difference in the absorbance excursion at these wavelengths the experiment is difficult to perform satisfactorily with very low saturations on the same sample. It did appear, however, that the 430-442 differences could be accounted for quantitatively by the contribution from the R-T deoxy difference spectrum, which has its maximal amplitudes of opposite sign at these wavelengths. The result is that binding to dimers (R form) is given a lower weight at 430 nm as compared to that at 442 nm, altering the shape of the overall curve. Presumably similar arguments could be applied to binding to R-deoxy tetramer, but these disappear very quickly after the flash to give T-deoxy hemoglobin, see Ref. 21.

The second modification of the basic model was suggested by the interesting observation of Ogawa and Shulman (22) that valence hybrid hemoglobins showed two forms (in nmr experiments) which were not in rapid equilibrium, and it was subsequently found that there were two functionally different forms, again not in rapid equilibrium (23, 24). Rather similar observations have been made on the high affinity mutant hemoglobins Chesapeake and Kempsey (25, 26) which show biphasic CU-binding kinetics at neutral pH in buffers of low
Laser Photolysis Study of T State of Human Oxyhemoglobin

FIG. 7 (left). Kinetic data points collected at 436 nm for 46 μM hemoglobin partially saturated with oxygen. The fractional saturation values at equilibrium and the free oxygen concentrations were: ●, 0.153 oxy with 8.8 μM; ■, 0.072 oxy with 5.4 μM; ▲, 0.051 oxy with 4.3 μM. Other experimental conditions were as in Fig. 3. The solid lines are the result of a simulation using the simple MWC model described in the text which neglects differences between the α and β chains. Parameters were set to the values suggested by Hopfield et al. (2). L = 1.45 × 10^9, k' = 32 μM^-1 s^-1, k_r = 12.5 s^-1, k_f = 500 s^-1, k'_f = 4.2 μM^-1 s^-1. Dimer contributions were included and K_c,D was set to 1 μM. The arrows connect simulated curves with the corresponding data points.

In a two-state model these functionally different forms can only be the R and T states, and by extension from the mutants and hybrids to hemoglobin A, the R and T states might not be at equilibrium throughout the ligand-binding reaction. In an attempt to see if such a change might improve the fit of the two-state model to the data, the rate of conversion of T to R was arbitrarily limited for the species Hb_(O2) and Hb_(O2). As with the alteration in the extinction coefficients, there was some improvement, chiefly because the kinetics and the equilibrium curve can be fitted largely independently of one another, but the distribution of the amplitudes of the fast and slow components continued to be systematically misrepresented, the model giving too large a fast phase for low oxygen saturation before the flash.

The third modification was to divide the T and R states between two distinct components, formally analogous to the α and β chains, i.e., present in equal amounts. The use of the two-state model was continued, but now with four different affinities and nine tetrameric species. The reactions assumed to occur are set out in Equations 3 to 11. R and T represent tetrameric R and T state hemoglobin. Dimers are represented by D. The distribution of bound oxygen is given in parentheses following each species. The subscripts on αs and βs denote the number of oxygen molecules bound to these chains in the hemoglobin tetramer. The subscripts on k_r and k_f give the molecular species R, T, or D, and the chain (α or β) which takes part in the reaction.

In order to run the program, 11 different parameters must be specified. Of these, the four values for the R state were taken from the literature and from Appendix 1 (27) and, as before, the dissociation velocities for the dimeric species were set equal to those for the corresponding reactions of the R tetramer. The tetramer-dimer dissociation constant is not affected by the change in model and was set at 1 × 10^-6 M. The remaining five parameters were allowed to vary freely under control of the minimization program. Excellent representations of the kinetic data were now obtained, as illustrated in Figs. 9 and 10 fitting simultaneously the families of kinetic curves and the lower half of the oxygen equilibrium curve. As expected from the qualitative considerations already outlined, the kinetic properties of the two components are quite widely different, but with smaller differences in affinity than in rates. The data shown in the figures do not embrace a complete experiment: in the experiment of Fig. 10 several different initial saturations were examined in addition to that shown for 515 μM hemoglobin. The additional data were fitted as satisfactorily as those shown. The fixed and best fit parameters are presented in Table I.

It is clear that the model is sufficient: the question of how far the data serve to define the parameters is less easy. When parameters are fixed, as has been done in this case, the standard errors of those varied are calculated from the error matrix (see Ref. 26) as if the fixed parameters were precisely known, which is, of course, not the case. An attempt has been made to deal with the problem by assigning standard errors...
to the six fixed parameters from the literature or from experience. The minimization program was then run repeatedly using randomly chosen values for the six parameters and optimizing the remaining five. With sufficient (about 80) runs, an estimate of the variance in the five optimized parameters due to uncertainty in the six fixed ones could be obtained. This has been added to the conventional variance to give the estimates included in Table I. The greatest uncertainty is in the value of \( L \); the four kinetic parameters are relatively well defined when several hemoglobin concentrations are examined in a single experiment. If an experiment is restricted to low hemoglobin concentrations of the order of 50 \( \mu \)M the slow pair of rates are poorly defined, although their ratio remains relatively constant. Numerical experimentation suggests that this is due in part to the relatively much greater contribution of dimers in the runs with low hemoglobin concentration (Fig. 9). At the oxygen concentrations and concentrations of dimers present, the observed rates of oxygen binding to the slow component of the tetramer and to dimers are similar. A second difficulty results from the near-invariance in shape of kinetic curves obtained with varying oxygen concentrations.

\begin{align}
R(a_n, B_m) + O_2 & \xrightarrow{k_{RB}} R(a_{n+1}, B_m) \quad [3] \\
R(a_n, B_m) + O_2 & \xrightarrow{k_{Ra}} R(a_{n+1}, B_m) \quad [4] \\
T(a_n, B_m) + O_2 & \xrightarrow{k_{TB}} T(a_{n+1}, B_m) \quad [5] \\
T(a_n, B_m) + O_2 & \xrightarrow{k_{Ta}} T(a_{n+1}, B_m) \quad [6] \\
R(a_n, B_m) & \xrightarrow{k_{Rn}} R(a_{n+1}, B_m) \quad [7] \\
R(a_n, B_m) & \xrightarrow{k_{Rn}} R(a_{n+1}, B_m) \quad [8] \\
D(a_n, B_m) + O_2 & \xrightarrow{k_{DB}} D(a_n, B_m) \quad [9] \\
D(a_n, B_m) + O_2 & \xrightarrow{k_{DB}} D(a_n, B_m) \quad [10] \\
D(a_n, B_m) + O_2 & \xrightarrow{k_{DB}} D(a_n, B_m) \quad [11]
\end{align}

Where 0 \leq n \leq 2, 0 \leq m \leq 1.

**Fig. 9.** Data points collected at 436 nm following photolysis of 41 \( \mu \)M hemoglobin partially saturated with oxygen in pH 7 phosphate buffer at 20°. The number above each set of points gives the fractional saturation of the sample at equilibrium. The dashed horizontal line connected to each set of data points gives the equilibrium absorbance level for that sample. The solid curves represent a fit of the MWC model including chain differences discussed in the text to the data points. The fixed parameter values used were: \( k_{Rn} = k_{Rn} = 59 \mu \text{M}^{-1} \text{s}^{-1}, k_{Rn} = 12 \text{s}^{-1}, k_{Rn} = 21 \text{s}^{-1}, k_{Rn} = 30 \mu \text{M}^{-1} \text{s}^{-1}. \) The best fit values for the parameters which were varied were: \( L = 1.55 \times 10^7, k_{Rn} = 1840 \text{s}^{-1}, k_{Rn} = 11.3 \mu \text{M}^{-1} \text{s}^{-1}, k_{Rn} = 1.1 \mu \text{M}^{-1} \text{s}^{-1}; \) and \( k_{Rn} = 88.6 \text{s}^{-1}. \) The equilibrium data for these samples was fitted in the same operation and the theoretical equilibrium curve obtained using the parameters listed above is given by the dotted line in Figs. 1 and 2.

**Fig. 10.** Data points collected at 436 nm following photolysis of partially saturated samples with various hemoglobin concentrations. The hemoglobin concentration in micromolar and the fractional saturation with oxygen in parentheses (number in parentheses) are given to the right of each set of points. The solid curves represent a fit of the MWC model including chain differences, discussed in the text to the data points. The fixed parameter values given in the legend to Fig. 9 were used in this fit. The best fitting values for the parameters which were varied were: \( L = 1.43 \times 10^7, k_{Rn} = 2480 \text{s}^{-1}, k_{Rn} = 11.8 \mu \text{M}^{-1} \text{s}^{-1}, k_{Rn} = 183 \text{s}^{-1}, \) and \( k_{Rn} = 2.86 \mu \text{M}^{-1} \text{s}^{-1}. \) The root mean square difference between the data points and the calculated curve was 0.0004 A.

\[
\Delta A
\]
concentration, for a fixed hemoglobin concentration (Fig. 4). This means that there is little advantage in fitting a family of curves such as those in Fig. 9 over fitting a single curve. Both of these difficulties are reflected in differences in the rate constants obtained by fitting the low concentration data (legend to Fig. 9) and the values obtained by fitting data for a range of hemoglobin concentrations (legend to Fig. 10).

The results of the detailed analysis of the binding reaction seem to agree well with the qualitative analysis presented earlier and definitely exclude the two-state model in the form employed by Hopfield et al. (2). This conclusion can be reached by direct examination of the results, and does not depend upon detailed analysis or upon small wavelength-dependent effects. The ability to reproduce the results does not, however, show that the present model is correct.

**Table I**

| Parameter values |
|------------------|
| Fixed parameters were: $\Delta v = 24 \text{ m/s}$, $\Delta v' = 29 \text{ m/s}$, $k_{rT} = 59 \pm 5$, $k_{rT} = 12 \pm 1.5$, $k_{oT} = 59 \pm 5$, $k_{oT} = 21 \pm 2$, $K_{vT} = 1 \times 10^{-5} \text{ M} \pm 0.3$, $k_{sT} = 30 \pm 3$. Second order constants ($k'$) are in units of $\mu M^{-1} \text{s}^{-1}$. First order constants ($k$) are in units of $s^{-1}$. The parameter values in the table were obtained from a fit to eight sets of kinetic data collected at 436 nm with hemoglobin concentration between 40 and 515 $\mu M$ and equilibrium fractional saturation between 0.02 and 0.15. Four sets of data and the best fit calculated lines are presented in Fig. 10. The root mean square residual was $\pm 0.0004$. Oxygen equilibrium data between 0.01 and 0.60 fractional saturation (solid triangles in these figures gives the fit to the equilibrium data. The V1 contribution to the variance was calculated from the error matrix while the V2 contribution was found by assuming errors in the fixed parameters as described in the text. S.D. denotes the standard deviation calculated from V1 and V2. |

| Constant | V1 | V2 | S.D. |
|----------|----|----|------|
| $k_{rT}$ | 2.9 | 0.04 | 0.05 | $\pm 0.30$ |
| $k_{oT}$ | 182 | 185 | 20 | $\pm 14$ |
| $k_{sT}$ | 11.8 | 0.04 | 0.08 | $\pm 0.35$ |
| $k_{oT}$ | 2480 | 11,700 | 400 | $\pm 110$ |
| $L \times 10^{-8}$ | 14.3 | 0.04 | 21 | $\pm 4.5$ |

**Fig. 11** (left). The time course of deoxygenation of hemoglobin at 20° in pH 7 phosphate buffer (points) observed following mixing of 46 $\mu M$ hemoglobin in equilibrium with 56 $\mu M$ oxygen with an 0.4% dithionite solution (all concentrations are before mixing). Experiments were performed using a Durrum stopped flow apparatus with a 2-cm light path cell and a dead time of 2 ms. Essentially identical results were observed at 540, 560, and 576 nm. The points represent the average of three experiments, one at each wavelength. The solid line is a simulation of the deoxygenation kinetics using the parameter values listed in Table I. The dashed curve gives the fraction of oxygenated $\alpha$ and $\beta$ chains as a function of time resulting from the simulation.

**DISCUSSION**

If the preceding analysis is accepted as plausible, it follows that the kinetics of the reaction of hemoglobin with oxygen is greatly influenced by differences between the $\alpha$ and $\beta$ chains as suggested by Gibson (12) whose interpretation of the oxygen pulse experiment and of low temperature stopped flow experiments is similar in principle. His suggestion of significant chain differences was not supported by an examination of hemoglobin Kansas made by Salhany et al. (29), but their experiments had to be performed in the presence of IHP, to suppress tetramer-dimer dissociation, and so cannot be closely compared with results obtained with hemoglobin A in the absence of inositol hexaphosphate. It appears that hemoglobin Kansas in the presence of IHP is not a good model for the T state of hemoglobin A.

The question next arises which chain is $\alpha$ and which $\beta$. Unfortunately, the present experiments do not provide any basis for answering it: the time course of oxygen binding may be almost equally well represented if the pairing of $R$ state $\alpha$ and $\beta$ chains with fast and slow T state components is reversed, although with some change in $L$. By default, it has been assumed that the $\beta$ chain is rapid in both R and T states, and the $\alpha$ chain is slower. Following this assignment, it has seemed logical to assume that the difference spectra are correspondingly located with the $\beta$ chain contribution at longer wavelengths (30), the fit to the data is appreciably better with this arrangement.

In much of the work described here, it has been assumed that the R and T states are at equilibrium. This is not an important matter in the low saturation range at present under study, but may become important if means can be found to extend the range of saturations used. It is not necessarily the case that the parameters of Table I would necessarily be equally well represented if the pairing of $R$ state $\alpha$ and $\beta$ chains with fast and slow T state components is reversed, although with some change in $L$. By default, it has been assumed that the $\beta$ chain is rapid in both R and T states, and the $\alpha$ chain is slower. Following this assignment, it has seemed logical to assume that the difference spectra are correspondingly located with the $\beta$ chain contribution at longer wavelengths (30). The fit to the data is appreciably better with this arrangement.

As a preliminary trial, the parameters of Table I have been used to compute the expected time course of deoxygenation of hemoglobin in the presence of dithionite. The result shown in Fig. 11 indicates that the values of the table together with the assumption of R-T equilibrium, give a reasonable repr-
mentation of the overall deoxygenation reaction. This figure is a simulation, and no optimization has been attempted.

The large value of $L$ of $1.4 \times 10^7$ which has been found indicates that the difference spectrum for R-liganded and T-liganded hemoglobins should be easily observed. In fact, in the Soret region, there is no large difference between the difference spectrum for 0 to 10% oxyhemoglobin and 0 to 100%, although a small difference may readily be observed in the immediate region of the oxyhemoglobin maximum. The conclusion is that spectrophotometric determinations of saturation should be reliable to 0.5% saturation or so when made at any wavelength in this region reasonably removed from the isosbestic point, and, since the difference spectrum is isosbestic at about 410 nm, determinations made using several wavelengths may be expected to be closer still.

The determinations made with the laser method agree well with the classic gasometric work of Roughton and Lyster (10) and have the advantage of showing clearly any contamination with CO. The importance of even small amounts of CO in shifting the curve significantly is illustrated in Fig. 1. In spite of these virtues, the laser method can scarcely be advocated as an everyday procedure, although provided that suitably thin films were prepared, concentrations up to 5 mm or so could be examined without producing a temperature rise sufficient to denature the hemoglobin. It seems that it is the inevitable association of a temperature jump with photophysical removal of oxygen which offers the main limitation to the use of the laser method for studying the kinetics and equilibria of the oxygen reaction. (See Appendix 2.)

The differences between the components of deoxyhemoglobin (presumed to be the $\alpha$ and $\beta$ chains) are the largest yet reported for a gaseous ligand and are of the same order as the differences reported earlier for the bulky ligand n-butyl isocyanide (31) and in the reaction of methylhemoglobin with diithionite (32). They are much too large to be neglected in analyses of hemoglobin kinetics, although in some circumstances a satisfactory approximation might be obtained by regarding the rates for the slower component to be zero. It is perhaps this effect which allowed Gibson (12) to describe his oxygen pulse experiments in terms of only two relaxations, the slower component simply did not contribute sufficiently to the observed kinetics to be detected in the presence of rapid T state and R state species.

The difference between the components derives from rate constants for combination (4-fold difference) and dissociation (12-fold difference). These constants combine so as to produce only a 3-fold difference in affinity. There is no immediately obvious correlation with structure, but with differences as small as 10-fold this could scarcely be expected.

Acknowledgment—It is a pleasure to thank Dr. Keith Moffat for a careful reading of this manuscript.

APPENDIX 1

Binding of Oxygen to R State Tetramer and to Dimers

In confirmation of earlier work (33) the rate of binding to R state tetramers was determined by partial flash photolysis of fully oxygenated (>200 $\mu$M free O$_2$) hemoglobin solutions. The rate constant obtained at 20°C was $59 \mu$M$^{-1}$ s$^{-1}$. The rate for binding to dimers was obtained by full photolysis of dilute (<1 $\mu$M heme) solutions of oxyhemoglobin. These solutions show a biphasic reaction the greater part of which can be attributed to the dimer on the basis of the concentration dependence of the amplitude of the rapid phase. The rate constant obtained by subtracting out the slow phase was $30 \mu$M$^{-1}$ s$^{-1}$ at 20°C. This is considerably less than the rate constants reported for isolated chains of $\alpha$ and $\delta$ for $\alpha$-$\text{SH}$ and 71 $\pm$ 6 $\mu$M$^{-1}$ s$^{-1}$ for $\beta$-$\text{SH}$ (34). The rate constants for the chains do not necessarily apply to the dimmer, particularly for the $\beta$ chain which is tetrameric in solution (35). No definite evidence of heterogeneity which might be attributed to chains was observed in these experiments.

APPENDIX 2

Temperature Rise on Photolysis of Oxyhemoglobin

The low quantum yield for the photolysis of oxyhemoglobin (36) requires that large amounts of light be used to obtain essentially complete photodissociation. Experiments and calculations performed earlier for 50 $\mu$M hemoglobin (16) suggested that 97 to 99% dissociation is associated with a temperature rise of about 0.3°C. In the present experiments it is important to use higher hemoglobin concentrations (up to 500 $\mu$M) in order to define the values of the kinetic parameters. The resultant temperature rise must be proportional to the hemoglobin concentration.

The temperature jump was determined using the rate of the $R \rightarrow T$ conformational change observed following photolysis of 500 $\mu$M carboxyhemoglobin in borate buffer at pH 9. This change has a large apparent activation energy (18 kcal/mol; see Ref. 21). The rate of the conformational change was observed following photolysis using a laser pulse of the same energy used in photolysis of the oxyhemoglobin samples. The experiment was then repeated using a filter which reduced the energy incident on the sample by a factor of 15 yet still allowed essentially complete photolysis because of the high quantum yield of COHb. The rates of the $R \rightarrow T$ change followed at 425 nm and 30°C for 500 $\mu$M COHb were 20,000 s$^{-1}$ for the full flash and 15,000 s$^{-1}$ for the attenuated flash, corresponding to a temperature difference of about 3°C in good agreement with the earlier work with oxyhemoglobin.

In order to determine rate constants for the reaction of deoxyhemoglobin with oxygen at 20°C the cell holder was maintained at 17°C before photolysis when 500 $\mu$M hemoglobin was used, and at 18.5°C for 250 $\mu$M. For lower concentrations a temperature of 20°C was employed. The change in temperature alters the oxygen equilibrium which is established before the flash. This was not taken into account in the calculations required to establish the initial concentration of dimers following the flash, but computation suggests that the effect of this neglect is unimportant at high hemoglobin concentration where a significant temperature jump occurs.

REFERENCES

1. Monod, J., Wyman, J., and Changeux, J. P. (1963) J. Mol. Biol. 12, 58-118
2. Hopfield, J. J., Shulman, R. G., and Ogawa, S. (1971) J. Mol. Biol. 61, 425-445
3. Edelstein, S. J. (1971) Nature 230, 224-227
4. Edelstein, S. J. (1975) Annu. Rev. Biochem. 44, 209-232
5. MacQuarrie, R., and Gibson, Q. H. (1972) J. Biol. Chem. 247, 5866-5894
6. Imai, K., and Yonetani, T. (1975) J. Biol. Chem. 250, 2227-2231
7. Mills, P. C., Johnson, M. L., and Ackers, G. K. (1976) Biochemistry 15, 5350-5362
8. Gibson, Q. H. (1970) J. Biol. Chem. 245, 3295-3288
9. Gibson, Q. H., and Roughton, F. J. W. (1955) Proc. R. Soc.
Laser Photolysis Study of T State of Human Oxyhemoglobin

10. Bansil, R., Herzfeld, J., and Stanley, E. (1976) J. Mol. Biol. 103, 89–126
11. Gibson, Q. H., Riggs, A., and Imamura, T. (1973) J. Biol. Chem. 248, 5876–5886
12. Gibson, Q. H. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 14
13. Ilgenfritz, G., and Schuster, T. M. (1974) J. Biol. Chem. 249, 2959–2973
14. Gibson, Q. H. (1966) J. Physiol. 131, 123–134
15. Seber, H. A., ed (1960) Handbook of Chemistry and Physics, 42nd Ed, p. 1706, Chemical Rubber Publishing Co., Cleveland, O.
16. Sawicki, C. A., and Gibson, Q. H. (1977) J. Biol. Chem. 252, 5783–5788
17. Kaszniak, F. C., and Gibson, Q. H. (1976) Anal. Biochem. 76, 458–486
18. Roughton, F. J. W., and Lyster, R. L. J. (1965) Hvalradets Skr. 48, 186–186
19. Gibson, Q. H. (1969) Biochem. J. 107, 293–303
20. Kellett, G. L., and Gutfreund, H. (1970) Nature 227, 921–926
21. Sawicki, C. A., and Gibson, Q. H. (1970) J. Biol. Chem. 251, 1533–1542
22. Ogawa, S., and Shulman, R. G. (1971) Biochem. Biophys. Res. Commun. 44, 193–194
23. Cassoly, R., Gibson, Q. H., Ogawa, S., and Shulman, R. G. (1971) Biochem. Biophys. Res. Commun. 44, 105–1021
24. Cassoly, R., and Gibson, Q. H. (1972) J. Biol. Chem. 247, 7332–7341
25. Gibson, Q. H., and Nagel, R. L. (1974) J. Biol. Chem. 249, 7265–7269
26. Bunn, H. F., Wohl, R. C., Bradley, T. B., Cooley, M., and Gibson, Q. H. (1974) J. Biol. Chem. 249, 7193–7196
27. Olson, J. S., and Gibson, Q. H. (1973) J. Biol. Chem. 248, 1623–1630
28. Bevington, C. R. (1969) Data Reduction and Error Analysis for the Physical Sciences, Chapt. 11, McGraw-Hill Book Co., New York, N. Y.
29. Salminen, J. M., Castillo, C. L., MacDonald, M. J., and Gibson, Q. H. (1969) Biochem. Biophys. Res. Commun. 44, 1015–1021
30. Banerjee, R., Alpert, Y., Lettieri, F., and Williams, R. J. P. (1969) Biochemistry 8, 2862–2867
31. Olson, J. S., and Gibson, Q. H. (1972) J. Biol. Chem. 247, 1713–1726
32. MacQuarrie, R. A., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 517–522
33. McCray, J. A. (1972) Biochem. Biophys. Res. Commun. 47, 187–190
34. Noble, R. W., Gibson, Q. H., Brunori, M., Antonini, E., and Wyman, J. (1969) J. Biol. Chem. 244, 3905–3908
35. Benesch, R. E., Ranney, H. M., Benesch, R., and Smith, G. M. (1961) J. Biol. Chem. 236, 2925–2930
36. Gibson, Q. H., and Ainsworth, S. (1967) Nature 180, 1416–1417
Properties of the T state of human oxyhemoglobin studies by laser photolysis.
C A Sawicki and Q H Gibson

J. Biol. Chem. 1977, 252:7538-7547.

Access the most updated version of this article at
http://www.jbc.org/content/252/21/7538.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/252/21/7538.citation.full.html#ref-list-1