Kinetics of Ligand Binding and Quaternary Conformational Change in the Homodimeric Hemoglobin from *Scapharca inaequivalvis* 

(Received for publication, October 31, 1983)

Eraldo Antonini†, Franca Ascoli‡, Maurizio Brunori, Emilia Chiancone, and Daniela Verzili

From the Consiglio Nazionale delle Ricerche Center of Molecular Biology, Institutes of Chemistry and Biological Chemistry, Faculty of Medicine, University of Rome and ‡Department of Cell Biology, University of Camerino, Italy

Roger J. Morris and Quentin H. Gibson§

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

The kinetics of the reaction with oxygen and carbon monoxide of the homodimeric hemoglobin from the bivalve mollusc *Scapharca inaequivalvis* has been extensively investigated by flash and dye-laser photolysis, temperature jump relaxation, and stopped flow methods. The results indicate that cooperativity in ligand binding, already observed for oxygen at equilibrium, finds its kinetic counterpart in a large decrease of the oxygen dissociation velocity in the second step of the binding reaction. In the case of carbon monoxide, cooperativity is clearly evident in the increase of the combination velocity constant as the reaction proceeds. Therefore, the ligand-binding kinetics of this dimeric hemoglobin shows the characteristic features of the corresponding reactions of tetrameric hemoglobins. Analysis of the data in terms of the allosteric model proposed by Monod et al. (Monod, J., Wyman, J., and Changeux, J. P. (1965) *J. Mol. Biol.* 12, 88–118) has shown that the values of the allosteric parameters cannot be fixed uniquely for a dimeric hemoglobin.

The rapid changes in absorbance observed at the isosbestic points of unliganded and liganded hemoglobin following laser photolysis provided a value of $7 \times 10^4$ s$^{-1}$ at 20 °C for the rate of the ligand-free quaternary conformational change, postulated on the basis of cooperative ligand binding. Comparison of the rapid absorbance changes observed during ligand rebinding in this hemoglobin with those observed in tuna hemoglobin indicate that, at full photolysis, binding to the T state is followed by further binding and conversion to the liganded R state; at partial photolysis, population of the liganded T state occurs immediately and is followed by a decay to the liganded R state upon further ligand binding.

These new results, in conjunction with previous equilibrium data on the same system, show unequivocally that the presence of two different types of chain is not an absolute prerequisite for cooperativity in hemoglobins, contrary to currently accepted ideas.

A correlation between the optical absorption spectrum of deoxyhemoglobin and its rate of reaction with ligand was first observed nearly 25 years ago (1). The original observations with sheep hemoglobin have since been extended to cover a range of mutant and chemically altered hemoglobins. As yet, no exception has been reported to the rule that a lowered Soret peak is associated with an increased rate of CO binding, although in some cases the spectral changes associated with a quickly reacting component are minute as compared to human hemoglobin A (2).

From the beginning, it was suggested that the change in spectrum was due to a change in conformation of the hemoglobin molecule, and following general acceptance of the two-state model (3) this could plausibly be identified with the R-Ta transition (4, 5). After the specification by x-ray crystallography of the differences between R and T state structures (6, 7), spectrum, function, and model were all reasonably associated with the relative motion of the chains in the tetramer on ligand binding. In detail, such a mechanism has been developed for a tetrameric hemoglobin for which the role of interactions between different types of chains has been considered essential. Although there are some reports of dimeric hemoglobins (Cucumaria miniata, Molpadia arenicola) with a Hill coefficient $n$ greater than 1, these have not been taken to imply the existence of cooperative dimers (8), but have been attributed to a change in subunit association on ligation which may produce cooperativity, as has been documented for lamprey hemoglobin (9).

It has been recently reported that the dimeric hemoglobin from molluscs of the Arcid family like *Anadara broughtoni* and *Scapharca inaequivalvis* show considerable cooperativity with $n$ of 1.5 (10, 11) and different rates of carbon monoxide binding following full and partial flash photolysis (12). These dimeric hemoglobins are composed of two identical chains, and do not undergo ligand-linked association-dissociation reactions (11, 13). It follows that cooperativity may be present in homodimers, in contrast with previous suggestions based on the behavior of human hemoglobin (14). This paper reports a detailed study of the kinetics of carbon monoxide and oxygen binding of the dimeric *S. inaequivalvis* hemoglobin (HbI$^*$), and of a rapid spectrophotometric change in the Soret region following photolysis of oxygen and carbon monoxide. The results are discussed in the framework of the allosteric two-state model.

**MATERIALS AND METHODS**

Specimens of the bivalve mollusc *S. inaequivalvis* were collected in the Middle Adriatic Sea. The dimeric component (HbI$^*$) was isolated...
from the hemolysate and purified by ion-exchange chromatography as previously described (11). Protein concentration was determined on the oxygenated derivative from the optical absorption at 578 nm, using the molar extinction coefficient on a heme basis, ε = 14,300 (11).

Samples of T. thynnus hemoglobin and of the isolated minor fraction of menhaden (Brevoortia tyrannus) hemoglobin, prepared as described in Refs. 15 and 16, were stored frozen in pellet form, under nitrogen.

Preparation of saturated HbCO, HbO₂, and deoxy-Hb was performed by flushing, in a slowly rotated tonometer, the appropriate gas for about 20 min. Except for a small number of control experiments as a function of pH, all measurements have been performed in 0.1 M phosphate buffer, pH 7.0.

The partition constant between oxygen and carbon monoxide was determined at 20 °C in a thin layer optical cell according to the method of Wyman et al. (17) by addition of increasing amounts of CO to the oxygenated protein at a concentration of 4.4 × 10⁻⁴ M in 0.1 M phosphate buffer, pH 7.0; after reaching equilibrium at each step, the optical density was recorded at the appropriate wavelengths.

Spectrophotometric measurements were carried out with a Cary 219 spectrophotometer; the pH was measured with a Radiometer PHM 64 pH meter.

Stopped flow experiments were performed with a Gibson-Durrum apparatus equipped with a 2-cm observation tube.

Temperature jump experiments were carried out with the single beam apparatus built by Messanlagen Gesellschaft (Göttingen, Germany); the experimental set-up and analysis of data are as given by Brunori et al. (18).

Both the dye-laser system, previously described (19), and the flash photolysis system (20) were used for photodissociation of the samples and observation of the consequences. The dye-laser system (capable of outputting 3 J in 500 ns at 540 nm), with its very short associated time constant (1 μs) allowed observation of very fast absorbance changes following photolysis of both O₂- and CO-ligated species. The conventional flash system permitted complete photolysis of HbCO samples at all ligand concentrations as well as partial photolysis of HbO₂ (up to 25%). Neutral density filters were used to attenuate the excitation light output as required. Interference filters of appropriate wavelength were used to minimize photolysis of the sample by the observation beam. Data were collected using a Biomatlon model 805 waveform recorder, with 2K memory, and transferred to a PDP9 computer for averaging and further analysis. Alternatively, the output to a conventional recorder for manual manipulation was employed.

A Hewlett-Packard model 9830A computer equipped with a model 8866 analog plotter was also used for the analysis of the data.

RESULTS

Reaction with Oxygen

Combination

The kinetics of oxygen binding to HbI was studied by means of several experimental techniques: partial or total photodissociation, temperature jump relaxation, and determination of the rate of oxygen displacement by carbon monoxide in flash photolysis and rapid mixing experiments.

Photodissociation Experiments—In conventional flash experiments, only partial photodissociation of HbI0₂ (4–23%) was achieved; the rate of the reaction showed no deviation from a simple pseudo-first order behavior and the rate of oxygen displacement by carbon monoxide in flash photolysis and rapid mixing experiments was very similar, if not identical.

Photodissociation experiments on fully saturated hemoglobin were also performed using a dye-laser system. The rate of recombination was closely proportional to oxygen concentration and there was no difference in rate after full and partial photolysis (98, 60, and 7% photodissociation) at high oxygen concentrations. The experiments reported in Fig. 1, where hemoglobin and free oxygen concentrations were 27 and 248 μM, respectively, show that the time course of recombination is accurately first order after full and partial photodissociation and yields a rate constant of 15 μM⁻¹ s⁻¹.

All the results at 20 °C are compiled in Table I from which it appears clearly that the rates of combination in the first and in the second step of the binding reaction are closely similar, indicating that cooperativity in oxygen binding to HbI (11, 21) does not manifest itself in the combination rate constants.

Temperature Jump Experiments—Temperature jump experiments on HbI equilibrated with oxygen show a single relaxation phase over the whole range of protein concentration (7–60 μM) and fractional saturation with oxygen (Y = 0.5–1.0) studied. The reciprocal relaxation time is linearly dependent on the concentration of the reagents in the higher constant for the overall oxygen combination varied between 12 and 15 μM⁻¹ s⁻¹. Thus, combination with oxygen of partially and totally photodissociated HbI0₂ yields values which are very similar, if not identical.

FIG. 1. Dye-laser photolysis of S. inaequivalvis HbI0₂. Protein concentration, 27 μM; free oxygen concentration, 248 μM. Optical path, 2 mm; observation wavelength, 546 nm; 0.1 M phosphate buffer, pH 7.0, 26 °C. Photolysis energy varied to produce: □, 98%; □, 60%; and ■, 7% breakdown. The results are presented as scaled semilogarithmic plots covering 90% of the reaction.
Ligand-binding Kinetics in a Homodimeric Hemoglobin

Experiments were performed by mixing in a stopped flow apparatus the Hbl solutions (1.8 \( \mu M \)) equilibrated with oxygen at different concentrations (60-140 \( \mu M \)) with a 1 mM CO solution. Alternatively, the same type of experiment was performed at low and high [O\(_2\)]/[CO] ratios by using the flash to perturb the system at equilibrium in the dark; the combination with oxygen (see above) and the subsequent displacement of oxygen by carbon monoxide were followed. The agreement between the flash photolysis and the stopped flow data is remarkably good. This is apparent from Fig. 3 where the data are plotted according to the following equation:

\[
\frac{1}{r} = \frac{2}{k_2} + 2 \frac{k_2}{k_1} \times \frac{[O_2]}{[CO]}
\]

where \( r \) is the observed rate of displacement, \( k_2 \) is the CO combination rate constant, and \( k_1 \) is the oxygen dissociation rate constant for the second step. This equation is applicable since on the basis of the data given below \( k_1 \) \( [02] \ll k_2 \) \( [CO] \) (22). Taking a value of \( l_2 = 2.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) and \( k_2 = 50 \text{ s}^{-1} \), a value of \( 5 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) for \( k_2 \) is obtained from the slope of the data reported in Fig. 3. As may be seen from the same figure, the intercept, which yields \( 2/k_1 \), cannot be determined with confidence, but is consistent with an intrinsic value of \( k_1 = 50 \text{ s}^{-1} \) (see below).

Displacement Experiments—The oxygen combination velocity constant was estimated also from experiments in which oxygen was displaced from HbI\(_2\) by CO (22).

saturation range (Fig. 2). Taking into account the oxygen dissociation constant obtained in the reaction with sodium dithionite (see below), the apparent combination rate constant obtained (\( k' = 13-15 \text{ M}^{-1} \text{s}^{-1} \)) is in good agreement with the data from flash photolysis.

From the relaxation amplitudes at high fractional saturation (\( Y = 0.77-0.99 \)), an apparent enthalpy change of \(-8.5 \text{ kcal/mol} \) is calculated for the binding process; this value compares well with the \( \Delta H \) value of the second binding step \((-7.4 \pm 0.5 \text{ kcal/mol} \)) estimated from the temperature dependence of the oxygen equilibrium curves (21), although over the saturation range covered more than a single state was populated.

Displacement Experiments—The oxygen combination velocity constant was estimated also from experiments in which oxygen was displaced from HbI\(_2\) by CO (22).

Fig. 2. Dependence of the reciprocal relaxation time on free oxygen and free binding sites in S. inaequivalvis HbI\(_2\). Protein concentration, 8-66 \( \mu M \); oxygen, 7-270 \( \mu M \); 0.1 M phosphate buffer, pH 7.0, 20 °C. Temperature jump, 5 °C. Observation wavelength, 430 nm. The fractional saturation values are reported for each experiment.

\[
\frac{[k_2 \cdot O_2]}{k_1} \times 10^6 \text{ M}
\]

Fig. 3. Reciprocal oxygen displacement rate by CO, at different oxygen:carbon monoxide ratios, in S. inaequivalvis HbI\(_2\). Stopped flow experiments; \( O_2 = 1.8 \mu M \) Hb before mixing. Full flash experiments: \( \Delta \), 4 \( \mu M \) Hb; \( \triangle \), 7 \( \mu M \) Hb; \( \blacktriangle \), 2 \( \mu M \) Hb. 0.1 M phosphate buffer, pH 7.0, 20 °C; observation wavelength, 436 nm.
constants are either statistically related or that \( k_1 \gg k_2 \). The absence of cooperativity in the combination process suggests that the second alternative is correct; thus, the intrinsic value of the dissociation rate constant from the fully saturated protein is \( k_2 = 50 \text{ s}^{-1} \). The velocity of the reaction is affected by temperature, the activation energy being 10 kcal/mol, corresponding to about half the value for human hemoglobin A (22).

The intrinsic values of the combination and dissociation rate constants for the two steps of the oxygen reaction in conjunction with the previously reported equilibrium constants (21) are shown in Table II. The data give a satisfactory picture of the oxygen-binding kinetics to HbI. The results indicate that cooperative oxygen binding in this homodimer finds its kinetic origin in the large decrease of the dissociation constant which occurs during the progress of the binding reaction similar to what is found for human hemoglobin A.

**Reaction with Carbon Monoxide**

The combination rate with CO was measured following the rebinding reaction after full and partial photodissociation of HbICO, obtained either by conventional flash photolysis, or by dye-laser photolysis. The combination of HbI with CO was also measured in rapid mixing experiments.

**Photodissociation Experiments**—The time course of the reaction was measured at different flash intensities, corresponding to different extents of photodissociation (100-5%); hemoglobin and CO concentrations were 1-75 and 7-100 \( \mu \text{M} \), respectively. An accelerating time course of binding was observed following full photodissociation at most wavelengths and [CO]/protein ratios, the initial rate constant being \( k'_1 = 7-10 \text{ \mu M}^{-1} \text{ s}^{-1} \). When photodissociation was less than 70%, the reaction was first order; the second order rate increases with decreasing photodissociation and reaches a limiting value at 10% photodissociation of \( k'_1 = 20 \text{ \mu M}^{-1} \text{ s}^{-1} \). This increase in the CO combination velocity constant as the reaction proceeds may be considered the kinetic counterpart of cooperativity in CO binding to HbI (see also below). Results of typical experiments performed at 4 \( \mu \text{M} \) HbI and 92 \( \mu \text{M} \) CO with the dye-laser system are reported in Fig. 4; identical results were obtained by conventional flash over a large CO concentration range (see inset to Fig. 4).

**Rapid Mixing Experiments**—The combination with CO was also measured in a stopped flow apparatus mixing CO solutions (0.1-1 nM) with deoxygenated hemoglobin (1-37 \( \mu \text{M} \)) obtained by addition of small amounts of sodium dithionite to the oxygenated derivative. Experiments were conducted at 9 and 20 °C. In agreement with the results of full photodissociation experiments, the time course of the reaction is autocatalytic at most wavelengths (410-450 nm), with an initial rate \( (l_d) \) of \( 9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) at 20 °C. The same behavior and initial rate constant were observed at pH 9.2 in 2% borate. The activation energy for the initial reaction is about 7.1 kcal/mol.

**Kinetic difference spectra** (HbI minus HbICO) obtained from the total changes in absorbance in both full flash and rapid mixing experiments are in good agreement with the static difference spectra of HbI, obtained with dithionite, minus HbICO (Fig. 5). In the same figure, the kinetic difference spectrum obtained upon partial photodissociation (25%) is presented. Its shape is similar to that of the kinetic difference spectrum obtained after full photodissociation. It should be mentioned that the static difference spectrum recorded using the deoxygenated derivative obtained by addition of dithionite is smaller than the difference spectrum measured under anaerobic conditions, in the absence of the reductant. The difference is more evident at some wavelengths, such as 420 nm.

**Dissociation**

The CO dissociation rate was measured by mixing HbICO (final concentration, 5-7 \( \mu \text{M} \)) with reduced microperoxidase (8-16 \( \mu \text{M} \)) in the absence of CO in excess, and recording the optical density change at 551 nm. This method was first described by Sharma et al. (23) and is based on the large value of the combination rate constant (20 \( \text{ M}^{-1} \text{ s}^{-1} \)) and of the equilibrium constant (2 \( \text{ M}^{-1} \)) of microperoxidase with CO. The time course of the reaction corresponds to a first order process and an apparent CO dissociation rate constant \( (l_d) \) of 0.009 s\(^{-1}\) is obtained at pH 7.0 and 20 °C.

CO dissociation from HbICO was also measured by taking advantage of the large combination rate constant of CO for HbA at pH 9.2 (13), and of the lack of a Bohr effect in HbI.
Ligand-binding Kinetics in a Homodimeric Hemoglobin

FIG. 5. Kinetic and static difference spectra of *S. inaequivalvis* HbI minus HbICO. Different symbols refer to different methods and protein concentrations (micromolar). Full flash: O, 7.27; △, 1.01; ▽, 6.5; ▼, 7.3. Stopped flow: O, 5.45; Δ, 5.55; O, 1.7; ▽, 5.6. Small flash (2.5% photodissociation): (- - -), 7.4. ◼, the static difference spectrum obtained in the presence of sodium dithionite. 0.1 M phosphate buffer, pH 7.0, 20 °C.

TABLE III
Kinetic and equilibrium data for CO binding to *S. inaequivalvis* HbI

Conditions were 0.1 M phosphate buffer, pH 7.0, 20 °C. All values are intrinsic, i.e., without statistical factors.

<table>
<thead>
<tr>
<th>Step</th>
<th>( t' )</th>
<th>( t )</th>
<th>( t'/t )</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second</td>
<td>0.2</td>
<td>0.005</td>
<td>40°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.003b</td>
<td>68°</td>
</tr>
</tbody>
</table>

* Calculated as \( t'/t \).*

* From 0.2 \( \mu M^{-1} \) s\(^{-1} \)/68 \( \mu M^{-1} \).

* From \( m \) and \( K_z \) (see Table II).

The experiment was performed at final concentrations of 20 \( \mu M \) for HbICO and 37 \( \mu M \) for reduced HbA. The reaction was followed at 560 nm and its time course was first order, with an apparent rate of 0.01 s\(^{-1} \). Thus, the time course of carbon monoxide dissociation is first order like that of oxygen dissociation by dithionite. However, in the case of CO, it is clear that cooperativity manifests itself in the combination process, and therefore the assessment of the intrinsic dissociation rate constant for the second step is less unequivocal. The value of 0.005 s\(^{-1} \) (i.e., \( \frac{t'_2}{2} \)) is, however, consistent with that calculated from \( t'_2 \) and from the equilibrium constant of the second step for carbon monoxide (La), obtained as shown in Table III taking into consideration the partition coefficient between oxygen and carbon monoxide (see below) and the value of \( K_z \) obtained from oxygen equilibrium experiments (21).

**Partition Constant between CO and O₂**

The partition constant CO/O₂ for HbI was determined spectrophotometrically at 20 °C using a thin layer optical cell according to the method reported by Wyman et al. (17) as described under "Materials and Methods." A value of \( m = 210 \) was obtained from the data of Fig. 6.

Table III gives the (intrinsic) kinetic and thermodynamic constants obtained from the experiments described above. Kinetically, cooperativity in CO binding manifests itself clearly in the increase of the combination rate constants as the reaction proceeds, although a contribution from the dissociation rate constants is very likely.

**Rapid Spectrophotometric Changes**

**At Ligand Isosbestic Points**

Experiments have been performed with two samples of *S. inaequivalvis* HbI using laser photolysis of either oxy- or CO-hemoglobin. The isosbestic point was located experimentally by cautious adjustment of the monochromator while observing the results of flashes on an oscilloscope. One may aim either to see no change on firing the flash, corresponding to the \( R_{oxy} \) isosbestic, or to locate the \( R_{CO} \) isosbestic by setting the monochromator so that there is no difference between the absorbance just before the flash and the absorbance just after the rapid excursion is over, i.e., 50 ps later. The very rapid absorbance change is substantially the same whichever method is used because the two isosbestic points are separated only by a nanometer or so. Some of the results obtained with HbI at the \( R_{oxy} \) isosbestic point are shown in Fig. 7. The excursion is small and very rapid, so control experiments to ensure that it is not due to artifact were undertaken using tuna and menhaden I hemoglobins as positive controls and \( \beta \)-p-mercuribenzoate chains, which indeed showed no change, as a negative control. As a further
Ligand-binding Kinetics in a Homodimeric Hemoglobin

Fast absorbance changes observed at \( R_{\text{CO}-T_{\text{isosbestic}}} \) isosbestic point following dye laser photolysis of liganded \( S. \text{inaequivalvis} \) HbI, tuna, and menhaden I hemoglobins. Protein concentration, \( \sim 15 \mu M \); 0.1 M phosphate buffer, pH 7.0. Observation wavelength: \( \sim 425 \) nm for the CO derivatives and \( 421 \) nm for the oxy derivatives. Total absorbance changes: for CO derivatives: 0.015, \( S. \text{inaequivalvis} \) HbI; 0.041, tuna; 0.043, menhaden I; oxy derivatives: 0.016, \( S. \text{inaequivalvis} \) HbI; 0.014, tuna; 0.014, menhaden I.

Near the Isosbestic Point during Ligand Rebinding

It was noticed that after the end of the rapid reaction (i.e., after 50 \( \mu \)s) there were small but definite absorbance changes at the wavelengths used for following the rapid reaction, particularly with \( S. \text{inaequivalvis} \) HbI, and that these were disproportionately large as compared with the corresponding changes seen with human hemoglobin (data not shown). A set of data was therefore collected over a time sufficient to permit about 90% completion of rebinding of CO, adjusting the monochromator so that the trace appeared to start from the same absorbance as that before the flash was fired. This wavelength is regarded as isosbestic for the change \( R_{\text{CO}-T_{\text{isosbestic}}} \) because on the 100-fold longer time scale used to follow ligand binding the rapid change illustrated in Fig. 7 is complete before absorbance recording begins effectively. Data were collected for full photolysis and for three other levels down to 22% breakdown. The results in Fig. 9 show that the amplitude of the absorbance change is not proportional to photolysis, and that its course is quite different in shape at high and low levels of photolysis. At high levels, the absorbance first increases and then decreases to reach its final level whereas with lower levels there is an initial jump in absorbance followed by a monotonic relaxation to the final level.

To help interpretation of the results, analogous experiments were performed with human hemoglobin A, with \( \beta-p \)-mercuribenzoate chains, and with menhaden hemoglobins I and IV.
Ligand-binding Kinetics in a Homodimeric Hemoglobin

Slow changes of similar form were seen in each case, except with the chains, but were of considerably smaller amplitude. The same experiment was also performed with hemoglobin from the bluefin tuna (T. thynnus), which has recently been studied by Morris and Gibson (24). They measured the absorption spectra for both chains in liganded T and liganded R forms, and concluded that only two difference spectra were required to describe ligand binding, one for binding to the higher affinity T state chain, the other for the low affinity T state chain and both R state chains. There are thus two isosbestic points, one for binding to either chain in the R state, which is shared also by one of the chains in the T state, and the second for binding to the other chain in the T state. Thus, one liganded T state chain is separately observable: it has a much higher affinity than the other. The explanation offered for the results in Fig. 9 gives a binomial distribution of intermediates, and those with one ligand remaining will relax rapidly from the R to the T state, giving the immediate absorbance excursion. Thereafter, the liganded T state may increase somewhat further and then decrease (70% photolysis), or decrease monotonically to zero (40% photolysis and 25% photolysis).

The absorbance changes with Scapharca depicted in Fig. 9 are qualitatively strikingly similar, and it is suggested that they have the same origin as in the experiment with tuna hemoglobin. At full photolysis, binding to the T state takes place at first, followed by further binding and conversion to the R-ligated form, while with partial photolysis the liganded T state is populated immediately by the flash itself, and decays to the liganded R state by the addition of further ligand molecules.

Assignment of Allosteric Parameters and Representation of Slow Changes by the Allosteric Model

If the origin of the changes at 425 nm is indeed that suggested in the previous paragraph, it should be possible to simulate the results of Fig. 9 by using the two-state model to calculate the time course of the T-ligated species during rebinding of CO. To do this, it is, of course, necessary to assign values to the allosteric parameters and. The data for CO binding shown in Fig. 4 are not sufficient for this purpose, in part because there are not sufficiently precise data for the dissociation velocities.

At first sight it would seem possible to complete the assignment of parameters by making use of data for the equilibrium and kinetics of the oxygen reaction. Equilibrium data have been given by Ikeda-Saito et al. (21) of 2.6 × 10^10 and 2.3 × 10^10 M^-1 for the intrinsic binding constants of the first and second molecules of oxygen. The kinetic experiments quoted earlier in this paper suggest a binding rate of 15 μM^-1 s^-1 for the binding of both oxygen molecules. The dissociation velocities are, therefore, 580 and 65 s^-1 (intrinsic) for the first and second molecules, respectively. The value for the second molecule is consistent with the results of replacement experiments which have given a rate of approximately 60 s^-1 for the dissociation of the first oxygen molecule from Hb(O2)2, in excellent agreement with the first estimate above.

It is, however, impossible to assign a single unique set of allosteric constants, even though both upper and lower asymptotes of a Hill plot have been defined by the oxygen data. The difficulty is that the lower asymptote, for instance, does not correspond to K_T, the binding constant for the T state, but is LK_T + (1 - L)K_R, where L is the allosteric constant for deoxyhemoglobin. An analogous expression, again involving K_T, K_R, and L, similarly defines the upper asymptote. That is, there are two equations with three unknowns, and an indefinitely large set of consistent values of the unknowns therefore exists, any one of which can represent the oxygen equilibrium precisely, but with different values of K_T, K_R, and L. The situation would not be helped by data at very low and very high saturations since asymptotes are already being considered. The problem, although particularly obvious in the case of a dimeric hemoglobin, applies also to tetramers, in principle, and may be practically significant if L is not large, as with some mutant hemoglobins.

To complete the analysis of the oxygen data, the equilibrium constant for binding of the first oxygen molecule was combined with the rate of oxygen binding, leading to a predicted
phenomenological rate of dissociation of oxygen from HbO₂ of 580/s. This rate has been visualized by performing flash photolysis experiments in which all oxygen was removed from partially saturated hemoglobin (Fig. 11). The relaxation at 580/s. This rate has been visualized by performing flash phenomenological rate of dissociation of oxygen from HbO₂ starting with saturated hemoglobin, in excellent agreement with the observed value of 100 s⁻¹.

In order to quantitate the binding of carbon monoxide to HbI, the set of data shown in Fig. 4 for full and partial photolysis was analyzed. A value of 10 was assumed for L which, as discussed earlier, falls in the range of equivalent values defined in the oxygen experiments. The family of curves was then fitted to a two-step two-state model, and an excellent fit obtained, the mean square residual over the whole family being only 0.3% in saturation. The rate parameters derived in this way were then used to generate the theoretical time course for the amount of T-liganded hemoglobin for full and 41% photodissociation. The form of the computed time courses is compared with experiments in Fig. 12, A and B, and, in view of the somewhat arbitrary assumptions involved, is regarded as good. It should perhaps be repeated here that repetition of the computations with another value of L, although defining different rates for binding to T and R than those given in Fig. 12, gave just the same computed time course for the concentration of the T-liganded species, for the reasons discussed in relation to the representation of the oxygen reactions. Overall, the result is consistent with the hypothesis that the slow changes seen at the ligand-binding isosbestic of 425 nm derive from the rise and fall in the population of the T-liganded species.

**DISCUSSION**

The experiments described in this paper are interesting and significant precisely because the results resemble so closely those obtained using mammalian hemoglobins, although *S. inaequivalvis* HbI is a homodimer. It seems difficult to escape the conclusion that there is more than one mechanism able to generate cooperativity. Description of this cooperativity in terms of the two-state model, although readily achieved, does not lead to unique values for the allostery parameters. The problem arises because their equilibrium behavior can be described by the Adair equation with two parameters. The corresponding two-state model, however, has three parameters, L, K_T, and K_R. It follows that these cannot be defined uniquely by a data set which is exhausted in providing a two-parameter fit, and as already discussed, there is a precise compensation between the parameters. In spite of the compensation between the parameters in *S. inaequivalvis* HbI, the range of values of allostery c defining the extent of cooperativity is surprisingly narrow. In fact, over a 100-fold range in L, c varies only 2.5 times. Its value corresponds to a 30- to 100-fold difference in affinity between the R and T states. The intrinsic cooperativity, as defined by c, is 3 to 10 times higher than the apparent cooperativity expressed by the Adair parameters. The value of c, although less than in mammalian hemoglobins, is quite comparable with reported results for trout I hemoglobin (25).

It is clearly impossible to specify a structural mechanism for cooperativity from the data for *S. inaequivalvis* HbI which are available at present, but some general comments are in order. First, cooperativity appears to have the same kinetic

**Fig. 11.** Dye-laser photolysis of *S. inaequivalvis* HbI₅. Protein concentration, ~16 μM; free oxygen concentrations (micromolar): O, 1.2; 2.4; C, 4.8; A, 9.6, giving saturations before flashing of 0.16, 0.25, 0.35, and 0.51, respectively. Path length, 2 mm; observation wavelength, 436 nm; 0.1 M phosphate buffer, pH 7.0, 20°C. The results are presented as semilogarithmic plots covering 99% of the rebinding reaction.

**Fig. 12.** Simulation of the concentration of the T-liganded form after flash photolysis of *S. inaequivalvis* HbICO using the allostery model described in the text. Data points are taken from the experiments reported in Fig. 4. A: full photolysis; B: 41% photolysis. Solid lines were calculated using the following constants: \( k_T = 0.086 \mu M^{-1} s^{-1} \); \( k_R = 0.114 s^{-1} \); \( k_K = 0.302 \mu M^{-1} s^{-1} \); \( k_H = 0.01 s^{-1} \); \( L = 10 \).
basis in *S. inaequivalvis* Hbl as in human hemoglobin and this seems to apply to both ligands, oxygen and carbon monoxide. However, at a structural level, the breakage of salt linkages between the subunits, an important term contributing to cooperativity in human hemoglobin, cannot be invoked for *S. inaequivalvis* Hbl whose functional properties are independent of pH. It may be envisaged that hydrogen bonding and/or hydrophobic interactions at the subunit interfaces contribute to the free energy of interaction, as in the case of trout HbI which does not show heterotropic effects in ligand binding either (26). Second, while in human hemoglobin the detailed knowledge of the quaternary structural change taking place upon ligation allows one to associate cooperativity with distinct molecular events, in *S. inaequivalvis* Hbl the relative motion of the chains following ligation is unknown. In the homodimeric hemoglobin from the related species *A. brough-tonii*, the oxygenated and deoxygenated derivatives differ in quaternary structure, since the reactivity toward iodoacetamide and 1-anilinonaphthalene-8-sulfonate is ligand-linked (27).

The body of these results indicates that a ligand-linked quaternary change, postulated on the basis of the cooperative oxygen binding, can actually be demonstrated although the lack of information on the three-dimensional structure does not allow one to define the structural change in any detail.

Finally the similarities in the behavior of the *S. inaequivalvis* homodimer and tetrameric hemoglobins, emphasized in this paper, show once and for all that the presence of different polypeptide chains is not a prerequisite for a cooperative hemoglobin. It is challenging to postulate that the homodimers of Arcid hemoglobins are a form of ancestral minimum cooperative unit whose mechanism of interaction may have been partially preserved during evolution. Hence, it is not impossible that a similar mechanism with preferential interactions within the dimers may also operate in mammalian hemoglobins, although the tetramer is essential for the heme-heme interactions. There is at present simply no reason to believe or to exclude this possibility, but if future studies succeed in defining the pathways of interaction in *S. inaequivalvis* Hbl, an answer may emerge.

Acknowledgment—We wish to thank Dr. Saverio Condò for performing the measurements of the partition coefficient between oxygen and carbon monoxide.