Functional Aspects of the Subunit Association-Dissociation Equilibria of Hemoglobin

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S. J. Edelstein,* Mark J. Rehmarr, John S. Olson,‡ and Quentin H. Gibson§

From the Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850

SUMMARY

Human hemoglobin has been studied in parallel experiments on structure and function. Structure has been examined in terms of the extent of dissociation into subunits in very dilute solutions by sedimentation velocity and equilibrium experiments with a scanning ultracentrifuge. Functional properties of the hemoglobin solutions have been evaluated by two rapid kinetic approaches, stopped flow mixing experiments with deoxyhemoglobin and carbon monoxide and flash photolysis experiments with CO-hemoglobin. The experiments indicate that the dissociation of tetrameric CO-hemoglobin into dimers is accompanied by a transition in kinetic properties from a slow rate of recombination with CO following a flash (k \( \approx 1.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1} \)) to a rapid rate of recombination (k \( \approx 6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} \)). The correlation of dissociation into dimeric units with enhancement in CO recombination rate is maintained under several conditions for which the tetramer-dimer equilibrium is considerably altered by the addition of salts, such as NaCl and triethylamine-HCl. In the standard buffer, 0.1 M phosphate, pH 7.0, the tetramer-dimer equilibrium is described by a dissociation constant of about \( \times 10^{-6} \text{ M} \) (in heme).

The dissociation of deoxyhemoglobin into subunits has also been studied by ultracentrifugation of dilute solutions under anaerobic conditions in the presence of dithionite. Compared to liganded hemoglobin, deoxyhemoglobin has a much lower tendency to dissociate, and the dissociation is much less sensitive to the addition of salts. In phosphate buffer alone, a dissociation constant of \( K_{d,1} \approx 10^{-7} \text{ M} \) is found. Values for the dissociation constant of about \( 5 \times 10^{-8} \text{ M} \) are obtained under conditions which facilitate dissociation (2 M triethylamine-HCl). Companion studies, on the functional properties of these solutions by stopped flow kinetic analysis, show no transition in kinetic properties in the range of protein concentration where dissociation to dimers occurs. The slowly reacting behavior typical of strong solutions of tetrameric hemoglobin (k \( \approx 1.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1} \)) is retained even at concentrations as low as \( 5 \times 10^{-8} \text{ M} \) where dissociation to dimers is extensive.

The observation that the dissociation of liganded and unliganded tetrameric hemoglobin gives rise to dimers with different functional properties may indicate the formation of different dimers, i.e. dissociation at different planes in the parent tetrameric molecules. The markedly lower sensitivity of the dissociation of unliganded hemoglobin to salts compared to liganded hemoglobin is consistent with this possibility. Further evidence is obtained by dissociation experiments on hemoglobin reacted partially or fully with \( \beta \)-mercuribenzoate. The results of these experiments, evaluated in terms of Perutz's atomic models of hemoglobin obtained by x-ray crystallography, suggest that liganded hemoglobin dissociates into dimers of the \( \alpha\beta \) type, whereas deoxyhemoglobin dissociates into dimers of the \( \alpha\beta_2 \) type.

In spite of the remarkable progress made in recent years in determining the detailed structure of hemoglobin by Perutz (1), the molecular mechanism of the cooperative binding of oxygen remains uncertain. One approach to resolving this mechanism involves determining whether or not cooperativity is uniquely associated with tetrameric hemoglobin. Were smaller units such as an \( \alpha\beta \) dimer also competent in generating cooperative binding of ligand, attention could be directed at resolving the essential interactions in the simpler two-chain units in order to understand cooperativity, as has been proposed especially by Wyman (2), Antonini (3), and Guidotti (4). Alternatively, if cooperativity is present solely at the tetrameric level, the various possible interactions between individual chains must be considered.

Many studies during the last several years have been directed at resolving the question of the cooperativity of dimeric hemoglobin. The Rome laboratory has been especially active in this area and has attributed a high degree of cooperativity to dimers. Their conclusions were reached on the basis of experiments showing that hemoglobin is apparently dissociated to dimers by the addition of large amounts of salt, with little or no loss in cooperativity (5, 6). Similar conclusions were reached in kinetic experiments. A rapidly reacting species (typical of non-cooperative hemoglobins), observed by flash photolysis experiments with dilute hemoglobin solutions, was attributed to single (monomeric) chains, with dimeric units assumed to retain the slowly reacting character (7, 8).

In contrast to the earlier studies of structure and function, the
experiments described here involve measurements of subunit dissociation in dilute solutions performed with the scanning ultracentrifuge (9, 10). With this method it is possible to obtain information on solutions at the low concentrations employed in the kinetic measurements. In addition the opportunity to perform the structural and functional studies in parallel and often on the same solutions has minimized sample variations that have been a major problem in the past. The importance of simultaneous structure-function work is revealed by the finding that the rapidly reacting form seen in flash experiments is now believed to be dimeric hemoglobin, not the monomeric form as had been concluded previously (7, 8). This observation, in conjunction with experiments which indicate that the dimeric form of deoxyhemoglobin is slowly reacting in stopped flow experiments, has led to the hypothesis that tetrameric hemoglobin dissociates into a noncooperative dimer when liganded and a cooperative dimer when present as deoxyhemoglobin. Presumably both dimers are of the $a_2b$ type although distinguished by different bonding surfaces at the interchain contacts. The possibility of two types of dimers was first suggested by Antonini et al. (11) in response to a preliminary report (12) of the work described in this paper.

**EXPERIMENTAL PROCEDURE**

**Materials**—Human hemoglobin, obtained as a pooled sample from the Cornell Clinic, was prepared from fresh blood by washing the erythrocytes with 0.9% NaCl and lysing with 3 volumes of distilled water. The preparation was then centrifuged to remove cell debris and stored in the cold. Solutions were discarded after 1 week of storage. In most instances, experiments were performed within 1 or 2 days of the preparation of the samples. All chemicals were reagent grade.

**Kinetic Analysis**—Flash photolysis experiments on CO-hemoglobin were performed with the apparatus described by Parkhurst and Gibson (13). In addition to following the course of the reaction on an oscilloscope, the output of the photomultiplier was directed to an analogue-digital converter and the digital values were collected in the memory bank of a small computer. For details of the computer system see DeSa and Gibson (14). From each kinetic progress curve hundreds of digital values were collected in the memory bank of a small computer. For details of the computer system see DeSa and Gibson (14). From each kinetic progress curve hundreds of digital values were collected in the memory bank of a small computer. For details of the computer system see DeSa and Gibson (14).

Stopped flow experiments were performed on the reactor of a spectrophotometer with a 2-cm light path and light of 432 or 418 m. With this arrangement hemoglobin solutions at concentrations as low as 0.1 to 0.2 $\mu$m could be examined. Solutions of CO were prepared by bubbling water with the pure gas and diluting the saturated solution into the appropriate buffers to give a final concentration of 5 to 10 $\mu$m. Where strong salt solutions were used, special care was taken to ensure equal concentrations of the salt in both syringes of the stopped flow apparatus to avoid artifacts due to refractive index gradients in the observation tube.

A standardized procedure for the preparation of deoxyhemoglobin solutions for the stopped flow experiments was followed. To prepare a very dilute solution of deoxyhemoglobin the deoxygenation was carried out with material at full strength (2 to 3 mM) by the addition of dithionite. The reduced solution was then diluted into deaerated buffers containing small amounts of dithionite in a closed system using syringes. This procedure was necessary to ensure reproducible results. If dithionite was added to solutions of oxyhemoglobin already diluted (~1 $\mu$m), a rapid component in the solutions could sometimes be observed in unpredictable amounts.

**Ultracentrifugation**—Sedimentation equilibrium and velocity experiments were performed with a Spinco model E analytical ultracentrifuge equipped with the absorption optical system and automatic scanning accessory. The system was focused and tested as described by Schachman and Edelstein (9). Linearity of the recording system (with the calibration step correction) was checked by cross-referencing concentration measurements on a protein solution at sedimentation equilibrium using the interference optical system as the independent variable (9). Myoglobin was employed for this purpose. For light in the Soret region, linearity was observed to within experimental limits between 0 and 1 optical density unit as long as the monochromator slit was set at 0.3 mm or less.

Sedimentation velocity experiments were performed with double sector cells (12 mm path length), examining cells singly or in combinations with the multiplex attachment and a multihole rotor. A number of experiments with very dilute solutions were performed in 30-mm cells. Data were recorded at fixed time intervals and the boundary position estimated from the half-height at the plateau concentration for the calculation of sedimentation coefficients. Experiments on deoxyhemoglobin were performed on solutions prepared by diluting a deoxygenated stock solution into buffers containing dithionite (3 mg per ml) with all transfers and loading cells conducted in a nitrogen glove bag.

Sedimentation equilibrium experiments were performed on liganded and ligand-free hemoglobin with short columns of solution (1 to 3 mm). Data obtained in the form of recorder deflection versus radial distance were entered into a program written for the Cornell IBM 360/65 computer which (a) converted the deflections into optical density units on the basis of calibration steps, (b) converted the radial distance into absolute distance in the rotor, and (c) fitted the data by standard matrix methods and least squares criteria to an equation of the form

$$C(r) = \sum C_i(r) = \sum a_i \exp \frac{M_i(1 - \varepsilon_i)2\pi r^2}{2RT}$$

In this equation, $C(r)$ represents the total concentration at the point $r$, and $C_i$, where $i = 1, 2, \text{or } 4$, represents monomers, dimers, and tetramers, respectively. In general, two exponential terms in the summation gave a satisfactory representation of the data with values of $M_i$ of 32,230 and 64,500, corresponding...
to dimers and tetramers. In a number of cases, dissociation was more extensive and values of $M$ of 16,125 and 32,250 were employed. In all cases the range of concentrations examined was sufficiently small so that a third term provided no additional physically meaningful information. While introduction of a third term of course lowered the calculated root mean square deviation of $C_1$, the coefficient of the term was either very small or negative. Therefore, only two terms were employed. The standard deviation of the experimental points in the two-term fitting procedure was close to 0.005 optical density in all cases described here.

The exponential fitting procedure was employed because of the ease in obtaining subunit dissociation constants from the fitting equation. Each term in the sum represents the total concentration (in optical density units) of the corresponding species (monomer, dimer, or tetramer) at the point $r$. Therefore, the equilibrium constant relating two species can be expressed in terms of the fitting coefficients, $a_i$. For example, the tetramer-dimer dissociation is given by

$$K_{4,2} = \frac{N_4}{N_2} = \frac{(C_4/M_4)^2}{C_2/M_2} = \frac{(a_4 \exp M_4/\theta^2)}{(a_2 \exp M_2/\theta^2)} = \frac{2(a_4)^2}{a_2M_2}$$

The $\beta$ represents the expression, $(1 - \theta^2)\theta^2/2RT$. The dissociation constant from the coefficients $a_i$ is expressed in optical density units and can be converted to molar units on the basis of the appropriate extinction coefficients (see below). Equilibrium constants are thus conveniently obtained along with the fitting. In addition a sensitive criterion for the homogeneity of the equilibrium system is provided when the values are examined for experiments at different initial concentrations or speeds (17).

In addition to fitting the entire distribution of protein in terms of the assumed species, data may be examined in a point-wise fashion by fitting small regions of the solution column independently (18, 19). This method is most desirable when non-ideality prohibits analysis of the data in terms of simple exponential sums. However, data treated in point-wise fashion generally are expressed in terms of $M_w$ versus $c$. Since this form is more familiar and simplifies comparison of the results of different experiments, the data reported here, while fitted exponentially, are expressed graphically as $M_w$ versus $c$. Values of $M_w$ are obtained from the differentiated form as shown in Equation 1.

$$M_w(r) = \frac{1}{C(r)} \sum M_i a_i \exp (M_i \theta^2)$$

Extinction Coefficients—To obtain the absolute concentrations of the solutions examined in the ultracentrifuge, effective extinction coefficients for the conditions of the measurements were determined. An aliquot of each solution examined in the ultracentrifuge was retained and its spectrum recorded in a Cary 14 spectrophotometer. In addition, scanner traces were recorded for various wave lengths as soon as the rotor containing the solution reached the speed of operation. By comparing the observed optical density at the appropriate monochromator and slit width settings with concentration determined from the measured spectrum and published extinction coefficients (20), the effective extinction coefficient to apply to the centrifugation data was obtained. The effective coefficients were generally different from the standard values by about 5 to 10%, and the corrected values were used in translating the dissociation constants into concentration units. The calibration operations were repeated with each experiment.

An additional consideration concerns the possibility of changes in absolute extinction of hemoglobin accompanying dissociation. While it is likely that liganded hemoglobin subunits effectively have the same absorption properties as the tetramer, a possibility exists of differences in the deoxy form between tetramers and dimers (21). Significant errors could be introduced in the analysis of dissociation with the ultracentrifuge since extinction differences would require weighting of the amount of dimer relative to tetramer. To establish whether or not such an extinction change occurs in passing from tetramer to dimer, concentration difference spectra and analysis of a dilution series in terms of Beer's Law were conducted with the Cary 14 recording spectrophotometer. To within the limit of a few per cent, no change in extinction in deoxyhemoglobin occurs in the range of 0.1 to 1.0 $\mu M$ where dissociation appears to occur (see “Results”). Therefore, a single extinction coefficient was applied to both dimers and tetramers of deoxyhemoglobin.

**RESULTS**

Studies on Liganded Hemoglobin

These studies began with an attempt to establish the identity of the fast reacting species observed in dilute solutions of CO-hemoglobin in flash photolysis experiments (16). The proportion of rapid material in solutions increases with dilution and hence must be a product of dissociation of the tetramer, presumably either dimers or monomers. The dissociation of a small molecule could also be responsible. While Antonini, Chiancone, and Brunori (7), and Antonini, Brunori, and Anderson (8) had concluded that the rapid behavior was due to monomers of hemoglobin, a consideration of the concentration at which the material represents about 50% of the population (about $\mu M$ in home) (7) with preliminary data on the subunit dissociation equilibria of liganded hemoglobin (22) suggested that dimers may be the rapid species. To settle this point a series of parallel sedimentation velocity and flash photolysis experiments were undertaken, with solutions of concentrations in the range 0.1 to 10 $\mu M$ examined simultaneously by the two methods. A typical flash photolysis curve is shown in Fig. 1. The curve is clearly biphasic, with a fitted value of the proportion of the rapidly reacting material of 50%. The same solution described in Fig. 1 was examined by sedimentation velocity. The position of the sedimentating boundary as a function of time is shown in Fig. 2. The optical density present, about 0.3, is sufficient to define very well the dependence of the boundary position on time. The data give a value of $s = 3.59 S$.

The observed value of the sedimentation coefficient is roughly midway between the values generally ascribed to dimers, 2.8 to 2.9 S and tetramers, 4.6 to 4.7 S. A value of 4.6 S has been suggested by Kuwabara, Kirschner, and Tanford (23) while Chiancone et al. (24) favor a value of 4.7 S. Taking an average value, 4.65 S for the tetramer and 2.85 S for the dimer, the proportion of the molecular population as dimer can be calculated by the method of Kirschner and Tanford (25). This method defines the degree of dissociation, $\alpha$, by

$$\alpha = \frac{4.65 S - 8(\text{observed})}{4.65 S - 2.85 S}$$

The proportion dimer for the experiment shown in Fig. 2 is indicated to be 59% with an uncertainty of $\pm 3\%$ due to the uncertainty of 0.1 S in the sedimentation coefficients of the tetramer.
Flash photolysis of carboxyhemoglobin. The change in optical density following the flash is given on the ordinate versus the time in milliseconds on the abscissa. The experiment was performed with a solution $1.5 \times 10^{-6} \text{M}$ in heme. The buffer was $0.1 \text{M}$ potassium phosphate, pH 7.0. The experiment was conducted at $22^\circ$.

Sedimentation velocity of carboxyhemoglobin. The logarithm of the radial distance of the half-height position of the boundary, $r$, versus time (in minutes). The experiment was performed with the same solution examined by flash photolysis (Fig. 1). Data were collected using light of 405 nm, rotor speed of 60,000 rpm, and a rotor temperature of $22^\circ$.

The possibility that the system is composed of only tetramers and monomers is eliminated by actual measurement of the appearance of monomers at even greater dilutions (22) and by the ability to fit a complete curve with a tetramer-dimer dissociation constant (see below).

The estimate of 59% dimer is sufficiently close to the value of 56% rapidly reacting material obtained for the same solution to suggest that the dimer is the rapidly reacting material. To test the correlation of rapid behavior with dimers, a number of other solutions were examined at different concentrations (and therefore different degrees of dissociation). For each solution examined from approximately 10% dissociation to 90% dissociation, a close parallel was observed between the proportion of dimers and the proportion of rapidly reacting material present in the solutions. These results are shown in Fig. 3 in terms of $\alpha$ versus concentration. The value of $\alpha$ is the fraction of rapidly reacting material in the kinetic studies and the fraction of dimers obtained by sedimentation. The position of the curve gives a tetramer-dimer dissociation constant of $K_{4,2} = 1.5 \times 10^{-6} \text{M}$.

Sedimentation equilibrium of CO-hemoglobin. Optical density on a logarithmic scale versus the square of distance in the rotor. The data are from an experiment on CO-hemoglobin at an initial concentration of $3 \times 10^{-6} \text{M}$ in a solution of $0.1 \text{M}$ phosphate, pH 7.0. Equilibrium was reached at 20,000 rpm at $20^\circ$ in an overnight experiment. Traces were recorded with light of 405 nm; the solution also contained 1 mg per ml of dithionite. See text for complete explanation.
Table I

<table>
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<th>Sample</th>
<th>Initial concentration $K_{4,2}$</th>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>$1.5 \times 10^{-6}$ M</td>
<td>$1.0$</td>
</tr>
</tbody>
</table>

* Experimental details as described in Fig. 4.

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Fig. 5. Sedimentation equilibrium of oxyhemoglobin. Weight average molecular weight versus concentration. Upper curve, solution in 0.1 M potassium phosphate; lower curve, solution in 0.1 M potassium phosphate plus 2 M triethylamine hydrochloride. The experiment was conducted at 20°, rotor speed 34,000 rpm with an initial concentration of hemoglobin of $3 \times 10^{-5}$ M in both cases. Dashed lines on the ordinate labeled $M_1$, $M_2$, and $M_4$ correspond to the values of monomers, dimers, and tetramers. Data were recorded at 405 nm. A value $v = 0.749$ cm per g was employed in the calculations.

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Table I, larger variations in the absolute value of the dissociation constant have been found among different samples, depending on the exact history of the sample. These effects will be treated in detail in a future publication. The data of the type shown and summarized in Table I and Fig. 4 may also be represented in terms of $M_w$ versus $c$ (Fig. 5). Since a dissociation constant is determined in a single sedimentation equilibrium experiment, the effects of various agents, e.g., salts, on the dissociation equilibrium can be conveniently studied. A number of experiments along these lines were undertaken to define conditions which alter $K_{4,2}$ so as to test whether the appearance of rapid material is shifted in a parallel manner. Experiments were undertaken principally with NaCl and triethylamine-HCl and also with MgCl$_2$. As indicated earlier by other workers (5, 25, 27), NaCl and especially MgCl$_2$ markedly increase the tendency of hemoglobin to dissociate into dimers. However, complications due to preferential hydration of the protein in these three-component systems make the exact interpretation of sedimentation results difficult (12). Work is in progress to solve all the necessary parameters of these systems by use of the density perturbation methods involving D$_2$O (10). To permit a preliminary examination of these systems, flash photolysis experiments in 2 M NaCl were compared with the estimate of dissociation taken from the osmotic pressure data of Guidotti (27). With the osmotic pressure method, problems due to preferential hydration are eliminated. Guidotti reported a value of $K_{4,2} = 75 \mu M$ for 2 M NaCl. Flash photolysis in 2 M NaCl indicates the appearance of the rapid form with a dissociation constant of about $70 \mu M$ (Fig. 6). Therefore, the correlation of the rapid species with dimeric hemoglobin also holds for 2 M NaCl. A similar correlation was obtained in 0.5 M MgCl$_2$ where the extent of dissociation is even greater.

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In contrast to the problems associated with NaCl and MgCl$_2$ in terms of defining the buoyant density of the protein, the salt triethylamine-HCl may be employed with no such difficulties since its density is essentially identical with that of water. Studies in this salt indicated that it is potent in dissociating hemoglobin; a 2 M solution produced a mixture of dimers and monomers in the concentration range of interest (see Fig. 5). The dimer-mono-
mer dissociation constant is found to be about 50 \mu M. With flash photolysis experiments in this concentration range, only rapid behavior is observed; therefore, in this salt, dimers also show rapid behavior. In contrast to the other experiments, some monomers, already established as rapid (20), are present.

Studies on Deoxyhemoglobin

In contrast to liganded hemoglobin, the dissociation of deoxyhemoglobin into subunits has been studied relatively little, principally because of difficulties in maintaining anaerobic conditions for the molecular weight measurements. Where results are available, the experiments were conducted in the presence of large quantities of salt (5, 27, 28), yet most functional studies are conducted in dilute buffers. To provide information on the behavior of deoxyhemoglobin under more physiological conditions, a series of measurements was undertaken, first by sedimentation velocity to establish the qualitative features of the system and second by sedimentation equilibrium to provide direct molecular weight information. Parallel function experiments using stopped flow kinetic methods are also reported.

In molecular weight experiments uncertainty has always existed as to whether it is indeed deoxyhemoglobin which is being examined in the ultracentrifuge. Fortunately, with the scanning ultracentrifuge, this concern can be eliminated by determining the spectra during the course of the experiment. This check was carried out for each experiment and most frequently with favorable results of the type shown in Fig. 7. Occasional samples showing less clearly defined deoxyhemoglobin spectra were abandoned.

The sedimentation results indicated that deoxyhemoglobin dissociates in 0.1 M phosphate, pH 7, to a measurable extent, but considerably less than liganded hemoglobin. The presence of dissociation is observed in experiments of the type reported in Figs. 8 and 9. A series of dilutions of deoxyhemoglobin, run singly or together in a multicell rotor, show a clear tendency for the sedimentation coefficient to decrease with decreasing concentration. Solutions examined in two identical buffers, with favorable results of the type shown in Fig. 7. Occasional samples showing less clearly defined deoxyhemoglobin spectra were abandoned.

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While the quantitative aspects of evaluating dissociation constants from the sedimentation velocity experiments are likely to be valid theoretically (25), sedimentation equilibrium experiments are more direct, and were also pursued. In addition to determining the absorption spectrum of each sample in the ultracentrifuge, data were collected and analyzed at both 435 and 405 nm (corresponding to emission maxima of the light source). If even a small amount of oxy- or methemoglobin were present, the data at 405 nm would be expected to indicate more dissociation than data at 435 nm, according to the pattern seen in Fig. 9. Alternatively, if only deoxyhemoglobin is present, the data at 405 and 435 nm should indicate the same dissociation constant,
Fig. 9. Summary of sedimentation coefficients for deoxyhemoglobin (Hb) and CO-hemoglobin (HbCO). The $s_{20,w}$ in Svedberg units is given on the ordinate versus concentration on the abscissa. The data for CO-hemoglobin is taken from Fig. 3 with several additional determinations also included. The line through the points of deoxyhemoglobin is calculated with $K_4 = 0.15 \times 10^{-4}$ M.

Fig. 10. Sedimentation equilibrium of deoxyhemoglobin. As in Fig. 5, with the following exceptions: data recorded at 435 nm; solutions contained 3 mg per ml of dithionite; lower curve, 2 M triethylamine-HCl added. Rotor speeds were 26,000 rpm for the solution without triethylamine-HCl and 40,000 rpm where triethylamine was present.

with higher observed molecular weights at 405 nm for comparable optical densities due to the lower extinction coefficient at that wave length. For each experiment reported these criteria for the presence of deoxyhemoglobin were satisfied.

Results of sedimentation equilibrium experiments on deoxyhemoglobin are shown in Fig. 10. The solution containing 0.1 M phosphate (upper curve) shows quite clearly that molecular weights lower than the value for the tetramer are present. However, the extent of dissociation is small, therefore, the dissociation constant is approximate. A value of about $10^{-7}$ M is found, in agreement with the estimates from sedimentation velocity.

Attempts were made to find conditions which enhanced the extent of dissociation of deoxyhemoglobin, so that more accurate values for the dissociation constant could be determined. The salt, triethylamine-HCl, at 2 M was tried in view of its potent effects on liganded hemoglobin (Fig. 5). As seen in Fig. 10 (lower curve) some enhancement of dissociation is found, but

Table II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of centrifugation (hrs)</th>
<th>Wave length for trace (nm)</th>
<th>$K_4$ (M)</th>
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<td>435</td>
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</table>

*Overall average: $K_{4,c} = 0.5 \mu M \pm 0.1 \mu M$ (S.D.).

Fig. 11. Stopped flow experiments on deoxyhemoglobin. The change in optical density on a logarithmic scale versus time (seconds). The experiments were performed on solutions of 2.3, 1.3, 0.6, and $0.3 \times 10^{-4}$ M (in heme), from top to bottom, respectively. The buffer was 0.1 M potassium phosphate, 2 M triethylamine-HCl, pH 7.0. The temperature was 22°C and light of 435 nm was employed. For other details see text.
not nearly to the degree observed with liganded hemoglobin. Nevertheless, the level of dissociation permitted more quantitative estimates of the dissociation constant. As seen in Table II, values quite close to \(5 \times 10^{-7}\) M are obtained in experiments which also demonstrate that these solutions are stable for long periods and relatively ideal in that determinations with different speeds and initial concentrations provide values of the dissociation constant in reasonably good agreement.

With the patterns of dissociation of tetrameric deoxyhemoglobin into dimers established, experiments were initiated to evaluate the kinetic properties of the deoxygen. Experiments on deoxyhemoglobin with and without added salts in 0.1 M phosphate were conducted with emphasis on solutions in the \(10^{-2}\) to \(10^{-4}\) M range. Although a structural transition (tetramer-dimer dissociation) occurs in this range, no change in kinetic properties was observed. For example in 2 M triethylamine-HCl, experiments in the range 0.3 to 2.3 \(\times 10^{-6}\) M give rise to essentially parallel curves with the usual slow rate (Fig. 11). At the lowest concentration in this range, the hemoglobin is about half-dissociated to dimers. At the highest concentration, less than a quarter of the population should be dissociated. The curves also show the progress acceleration typical of the reaction of deoxyhemoglobin with ligands.

**Studies on p-Mercuribenzoate Derivatives of Hemoglobin**

Since the dimer produced from liganded hemoglobin reacts rapidly in kinetic experiments, while the dimer generated from deoxyhemoglobin retains the slow behavior, the possibility that structurally different kinetic species are involved was explored. Should different planes of tetrameric hemoglobin be involved in the dissociation of the liganded and unliganded forms, the effects of reaction with PMB\(^1\) could be expected to differ for oxy- and deoxyhemoglobin. Two degrees of reaction are possible. First, oxyhemoglobin can be reacted with 1 mole of PMB per \(\beta\) chain. In this case, the reaction is stoichiometric with the \(\beta 83\) sulfhydryl group and rapid (30). Prolonged incubation with a large excess of PMB results in derivitization of the remaining sulfhydryl groups at the \(\alpha 104\) and \(\beta 112\) positions (30). The \(\beta 83\) group is located at the \(\alpha 1-\beta 1\) interface whereas the two less reactive groups lie at the \(\alpha 1-\beta 1\) interface (1). Since one or the other of these two interfaces is likely to be involved in the dissociation of either liganded or unliganded hemoglobin, comparison of the effects of full or partial reactions of PMB with oxy- and deoxyhemoglobin could provide indications of the domains of subunit bonding involved in each dissociation. Therefore, deoxyhemoglobin partially and fully reacted with PMB was prepared and examined in both the liganded and unliganded forms. As seen in Fig. 19, reaction at the \(\beta 83\) position leads to a marked enhancement of the dissociation of oxyhemoglobin with relatively little or no effect on deoxyhemoglobin. Rosemeyer and Huehns (31) also observed facilitation of dissociation of oxyhemoglobin with PMB although deoxyhemoglobin was not examined.

Extended reaction of hemoglobin with PMB has only a small additional effect on the dissociation of either deoxyhemoglobin or oxyhemoglobin. This finding is surprising in view of the ease of separating the chains of fully reacted hemoglobin (30) and the location of the relatively unreactive \(-SH\) groups deep in the \(\alpha 1-\beta 1\) interface (1). Other workers have also observed an equi-

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\(^1\) The abbreviation used is: PMB, p-mercuribenzoate.
with CO, the other slowly reacting with CO. While hemoglobin has been generally held to dissociate into dimers of the α1-β2 type, dissociation into α1-β3 dimers is also feasible considering the structure of hemoglobin as seen in the atomic models (1). The α1-β3 contact is the most extensive interaction between subunits involving some 34 residues with contributions from the B, C, G, and H helices of the α chain and the C, D, G, and H helices of the β chain as well as nonhelical regions. In comparison, only 19 residues participate at the α1-β2 contact being derived from the C, G, and H helices of the α chain and the G and C helices of the β chains and additional nonhelical regions (1). However, when the number of atomic contacts is considered, the differences appear diminished; the α1-β2 contact involves some 110 atoms, whereas about 80 atoms are within contact distance (4 Å) at the α1-β3 interface. Since the absolute energies of the contacts are difficult to estimate, the possibility cannot be excluded that the α1-β3 contact, although involving fewer atoms than the α1-β2 contact, represents an association as stable or even more stable than the α1-β2 interface. Moreover, the transition from oxyhemoglobin to deoxyhemoglobin involves a rearrangement that brings additional atoms into contact in the α1-β3 interface. Therefore, the crystallographic results are consistent with the view that deoxyhemoglobin is predominantly stabilized at the α1-β3 interface whereas oxyhemoglobin is predominantly stabilized at the α1-β2 interface. The α1-β2 contact, located very near the oxygen-binding heme sites, would then be visualized as constraining the initial ligand-binding reaction. As the molecule becomes progressively saturated with ligand, a transition to the α1-β1 stabilized form occurs with a relaxation of the constraint created by the α1-β2 interface. This α1-β1 stabilized form would be the high affinity tetramer observed in partial flash photolysis experiments (33). Whether the structural transition occurs in all-or-none fashion (34) or gradually (35, 36) remains to be resolved. The importance of the α1-β2 interface in providing the constraint which ultimately leads to cooperative properties is also reflected in the marked changes in cooperativity of mutants located in that region of the molecule (37). Some minor interactions between like chains of hemoglobin probably contribute additional stability to the tetramer, especially in the deoxy form and these interactions have been implicated in the Bohr effect (38).

With the products of dissociation of deoxyhemoglobin and CO-hemoglobin tentatively identified as α1-β2 and α1-β1 (CO)2, respectively (with oxygen equivalent to CO), the structure-function experiments can be given a more precise interpretation. The various aspects of the subunit dissociation and ligand-binding reactions are summarized in Fig. 13. Both Hb4 (deoxyhemoglobin) and its dimeric subunits react slowly with CO. To signify this aspect of the dimers produced from deoxyhemoglobin, we refer to the α1-β2 stabilized dimer as slow or S-dimers (S-Hb4). The dimer resulting from dissociation of Hb4(CO)2, the α1-β1 stabilized dimer, is referred to as the rapid or R-dimer (R-Hb4).

The various dimeric and tetrameric species may also be related by other paths of conversion. The transition of rapidly recombining to slowly recombining material seen upon prolonged illumination of CO-hemoglobin (5) may now be interpreted as resulting from the formation of Hb4 from R-Hb4 (dashed pathway labeled 1 in Fig. 13), rather than reflecting association of monomers as suggested by Antonini et al. (8). In addition, the transformation of S-Hb4(CO)2 into R-Hb4(CO)2 (Fig. 13, dashed pathway 2) could be monitored in principle in appropriate flow-flash experiments. Some liganded monomers would also be expected to be present in equilibrium with the carboxy R-dimers.

In conclusion, it may be pointed out that although the interpretation of the kinetic and ultracentrifuge studies described here differ significantly from earlier ideas, and in particular deny a significant role to the monomer in the concentration range examined, few of the actual experimental findings differ importantly from those of earlier workers. Thus, an effect of dilution on kinetic behavior in photochemical experiments was reported more than 10 years ago (39) and the apparently normal behavior of deoxyhemoglobin in very dilute solutions both with high and low salt concentrations has also been described (7). The essential new feature is the use of the scanning ultracentrifuge which has supplied information on the state of aggregation of hemoglobin, not only in the same concentration range, but frequently on the same solutions as those used in the kinetic experiments and has permitted more incisive interpretations of the results.

Note Added in Proof—A number of recent experiments combine to cast doubt on the idea that there may be two types of dimers.

1. Dr. C. M. Park (J. Biol. Chem., 245, in press (1970)) has carried out detailed hybridization studies which suggest that dissociation of oxy- and deoxyhemoglobins, if it occurs, must do so at the same interface.

2. Unpublished kinetic studies by Andersen, Moffat, and Gibson and ultracentrifuge studies by Moffat and Edelstein on the behavior of hemoglobin at high pH, where extensive dissociation of deoxyhemoglobin to dimers occurs, show that the deoxy-dimer then formed reacts rapidly with CO.

3. The most recent interpretation of the atomic models of hemoglobin does not suggest that the preferred plane of splitting changes on ligand binding (Dr. H. Muirhead, personal communication).

There does not seem to be room for doubt of the accuracy of the experimental findings reported in this paper, and we suggest that perhaps the observed dissociation of deoxyhemoglobin in the ultracentrifuge is due to the combination of great dilution, presence of large amounts of dithionite, and prolonged contact with the centrifuge cell. In this view deoxyhemoglobin is always

\[
\begin{align*}
2 \text{S-Hb}_4 + 4\text{CO} & \xrightleftharpoons{\text{slow, accelerating}} 2 \text{S-Hb}_4(\text{CO})_2 \\
\text{K}_{d1} \approx 10^{-7} \text{M} & \text{R-Hb}_4 + 4\text{CO} \xrightleftharpoons{\text{slow accelerating}} \text{Hb}_4(\text{CO})_4 \\
\text{K}_{d2} = 2 \times 10^{-4} \text{M} & \text{R-Hb}_4 + 4\text{CO} \xrightleftharpoons{\text{rapid, homogeneous}} 2 \text{R-Hb}_4(\text{CO})_2
\end{align*}
\]

**Fig. 13.** Descriptive scheme for the subunit dissociations and ligand binding reactions of hemoglobin. Details are discussed in the text.

\[
2 \text{R-Hb}_4 + 4\text{CO} \rightarrow 2 \text{R-Hb}_4(\text{CO})_2
\]
tetrameric under the less severe conditions of the stopped-flow mixing experiments with CO, explaining the slow kinetic behavior observed without recourse to a $\alpha_2\beta_2$ type dimer.

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