Kinetics of Deoxyhemoglobin Subunit Dissociation Determined by Haptoglobin Binding: Estimation of the Equilibrium Constant from Forward and Reverse Rates†

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ABSTRACT: Deoxyhemoglobin tetramers dissociate into dimers very slowly, with half-times on the order of several hours. It is demonstrated that absorbance changes in the Soret region which accompany this dissociation and persist upon binding of haptoglobin 1-1 to the dissociated dimers can be used for accurate kinetic determinations over the necessarily long periods required for study. This method of study for the slow reactions depends upon long-term spectral integrity of the reaction mixtures and upon accurate measurement. The variation in rate constants determined by this procedure has been correlated with variations in structural constraints at the dimer-dimer contact region. In the presence of 2,3-diphosphoglycerate the rate constant is decreased, consistent with the role of this effector in binding to both β chains and stabilizing the constrained deoxy tetramer against dissociation into $\alpha\beta$ dimers. With hemoglobin specifically modified (des-Arg-141α) to eliminate half the constraining salt links within the dimer-dimer contact region, the dissociation rate is increased by approximately three orders of magnitude. In hemoglobin S where the amino acid substitution is not directly in the intersubunit contact region of interest, the dissociation rate is found to be approximately the same as that for hemoglobin A. Combination of the dissociation rate constants determined by haptoglobin binding with stopped-flow determinations of the rate constant for reassociation of dissociated dimers provides an estimate of the equilibrium constant, ${}^0K_2$, for the deoxyhemoglobin dimer–tetramer equilibrium. This estimate is independent of any assumptions regarding other energetic quantities, and yields a value of $2.54 \pm 0.7 \times 10^{10}$ M^{-1} (heme) in 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM EDTA, pH 7.4, 21.5 °C. Thus the intersubunit contact energy is -14.0 ± 0.2 kcal/mol of heme. The stabilization energy between deoxy and oxy tetramers is found to be approximately 6.4 kcal/mol, under these conditions.

Dissociation of hemoglobin tetramers into $\alpha\beta$ dimers near neutral pH eliminates the intersubunit contact plane where major changes in quaternary structure are known to occur upon oxygenation. This contact region contains a number of specific chemical linkages (e.g., the critical salt bridges and hydrogen bonds) which are believed responsible for stabilizing the deoxy quaternary complex and which are destroyed upon oxygenation (Perutz, 1970; Perutz and Ten Eyck, 1971). Experimental studies on the kinetics and thermodynamics of dissociation processes affecting this contact region can therefore provide basic information regarding the molecular events which accompany cooperative ligand binding (Noble, 1969; Weber, 1972; Thomas and Edelstein, 1972, 1973; Ackers and Halvorson, 1974). In this paper we report results of measurements on the kinetics of deoxyhemoglobin subunit dissociation determined by haptoglobin binding. We have also carried out stopped-flow determinations of the rate constant for reassociation of dissociated dimers. Combination of these forward and reverse rate constants permits us to estimate the equilibrium constant and free energy for dissociation of unliganded tetramers into dimeric species. We report here the first determination of this constant which is independent of any assumptions regarding other energetic quantities to which the dissociation is linked.

Previous studies (Nagel and Gibson, 1971) have shown that human haptoglobin 1-1 binds very rapidly to hemoglobin dimers, but not to tetramers. Using stopped-flow experiments Nagel and Gibson have demonstrated the utility of this binding reaction for probing significant hemoglobin structural variations (Nagel and Gibson, 1972). From measurements of fluorescence quenching or absorbance changes with time, it has been possible to study the binding rates of haptoglobin in solutions of liganded hemoglobins and with certain rapidly dissociating unliganded hemoglobins having structural modifications within the $\alpha_1\beta_2$ contact region (Bunn et al., 1974; Nagel and Gibson, 1972). In these stopped-flow experiments haptoglobin binding by unliganded hemoglobin A has usually not been detectable (Nagel and Gibson, 1971). In one instance, however, a very slow haptoglobin binding has been reported in solutions of stripped deoxyhemoglobin A (Nagel and Gibson, 1972). This observation is consistent with a rate-limiting slow dissociation of the deoxy tetramers into dimeric species.

Hybridization studies using anaerobic isoelectric focusing in polyacrylamide gels (Park, 1973; Bunn and McDonough, 1974) and anaerobic ion-exchange chromatography (Williams and Kim, 1975) have established the existence of slow dissociation (i.e., with half-times of several hours) by deoxyhemoglobin tetramers. The present study is an exploration of this slow process. We will demonstrate that absorbance changes which accompany the haptoglobin binding reaction can be used for accurate kinetic determinations over the necessarily long periods required for study, and that the rate constants measured can be correlated with

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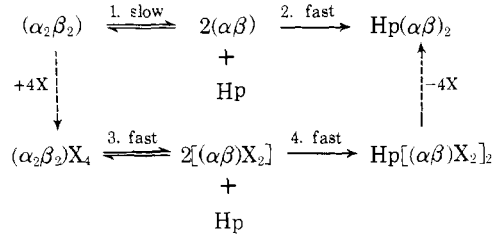
variations in structural constraints at the dimer–dimer contact region. The reactions to be considered are depicted by Scheme I:

Scheme I



In this scheme the upper and lower reaction sequences denote the reversible dissociation of hemoglobin tetramers, followed by (essentially irreversible) haptoglobin binding in the unliganded (upper) and fully liganded (lower) states. Vertical arrows denote steps of oxygenation of hemoglobin tetramers (left) and deoxygenation of the complex formed between oxygenated hemoglobin and haptoglobin (right).

It is reaction 1 that we are interested in studying. The optical property to be used is primarily the absorbance change in the Soret which is associated with the transition between the constrained quaternary structure of deoxy tetramers and the unconstrained $\alpha\beta$ dimers in either their free dissociated state, or as part of the $[Hp(\alpha\beta)_2]$ complex (Brunori et al., 1968; Kellett and Gutfreund, 1970; Anderson et al., 1971; Perutz et al., 1974; Kawamura et al., 1972). A second property is fluorescence quenching of haptoglobin associated with formation of the $Hp(\alpha\beta)_2$ complex (Chiancone et al., 1968). Use of these optical properties to study reaction 1 under deoxy conditions requires a rapid rate for (essentially irreversible) reaction 2 in comparison with reaction 1. Reactions 2–4 have previously been characterized kinetically (Nagel and Gibson, 1971; Anderson et al., 1971) and found to have rate constants of: $5.5 \times 10^5\ M^{-1}\ s^{-1}$ (reaction 2), $6.5 \times 10^5\ M^{-1}\ s^{-1}$ (reaction 4 with CO as ligand), and $2\ s^{-1}$ (reaction 3) under conditions of neutral pH and low salt. Previous estimates for the rate constant of reaction 1 in the absence of DPG[1] at neutral pH have been 0.018–0.054 h^{-1} (Park, 1973) and 0.30 h^{-1} (Bunn and McDonough, 1974). These reactions thus satisfy the criteria for use of the optical properties mentioned above in studying reaction 1.

Materials and Methods

Purification of Haptoglobin. After screening a number of individuals according to haptoglobin type (Smithies et al., 1962), a sample of fresh human serum was obtained from which haptoglobin 1-1 was isolated according to the method of Connell and Shaw (Connell and Shaw, 1961) with minor modifications. The pH of the serum was adjusted to 4.7 with 1.0 M acetic acid and subsequently desalted on a Sephadex G-25 column in distilled H_2O. The salt-free serum was applied to a DEAE-cellulose column previously equilibrated with 0.01 M NaOAc buffer (pH 4.7) and washed with the same buffer until the absorbance of the effluent was less than 0.01. Haptoglobin was eluted with 0.09 M NaCl in water. The effluent was twice precipitated with ammonium sulfate at pH 7.0. The fraction precipitating between 45 and 55% saturation was collected, and dissolved in distilled H_2O. After dialysis against several changes of distilled water, the protein was lyophilized and stored at −20 °C.

Hemoglobins. Hemoglobin A was purified from freshly drawn blood by the method of Williams and Tsay (Williams and Tsay, 1973) and stored in liquid nitrogen until use. Hemoglobin S was a gift of Dr. Robley S. Williams, Jr. (Department of Biology, Yale University) and was purified similarly to the hemoglobin A.

Des-Arg-141α hemoglobin was a gift of Dr. Ronald Liem (Department of Biochemistry, University of Pennsylvania School of Medicine) and was prepared according to the method of Antonini et al. (1961). Electrophoresis of the des-Arg sample on polyacrylamide gel indicated high purity with minor components comprising less than 5% of the protein. An oxygenation curve obtained on this preparation with the Imai automatic oxygenation apparatus (Imai et al., 1970) yielded a P_{50} of 0.7 Torr at 20 °C in the buffer conditions of these experiments and a hemoglobin concentration of 2.3×10^{-5} M.

Hemoglobin concentrations are referred throughout this paper to an extinction coefficient ϵ_{430}(1 cm) 1.33×10^5/M heme in the deoxy state (Antonini and Brunori, 1971).

Buffer Conditions. Except when specified all experiments were carried out in a "standard buffer" consisting of 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM Na_2EDTA, pH 7.4, 21.5 °C.

Fluorescence Titration. Titrations using both fluorescence quenching and absorbance changes (described below) were performed to determine whether all the hemoglobin preparations used exhibited tight binding to the haptoglobin.

For the fluorescence experiments a Perkin-Elmer MPF-3 fluorescence spectrophotometer was used, operating at an 8 nm/8 nm spectral band width. Haptoglobin samples were excited at 284 nm and fluorescence emission was measured at 330 nm.

For each experiment, a 3-ml sample of haptoglobin solution with a concentration between 0.064 and 0.072 mg/ml was used. Fluorescent intensity was recorded upon successive addition of 10-μl increments of a concentrated stock hemoglobin solution. Baselines were taken before and after each addition of hemoglobin. The absorbances at 280 and 284 nm were determined after each addition of 10 μl of hemoglobin to the 3-ml haptoglobin solution in a Gilford 2000 spectrophotometer.

The measured fluorescence intensity F_{obsd} was corrected for self-absorption of incident light to yield the true sample fluorescence F_0, by the following equation (Parker, 1968):

$$F_0/F_{obsd} = 2.303A_{284}/1 - 10^{-A_{284}}$$

where A_{284} is the absorbance of the hemoglobin–haptoglobin complex at the exciting wavelength.

Absorbance Titration. Similar titrations were carried out measuring a different property, the absorbance change at 430 nm. These measurements were done using a Cary 118C spectrophotometer. To each of 11 tubes containing a known amount of stock hemoglobin (approximately 3 ml each), a small weighed amount of stock haptoglobin was added. The resulting mixtures of Hp–Hb were deoxygenated in a glove bag under nitrogen, followed by addition of a small amount (~0.3 mg) of sodium dithionite. The absorbances at 430 nm were then determined. The absorbance at 430 nm of a reference deoxygenated Hb solution with no Hp was also measured.

After normalizing all the samples to the same concentra-

[1] Abbreviations used are: DPG, diphosphoglycerate; Hb, hemoglobin; Hp, haptoglobin.

tion of Hb (a very small adjustment), the percentage decrease in absorbance at 430 nm was calculated:

$$\% \Delta A_{430} = \frac{A_{430}^{ref} - A_{430}^{mix}}{A_{430}^{ref}} \times 100$$

where A_{430}^{ref} and A_{430}^{mix} are absorbances of the reference deoxygenated hemoglobin and mixture of Hp–Hb, respectively.

Spectrophotometric Rate Measurements. Measurements were made of the rate of decrease in absorbance at 430 nm both in the presence and absence of sodium dithionite. The two procedures differ in the method of deoxygenation.

A. With Dithionite. Inside a glovebag, 0.2–0.5 mg of sodium dithionite was added to 3 ml of haptoglobin solution in a cuvette. Similarly, 0.4–0.7 mg of sodium dithionite was added to 2–3 ml of concentrated hemoglobin solution in a separate test tube. After 3–5 min, a few drops of the mM deoxyhemoglobin solution were transferred into the cuvette containing deoxygenated haptoglobin. A reference cuvette containing buffer without haptoglobin was made up at the same time. Both cuvettes were purged with nitrogen and sealed with glass stoppers.

B. Without Dithionite. Flasks of hemoglobin and haptoglobin (5–10 ml each) were purged with nitrogen for 45 min to 1 h on a gently rotating platform. After the hemoglobin samples were deoxygenated (as later verified from spectra), the samples were mixed inside a N_2-filled glovebag as in method A.

After the samples were mixed by either method A or B, spectra of the sealed cuvettes were measured in the Soret region (370–470 nm) to ensure total deoxygenation. A spectrum of the reference cuvette was also taken. Kinetic data were then obtained by recording absorbance at 430 nm using a constant chart speed. During the course of the experiment, spectra were taken at various intervals.

Stopped-Flow Measurements. Measurements of transmittance changes at 430 nm were made with a Durrum-Gibson stopped-flow apparatus. Transmittance as a function of time was recorded on a storage oscilloscope and then photographed. Data points taken from traces on the photos were analyzed by nonlinear least-squares fitting to the following equation:

$$\text{transmittance} = 10^{-(A_a \exp(-kt)+A_\infty)}$$

In this equation A_a corresponds to the optical density change during the reaction, k is the first-order rate constant, and A_∞ corresponds to the baseline absorbance measured after the reaction has taken place.

A. Des-Arg Hemoglobin Dissociation. The des-Arg-141α hemoglobin was equilibrated with buffer by passage through a small (2.5 cm × 10 cm) Sephadex G-25 (medium) column. The stock hemoglobin solution was diluted approximately 100:1 into buffer containing 0.3 mg/ml of sodium dithionite. From spectral measurements the hemoglobin samples prepared in this way were seen to be completely deoxygenated, and the spectra were stable with time. Haptoglobin solutions were deoxygenated by adding 0.3 mg/ml of sodium dithionite.

B. Deoxyhemoglobin A Dimer Reassociation. In these experiments a dilute oxygenated hemoglobin solution containing appreciable dimeric species is deoxygenated in the stopped-flow apparatus. This shifts the equilibrium almost completely in favor of the tetramer and consequently the reassociation of deoxygenated dimers can be monitored at 430 nm (Kellett and Gutfreund, 1970; Anderson et al., 1971). The hemoglobin stock solution was diluted in 0.1 M Tris-HCl, 0.2 M NaCl, and 1 mM Na_2EDTA (pH 7.4). This was then mixed in the stopped-flow apparatus with an equal volume of 0.1 M Tris-HCl, and 1 mM Na_2EDTA (pH 7.4) containing approximately 0.3 mg/ml of sodium dithionite. The final condition after mixing is our "standard buffer" with a small amount of sodium dithionite.

Since the reaction rate is dependent on concentration of dimers the association equilibrium constant had to be determined for oxygenated hemoglobin A in 0.1 M Tris-HCl, 0.2 M NaCl, and 1 mM Na_2EDTA, pH 7.4, 21.5 °C. This was done by measuring the apparent weight average sedimentation rate (from second moments of photoelectric scanner data using a Spinco Model E analytical ultracentrifuge) as a function of protein concentration. A nonlinear fitting procedure was then used to determine the molar association constant. This determination is rapid, minimizing the formation of methemoglobin. The equilibrium constant $^4K_2$ was found to be $3.6 \pm 0.6 \times 10^5$ l./mol of heme under these conditions.

Results

The use of haptoglobin binding for accurate determination of kinetic parameters from spectral changes over many hours requires exceptionally high stability both in the measuring instrument and in the reaction mixtures employed. We present results which show that both of these requirements are met in the present study, so that accurate kinetic constants are obtained. The rate constants are found to be a sensitive probe of constraints within the dimer–dimer contact region separating $\alpha_1\beta_1$ from $\alpha_2\beta_2$. In addition, these dissociation constants may be combined with dimer reassociation rate constants to yield the equilibrium constant for formation of deoxyhemoglobin tetramers from deoxy dimers. The exceedingly high stability of deoxyhemoglobin has to date prevented any direct experimental determination of this equilibrium constant. We present results of such a determination.

Titration of Hemoglobins with Haptoglobin. Hemoglobins A, S, and des-Arg all exhibited linear titration behavior with sharp end points as shown in Figures 1 and 2. The same end point, corresponding within experimental error to a Hb/Hp ratio of unity, was found for both fluorescence and ΔA_{430} titrations. The sharpness of these end points indicated very tight binding of the haptoglobin to all of the hemoglobin preparations studied. The percentage change in A_{430} was found to be 17% (Figure 1) whereas the fluorescence quenching end point corresponded to 50% (Figure 2). These fluorescence results are in agreement with the titration studies of Chiancone et al. (1968).

Spectral Changes of Deoxyhemoglobin–Haptoglobin Reaction Mixtures. When deoxygenated solutions of hemoglobin and haptoglobin were mixed the absorbance spectrum in the Soret region was found to change slowly with time as shown in Figure 3a. The position of the Soret maximum remains nearly constant, changing from 430 to 429 nm, whereas the intensity decreases considerably during the reaction. This small wavelength shift and decrease in intensity is also observed when the hemoglobin sample is first oxygenated, reacted (rapidly) with haptoglobin, and then deoxygenated (Figure 3b). The spectrum of the deoxyhemoglobin–haptoglobin complex is very similar to the spectrum of isolated chains (Brunori et al., 1968). This decrease in intensity of the Soret has been correlated extensively with the change from cooperative constrained tetrameric hemo-

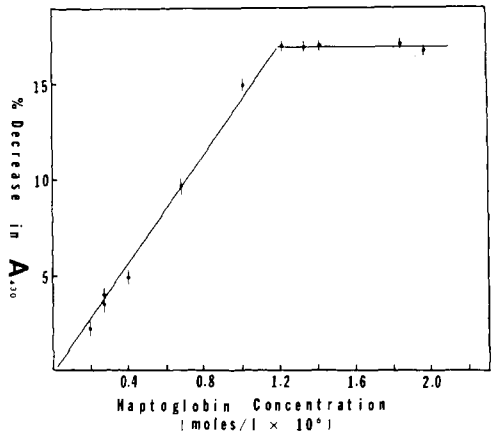


FIGURE 1: Titration of human hemoglobin A solution with haptoglobin 1-1 determined by absorbance change at 430 nm. To each of 11 tubes containing a solution of 1.2×10^{-6} M hemoglobin (in terms of tetramers), a small accurately weighed amount of haptoglobin was added. The resulting mixtures were deoxygenated in a glovebag under nitrogen and 0.3 mg of sodium dithionite was added. Absorbances relative to a reference containing no haptoglobin are plotted. Buffer conditions were: 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM Na_2EDTA, pH 7.4, 21.5 °C.

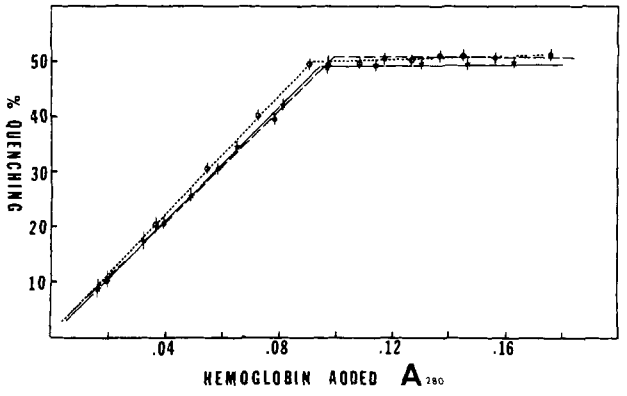


FIGURE 2: Fluorescence titration of haptoglobin binding by human oxyhemoglobin preparations. For each titration curve the fluorescence quenching was determined corresponding to 10-μl increments of a concentrated stock hemoglobin solution to a 3-ml sample of haptoglobin (6.5×10^{-7} M). The sharp titration end points correspond within experimental error to a haptoglobin/hemoglobin tetramer ratio of unity, and indicate tight binding in all the hemoglobin preparations (★) HbA, (●) HbS, (○) des-Arg Hb.

globin to unconstrained noncooperative states (Brunori et al., 1968; Kellet and Gutfreund, 1970; Anderson et al., 1971; Perutz et al., 1974).[2]

The characteristic spectra (Figure 3b) for the deoxyHb–Hp complex solutions, sealed in the presence of sodium dithionite, were found to be stable at 21.5 °C over periods of at least 48 h, permitting the time course of the reaction to be monitored accurately over an extended range, as shown in Figure 4.

Kinetic Determinations of Hemoglobin Dissociation. The absorbance data were fit by least squares to the expression for first-order reaction:

$$A = A_a e^{-kt} + A_\infty$$

[2] It should be noted again in this regard that the changes in spectral properties do not necessarily "accompany" haptoglobin binding per se; rather the ($\alpha\beta$) dimers may already have decreased spectral intensity prior to their interaction with haptoglobin.

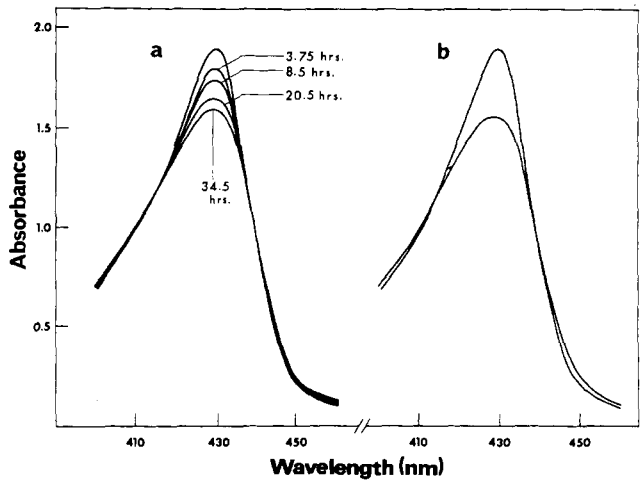


FIGURE 3: Spectral changes in hemoglobin associated with haptoglobin binding. (a) Successive spectra taken over long time intervals on a reaction mixture containing hemoglobin and haptoglobin, showing the decrease with time in intensity of the Soret band under deoxygenated conditions. (b) Spectra of deoxyhemoglobin (upper curve) and deoxyhemoglobin haptoglobin complex (lower curve).

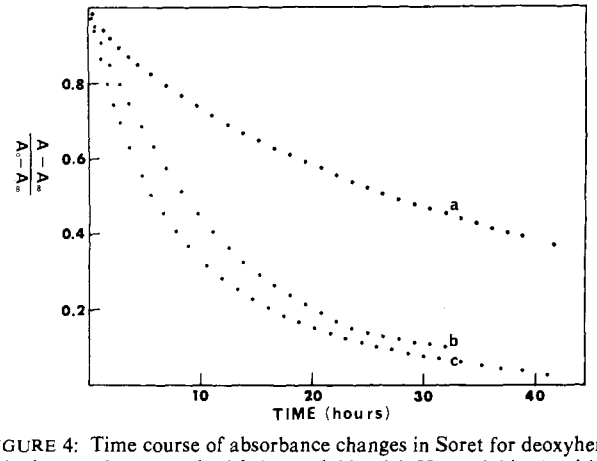


FIGURE 4: Time course of absorbance changes in Soret for deoxyhemoglobin samples reacted with haptoglobin. (a) Hemoglobin A with 2 mM DPG; (b) hemoglobin A; (c) hemoglobin S.

Table I: Kinetic Determinations of Deoxyhemoglobin Dissociation by Haptoglobin Binding.

	Hb Concn A_{430}	[Hb]/[Hp]	Fitted k (h^{-1})	Fitted % ΔA_{430}
HbA	0.797	0.65	0.084	19.7
	1.890	1.10	0.078	17.0
HbA with 2 mM DPG	1.280	0.54	0.029	12.2
HbS	1.243	0.61	0.116	16.8
HbA + HbS	1.183	0.54	0.110	16.8
des-Arg-141α Hb	1.05	1.10	107.5	

This analysis yields both A_a and A_∞ as well as the rate constant k. Representative results are listed in Table I. The two sets of values listed for hemoglobin A are typical of the reproducibility obtained over widely differing ratios of hemoglobin to haptoglobin. From the fitted values of A_a and A_∞ it is possible to calculate the total percentage change in absorbance at 430 nm upon formation of the hemoglobin–haptoglobin complex under deoxy conditions as

$$\% \Delta A_{430} = 100 A_a/(A_a + A_\infty)$$

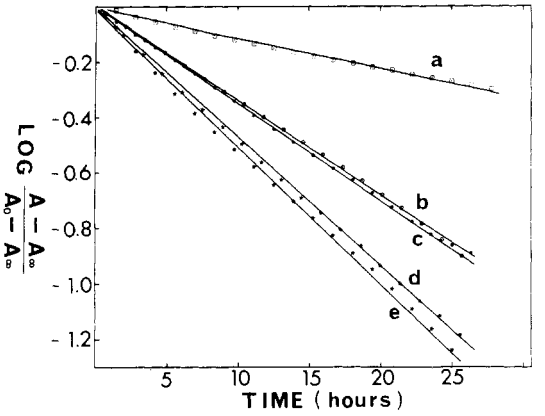


FIGURE 5: First-order plots of kinetic data for dissociation of deoxyhemoglobins in 0.1 M Tris-HCl, 0.1 M NaCl/1 mM Na_2EDTA, pH 7.4, 21.5 °C. (a) Hemoglobin A (9.6×10^{-6} M) in 2 mM DPG; (b) hemoglobin A (1.4×10^{-5} M); (c) hemoglobin A (6.0×10^{-6} M); (d) curve resulting from an equimolar mixture of hemoglobins A and S; (e) hemoglobin S (see Table I).

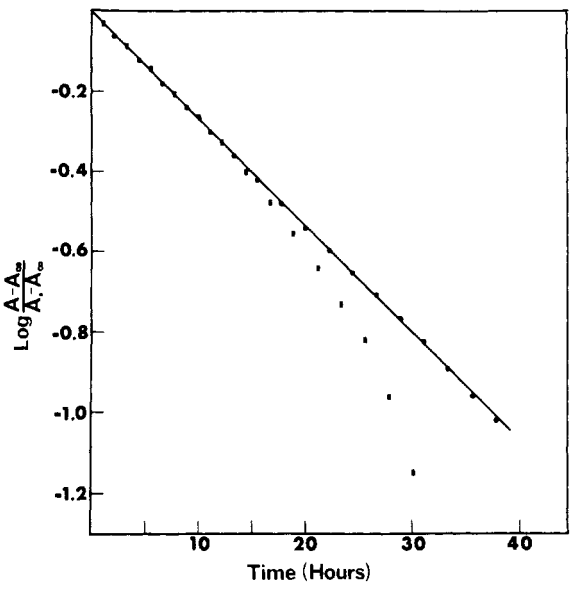


FIGURE 6: Effect of dithionite on kinetic curves. Solid line represents data taken in the standard manner with dithionite present. Lower data points are from an identical sample but without dithionite.

Estimates of this value were subject to considerably greater error than the estimates of k, but were found to be in good agreement with the 17% value obtained in the titration experiments of hemoglobin A whenever the sample concentration and rate were sufficiently high so that a substantial fraction of the amplitude could be sampled with good accuracy. This agreement is consistent with the assumptions (i) that the Hb–Hp complex is the same whether formed (slowly) in the deoxy state or formed first (rapidly) in the oxy state and subsequently deoxygenated (see Scheme I), and (ii) that the spectral changes observed in the Soret (Figure 4) do indeed measure the rate of complex formation and are not determined by other processes.

Figure 5 shows first-order plots of the data, where A_0 is the initial absorbance:

$$\log \frac{A - A_\infty}{A_0 - A_\infty} = -kt + \text{constant}$$

It is seen (Figure 5) that the first-order character of these spectral changes is very high. Good reproducibility of the slopes found in these experiments is illustrated by curves b and c of Figure 5 which are from two separate experiments with hemoglobin A.

Results of an experiment without sodium dithionite are shown in Figure 6 in comparison with a sodium dithionite containing reaction mixture. It can be seen that whereas the early stages of the reaction are linear and closely parallel that of the sodium dithionite containing reaction mixture, large deviations begin to occur after about 10 h. Spectral scans taken at successive intervals indicated appreciable oxidation under these conditions. Since methemoglobin reacts very rapidly with haptoglobin, the increasing negative slope of the first-order plot for the no-dithionite curve of Figure 6 is expected.

Correlation of Dissociation Rates with Structural Constraints. In order to explore the concept that we are measuring a property related to constraints at the contact region of interest we carried out experiments with hemoglobin A in the presence of DPG, known to increase such constraints, and with a hemoglobin specifically modified (des-Arg-141α) to eliminate half of the constraining salt links within the contact region (Kilmartin and Hewitt, 1971).

The effect of 2 mM DPG on the deoxyhemoglobin A rate is illustrated in Figure 5. Comparing curves a with b and c it can be seen that the dissociation rate is decreased in the presence of this effector. This decrease is consistent with the role of DPG in binding to β chains of both dimer pairs and stabilizing the constrained deoxy conformation of hemoglobin tetramers (Benesch et al., 1971; Benesch and Benesch, 1974).

With the des-Arg-141α hemoglobin the observed rates were too fast to be effectively studied except by stopped-flow experiments. The stopped-flow results also exhibited first-order character with a half-time on the order of 33.5 ± 1.5 s. A control experiment carried out under the same conditions with hemoglobin A resulted in no detectable change of transmittance for several minutes.

Hemoglobin S and A–S Mixtures. Since hybridization studies have utilized A–S mixtures extensively (Park, 1973; Bunn and McDonough, 1974; Williams and Kim, 1975) it was of interest to determine whether this hemoglobin having a modification which does not lie within the contact region would exhibit the same rate of dissociation as that of hemoglobin A. Data obtained with hemoglobin S are shown in Figure 5 and Table I, along with results from mixtures of hemoglobins A and S in equal proportions. It was found that both S and AS mixtures consistently exhibited slightly higher rates of dissociation than that of hemoglobin A. The differences may be barely significant at most. Linearities of first-order plots with both S and AS were found to be not quite as good as those obtained with hemoglobin A. It is not clear whether the slightly sigmoidal appearance of these plots may represent a "mixed reaction" as a result of species heterogeneity as opposed to systematic errors in the experiment.

Determination of Association Rate for Hemoglobin A Dimers. Using the equilibrium constant (3.6×10^5 M^{-1}) determined from the sedimentation experiments, initial concentrations of dimeric species in the stopped-flow reassociation experiments were estimated. These values, combined with the stopped-flow results, were analyzed in ten separate experiments to yield the association rate constant. The value obtained was $5.7 \pm 0.5 \times 10^5$ M^{-1} s^{-1} (heme). Under slightly different conditions, Anderson et al. (1971) have estimated this constant to be 6.3×10^5 M^{-1} heme s^{-1}.

Estimation of the Equilibrium Constant for Deoxyhemoglobin. The equilibrium constant for association of deoxy tetramers into dimers was calculated as the ratio of the rate constants for forward and reverse steps of the reaction. Taking into account the error propagation arising from the two types of kinetic determinations and the additional error arising in the independent determination of the oxy dissociation constant $^4K_2$ used, we find a value of $2.54 \pm 1.0 \times 10^{10}$ M^{-1} (heme) for $^0K_2$ in 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM Na_2EDTA, pH 7.4, 21.5 °C. This leads to a value of -14.0 ± 0.2 kcal/mol of heme for the free energy of association between dimer pairs in the deoxyhemoglobin tetramer. Subtracting from this the corresponding free energy of association $^4\Delta G_2$ in the oxygenated form (−7.6 kcal/mol of heme) determined by analytical gel chromatography (Ackers, 1975) we find that the total stabilization energy of deoxyhemoglobin relative to oxyhemoglobin is −6.5 kcal/mol of heme under the conditions of these experiments.

Discussion

Results of this study establish the utility of haptoglobin binding reactions as a means of studying slow deoxyhemoglobin dissociation processes. They thus extend the elegant studies of Nagel and Gibson on the faster reactions using stopped-flow methods. The combination of rapid reaction techniques with long time measurements using a highly accurate spectrophotometer such as the Cary 118C provides a means of studying these processes over a very wide range of conditions.

Compared with other methods for measurement of deoxyhemoglobin rates the one used here offers advantages of simplicity and minimization of additional chemical agents, such as ampholytes, which might interact with the hemoglobin (Shimizu and Bucci, 1974). Considering the differences in experimental conditions the agreement between results obtained by the different techniques is quite good.

In the present study we have measured the dissociation rate over three orders of magnitude. The total variation in this function appears to be five orders of magnitude, a value nearly identical with the range for the equilibrium constant. The simplest interpretation would be that most or all of the five orders magnitude variation in the equilibrium constant is attributable to the variation in dissociation rate constant, the reassociation rate constants being essentially diffusion controlled with approximately the same value. If we make this assumption then the decrease in negative free energy for stabilization in going from hemoglobin A to des-Arg hemoglobin would be approximately 4 kcal/mol (out of a total stabilization energy of ~6.4 kcal/mol). Likewise, the increase in stabilization energy with DPG would be only 0.64 kcal/mol. These estimates illustrate how extremely sensitive the measurements are in terms of small energy differences. For hemoglobin S the 30% difference in measured rate constant would correspond to an energy difference of only 150 cal, which is near the limit of sensitivity of the method.

It seems likely that rate studies of dissociation by deoxyhemoglobin tetramers may be used as a sensitive test for integrity and overall identity of state between hemoglobin preparations. The degree of first-order character to the reaction provides a means to assess heterogeneity, whereas the overall first-order rate provides a highly sensitive overall characterization. Both of these aspects warrant further exploration.

Experiments with the effector DPG and with variant and chemically modified hemoglobins (S and des-Arg) illustrate the important types of correlation between kinetic parameters and structural features of the deoxyhemoglobin molecule. The decrease in dissociation rate for hemoglobin A in the presence of DPG is consistent with a role of this effector in stabilizing the constrained tetrameric structure (Benesch and Benesch, 1974). The increased rate for the des-Arg-141 hemoglobin is particularly striking, and is consistent with the proposed role of the four deleted salt bridges in stabilizing the constrained deoxy structure.

The results with hemoglobin S and AS mixtures imply a near identity in the intersubunit interactions with hemoglobin A. Although a small difference is consistently indicated this may not be significant. The AS hybrid appears more similar to S than to A in this regard. The rate constant obtained for these hemoglobins permits an estimate of the extent to which the fraction of hybrid may have been underestimated in the experiments of Williams and Kim (1975). It appears that their lower limit estimate may have differed from the true value by a factor of approximately two, as they suggest.

Estimation of the equilibrium constant for association of deoxy dimers into tetramers by combination of the forward and reverse rate constants provides the most direct method to date for experimental determination of this important quantity. Previous estimates (Thomas and Edelstein, 1972, 1973) used an indirect approach based upon the assumption that $\alpha\beta$ dimers were totally noncooperative and bound oxygen with the same affinity as the isolated chains. Then $^0K_2$ was estimated from measurements of the variation in median ligand activity with hemoglobin concentration, assuming no doubly or triply liganded tetramers. The present estimate does not depend upon such assumptions. Our estimate of the association energy is lower by approximately 1.5 kcal/mol that estimated by Thomas and Edelstein (1972) in 0.1 M phosphate (pH 7). Although this difference is considerably outside our range of error, it is not possible to make a valid comparison due to differences in salt, EDTA, and pH conditions. In a subsequent paper we will describe the use of procedures developed here to provide, in combination with other techniques, a more complete energetic picture of the linkage between oxygenation and subunit interactions in human hemoglobin.

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Preparation of Cross-Linked Dimers of Pancreatic Ribonuclease†

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ABSTRACT: The cross-linking reaction between diimido esters and ribonuclease has been studied in terms of the yield of cross-linked dimer with optimum activity toward double-stranded RNA. With dimethyl suberimidate the most satisfactory conditions were condensation for 15 min at pH 7.5–8.0 at 21 °C with 1.25 mol equiv of the diimido ester and a protein concentration of 6%. The dimer (yield 20%) had 19 unmodified NH_2 groups out of a theoretical 20 for a molecule in which two such groups are involved in the cross-linkage; the activity toward poly(A)·poly(U) in 0.14 M salt solution by spectrophotometric assay was 8.5 times that of the monomeric enzyme toward the same substrate.

The reaction of dimethyl adipimidate with amino groups of bovine pancreatic ribonuclease to give a cross-linked dimer was first studied by Hartman and Wold (1967). Interest in the enzymic properties of dimeric RNases has been stimulated by the finding of D'Alessio and Leone and their colleagues (1972b, 1975) that the ribonuclease of bovine seminal plasma is a dimer in which the identical halves (each a homologue of the pancreatic enzyme) are linked by two disulfide bridges. Libonati and Floridi (1969) found that the seminal enzyme was more active than the pancreatic enzyme against double-stranded RNAs; they also noted that a non-cross-linked dimer prepared according to Crestfield et al. (1962) by aggregation of the pancreatic monomer had this property (Libonati, 1969). These observations prompted Bartholeyns and Moore (1974) to undertake a preliminary study of enzymic and physiological properties of a cross-linked dimer of the pancreatic enzyme. The desire to prepare such dimers in maximum yield and with optimum activity led to the present investigation of the chemistry of the cross-linking reaction with reagents of different lengths and of the activities of the products toward double-stranded ribonucleic acids.

Experimental Section

Materials and Methods

Bovine pancreatic ribonuclease A (Type IIA) was purchased from Sigma. Yeast RNA (Sigma VI) was dissolved in 0.025 M EDTA to give a 6% solution which was adjusted to pH 6.5–7.0 with NaOH and dialyzed twice for 6 h

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