The Kinetics of Ligand Binding and of the Association-Dissociation Reactions of Human Hemoglobin

PROPERTIES OF DEOXYHEMOGLOBIN DIMERS*

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SUMMARY

Dissociation of both human deoxyhemoglobin (deoxy-Hb) and carboxyhemoglobin (HbCO) at alkaline pH exposes previously buried tyrosine residues to the environment and alters their pK. Subunit association and dissociation of deoxy-Hb and HbCO can be followed through spectral changes which arise from these changes in tyrosine ionization above pH 10.0. The difference spectrum of the dissociation reaction contains a contribution from both tyrosine and tryptophan. Ultracentrifugation results confirm that a tetramer-dimer equilibrium exists at these high pH values. The tetramer-dimer dissociation constant, $K_{4,2}$, of both HbCO and deoxy-Hb increases dramatically above pH 10.0 and the value of $K_{4,2}$ for HbCO is significantly greater than the value of $K_{4,2}$ for deoxy-Hb in the pH range of 7 to 11. The groups responsible for the spectral change on dissociation have been tentatively identified with Tyr C7(42)$\alpha$ and Trp C3(37)$\beta$, which lie in the $\alpha_{1}\beta_{1}$ interface.

Substantial quantities of deoxy-Hb dimers are produced at high pH. The properties of these dimers have been examined both at high pH and at pH 7.0 after rapid pH drop in the stopped flow apparatus. Association of deoxy-Hb dimers to the tetramer is accompanied by a spectral change in the Soret region, which has allowed us to follow the association reaction at pH 7.0. Deoxy-Hb dimers react rapidly with CO at a rate characteristic of noncooperative species ($t' = 6.5 \times 10^8$ m$^{-1}$ sec$^{-1}$). Further, human haptoglobin 1-1 binds these deoxy-Hb dimers at pH 7.0 at a rate similar to that observed with HbCO dimers ($6.3 \times 10^8$ m$^{-1}$ sec$^{-1}$ and $5.5 \times 10^8$ m$^{-1}$ sec$^{-1}$, respectively). We conclude that the deoxy-Hb dimer, the unliganded dimer derived from liganded hemoglobin, and the HbCO dimer are identical in conformation, $\alpha_{1}\beta_{1}$; the first two are noncooperative in their ligand-binding properties.

The hemoglobin tetramer is therefore the minimum unit capable of full cooperativity in ligand binding. Models in which the free hemoglobin dimer is assumed to possess basically the same functional properties as the tetramer are untenable.

Recent x-ray studies on human and horse hemoglobins have shown that the conformation of the penultimate tyrosine residues of the $\alpha$ and $\beta$ chains differs in HbO$_2$ and deoxy-Hb; Tyr HC2(145)$\beta$ and Tyr HC3(140)$\alpha$ are more strongly hydrogen bonded to peptide carbonyl groups in deoxy-Hb than in HbO$_2$ (1-3). X-ray and functional studies by Moffat, Simon, and Konigsberg (4) indicated that cooperativity in ligand binding depends in some way on the conformation of Tyr HC2(145)$\beta$ since the irreversible replacement of this tyrosine or its removal by digestion with carboxypeptidase A abolished cooperativity. Spectral evidence, apparently related to these differences in tyrosine conformation in HbO$_2$ and deoxy-Hb, comes from the HbO$_2$-deoxy-Hb difference spectrum at pH 10.65 reported by Nagel, Ranney, and Kucinskis (5). This spectrum contained a contribution which resembled a tyrosine ionization difference spectrum (6), with a peak at 245 nm. Nagel et al. suggested that this contribution was due to a shift in the pK of 1 tyrosine residue on ligand binding, from a pK of > 12, a value typical of a tyrosine completely inaccessible to solvent, to a pK of $\sim$10.6, a value typical of a tyrosine in an aqueous environment. They tentatively identified this tyrosine residue as Tyr HC2(145)$\beta$.

We initially intended to examine the kinetic characteristics of changes in tyrosine conformation within the tetramer on ligand binding to deoxy-Hb, and to relate these to changes in heme conformation. However, at an early stage it became apparent that the spectral change at 245 nm was not a direct consequence of ligand binding to the tetramer, but arose from dissociation of hemoglobin tetramers to dimers. This dissociation subsequent to ligand binding converts the equivalent of 1 tyrosine per dimer with an abnormally high pK to a normally ionizing tyrosine. Thus, the change in tyrosine ionization is not a measure of a change in protein conformation within the tetramer, but of the association-dissociation reactions of hemoglobin at these pH values. Furthermore, since the tetramer-dimer dissociation constants, $K_{4,2}$, of both forms of hemoglobin increase markedly at pH values above 10.0 (7, 8), substantial quantities of stable dimers of deoxy-Hb and HbCO can be produced at these high pH values. The examination of the properties of the stable dimers is therefore of interest.

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1 Amino acid residues in hemoglobin are identified by the notation of Perutz (3).
dimers of deoxy-Hb and HbCO has enabled us to test the dimer hypothesis of hemoglobin function (9-11).

MATERIALS AND METHODS

Stock solutions of human HbO2 and deoxy-Hb were prepared according to the method of Gibson (12). These solutions were stored at 4°C and used within 1 week. Human haptoglobin 1-1 (13) was a gift of Dr. R. L. Nagel. The buffers used were glycine-NaOH in the pH range 9.0 to 10.4, e-aminocaproate-NaOH in the pH range 10.4 to 11.0, and potassium phosphate at pH 7.0. Buffers were deoxygenated by bubbling with nitrogen (Airco, New York, New York). CO (The Matheson Company, Rutherford, New Jersey) solutions were prepared by diluting CO-saturated buffer with deoxygenated buffer. Sodium dithionate was Manox brand (Hardman and Holden, Miles Platting, Manchester, England).

Concentrations of hemoglobin were determined using a Zeiss PMQ II spectrophotometer, and are given on a heme basis throughout. Difference spectra were obtained on a Beckman DK-2A recording spectrophotometer made available by Dr. D. C. Wharton, or on a Cary model 14.

pH measurements were made on a Radiometer model 22 pH meter. The reaction of dithionite with oxygen releases protons; this may change the pH appreciably. This represents the largest source of error in pH measurements on deoxy-Hb and was particularly evident in the ultracentrifuge studies.

The stopped flow apparatus, visible light source, and data acquisition system have been described by Gibson (12). The spectrophotometer, and are given on a heme basis throughout. Difference spectra were obtained on a Beckman DK-2A recording spectrophotometer made available by Dr. D. C. Wharton, or on a Cary model 14.

The estimated error in the log of the tetramer-dimer dissociation constant, log K4,2, is ±0.3 for both techniques.

RESULTS

Static Difference Spectra

Nagel et al. (5) have described the HbO2-deoxy-Hb difference spectrum at pH 10.55. Under certain circumstances CO is a more convenient ligand for kinetic analysis than O2. CO binds more slowly than O2, and under the appropriate conditions CO binding to deoxy-Hb can be treated as an irreversible reaction. This avoids the problems involved in treating an equilibrium reaction such as the O2 binding reaction. HbCO is also photosensitive, thus allowing photolytic investigation of the recombination reaction. For these reasons CO was used in this study.

The difference spectra of HbCO-deoxy-Hb at pH 10.6 and 8.9 are shown in Fig. 1a. A pronounced shoulder at 245 nm, the tyrosine ionization peak, is seen at pH 10.6, but is absent at pH 8.9.

\[ \frac{\alpha}{D + T} \approx 0.3 \quad \text{for both techniques.} \]

\[ \log K_{4,2} = \frac{H[(\alpha^2)/(1 - \alpha)]}{1.41 \times 10^{-9}} \]

\[ \frac{\alpha}{D + T} = \frac{H[D + T]}{1.41 \times 10^{-9}} \]

\[ \log K_{4,2} = \frac{H[(\alpha^2)/(1 - \alpha)]}{1.41 \times 10^{-9}} \]

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\[ \log K_{4,2} = \frac{H[(\alpha^2)/(1 - \alpha)]}{1.41 \times 10^{-9}} \]

\[ \frac{\alpha}{D + T} = \frac{H[D + T]}{1.41 \times 10^{-9}} \]

\[ \log K_{4,2} = \frac{H[(\alpha^2)/(1 - \alpha)]}{1.41 \times 10^{-9}} \]

\[ \frac{\alpha}{D + T} \approx 0.3 \quad \text{for both techniques.} \]
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A coordinate, which the ligand-binding reaction is carried out. deoxy-Hb = 20 μM before mixing in the appropriate 0.1 M buffer. Ordinate, Δε, M⁻¹ cm⁻¹ × 10⁻³.

Fig. 2. The variation in the observed Δε with the pH at which the ligand-binding reaction is carried out. CO = 100 μM; deoxy-Hb = 20 μM before mixing in the appropriate 0.1 M buffer.

FIG. 3. Time course of the CO-binding reaction to deoxy-Hb at the pH values indicated. λ = 430 nm; CO = 100 μM before mixing; deoxy-Hb = 20 μM before mixing in the appropriate 0.1 M buffer.

8.9. Hermans (6) obtained a value for the difference in molar extinction coefficient at 245 nm between fully ionized and fully protonated tyrosine of 12 × 10⁶ M⁻¹ cm⁻¹. At pH 10.6 in the HbCO-deoxy-Hb difference spectrum, the change in extinction coefficient at 245 nm, Δε₂₄₅, is 9.2 × 10⁶ cm⁻¹ per mole of hemoglobin dimer per liter, which thus corresponds to the ionization of 0.77 more tyrosines per dimer in HbCO than in deoxy-Hb at this pH.

Preliminary Kinetic Investigations

Flash Photolysis—Gray (20) measured the rate of proton uptake by deoxy-Hb produced by rapid photodissociation of HbCO. This uptake had a rate of 8000 sec⁻¹ at pH 7.0 and it was assumed to represent the conversion of unliganded hemoglobin from the liganded to the stable deoxy-Hb conformation. The conformation change which gives rise to the tyrosine-like spectral change was expected to proceed at a similar rate. In an attempt to measure the rate of this tyrosine-like spectral change at 245 nm, flash photolysis experiments were performed at pH 9.1, 9.6, 10.5, and 10.8 and at 3° and 20°. However, no spectral change at 245 nm was found. At the two lower pH values the reaction is biphasic as observed by Gibson (21), but at the higher pH values the reaction is monophasic and extremely rapid (τ = 2 × 10⁶ M⁻¹ sec⁻¹ at 3°). The kinetic difference spectrum for this reaction (HbCO-deoxy-Hb) shows none of the tyrosine-like contribution observed in the static difference spectrum at pH 10.5. Indeed, this spectrum measured after flash photolysis is identical with the pH 8.9 static difference spectrum (HbCO-deoxy-Hb) shown in Fig. 1a.

Stopped Flow—The absence of a tyrosine-like contribution in this kinetic difference spectrum shows that the unliganded hemoglobin produced by removing ligand rapidly from HbCO differs in some respect from an equilibrium solution of deoxy-Hb at this pH. Stopped flow experiments were undertaken to study a system in which an equilibrium solution of deoxy-Hb was reacted with CO. When this deoxy-Hb is rapidly mixed with CO, a spectral change at 245 nm is apparent. The kinetic character of this change is very different from the ligand-binding reaction followed at 270 nm or in the Soret region of the spectrum. The ligand-binding reaction measured at 270 nm is biphasic with rates of 2.5 × 10⁶ M⁻¹ sec⁻¹ and 6.5 × 10⁶ M⁻¹ sec⁻¹, while the reaction measured at 245 nm has a rate of 0.45 sec⁻¹ which is independent of the CO concentration. The kinetic difference spectra of both the ligand binding reaction and the slow reaction observed at 245 nm are shown in Fig. 1b. The sum of these two kinetic spectra corresponds to the static difference spectrum (Fig. 1a). The spectrum of the slow reaction resembles a tyrosine ionization difference spectrum. However, the rate of this process excludes its identification with a rapid conformational change within the tetramer subsequent to ligand binding.

Other experiments also indicated that this tyrosine-like spectral change did not represent a simple conformational difference between liganded and deoxy-Hb tetramers. When the ligand-binding reaction was carried out at several alkaline pH values, the magnitude of Δε₂₄₅ was found to vary with pH (Fig. 2). The bell shaped curve obtained is completely unlike a normal titration curve. Thus, the tyrosine-like contribution to the HbCO-deoxy-Hb difference spectrum almost disappears at pH 11.0. The time course of CO binding, measured at 430 nm, was also followed at various pH values (Fig. 3). The disappearance of the spectral change at 245 nm is accompanied by a change in the ligand-binding reaction from a predominantly slow rate at pH 10.2 to a predominately rapid rate at pH 11.0. The unusual characteristics of this spectral change, its absence in flash photolysis experiments, its slow rate, and its peculiar variation with pH (Fig. 2), led us to suspect that this spectral change represented a subunit dissociation process.

Ultracentrifuge Studies

Preliminary ultracentrifuge studies had reported that the sedimentation coefficient, s₂₄₀, for human HbCO (7), HbO₂, and deoxy-Hb (8) decreases markedly above pH 10.0. This decrease was attributed to dissociation of the tetramer to the dimer. Further dissociation to monomers was only observed at pH values greater than 11.0 (7). Rossi-Fanelli, Antonini, and Caputo (8) showed that HbO₂ and deoxy-Hb dissociate to the same extent at these high pH values. However, the slow change in tyrosine ionization which occurred subsequent to ligand bind-
ing suggested that the extent of dissociation differed in deoxy-Hb and HbCO at alkaline pH.

Ultracentrifuge experiments in collaboration with Dr. S. J. Edelstein were performed to establish the nature of the dissociation process of both forms of hemoglobin at alkaline pH. We did not study the concentration dependence of $e_{280}$ in detail since the chief object was to demonstrate the presence of tetramers and dimers at alkaline pH and the absence of significant amounts of monomers. Once the nature of the dissociation had been established, the existence of a reversible tetramer-dimer equilibrium and more precise values of the tetramer-dimer dissociation constant, $K_{4,2}$, were obtained from the kinetic experiments reported below.

Dissociation of HbCO and deoxy-Hb was studied by both sedimentation equilibrium and sedimentation velocity techniques. Equilibrium studies on deoxy-Hb above pH 10.4 were extremely difficult due to denaturation of the protein which occurred on exposure of the hemoglobin to alkali for 12 hours, the time necessary to reach equilibrium. Care also had to be taken to ensure that such denaturation, as evidenced by spreading and skewing of the boundary, did not occur during the 2 hours required for sedimentation velocity experiments. In our experiments on both HbCO and deoxy-Hb at pH values less than 11.0, no $e_{280}$ values less than 2.85 (sedimentation velocity) or weight average molecular weight values at the meniscus of less than 32,000 (sedimentation equilibrium) were found. This evidence as well as the kinetic evidence to be described later excludes the presence of measurable amounts of monomers below pH 11.0. All experiments were therefore interpreted in terms of tetramer-dimer equilibria. The results are summarized in Table 1. The value of $K_{4,2}$ for both HbCO and deoxy-Hb increases dramatically above pH 10.0. Further, $K_{4,2}$ for HbCO is considerably larger than $K_{4,2}$ for deoxy-Hb at all alkaline pH values. At the protein concentrations used in the kinetic experiments, an appreciable fraction of the hemoglobin is dimeric at pH values above 10.4 for deoxy-Hb and at pH values above 10.0 for HbCO. These results confirm that extensive tetramer-dimer dissociation occurs at high pH and establish that HbCO is substantially more dissociated than deoxy-Hb at these pH values.

**pH Relaxation Experiments**

Carboxyhemoglobin—A consequence of the pH dependence of $K_{4,2}$ is that the tetramer-dimer equilibrium may be perturbed by altering the pH of a hemoglobin solution rapidly in the stopped flow apparatus. The subsequent re-equilibration can then be followed at the new pH. On mixing a weakly buffered solution of HbCO (32 μM) at pH 10.0 with a strong, CO-saturated buffer at pH 10.6, a first order reaction with a rate of 0.45 sec$^{-1}$ is seen at 245 nm. This rate is identical with the rate observed above for the slow reaction which accompanied CO binding in the stopped flow apparatus. Since no change in heme conformation occurs, this experiment shows conclusively that ligand binding is not necessary to produce this spectral change at 245 nm; dissociation alone is sufficient to cause it. The ultraviolet difference spectrum of the dissociation reaction of HbCO at pH 10.6 is shown in Fig. 4. It is almost identical with a tyrosine ionization difference spectrum; the slight differences in the 270- to 300-nm region are referred to under "Discussion." Several pH jump experiments were performed, each starting from pH 9.54 in weak borate buffer and ending at various higher pH values.

### Table I

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration (μM)</th>
<th>pH</th>
<th>$e_{280}$</th>
<th>$K_{4,2}$ (μM)</th>
<th>Method</th>
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<td>4.00</td>
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<td>2.3</td>
<td>V</td>
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<tr>
<td></td>
<td>0.40</td>
<td>10.60</td>
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<td>8.2</td>
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<td>54.3</td>
<td>10.75</td>
<td>3.08</td>
<td>301.0</td>
<td>V</td>
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</tbody>
</table>

![Fig. 4](http://www.jbc.org/)

In Fig. 5 the resultant $A_{280}$ of this slow reaction is shown as a function of the pH value. This experiment determines the total excursion under conditions in which tetramers predominate in the starting material (pH 0.54) and dimers predominate after...
the pH jump (pH 11.0). This value is $\Delta e_{245} = 6.6 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$, equivalent to 1.1 tyrosine ionized per dimer.

The reverse experiment in which the pH is dropped from pH 10.6 to pH 10.0 gives information concerning the kinetic parameters of the reassociation process, and an approximate value of the pK for tyrosine ionization. The pH relaxation from pH 10.6 to pH 10.0 produces a biphasic decrease in absorbance at 245 nm. A rapid phase must occur as all exposed tyrosines become more protonated, but this is not observable directly as it is complete within the dead time of the stopped flow apparatus. A slower phase follows as the dimers reassociate to the tetramer with an initial first order rate of 0.56 sec$^{-1}$ at 32 PM. This association reaction at high pH is an approach to equilibrium process and not an irreversible second order reaction. Since the observed rate depends on both the dissociation and the association rates, this reaction is described phenomenologically here by the initial first order rate constant at a specified pH and protein concentration.

The pK of this tyrosine group can be estimated by observing the percentage changes in tyrosine ionization that occur upon pH drop, and comparing them with the percentages derived from a single proton ionization curve. This method estimates the pK as 10.1 ± 0.15, a value typical of tyrosine in an aqueous environment.

It is unfortunately not possible to study this dissociation process kinetically at pH 7.0 with the liganded species since we are restricted to pH values at which the dissociation-linked change in tyrosine ionization can be followed (pH ≥ 10.0). This restriction does not apply in the pH relaxation experiments with deoxy-Hb.

**Deoxyhemoglobin**—The unusual variation of $\Delta e_{245}$ in the ligand-binding experiment carried out at various pH values (Fig. 2) and the ultracentrifuge data (Table I) indicate that deoxy-Hb is also extensively dissociated at high pH. Experiments similar to those just described for HbCO were carried out with deoxy-Hb at 32 PM by dropping the pH from pH 10.7 to pH 10.0. A tyrosine-like spectral change was again observed with an initial first order rate constant of 0.7 sec$^{-1}$. When interpreted in terms of the ultracentrifuge results the data shown in Fig. 3 suggest that at high pH the species which reacts rapidly with CO ($P = 6.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) is the deoxy-Hb dimer. This rapid rate of ligand binding is characteristic of noncooperative species such as isolated chains.

All hemoglobins thus far examined which are noncooperative in ligand binding have a different Soret absorption spectrum in the deoxy form than cooperative species. The rapidly reacting hemoglobin produced on complete photodissociation of HbCO (Hb$^+$), hemoglobin H, hemoglobin digested with carboxypeptidase A, and hemoglobin Gun Hill are noncooperative in ligand binding and have a lower $e_{380}$ than does hemoglobin A (11, 21, 23, 24). This characteristic depression of the Soret absorption spectrum in the deoxy form is believed to be a general property of all noncooperative hemoglobins (11).

Brunori et al. (25) showed that the association of deoxy-Hb chains to the cooperative tetramer produced this characteristic change in the spectrum in the Soret region. Since deoxy-Hb dimers appeared to be noncooperative in ligand binding, the association of these dimers to the tetramer should produce changes in the heme absorption region as well as at 245 nm. At 32 PM in a 20-mm cell, the heme absorption in the Soret is too intense to allow the investigation of this association reaction. However, the difference spectrum (deoxy-Hb tetramer-deoxy-Hb chains) observed on association of deoxy-Hb chains (25) extends to wave lengths lower than 430 nm where the heme absorption is much less intense. Fig. 6 shows that the time course of the spectral change observed in the Soret region on dropping the pH from pH 10.7 to pH 10.0 parallels the change at 245 nm. The identity of the time course of these reactions suggests that the reassociation process is the rate-limiting step in the production of spectral changes at these wave lengths. The association-dissociation equilibrium can then be followed at neutral pH through this spectral change in the Soret region. Since deoxy-Hb dimers do not have a different Soret absorption spectrum in the deoxy form than does hemoglobin A (11, 21, 23, 24), these reactions suggest that the reassociation process is the rate-limiting step in the production of spectral changes at these wave lengths.

The effect of hemoglobin concentration on the association rate after pH relaxation from pH 10.6 to pH 7.0 is shown in Fig. 7. This spectrum is very similar to those observed with the other noncooperative hemoglobins. Since at pH 10.6 and 8 PM heme only 35% dimer is present, the observed extinction coefficients were corrected to the value which would be obtained if all the hemoglobin were dimeric at pH 10.6 and tetrameric at pH 7.0.
plot of initial first order rate constant against hemoglobin concentration gives a straight line which passes through the origin. The association rate constant $k_a$ calculated by plotting the reciprocal of the heme concentration against time, gives a second order rate constant of $6.3 \times 10^4$ $\text{m}^{-1} \text{sec}^{-1}$ at pH 7.0.

The dissociation rate at pH 10.6 was calculated by jumping the pH from pH 7.0 to pH 10.6. In contrast to the dissociation of HbCO, this dissociation occurs very slowly ($0.008$ sec$^{-1}$ at pH 10.6). The kinetic difference spectrum (deoxy-Hb tetramer-deoxy-Hb dimer) of this dissociation reaction is also shown in Fig. 7; the two spectra in this figure agree to within experimental error. The low rate of dissociation requires incubation of deoxy-Hb at high pH for at least 5 min to allow the new dissociation equilibrium to be established. The constancy of kinetic and spectral properties was taken as an indication that equilibrium had been reached and that denaturation had not occurred during this brief exposure to high pH. Deoxy-Hb is stable for several hours at pH 10.6 and for at least 10 min at pH 11.0.

The apparent $\Delta A_{430}$ on jumping the pH of deoxy-Hb from pH 7.0 to pH 10.6 depends on the initial hemoglobin concentration. Increasing dilution produces a greater apparent $\Delta A_{430}$ since a larger percentage of the hemoglobin is dimeric at pH 10.6, whereas the tetramer content at pH 7.0 is constant at $\sim 100\%$. If a sufficiently low hemoglobin concentration could be reached at which deoxy-Hb at pH 7.0 were significantly dissociated, the concentration dependence of the apparent $\Delta A_{430}$ would be altered. In such experiments at varying hemoglobin concentrations, no evidence for dissociation at pH 7.0 was found even at hemoglobin concentrations as low as 1 $\mu\text{M}$ after mixing. The value of $K_{4,2}$ for deoxy-Hb at pH 7.0 must then be less than $5 \times 10^{-4}$ $\text{M}$.

Ligand-binding Reactions

The results shown in Fig. 3 indicate the existence of two species which bind ligand at different rates. The ultracentrifuge studies identify these two species as the deoxy-Hb dimer and tetramer. If this distribution of species is due to an aggregation phenomenon, the fraction of slowly reacting material (the tetramer) will decrease as the hemoglobin concentration is decreased at constant pH. Table II includes the results of such an experiment at pH 10.6. The distribution of these two species in the ligand-binding reaction has been corrected for the difference in $\Delta A_{430}$ (HbCO-deoxy-Hb) for the dimer and the tetramer; the value of $\Delta A_{430}$ for the tetramer is $15\%$ larger than the value for the noncooperative species (25). The fraction of slowly reacting material, the tetramer, does indeed increase with increasing hemoglobin concentration. Table II also gives the values of $K_{4,2}$ at various pH values calculated from the distribution of rapidly and slowly reacting species in Fig. 3.

The CO-binding reaction was also carried out in conjunction with a pH drop experiment by mixing weakly buffered deoxy-Hb at pH 10.6 with strong buffer at pH 7.0 which contained a known concentration of CO. The ligand-binding reaction was complete before reassociation of deoxy-Hb dimers to the tetramer became significant. It is therefore possible to study the ligand-binding reactions of the deoxy-Hb dimer at neutral pH. In this experiment, as in the ligand-binding experiment at high pH, the dimer is rapidly reacting ($l' = 6.5 \times 10^6$ $\text{m}^{-1} \text{sec}^{-1}$), and the fraction of rapidly reacting species is the same as that found at pH 10.6. The pH drop by itself thus does not alter the fraction of rapidly reacting material.

The fraction of the rapidly reacting species on ligand binding and the fraction of the total Soret change at 430 nm upon aggregation to the tetramer were determined on the same solution of deoxy-Hb by dropping the pH from values between pH 10.0 and pH 11.0 to pH 7.0, with and without CO present. The correction for the different value of $\Delta A_{430}$ for the dimer and the tetramer on ligand binding was again used to calculate the distribution between rapid and slow species. The distribution of species in the ligand-binding reaction depends on the concentration before mixing (7.5 $\mu\text{M}$) and the size of the change at 430 nm depends on the concentration after mixing (3.75 $\mu\text{M}$). The fraction of rapidly reacting species on ligand binding was therefore further corrected to that fraction which would be present at 3.75 $\mu\text{M}$. A plot of the corrected fraction of rapid species against the observed absorbance change at 430 nm yielded a straight line from

### Table II

<table>
<thead>
<tr>
<th>pH before mixing</th>
<th>pH after mixing</th>
<th>Fraction slow</th>
<th>$K_{4,2}$</th>
</tr>
</thead>
<tbody>
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<td>7.0</td>
<td>10.6</td>
<td>0.85 (per cent slow)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.85 (per cent slow observed) + (per cent rapid)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha = 1 - \text{fraction slow}$</td>
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<tr>
<td></td>
<td></td>
<td>$K_{4,2}$</td>
<td></td>
</tr>
<tr>
<td>17.0</td>
<td>10.6</td>
<td>51</td>
<td>8.0</td>
</tr>
<tr>
<td>8.5</td>
<td>10.6</td>
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<tr>
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<td>6.25</td>
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<tr>
<td>20.0</td>
<td>10.2</td>
<td>88</td>
<td>0.29</td>
</tr>
<tr>
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<td>10.4</td>
<td>84</td>
<td>0.63</td>
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<tr>
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<td>20.0</td>
<td>10.8</td>
<td>25</td>
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</table>
FIG. 8. Correspondence of the percentage of deoxy-Hb which reacts rapidly with CO and of the percentage of change in extinction coefficient at 430 nm on association of deoxy-Hb dimers at pH 7.0. •, pH drop from a series of high pH values (appropriate buffer, 0.01 M) to pH 7.0 (0.1 M phosphate) to determine the percentage of the Soret change. †, similar experiment in which the pH 7.0 buffer contains 24 μM CO to determine the percentage of the rapidly reacting species. Both reactions followed at 430 nm. Deoxy-Hb = 7.5 μM before mixing. Data corrected as described in the text. -- - - , smooth curve through the data points.

FIG. 9. The variation of log $K_{4,2}$ with pH, for deoxy-Hb and HbCO. •, ultracentrifuge results (Table I); ○, kinetic results (Table II). The single kinetic result on the upper curve was obtained from the data in Fig. 10 and is a value for HbO2. The lines are theoretical curves given by Equation 6, using values of log $K_D = -6.1; \log K'_D = -7.5; K_o = K'_o = 1.24 \times 10^{-15}; m = m' = 1.5$. $K_{4,2}$ is in molar units.

FIG. 10. CO binding to hemoglobin rapidly deoxygenated with dithionite. •, HbO2 at pH 10.6 (0.01 M ε-aminocaproate-NaOH) reacted with dithionite and CO at pH 7.0 (0.1 M phosphate). (Encircled asterisk), HbO2 at pH 7.0 (0.1 M phosphate) reacted with dithionite and CO at pH 7.0 (0.1 M phosphate). Final pH = 7.0 in both; λ = 422 nm; HbO2 = 8 μM before mixing; CO = 24 μM before mixing. Ordinate: 1.0 minus fraction HbCO.

which the value for 100% change at 430 nm was established. From this value the fractional change in absorbance at 430 nm was calculated at each pH value. The agreement between the two sets of data shown in Fig. 8 is excellent.

The value of $K_{4,2}$ at each initial pH value can be calculated from the curve in Fig. 8. These values of log $K_{4,2}$, those obtained from the ultracentrifuge studies (Table I), and those from other kinetic experiments (Table II) are compared in Fig. 9. The internal agreement of the values obtained by these various techniques is remarkably good. This is further evidence that measurable amounts of deoxy-Hb monomers are not formed at high pH. Deoxy-Hb monomers, because of their rapid rate of ligand binding, would be identified as dimers in the kinetic experiments. Although this would underestimate the amount of dimer present, it would underestimate the degree of dissociation by treating the monomer population as if it were dimeric. The apparent value of $K_{4,2}$ from kinetic experiments would be smaller than the ultracentrifuge value, contrary to our results.

The excellent agreement between these kinetic and ultracentrifuge estimates of $K_{4,2}$ establishes that the rapid rate of CO binding and the changes in Soret absorbance on association can be quantitatively associated with the deoxy-Hb dimer.

The properties of both dimeric and tetrameric hemoglobin were examined by mixing HbO2 with a dithionite solution containing 24 μM CO. In the first experiment, predominantly tetrameric HbO2 at pH 7.0 was used. In the second, predominantly dimeric HbO2 was produced by incubation of the HbO2 at pH 10.6, before mixing it with the dithionite and CO in strongly buffered pH 7.0 solution. The CO-binding reaction to
the freshly formed unliganded hemoglobin was followed at 422 nm, an isosbestic point for deoxy-Hb and HbO₂. These unliganded dimers derived from liganded hemoglobin also combine rapidly with CO (Fig. 10). HbO₂ at pH 7.0 is also partially jump experiments on sheep HbO₂ at pH 9.2 (29) suggest, however, the deoxy-Hb dimers produced by incubation of HbO₂ at high pH are incubated at pH 7.0 for several minutes before mixing with Hp. Deoxy-Hb = Hp = 12 μM before mixing. Protein fluorescence excited at 285 nm and measured at 330 nm through a Corning 760 filter. This apparent lag is an artifact of the freshly formed unliganded hemoglobin was followed at 422 nm, an isosbestic point for deoxy-Hb and HbO₂. These unliganded dimers derived from liganded hemoglobin also combine rapidly with CO (Fig. 10). HbO₂ at pH 7.0 is also partially dissociated at this hemoglobin concentration, since a fraction of the unliganded hemoglobin also combines rapidly with CO (Fig. 10).

**Haptoglobin Binding**

Haptoglobin 1-l binds liganded hemoglobin dimers with a rate of 5.5 × 10⁵ M⁻¹ sec⁻¹ at pH 7.0 in an essentially irreversible reaction, but does not bind to liganded tetramers (15). Dissociation of the liganded tetramer to the dimer is a prerequisite for Hp binding. However, no interaction at all is observed between deoxy-Hb and Hp at this pH (26). The apparent inability of Hp to bind deoxy-Hb may be due either to a structural difference between the liganded and the deoxy-Hb dimers, or to the very low dissociation constant of deoxy-Hb at pH 7.0. Hp does not bind to the liganded dimers at high pH, presumably because of an alteration in Hp conformation at this pH. It is therefore not possible to react these deoxy-Hb dimers with Hp at high pH. However, the deoxy-Hb dimers produced by incubation of deoxy-Hb at high pH were reacted with Hp by mixing with a deoxygenated Hp solution strongly buffered at pH 7.0. Hp does indeed bind to these deoxy-Hb dimers (Fig. 11), with a rate of 6.5 × 10⁵ M⁻¹ sec⁻¹. Furthermore, if these dimers generated at high pH are incubated at pH 7.0 for several minutes before mixing with Hp, no binding is observed. On adding CO or O₂ to this solution of deoxy-Hb previously incubated at pH 7.0, binding to Hp is restored (Fig. 11), which confirms that no irreversible changes occur in deoxy-Hb on incubation for a few minutes at high pH. The necessary condition for hemoglobin binding by Hp is then not the presence of ligand, but the ability of the tetramer to dissociate to dimers.

**Discussion**

In view of the varied experimental results presented here, “Discussion” is divided into several sections. We first present a general scheme describing both the association-dissociation reactions and the ligand-binding reactions, then discuss the conformation and properties of liganded and unliganded dimers, and finally, discuss the dimer hypothesis (11) in the light of these results.

**General Scheme**—Eigen (27) has shown that rates of proton uptake and release are extremely rapid by comparison with the rates of any association or dissociation process of the protein. Although these proton equilibration steps are directly responsible for all tyrosine-linked spectral changes at 245 nm, they cannot be rate limiting.

The dissociation of HbCO tetramers to dimers induced by pH jump is accompanied by a first order reaction (0.45 sec⁻¹ at pH 7.0, 10.6, 245 nm). Gibson and Antonini (28) studied the appearance of a rapidly reacting species in flow flash experiments on dilute solutions of HbCO at pH 7.0. The rate of formation of this rapidly reacting species was 0.6 sec⁻¹. Although they could not determine the state of aggregation of this molecular species, recent ultracentrifuge data (19) show that it is dimeric. This rate then corresponds to k₂, the tetramer-dimer dissociation rate constant. From studies of the binding of HbCO by Hp, Nagel and Gibson (15) calculated a value of K₂ of 2 sec⁻¹ at pH 7.0. Both of these values agree well with the observed rate of dissociation measured by subsequent tyrosine ionization. Temperature jump experiments on sheep HbO₂ at pH 9.2 (29) suggest, however, a much more rapid rate of dissociation of 27 sec⁻¹. This more rapid rate may reflect species, ligand, or pH differences, or it may represent some relaxation process other than dissociation.

Parallel pH jump experiments on deoxy-Hb and HbO₂ produced similar spectral changes at 245 nm. This change in tyrosine ionization is therefore intimately linked to dissociation in HbCO, deoxy-Hb, and HbO₂. The dissociation rate constant for deoxy-Hb (0.008 sec⁻¹ at 8 μM) is, however, substantially lower than that for the liganded species (0.45 sec⁻¹ at 32 μM). Association of dimers to the tetramer on pH drop also produces a slow change in absorbance at 245 nm for both HbCO and deoxy-Hb. Association of dimers of deoxy-Hb produces a further spectral change in the Soret region.

These reactions are summarized in the following scheme which, although it may not be unique, is certainly consistent with all of our results.

\[
H₄₋₄Hb⁴(CO)₄ ⇌ \frac{k₄}{k₆} 2H₂₋₄Hb₂(CO)₂
\]  

(1)

\[
H₄₋₄Hb⁴(CO)₄ ⇌ \frac{k₈}{k₉} mH + H₄₋₄Hb⁴(CO)₄
\]  

(2)

\[
H₄₋₄Hb⁴ ⇌ \frac{k₄}{k₆} 2H₂₋₄Hb₂
\]  

(3)

\[
H₄₋₄Hb⁴ ⇌ \frac{k₈}{k₉} mH + H₄₋₄Hb⁴
\]  

(4)

1 R. L. Nagel and Q. H. Gibson, unpublished results.
K4,2 reaction is unaffected by pH drop to pH 7.0. This shows that reaction is biphasic above pH 10.0, and the kinetic profile of this reaction with CO is complete before significant association of the dimer. At high CO concentrations, ligand binding and deprotonization of a singly ionizing species would be found if this As2ds arose from the change in the environment of other ionizable groups on dissociation. These other ionizable groups which might be cysteine or lysine must also have pK values around 10.

The parallel curves in Fig. 9 were calculated from Equation 6 (and the corresponding equation for HbCO) using values of log Kd = -6.1; log Kp = -7.5; K = Kp = 1.24 x 10^-14 and m = m' = 1.5. The curve for HbCO is 1.4 log units higher than the curve for deoxy-Hb at all pH values above pH 8.5. This corresponds to a stabilization of the deoxy-Hb tetramer of 1.87 kcal per mole of tetramer relative to the HbCO tetramer.

In this scheme, Reactions 3 and 4 imply that dissociation of the deoxy-Hb tetramer to the dimer precedes the release of protons. A full description of the process must include the alternative sequences, that is, proton release followed by dissociation, as shown by Reactions 8 and 9.

\[
\frac{d \log (K_{4,2})}{dpH} = -4.906(H^+) + 2m(pH)
\]
the same conformation. Park (36) has similarly concluded on
the basis of hybridization experiments on human and canine
HbO₂ and deoxy-Hb at pH 7.0 that dissociation occurs across
the same α₂β₂ interface in both deoxy-Hb and HbCO. It is
likely. The recent x-ray results on deoxy-Hb (1), when com-
pared with those on HbO₂ (3), provided no strong evidence
to support this hypothesis. It thus appears that dissociation
of deoxy-Hb occurs across the α₂β₂ interface to give αβ₂ dimers
at both neutral and high pH.

The observed pH dependence of log K4,2 agrees well with the
dependence predicted by Equation 6, which was derived on the assumption that a single mechanism of dissociation
holds at all pH values between pH 7.0 and 11.0 for HbCO and
dehy-Hb. If at some intermediate pH, both interfaces
were equally stable, monomers would be formed and the apparent
value of log K4,2 versus pH would show a pronounced shoulder.
The monotonic increase of log K4,2 with pH and the good fit of
the experimental values to the theoretical curve indicate that at
pH values between 7.0 and 11.0 for HbCO, and at pH values be-
 tween 8.5 and 11.0 for deoxy-Hb, the dimers have the αβ₂ con-
formation.

Calculation of K4,2 for deoxy-Hb at pH 7.0 using Equation 6
gives a value of 0.032 μμ. Certain other results suggest that the
actual value is lower and that Equation 6 is not applicable below
pH 8.5 for deoxy-Hb. Indeed, CO binding at pH 7.0 to 0.3
μμ deoxy-Hb, even in 2 M tritylamine-HCl, which favors
dissociation of the tetramer, proceeds at a single slow rate charac-
teristic of the tetramer (10); no rapid phase attributable to the
dimer is observed. The total amount of free deoxy-Hb dimer
bound to binding to Hp, α(deoxy-Hb), increases with increasing
hemoglobin concentration and decreasing α, since α(deoxy-
Hp) = K4,2[(1/α) – 1]. Hp binding to concentrated solutions
of deoxy-Hb has nevertheless not been observed (26) which
requires a value of K4,2 below 10⁻⁸ μμ. Preliminary experiments
by the present authors on CO binding to deoxy-Hb at CO ω
hemoglobin ratios around 0.2 in the pH range 9.5 to 7.5 show
that the amount of rapidly reacting species, the dimer, is con-
stant from pH 9.5 to pH 8.5 as predicted by Equation 6. At
lower pH values, at which the alkaline Bohr effect becomes
appreciable, the amount drops to zero. These results suggest that
the binding of Bohr protons to deoxy-Hb contributes to the
stabilization of the tetramer and lowers the value of K4,2 below
that predicted by Equation 6. The interactions in deoxy-Hb
which are the source of some of the Bohr proton have been iden-
tified by comparison of the structure of HbO₂ and deoxy-Hb
(1, 3, 37). Three of these, those involving Asp Hb(126)α-Arg
HC(314)α, NH₂⁺ of Val NA(1)α-COO⁻ of Arg HC(314)α, and
(indirectly) COO⁻ of His HC(146)β-Lys C₈(40)α, form
interchain bonds which must further stabilize the deoxy-Hb
tetramer. Kinetic experiments which measure either CO or Hp binding
to deoxy-Hb at pH 7.0 fail to detect the presence of deoxy-Hb
dimers (19, 26). By contrast, ultracentrifuge data appear to show that deoxy-Hb at neutral pH dissociates to such an extent
that the presence of dimers should be readily detected (19, 38).
However, the interpretation of these ultracentrifuge experiments
is obscured by several factors; it is necessary to use very low
protein concentrations and high dithionite concentrations, and to
allow the solution to stand in contact with the ultracentrifuge
cell for a considerable length of time. Attempts to increase the
dissociation of deoxy-Hb by addition of salt, which greatly facili-
tates dissociation of liganded hemoglobin (8), have further com-
plicated the problem. These experiments show that salt has
little effect on the value of K4,2 when the results are corrected for
the presence of bound water (39). If this correction is not applied, a gross overestimate of the value of K4,2 results. A
recent report (40) shows that when suitable experimental pre-
cautions are taken, no dissociation of deoxy-Hb can be detected
even in the presence of salt. The kinetic experiments are more
readily interpreted within the framework of these later findings,
which suggest that deoxy-Hb is tetrameric at all concentrations
accessible to investigation by spectrophotometric methods. An
alternative, that dissociation at pH 7.0 produces a dimer which differs in
conformation and ligand-binding properties (19, 35) from the
dimer formed at alkaline pH, seems unlikely both from the present
data and that of Park (36).

The present ultracentrifuge results at alkaline pH are less
subject to error, since the large increase in the value of K4,2 allows
the use of much higher hemoglobin concentrations (1 to 50 μμ)
which minimizes artifacts arising from the presence of impurities
or denatured protein (irreversible dissociation). Reversibility
of the tetramer-dimer equilibrium is clearly shown by the loss of
certain characteristics when the pH of an alkaline solution of
dehy-Hb is lowered to pH 7.0. Moreover, the studies on deoxy-
Hp at pH 7.0 (19) required an extrapolation to obtain the disso-
ciation constant since the experiments were performed at concentrations about an order of magnitude above the calcu-
lated dissociation constant. The present experiments at alkaline
pH could be conducted at concentrations in the region of the
dissociation constant, and under these conditions the experi-
mental errors are likely to be much less significant than at pH 7.0.
Finally, the reliability of the present ultracentrifuge data
is strongly indicated by the excellent agreement of these values for
K4,2 with those determined by kinetic methods.

It is tempting to speculate on the identity of the tyrosine
residue whose pK is altered on dissociation of HbCO and deoxy-
Hp across the α₂β₂ interface. Perutz (3) pointed out that this
dissociation would bring Tyr C(749)α and Trp C(37)β into
contact with solvent. In the tetramer these residues are buried
and presumably inaccessible to solvent; the tyrosine is hydrogen
bonded to Asn G(97)α in HbO₂. The kinetic difference spec-
trum of HbCO (pH 10.6 to pH 10.0) in Fig. 4 closely resembles
a tyrosine difference spectrum (6) but has an additional shoulder
at 290 nm and a slightly positive value at 270 nm. These features
suggest that a change in tryptophan environment (11) also
takes place on dissociation. Although unambiguous identifica-
tion cannot be made, all the present results are consistent with
the suggestion that Tyr C(749)α and Trp C(37)β are responsible
for the spectral changes in the ultraviolet on association and dis-
sociation of deoxy-Hb and HbCO.

Briehl and Hobbs (42) have shown that the HbO₂ minus
dehy-Hb spectrum in the ultraviolet has contributions from
tryptophan perturbations, and have interpreted this to represent
a change in the environment of Trp C3(37)β, a residue in the α1-β2 interface, on oxygenation. The persistence of this spectrum in 3 M NaCl, with the assumption that both forms of hemoglobin are extensively dissociated in high salt, was interpreted to mean that this interface was still intact in the dimer, which must then have the composition αββ. However, if deoxy-Hb does not dissociate under these conditions, then the spectrum will persist irrespective of the interface split in HbO2. The only conclusion which can be drawn with certainty from these results is that structural changes occur in the αββ interface on ligand binding, in agreement with the present results.

Properties of Deoxy-Hb Dimers—Because the value of K₄,4 is considerably lower than the concentration at which equilibrium and kinetic measurements can be made, appreciable quantities of deoxy-Hb dimers cannot be produced readily by dilution at pH 7.0. This investigation has taken advantage of the large increase in K₄,4 for deoxy-Hb at alkaline pH to produce substantial quantities of stable deoxy-Hb dimers and to study the properties of these dimers for the first time.

Transient deoxygenated species have been produced previously by removal of ligand from HbO2 with dithionite (40, 43) or by flash photolysis of HbCO (28, 43). The ligand-binding reaction of these deoxygenated species contains a rapid component whose rate of CO binding is identical with that observed on CO binding to the deoxy-Hb dimer produced at high pH and subsequently studied at pH 7.0. The recent ultracentrifuge results (19) make it clear that this rapid component produced on ligand removal is dimeric and not monomeric as was assumed previously (28, 43). The ligand-binding properties of these transient, unliganded dimers and the stable deoxy-Hb dimer are therefore identical. In short, the results of Antonini, Brunori, and Anderson (43) are consistent with the present interpretation that deoxy-Hb and HbCO dimers have the same conformation and that where their properties overlap they are identical. Both unliganded dimers are devoid of cooperativity in ligand binding. The sole difference between HbCO and deoxy-Hb dimers (apart from the presence of ligand) is that association of deoxy-Hb dimers to the tetramer is accompanied by a characteristic spectral change in the Soret region which is absent on association of HbCO dimers. This result represents the conversion of a noncooperative to a cooperative species. As noted previously, the same characteristic differences are seen when the Soret spectra of other noncooperative species—hemoglobin H, carboxypeptidase A-digested hemoglobin, hemoglobin Gun Hill, free deoxy-Hb chains, unliganded dimers derived from liganded hemoglobin, and the rapidly reacting hemoglobin, Hbαβ—are compared with the Soret spectrum of native deoxy-Hb. These spectral differences appear to arise because all these hemoglobins retain the liganded conformation even in the absence of ligand. We have established this for the dimeric species, and it is known to be true for horse hemoglobin digested with carboxypeptidase A (44).

The magnitude of the spectral change in the Soret region on association of deoxy-Hb dimers to the tetramer (Fig. 7) corresponds to the entire change seen on association of deoxy-Hb chains (25). Association of α and β chains to the dimer cannot produce a spectral change in the Soret region. This result partially explains the complex reaction kinetics observed as deoxy-Hb chains associate to the tetramers (45), since this reaction would require, in addition to other processes, two consecutive second order reactions, the second of which gives the spectral change. The fact that the association of dimers is accurately second order and shows no such kinetic complexities provides the clearest kinetic evidence that measurable amounts of deoxy-Hb monomers are not present at high pH.

The dimer-tetramer reactions are summarized in the following two reactions

\[ D_d + D_d \rightarrow T_d \]  
\[ D_l(CO)_2 + D_l(CO)_2 \rightarrow T_l(CO)_4 \]

where \( D \) and \( T \) denote the dimer and the tetramer and the subscripts \( d \) and \( l \) denote unliganded and liganded conformations, respectively. Both reactions are accompanied by equivalent spectral changes in the ultraviolet, and, in Reaction 10, by the additional spectral change in the Soret region. As established above, \( D_d \) and \( D_l(CO)_2 \) differ only in the presence of ligand on \( D_l(CO)_2 \). The rates of association of \( D_d \) and \( D_l(CO)_2 \) are approximately the same at pH 10.0, but the rates of dissociation of \( T_d \) and \( T_l(CO)_4 \) differ widely.

Kellett and Gutfreund (40) studied the reactions of dimers produced by reacting dilute solutions of HbO2 with dithionite. They identified two competing processes with identical difference spectra in the Soret region for the removal of the unliganded \( D_l \) dimers. One was a first order process with a rate of 0.2 sec⁻¹ which they assigned to a conformational change within the dimer, and the other was a second order process involving reassociation to the tetramer (\( k = 4.35 \times 10^7 \text{ M}^{-1} \text{sec}^{-2} \)) which was followed by a rapid conformational change within the tetramer. In our notation these are represented by

\[ D_l \rightarrow D_d \]  
\[ D_l + D_l \rightarrow T_l \rightarrow T_d \]

where \( D_l \) denotes the unliganded dimer produced from \( D_l(O_2)_2 \) by removal of ligand. Our results suggest that \( D_l \) and \( D_d \) are identical.

These two schemes are not as different as they appear. Although we find no first order process such as Reaction 12, this reaction only contributes to the removal of \( D_d \) at hemoglobin concentrations below 1.5 \( \mu \text{M} \) where the absorbance changes measured are very small. Since side reactions due to the use of dithionite will be especially significant at these small absorbance changes (46), accurate measurements of the reaction at these low concentrations are very difficult. Therefore, this discrepancy with the present interpretation is felt unlikely to be serious.

Dimer Hypothesis—One reaction scheme which has been proposed to account for the functional properties of hemoglobin is the so-called dimer hypothesis (11). This hypothesis was advanced to account for the retention of full functional behavior under conditions in which extensive dissociation of liganded and deoxy-Hb to dimers was believed to have occurred (9, 43, 47). In its most general form, this hypothesis states that the dimer is the basic unit of function in hemoglobin and that it possesses most of the functional characteristics of the tetramer. The behavior of the tetramer can then be largely approximated by that of the free dimer (11). It is now recognized that the dimer derived from liganded hemoglobin is noncooperative (19). In view of this result it has been suggested (19, 35) that there are two types of dimer: the αββ dimer derived from liganded hemoglobin, and the αβα dimer derived from deoxy-Hb, which are assumed to be cooperative and noncooperative in ligand binding, respectively. The dimer referred to in the dimer hypothesis is then the αββ dimer.
The simplest interpretation of the results presented here, and those of Park (38), is that there is only one type of hemoglobin dimer, the $\alpha_2\beta_2$ dimer, at all pH values greater than pH 7.6, irrespective of the presence or absence of ligand. This free dimer is noncooperative by two criteria; the rate of ligand binding is rapid, and association of the free dimer to the deoxy-Hb tetramer is accompanied by the characteristic spectral change in the Soret region. Furthermore, in equilibrium studies of oxygen binding it was found that the oxygen affinity rose precipitously at pH values above 10.0 (48), where extensive dissociation of both deoxy-Hb and liganded hemoglobin to dimers has been shown to occur. Recent ultracentrifuge investigations have established that the experiments on which the dimer hypothesis was founded were incorrectly interpreted; the extent of dissociation to the dimer is predominantly tetrameric (39).

It must be concluded that the intact tetramer is the minimum unit able to exhibit normal functional behavior. Association of free dimers to the deoxy-Hb tetramer is accompanied by a conformational readjustment within the tetramer to give the cooperative species. A full description of the functional behavior of hemoglobin must then be based on the tetramer, and must incorporate the effect of organic phosphates (49-51) and the inherent differences in reactivity of the $\alpha$ and $\beta$ chains (51, 52).

Acknowledgments—It is a pleasure to thank Dr. S. J. Edelstein and Mark J. Rehm for making the ultracentrifuge and data processing programs available and for guidance in their use. We thank Dr. R. L. Nagel for the gift of the haptoglobin, Dr. R. MacQuarrie for helpful discussions, and Christa Molloy for technical assistance.

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