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Subunit Interactions in Hemoglobin Probed by Fluorescence and High-Pressure Techniques†

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ABSTRACT: The dissociation of the subunits of human adult oxyhemoglobin has been investigated by using steady-state fluorescence anisotropy, multifrequency phase fluorometry, and high hydrostatic pressure. Human hemoglobin obtained by using two purification procedures (bulk preparation by centrifugation or further fractionation using anion-exchange chromatography) was labeled with an extrinsic fluorescent probe, 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNS-Cl). The long fluorescence lifetime of this probe allows for the observation of the macromolecular tumbling, and thus provides a method for observing changes in the size of the complex upon subunit dissociation under differing solution conditions of proton and organic phosphate concentration. At pH 7, the dansylated preparations of bulk and fractionated hemoglobin showed a concentration-dependent decrease in the anisotropy which though not identical can only arise from the tetramer to dimer dissociation. We observed primarily the dimer at pH 9 and a small destabilization of the tetramer in the presence of saturating inositol hexaphosphate (IHP). High-pressure experiments allowed for the observation of the dissociation of the hemoglobin dimer into monomers. From these measurements, we estimate the dimer dissociation constant to be between 0.1 and 1 nM. We compare the present results on the subunit affinities in hemoglobin obtained from steady-state and time-resolved fluorescence data with those obtained previously by using gel filtration, sedimentation, and kinetic techniques. These comparisons are indicative of a certain degree of conformational heterogeneity in the hemoglobin preparations.

The regulation of the activity of oligomeric proteins is based upon free energy linkages between the various molecules of the system (Wymann, 1948, 1964). While the values of these cooperative or coupling free energies are relatively small (~1–2 kcal), they are responsible for the activation or inhibition of the biological process upon changes in metabolic conditions. In the oligomeric proteins, ligation effects are modulated through free energy couplings of ligand binding with those of the subunit interactions. Thus, monitoring changes in the subunit affinity in such systems is equivalent, from an energetic point of view, to monitoring changes in the ligand binding equilibria (Weber, 1972; Ackers et al., 1976). The hemoglobin tetramer has often served as a model system for the study of the physical basis for these coupled interactions. The destabilization of the hemoglobin tetramer by the binding of oxygen, as well as the effects of the allosteric ligands on the dimer affinity, has been studied by gel filtration, osmotic pressure, sedimentation, and kinetic techniques (Ackers & Thompson, 1965; Guidotti, 1967; Chiancone et al., 1968; Kellet & Schachman, 1971; Thomas & Edelstein, 1972; Atha & Riggs, 1976; Antonini & Chiancone, 1977; Chu & Ackers, 1981). Techniques such as sedimentation and gel filtration are not entirely passive and involve a certain degree of perturbation (i.e., chemical interactions with column resins and pressure and gradient effects in the ultracentrifuge).

In the present work, we have undertaken the study of the subunit interactions (and the effect of allosteric ligands thereon) in hemoglobin using fluorescence spectroscopy and high hydrostatic pressure. These methods permit the direct observation of variation in the size of the macromolecular complex upon dissociation through the measurement of the depolarization of fluorescence due to brownian tumbling. The fluorescence lifetime of the covalent probe 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNS-Cl) is sufficiently long for the observation of the global tumbling of proteins with the molecular weight of hemoglobin. In order to minimize the effects of the chemical modification, we have worked under conditions of low probe/protein ratios (<1 DNS/heme). The tetramer–dimer equilibrium of hemoglobin was studied by dilution. The combination of fluorescence techniques with high hydrostatic pressure has made possible the direct observation of the dissociation of the dimers into monomers. The use of hydrostatic pressure to study high-affinity equilibria in oligomeric proteins is based on Le Chatelier's principle of equilibrium perturbation (Paladini & Weber, 1981a; Heremans, 1982; Weber & Drickamer, 1983, Weber, 1987). The destabilizing effect of pressure stems from the fact that hydration is more efficient for the dissociated subunits than for the oligomeric species due to imperfect interfacial packing in the oligomer and electrostriction effects resulting in a smaller volume for the dissociated form.

In addition to its perturbing effects, each particular technique presents a unique correlation between the signal and the population of molecules under study. In the case of steady-state fluorescence anisotropy, the weight of each molecular species in solution is dependent upon its decay rate. Heterogeneity in hemoglobin preparations has been reported from observations of the intrinsic tryptophan fluorescence in which the long-lived tryptophan decay is essentially eliminated by anion exchange (Bucci et al., 1988a,b; Szabo et al., 1989). It was of interest to determine whether any relation existed between the heterogeneity of a given preparation and the apparent subunit affinity as determined from the anisotropy
measurements. Therefore, we have investigated the subunit equilibria for two preparations of DNS-labeled hemoglobin. One consisted of hemoglobin purified by standard procedures, HB, and the second from a further fractionation using anion-exchange chromatography, MC.

**Materials and Methods**

**Fluorescence Measurements**

**Polarization.** Steady-state fluorescence polarization measurements on the DNS-labeled hemoglobin samples were carried out in the photon counting mode in L-format using a Greg PC fluorometer (ISS, Inc., Champaign, IL). Excitation was at 340 nm, and the emission was monitored at 500 nm with a Y-44 cutoff filter (Hoya Optics, Inc., Freemont, CA) to eliminate any scattered light intensity. For the very high hemoglobin concentrations, observation of the fluorescence was from the front face of a triangular cuvette to avoid trivial reabsorption effects. The average standard deviation of the polarization measurements was ±0.005 or less. Dilution and Perrin plot polarization measurements were corrected for any contribution of buffer or sucrose background fluorescence to the total signal.

**Lifetime.** The frequency response of the DNS-labeled hemoglobin emission was measured by the multifrequency phase modulation fluorometer described by Gratton and Linkemman (1983). The exciting light, the 325-nm line from a Liconix HeCd continuous-wave laser, was modulated with the acousto-optic modulator system described by Piston and co-workers (Piston et al., 1989). A Y-44 cutoff filter was used to monitor the fluorescence emission. The instrumental phase delay and demodulation were corrected for by measuring the relative phase and demodulation between the hemoglobin samples and that of the standard fluorophore 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP; Eastman Kodak, Syracuse, NY) in ethanol with a fluorescence lifetime of 1.32 ns (Gratton et al., 1984). The standard phase error was ±0.2° or less, while that of the modulation ratio was ±0.004 for the 10-12 frequencies between 2 and 100 MHz monitored for each sample.

**Pressure Experiments.** High-pressure fluorescence measurements were carried out by using a high-pressure cell and generator similar to that described by Paladini and Weber (1981b). The high-pressure polarization data were corrected for the slight pressure-induced birefringence in the quartz windows of the cell using a modified version of the method of Paladini and Weber (1981b) which takes into account the wavelength dependence of the birefringence (Royer and Scarlata, unpublished results). The reference lifetime used to correct for the instrument response in lifetime measurements was the zero value for the scatter of the exciting light from the protein particles. This light (325 nm) was monitored by using a Corion 320-nm interference filter (band-pass 10 nm) (Corion Corp., Holliston, MA). The sample was allowed to equilibrate for 5 min at each pressure before data were acquired. All pressure transitions were found to be ≥95% reversible from comparison of the polarization values and absorption spectra measured before application of pressure and approximately 30 min after release.

**Analysis**

**Lifetime Analysis.** Multifrequency phase and modulation data sets of DNS-labeled hemoglobin as a function of either concentration or pressure were simultaneously analyzed by using the global analysis software package Globals Unlimited which is based on the theory and algorithms described elsewhere (Knutson et al., 1983; Beechem et al., 1983, 1989; Beechem & Gratton, 1988). These algorithms allow for the simultaneous analysis of multiple data sets in terms of an internally consistent kinetic model, which in this case was the linkage of lifetime values between all data sets (dilution or pressure) of each analysis, with preexponential factors allowed to vary. Such a linkage scheme describes a ground-state equilibrium which is perturbed by dilution or pressure. Average lifetime values were obtained from the linear combination of the lifetime values recovered from the global fits weighted for their recovered fractional contribution to the decay.

**Molecular Volume Calculations.** Polarization values, \( p \), were converted to emission anisotropy, \( A \), by using the relation

\[
A = (2/3)(1/p - 1/3)^{-1}
\]

The average molecular volume, \( V_m \), was calculated from the Perrin equation (Perrin, 1926):

\[
A_0/A - 1 = RT\langle\tau\rangle/\eta V_m
\]

where \( A_0 \) is the limiting anisotropy of the fluorophore, \( \langle\tau\rangle \) is the average lifetime, \( R \) is the gas constant, \( T \) is the kelvin temperature, and \( \eta \) is the viscosity of the solution. The slopes and intercepts of the Perrin plots were obtained from linear least-squares regression of the data. The average molecular volumes for the MC hemoglobin preparations were calculated by using the value of \( A_0 \) (0.35) obtained from the intercept of the Perrin plot \((1/p_0 - 1/3)\) carried out on these samples. For the HB standard samples, the value of 0.31 (Weber, 1951) was used in these calculations. Use of a higher limiting anisotropy in the calculations of the average molecular volumes for the HB samples would simply shift the volumes to lower values by implying more local fluorophore mobility, but has no effect upon the dilution profile, itself.

**High-Pressure Subunit Dissociation Calculations.** The dissociation constant for the hemoglobin dimer–monomer equilibrium was calculated from the average molecular volumes under pressure in the following manner. The degree of dimer dissociation at each pressure, \( \alpha_\rho \), was obtained from the equation:

\[
\alpha_\rho = \left[ V_{mD} - V_{m(p)} \right] / \left[ V_{mD} - V_{mM} \right]
\]

where \( V_{m(p)} \) is the average molecular volume at pressure, \( p \), \( V_{mD} \) is that of the dimeric hemoglobin molecule, and \( V_{mM} \) is that of the monomeric hemoglobin subunit, taken as the low- and high-pressure plateau values, respectively. The dissociation constant at each pressure, \( K_{d(p)} \), was calculated following Paladini and Weber (1981a):

\[
K_{d(p)} = 4[D]_0 \alpha_\rho^2 / [1 - \alpha_\rho]
\]

where \([D]_0\) is the total protein concentration expressed as dimer. The volume change \( \Delta V_p \) for the association of monomers to dimer and the dissociation constant for that equilibrium at atmospheric pressure were obtained from the slope and the intercept, respectively, of the plots of \( \ln [\alpha^2/(1 - \alpha)] \) vs pressure.

**Preparation of Hemoglobin Samples**

**Standard Preparation of Hemoglobin.** All of the buffer components utilized in the following experiments were of high purity and showed no evidence of fluorescence contaminants. Sodium chloride and sodium borate, potassium phosphates, and Tris Ultra Pure were purchased from Fisher Scientific (Fair Lawn, NJ), Mallinckrodt, Inc. (Paris, KY), and Schwarz/Mann Biotech (Cleveland, OH), respectively. Several grades of sucrose from several vendors were tested.
and the purest was found to be the ultra-pure sucrose from Schwarz/Mann. Even this grade of purity contained a small amount of fluorescent contaminant which was subtracted from the Perrin plot signals. The water used in all buffers was soaked in 50% ethanol, rinsed with 10 mM sodium bideionized and further purified with a NANOpure system (Barnstead/Sybron Corp., Boston, MA). To eliminate carbonate/ 1 mM Na2EDTA, and boiled in the same buffer for 30 min and then for another 30 min in water. Finally, the tubing was rinsed with water and stored at 4 °C. Adult human hemoglobin was purified from fresh blood provided by Carle Foundation Hospital (Urbana, IL) following the method of Perutz (1968) with precipitation of the erythrocyte membranes using 280 mM phosphate buffer, pH 7. The resulting sample was dialyzed overnight at 4 °C against pure water. Organic phosphates were stripped from the hemoglobin sample by elution over an AG501-X8(D) column (Bio-Rad Laboratories, Richmond, CA) (Jelkmann & Bauer, 1976). The resultant protein sample, which we will refer to as HB, was either labeled with the fluorescent dye for direct use in depolarization experiments or subjected to further fractionation by a modification of the methods of Bucci and co-workers (Bucci et al., 1988a,b) and Szabo and co-workers (Szabo et al., 1989) as described below. Absorbance measurements were carried out with a Perkin-Elmer Lambda VI spectrophotometer (PerkinElmer, Norwalk, CT). Hemoglobin concentrations were calculated from the absorbance at 576 nm using an absorption coefficient of 15 150 cm² mol⁻¹ (Banerjee et al., 1969).

Fractionation of the HB Sample. The HB sample was diluted to a concentration of approximately 10⁻³ M in heme into a 20 mM Tris-HCl buffer, pH 8.4, and applied to an FPLC (Pharmacia Corp., Uppsala, Sweden) DE52 anion-exchange column (Whatman Biosystems Ltd., Maidstone, England) which had been previously equilibrated with the same buffer. The different fractions of hemoglobin were eluted with three discontinuous buffers which were 20%, 30%, and 100% mixtures of a 20 mM Tris-HCl/200 mM sodium acetate buffer at pH 8.4 with the 20 mM Tris-HCl starting buffer. The elution profile of the above fractionation is shown in Figure 1. Four eluted peaks are evident. The hemoglobin sample which we will refer to as the major component, MC, corresponds to the fraction of the principal peak (in total peak volume, not optical density) after the first 30% and the last 40% were discarded. The fourth peak, which was found to contain two different hemoglobin fractions, was also seen by Bucci and co-workers (Bucci et al., 1988a). It was not systematically further fractioned in our preparations since we were interested only in working with the MC sample. The MC sample was dialyzed overnight at 4 °C against pure water to eliminate the sodium acetate and Tris. The MC sample was then concentrated at 4 °C under vacuum to that of the HB sample [(2-4) × 10⁻⁴ M in tetramer] in order to carry out the labeling reaction under identical conditions.

Labeling Reaction. The HB and MC samples were diluted to a final concentration of (1-2) × 10⁻⁴ M into sodium borate buffer, pH 8.0, final concentration 240 mM. The solution containing the fluorescent probe, 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNS-Cl) (Molecular Probes, Inc., Portland, OR), at 20 mM in N,N-dimethylformamide, was added to the hemoglobin sample to obtain a final concentration of DNS-Cl of approximately 1 mM. The reaction was allowed to proceed at 4 °C for 2 h for the MC sample and for 4 h for the HB sample. The samples were then applied to a Sephadex G-25 Superfine desalting column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in 100 mM Tris-HCl, pH 7.0, to stop the reaction and separate the labeled protein material from the free dye. The protein-containing fractions from the desalting column were pooled and then divided into three samples of equal volume. These samples (1-2 mL) were then dialyzed at 4 °C, 3 times against 300 mL of 100 mM Tris-HCl either at pH 7.0, at pH 9, or at pH 7.0 with 1 mM inositol hexaphosphate (IHP) from Sigma Chemical Co. (St. Louis, MO). There was no evidence of any free dye in the last dialysis buffer either by absorption or by fluorescence emission measurements. All measurements were carried out at 20 °C, and the final experimental pH was directly verified in the cuvette using a micro-electrode (Micro-electrodes, Inc., Londonderry, NH).

RESULTS

Characterization of DNS-Hemoglobin

Calculation of the Labeling Ratios. For the calculation of the ratio of dye per tetrameric hemoglobin, it was assumed that the absorbance at 340 nm was the sum of the absorbance of the dye and of the protein. The contribution of the protein absorbance at 340 nm was deduced from the ratio of the absorbance at 576 nm to that at 340 nm in the absence of the dye and the absorbance of the labeled protein at 576 nm. The molar extinction coefficient for DNS at 340 nm was taken to be 4500 cm² mol⁻¹ (Haugland, 1983). The average labeling ratios in DNS molecules per tetramer for the seven HB preparations were systematically smaller (1.6-2.0) than those obtained for the six MC preparations (2.0-2.8). It is interesting to note that for each HB preparation the apparent calculated labeling ratio was largest for the solutions dialyzed in the presence of IHP (1.98 ± 0.40) and smallest for those dialyzed at pH 7 (1.56 ± 0.43). The average value for the labeled hemoglobin solutions dialyzed at pH 9 was found to be intermediate (1.68 ± 0.47). There was also a systematic difference between the apparent calculated labeling ratios for the MC samples dialyzed under the same three conditions, although the order was not identical with that found for the HB samples. In the case of the MC samples, it was the solutions dialyzed at pH 9 that gave the largest labeling ratio (2.76 ± 0.58). Those dialyzed against the pH 7 buffer again yielded the lowest labeling ratio (2.00 ± 0.41). The MC samples dialyzed against the buffer containing IHP yielded intermediate apparent labeling ratios (2.38 ± 0.49). Since the labeling of these three samples was in fact carried out on one pooled sample, the reaction of course was identical. We thus conclude that the small differences in labeling ratios (calculated from the dye and protein absorbance) for the three different dialysis conditions result from small alterations in
the extinction coefficient of the either the dye (340 nm) or the heme (576 nm) when the protein is in the presence of the allosteric effectors, protons or organic phosphates. The fluorescence measurements were carried out on a total of 13 different preparations. The extent of labeling varied for these preparations between one and three DNS molecules per tetramer. There was no correlation found between the number of dansyl molecules per tetramer and the measured subunit affinities for these different samples. In addition, the systematic reproducibility of the order of these calculated ratios for the three dialysis conditions served as a control for the reproducibility of our preparations.

Control Parameters for the HB and MC Preparations. Before labeling, the reproducibility of the preparations was determined by the measurement of the absorbance at 576 nm to that at 340 nm. For the HB sample, the average value was 0.561, and the standard deviation was extremely low, ±0.006. The value for the MC samples was 0.554, also with the same standard deviation. After labeling, the ratio of the two visible peaks, 576 and 542 nm, was measured as an indication of the degree of formation of methemoglobin. The measured values (between 1.030 and 1.052) were consistent with the absence of any methemoglobin. In addition, we found that the values for the HB samples at pH 7 and 9 were consistently higher (1.052 ± 0.004) than those calculated for the MC samples (1.036 ± 0.008) under the same conditions. The addition of IHP in both cases resulted in a decrease in the average peak ratio (1.043 ± 0.003 for HB and 1.030 ± 0.009 for MC) although the HB value remained larger than the MC value.

We interpret the fact that slightly lower values for both two-peak ratios (576/340 and 576/542 nm) for the MC samples, as compared to HB, is indicative of subtle differences in the heme environments of the molecules present in the total hemoglobin preparation, HB. The component (MC) separated on the basis of anion interaction with the DEAE column thus represents a certain subpopulation of the hemoglobin and is composed uniquely of hemoglobin Aα as determined by Bucci and co-workers (Bucci et al., 1981a) by electrophoretic methods. The other DEAE peaks revealed heme absorption characteristics identical with those of hemoglobin. Their number, a total of 3 (the last containing two components), excludes the possibility that they correspond uniquely to Aα, Aγ, and Aγ. Reproducible chromatograms from multiple donors also exclude the possibility that these fractions correspond to abnormal hemoglobins.

Dilution Studies on DNS-Hemoglobin

Fluorescence Polarization Measurements. The DNS-HB and DNS-MC samples at pH 7, at pH 7 in the presence of 1 mM IHP, and at pH 9 were diluted in small steps from approximately 10^{-4} to 10^{-6} M in hemoglobin tetramer. The fluorescence anisotropy was measured as described under Material and Methods as a function of protein concentration. These results are shown in Figure 2. In all cases, we observe a concentration-dependent decrease in the anisotropy of the DNS emission, consistent with oligomer dissociation. A plateau at low concentrations is observed for all samples studied except the MC sample at pH 7. For both the HB and MC fractions, the curves at pH 9 or in the presence of IHP are shifted to higher protein concentrations than those obtained at pH 7. Additionally, the values for the DNS anisotropy for the MC samples were in all cases higher than those observed for the HB samples.

Fluorescence Lifetime Measurements. The above results were highly suggestive of protein oligomer dissociation by dilution. However, the steady-state fluorescence anisotropy value is determined not only by the rotational rate of the fluorophore but also by the weights and values of the fluorescence decay rates of the sample. For this reason, we carried out a dilution study of the decay of the DNS-hemoglobin fluorescence for the two fractions, HB and MC, under the three experimental solution conditions as a function of protein concentration. The data from the measurement of the frequency response of the DNS-hemoglobin samples were analyzed globally assuming a ground-state equilibrium as described under Materials and Methods. The best fits were to a linked triple-exponential decay. Neither a double-exponential decay nor a double or single distribution of lifetimes gave an adequate fit to the data. An example of the data for the DNS-hemoglobin at pH 7 for the HB sample is shown in Figure 3. The global χ² for this fit was 2.5. The global χ² values for the other samples were between 3.1 and 5.2. Since the data were fit to a linked model in which dilution resulted in a change in the fractional contribution for a constant lifetime value, we recover three lifetime components for each dilution curve. These values are given in Table I. For both the HB and MC preparations, the very short value for the shortest decay, between 120 and 340 ps, is indicative of efficient energy transfer of the DNS emission to the heme. It can be seen from the values in Table I that the lifetimes for the HB preparation were longer than those recovered for MC at pH 7. For both HB and MC preparations, the addition of IHP had very little effect on the lifetimes values.

In Table II are given the recovered fractional intensities (f_i) and molecular percentages (α_i) for the long-lived component at four protein concentrations. The contribution (f_2 and α_2)
of the intermediate lifetime (~15% for HB and ~26% for MC) was independent of concentration. The variation of the contribution of the highly quenched component is the result of the subtraction from 1 of the sum of the fractional weights of the long and intermediate species. From the values in Table II, it can be seen that between approximately 30 and 80% of the total intensity, as indicated by the fractional amplitude, originate from only 1 to 10% of the molecular population, as calculated from the \( \alpha \) values. If the population of molecules is homogeneous, the percentage of molecules observed in the time-averaged experiments is unimportant. However, if the solution contains a heterogeneous population of hemoglobin molecules, predating the excited state and differing in their structural interactions and/or dynamic properties, the weighting of the different species in the observable parameters in the steady-state measurements may not be evenly distributed. This may well be the case in the above steady-state anisotropy measurements, given that the molecules observed are primarily those with a long fluorescence lifetime. A second interesting observation is that the fractionation by the DEAE column results in a decrease in the number of molecules emitting with the long lifetime and a concomitant increase in those having an intermediate decay under a given set of experimental conditions.

In Figure 4 (bottom) are plotted the molecular percentages of the long component of each sample under the three experimental conditions. The effect of dilution on the emitting population of both the HB and MC preparations is to decrease the highly quenched population to the benefit of the long-lived emitters. For the MC preparation, the change is observed for the first dilution step from approximtely 3.0 \( \times 10^{-4} \) to 1.3 \( \times 10^{-3} \) M tetramer. The change observed for the first dilution step at high concentration is interpreted as resulting from a decrease in energy transfer of the DNS emission to the heme moieties between tetrameric molecules (intramolecular effect). The second dilution step results in no change, whereas between 4 \( \times 10^{-3} \) and 1 \( \times 10^{-4} \) M, a small increase in \( f_1 \) and \( \alpha_1 \) is observed. This loss of the very short component to the very long one is interpreted as arising from a decrease in energy transfer between subunits upon tetramer dissociation, an intramolecular effect. The concentration dependence of the molecular percentages of the HB samples is more gradual than that observed for MC. This cannot result from a shift to lower concentrations of the dilution effects on intertetramer energy transfer. The implication then is a shift to higher concentrations of the tetramer dissociation of the HB preparation. Thus, in contrast to the steady-state measurements, in the concentration range where trivial intermolecular transfer is eliminated, the changes in the molecular fractions of the lifetime components can be correlated with the effect of dilution upon the entire population of molecules.

The values of the recovered lifetimes for all six samples were used in conjunction with the concentration-dependent fractional contributions to calculate the average fluorescence lifetime as a function of protein concentration as described under Materials and Methods. These results are also shown in Figure 4 (top). The values are distributed between 4.5 and 11.5 ns over the four concentrations for the three solution conditions tested for the HB and MC preparations. The values for the HB samples are systematically larger than those calculated for the MC samples, resulting from larger values for both the long component, itself, and its fractional contribution in the HB samples. These differences range between 2 and 3 ns. Since the value of the average lifetime is essentially determined by the fractional contribution and value of the long component, the dilution profiles for the average lifetime closely follow the trends of the values of the molecular percentages.

**Average Molecular Volumes.** Using eq 2, the anisotropy and average lifetime values obtained from the fractional intensities and decay values of each species, we have calculated the average molecular volume for the HB and MC samples as a function of the tetramer concentration at pH 7, at pH 9.

### Table I: DNS-Hemoglobin Lifetime Values Recovered from Global Analysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>HB</th>
<th>MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7</td>
<td>26</td>
<td>34</td>
</tr>
<tr>
<td>IHP</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>pH 9</td>
<td>27</td>
<td>34</td>
</tr>
</tbody>
</table>

*The lifetime values (nanoseconds) of the triple-exponential decay model were linked across the data sets at each protein concentration. The contribution of \( \tau_2 \), ~15% for HB and ~26% for MC, was independent of concentration. The variation in the fractional intensities and preexponential factors for \( \tau_1 \) was simply the results of the subtraction from one of the sum of the other two.*
Hemoglobin Subunit Dissociation

FIGURE 5: Average molecular volumes calculated from the data in Figures 2 and 4 according to eq 2 as a function of $-\log [\text{DNS-HB}]$ in tetramer for DNS-HB at pH 7 (O), at pH 7 in the presence of 1 mM IHP (l), and at pH 9 (A) and for DNS-MC at pH 7 (O), at pH 7 in the presence of 1 mM IHP (l), and at pH 9 (A). Lines drawn through the points do not represent a fit of the data.

FIGURE 6: Perrin plots for the DNS-MC preparation at pH 7 and pH 9. Three protein concentrations were measured at pH 7: 1.65 $\times 10^{-5}$ M ([A] and solid line), 1.0 $\times 10^{-2}$ M ([X] and dashed line), and 4.0 $\times 10^{-4}$ M ([O] and dotted line in tetramer)]. At pH 9, the concentrations examined were 2.0 $\times 10^{-5}$ M ([A] and solid line), 1.0 $\times 10^{-3}$ M ([X] and dashed line), and 4.0 $\times 10^{-5}$ M ([O] and dotted line in tetramer)]. Aliquots of 60% sucrose were added to the solutions, and background sucrose fluorescence was subtracted using blank containing no protein. Lines represent the result of a linear least-squares reduction of the data.

section and showed no change upon addition of sucrose. The values for the molecular volumes extracted from the Perrin plots in Figure 6 are given in Table III. Those found for pH 9 (~26,000 mL/mol) agree reasonably well with the theoretical volume (35,200 mL/mol) calculated for a dimer of molecular weight 32,000 and a hydration of 0.38 g/g (Cantor & Schimmel, 1980). The observed lack of concentration dependence as well as the actual plateau value lead us to conclude that at pH 9, the observed populations of both preparations of hemoglobin are essentially dimeric below $10^{-5}$ M in tetramer. A destabilization of the tetramer at pH 9 is compatible with ionic repulsion due to titration of the amino acid side chains. The molecular volume for the MC solution at pH 7 decreased from 43,400 to 33,400 mL/mol upon dilution, indicative of tetramer dissociation toward dimer in this range.

As described under Materials and Methods, the molecular volumes were calculated by using the average lifetime weighted with the fractional intensities. We have verified that these values did not artifically result from the lifetimes heterogeneity by performing simulations of the Perrin plots using the lifetime values and limiting anisotropy obtained from the data and the theoretical rotational correlation time of hemoglobin. No significant curvature of the plots was found, justifying the calculation of the average lifetime from the fractional intensities. In addition, one can calculate the average lifetime value necessary for the observation of a given rotational rate, $\phi$, using the following relation derived from the additivity of anisotropy and the Perrin equation:

$$\langle \tau \rangle = \left( \frac{f_1 \tau_1 \phi + f_2 \tau_2 \phi + \tau_1 \tau_2}{f_1 \tau_1 + f_2 \tau_2 + \phi} \right)$$  \hspace{1cm} (5)$$

The average $\tau$ values calculated in this manner (averaging the two short components) were almost identical with those calculated from the fractional intensities and used in the Perrin equation calculations of the volumes.

The values for the dissociation constants were estimated from the midpoint of these dilution curves. It was assumed that the molecular volume for the tetrameric species was approximately twice that found at the dimer plateau. The dissociation constant for all of the HB samples indicated that the population of hemoglobin molecules observed in the HB sample presents a relatively low affinity between the dimers. The estimated dimer affinity for the HB solution at pH 7 was found to be approximately $3 \times 10^{-4}$ M. The profile in the presence of IHP for the HB preparations was quite similar to that in the absence of the effector, indicating no major effect of IHP on the observed tetramer dissociation. The dissociation constant for the MC sample at pH 7 was found to be much lower than for HB (~4 $\times 10^{-6}$ M), and a destabilization of the tetramer by IHP was observed in this case.

High-Pressure Studies

Due to the high affinity between the monomeric subunits in the hemoglobin dimer, its dissociation to monomers had not been previously directly measured. The observation of dimer

Table III: Molecular Volumes for the Major Component of the DEAE Fractionation of DNS-Hemoglobin Calculated from the Perrin Plots

<table>
<thead>
<tr>
<th>pH 7</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb-DNS]$_T$ (mM)</td>
<td>[Hb-DNS]$_T$ (mM)</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
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<tr>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
dissociation by dilution necessitates concentrations which are too low, even using fluorescence techniques. For this reason, high hydrostatic pressure was used to perturb the equilibrium. The pressure dependence of the anisotropy of the DNS-hemoglobin fluorescence emission was measured for both the HB and MC preparations at two or three protein concentrations and under the three experimental conditions, at pH 7, at pH 7 in the presence of IHP, and at pH 9. In all cases, a concentration-dependent, pressure-dependent decrease in the anisotropy was observed, indicative of oligomer dissociation. The variation in the fluorescence lifetime as a function of pressure (in 500-bar increments from atmospheric pressure to 2 kbar) was also investigated. The average fluorescence lifetime for all samples increased linearly by approximately 1.3 ns over 2 kbar. As in the dilution experiments, this increase is interpreted as being due to a loss of energy transfer between the subunits upon dissociation. Owing to the difficulty of the high-pressure lifetime experiments, the quality of the data do not permit a more detailed analysis or interpretation ($\chi^2 \sim 8$–$10$ compared to $\sim 2$–$5$ for dilution curves at equivalent concentrations).

From the values of the anisotropy and the average lifetime, the average molecular volume as a function of pressure was calculated by using eq 2 and is presented in Figures 7 and 8, for HB and MC, respectively. The curves measured for the highest concentration ($3.2 \times 10^{-5}$ tetramer) for both preparations are indicative of the superposition of the tetramer–dimer and the dimer–monomer equilibria. Dissociation constants could not be extracted from these data due to the absence of an end point and to the linked equilibria. Equilibrium constants for the dimer–monomer equilibrium from the data obtained at $10^{-6}$ M tetramer for the HB samples at pH 7 in the absence and in the presence of IHP were calculated from the linear regression of the plots of ln $(\alpha^2/(1 - \alpha))$ vs pressure (Figure 9) (see Materials and Methods). This was straightforward since both a low- and high-pressure plateau were evident. As was the case in the dilution experiments, surprisingly high values for these subunit dissociation constants were obtained and are given in Table IV. The $K_d$ at pH 7 for HB was found to be $4.8 \times 10^{-6}$ M (dimer) and for IHP, $1.2 \times 10^{-9}$ M (dimer). The constant at pH 9, estimated by assuming the same total decrease in molecular volume as observed for the two experiments at pH 7, was found to be much lower, at $6 \times 10^{-9}$ M (dimer).

For the HB preparations, the curves taken at the middle and lower concentrations ($4 \times 10^{-6}$ and $1 \times 10^{-6}$ M tetramer) converge to the same molecular volume value at high pressure ($12,000$ mL/mol). Dissociation constants were calculated only for the curves at the lowest concentrations, since contamination of the observed dimer–monomer equilibrium by residual tetramer dissociation was at a minimum. The end point for these transitions was considered as the point at which all curves converged at high pressure. The logarithmic plots may be found in Figure 9 and the resultant $K_d$ values in Table IV. At pH 7, the value was much lower than that found for the HB solution at the same pH, $2.8 \times 10^{-9}$ M (dimer) (a factor of 17). However, the sample in the presence of IHP presents approximately the same value for the two

**Table IV: High-Pressure Dissociation Parameters of DNS-Hemoglobin**

<table>
<thead>
<tr>
<th>Condition</th>
<th>HB $K_d$ (M)</th>
<th>$\Delta V_d$ (mL/mol)</th>
<th>MC $K_d$ (M)</th>
<th>$\Delta V_d$ (mL/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7</td>
<td>$4.8 \times 10^{-6}$</td>
<td>91</td>
<td>$2.8 \times 10^{-6}$</td>
<td>93</td>
</tr>
<tr>
<td>+IHP</td>
<td>$1.2 \times 10^{-9}$</td>
<td>90</td>
<td>$7.4 \times 10^{-9}$</td>
<td>71</td>
</tr>
<tr>
<td>pH 9</td>
<td>$6.0 \times 10^{-9}$</td>
<td>78</td>
<td>$7.7 \times 10^{-8}$</td>
<td>78</td>
</tr>
</tbody>
</table>

*These values were calculated by assuming a dimer to monomer dissociation based on the concentrations for dimer formation from tetramer obtained from the dilution curves.*
preparations $7.4 \times 10^{-8}$ M (dimer) (a factor of 1.6). At pH 9, a destabilization of the dimer was found as compared to the HB sample, with a $K_d$ of $7.7 \times 10^{-8}$ M (dimer) (a factor of 13). The volume changes for monomer association to dimer, $\Delta V_m$, are typical for a dimer of molecular weight of approximately 30,000 (Paladini & Weber, 1981a; Silva et al., 1986; Harrigan et al., 1989) and are also given in Table IV.

**DISCUSSION**

We have investigated the subunit affinities in two different human hemoglobin preparations (HB and MC) using fluorescence spectroscopy and high-pressure techniques at pH 9 and at pH 7 in the absence and in the presence of organic phosphates. We have measured the emission anisotropy and lifetime of a long-lived covalent extrinsic fluorescence probe as a function of protein concentration to monitor the dissociation of the tetrameric species to dimer. In addition, the application of high hydrostatic pressure was used to dissociate the dimeric species into monomers.

Despite the high affinity between the subunits in the hemoglobin dimer, we have observed close to 100% monomer formation at pressures below 2.4 kbar. The dimer–monomer $K_d$ calculated for the MC sample at pH 7 was $\sim 3 \times 10^{-9}$ M in dimer, while that for the HB sample was 15-fold higher. Kellet and Schachman (1971) by sedimentation estimated the value at $\pm 3.1 \times 10^{-9}$ M in dimer. The values we have obtained are quite comparable to those reported for a number of high-affinity dimers such as the enolase dimer (10$^{-7}$ M) (Paladini & Weber, 1981a; Xu & Weber, 1982), the $\lambda$ repressor (2 \times 10$^{-8}$ M) (Seneac & Ackers, 1988), tryptophan synthase (10$^{-9}$ M) (Silva et al., 1986), trp repressor (10$^{-9}$–10$^{-8}$ M) (unpublished results), human sex steroid binding protein (10$^{-10}$–10$^{-11}$ M) (Ross et al., 1989), and yeast hexokinase (10$^{-10}$–10$^{-11}$ M) (Ruan & Weber, 1988). Mrabet and co-workers (Mrabet et al., 1986), using kinetics of radiolabeled $\alpha$-chain exchange, and the dimer association rate reported by Kawamura and Nakamura (1983), estimated a subpicomolar affinity. Since typical measured values for dimer affinity range between $10^{-8}$ and $10^{-11}$ M, we find it highly unlikely that the hemoglobin dimer affinity estimated from the present results ($\sim 10^{-9}$ M) would be incorrect by 4 orders of magnitude.

Our bulk HB preparation yielded tetramer–dimer dissociation constants from the molecular volume curves of $\sim 3 \times 10^{-5}$ M, whereas the MC sample at pH 7 dissociates to dimer with a $K_d$ of approximately $4 \times 10^{-6}$ M. We have compared the molecular volume dilution profiles with the results of a number of studies of the oxygen-induced tetramer dissociation in hemoglobin using gel filtration and sedimentation techniques (Chiancone et al., 1968; Kellett & Schachman, 1971; Atha & Riggs, 1976; Antonini & Chiancone, 1977; Chu & Ackers, 1981). The dissociation constant obtained from the bulk HB preparation at pH 7 was approximately 1 order of magnitude higher than the values given by the above investigators. However, the dissociation of the MC sample was found to be in approximately the same concentration range.

Whereas for the HB sample the dilution effects on anisotropy and lifetime were quite dissimilar, this was not the case for the MC preparation. It should be remembered that the fractionation over an anion-exchange column increased the apparent homogeneity of the DNS-hemoglobin emission, as evidenced by the decrease in the contribution of the long-lived fluorescence decay from 3–10% for HB to 1–5% for MC. There is thus an underlying heterogeneity of hemoglobin molecules in the HB preparation which can be diminished by chromatographic techniques. In addition, it appears that the molecular species exhibiting the long lifetime also exhibit a low affinity between subunits and that most of these molecules are eliminated on the DEAE column. The preparation obtained from the DEAE fractionation displays a higher apparent subunit affinity than that observed for the HB samples due to the difference in the weighting of each molecular species to the total fluorescence signal. Since the observed signal in centrifugation (absorption or interference) and gel filtration (absorption or radioisotopes) maps to the majority of the population, small amounts of oligomer exhibiting altered subunit affinities are not detected. In the present experiments, the signal is highly weighted for the small fraction of hemoglobin molecules presenting long fluorescence lifetimes and lower subunit affinities.

The question arises as to the character of this fraction of hemoglobin molecules. They cannot arise from abnormal hemoglobins, since identical profiles were obtained with hemoglobin extracted from the blood of multiple donors. Besides the simple fact that certain hemoglobin molecules are eliminated from the HB preparation by anion exchange, the significant and systematically reproducible differences in the UV and visible absorption spectra of the MC as compared to the HB samples before the labeling reaction as well as their different chemical reactivities with DNS (4 h for HB, 2 h for MC) constitute evidence that the heterogeneity is not labeling-induced. Similar results of heterogeneous fluorescence emission from hemoglobin preparations observing tryptophan decay were obtained by Bucci and co-workers (Bucci et al., 1988a,b) and Szabo and co-workers (Szabo et al., 1989). Chromatography evidently selects conformational states of hemoglobin with different fluorescence lifetimes and, in this case, subunit affinity. Although we have observed no changes in our data over the limited period of the experiments (2 days), we cannot discount a time-dependent property of these conformational states.

Interpretation of the present results in the presence of organic phosphate and at high pH is not as straightforward as for those at pH 7. The addition of saturating organic phosphate leads to a certain degree of destabilization of the tetrameric and dimeric species in all cases. In the experiments at pH 9, we observed very little tetramer, even at high concentration. Previous studies (Weidemann & Olson, 1975; Chu & Ackers, 1981; Benesch et al., 1986) have shown that the coupling between these effectors and the subunit interactions in hemoglobin are bimodal. As the pH or organic phosphate concentration is increased, a stabilization of the tetramer is observed. However, at a given concentration of organic phosphate (depending upon the phosphate used) and above pH 8.5, this trend reverses, and the dimeric species is favored. It is possible that the molecules which we observe using fluorescence spectroscopic methods present a shift to lower phosphate and higher proton concentrations of this bimodal behavior.

In conclusion, due to the high sensitivity of fluorescence and the destabilizing effect of pressure on protein subunit interactions, we have observed the formation of the hemoglobin monomer, and from the high-pressure data, we estimated a nanomolar dissociation constant for the dimer–monomer equilibrium. We have also studied the dilution profile of the tetrameric species and have observed its dissociation to dimer. We have compared these data with those obtained by a variety of techniques and have concluded that the hemoglobin solution contains molecules presenting different subunit affinities. The heterogeneity observed in this study for the hemoglobin molecule may well represent a general phenomenon in the in vitro study of proteins with important implications for the
interpretation of past and future physical measurements of macromolecules. Polymorphism in hemoglobin and myoglobin has been observed by using a number of techniques (Theorell & Akeson, 1955; Rossi-Fanelli & Antonini, 1956; Perkoff et al., 1962; Atassi & Saplin, 1966; Austin et al., 1975; Yamamoto & La Mar, 1986; Bucco et al., 1988a,b; Szabo et al., 1989). Acalca and co-workers (Acalca et al., 1987) have interpreted the distributed fluorescence decay in a number of proteins as arising from ground-state conformational heterogeneity. Recently, Ruan and Weber (1989) have concluded from their high-pressure dissociation studies that the subunit affinities in yeast glyceraldehyde phosphate dehydrogenase tetramers presents considerable heterogeneity. These observations concerning the heterogeneity of the physical states of macromolecules in vitro raise additional questions as to the existence and eventual role of such heterogeneity in vivo.

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