Electrophoretic Mobilities and Diffusion Coefficients of Hemoglobin at High pH†

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ABSTRACT: Diffusion studies by photon correlation of scattered laser light confirm the dissociation of the tetrameric form of human carboxyhemoglobin to dimers above pH 10 and provide new estimates of the subunit dissociation equilibrium constants in this pH range. Electrophoretic light-scattering experiments under the same conditions reveal that the electrophoretic mobilities of tetramers and dimers are indistinguishable to within instrumental resolution (ca. 7% in these experiments). The data imply an increase of the electrical charge on the dimer of at least 2.8 to 4.4 net negative charges upon dissociation. Mechanisms for the accumulation of negative charge by the dimer upon dissociation of the tetramer are proposed.

We have used the techniques of electrophoretic light scattering (Ware & Flygare, 1971; Ware, 1974) and photon correlation spectroscopy (Chu, 1974; Berne & Pecora, 1976) to study the dissociation of the liganded hemoglobin tetramer to dimers at high pH. Dissociation of hemoglobin tetramers to dimers has been studied by several experimental techniques (see Herskovits et al., 1978, and references therein), and the effects of this dissociation upon estimated values of ligand-binding constants have been considered (Ackers & Halvorson, 1974). Proton release accompanying the transition from tetramer to dimers has been inferred from linked-function analysis of the dissociation equilibrium constant's dependence upon pH (Anderson et al., 1971; Atha & Riggs, 1976). In an attempt to characterize further the extent of dissociation at high pH and the electrical charge properties of the dimer and tetramer, we have measured the diffusion coefficients and electrophoretic mobilities as a function of pH and hemoglobin concentration. Our electrophoretic light-scattering spectra of human carboxyhemoglobin (HbCO) imply that the electrophoretic mobility of the dimeric form is within about 7% of that of the tetrameric form in the vicinity of pH 10.2. Our z-average diffusion coefficients measured for similar solutions confirm the presence of substantial proportions of both dimers and tetramers under these experimental conditions. The proposal that the free dimer acquires an average of at least 2.8 to 4.4 additional negative charges is consistent with our data.

Materials and Methods

All hemoglobin was prepared by the method of Williams & Tsay (1973) with the substitutions of gravity-driven flow of the DEAE[1]-Sephadex A-50 chromatography columns and of mixing pH 8.6 Tris buffer with the main-column eluant prior to application of the hemoglobin A_0 fraction to the collection column (Williams & Tsay, 1973). The hemoglobin was dialyzed against four changes of 500 mL of 0.005 M glycine–0.005 M NaOH–0.005 M NaCl—0.0001 M EDTA buffer (pH 10.3) and stored as 1.5 mM (heme) stock solutions under a CO atmosphere at 4 °C. The sample of desired heme concentration, pH, and ionic strength was prepared on the day of the experiment by dilution of stock solution with an appropriate amount of buffer which had been previously titrated with HCl or NaOH. The concentration of methemoglobin was less than 3% of the total hemoglobin in the samples for which data are reported. All light-scattering experiments were conducted at a thermostating bath temperature of 20.0 °C.

The samples were partially degassed by placing a 50-mL glass syringe with plugged nozzle containing 25 mL of sample and a 1-mL bubble of CO inside a bell jar and then gently evacuating the bell jar to 0.1 atm for about 0.5 h. After returning the interior of the bell jar to ambient pressure, the syringe was unplugged and the bubble was expressed from the syringe. The nozzle of the sample syringe was attached to a buffer-filled filter holder containing two 0.050-μm pore-size (N005 025 00 CPR), three 0.10-μm pore-size (N010 025 00 CPR), and three 0.20-μm pore-size (N020 025 00 CPR) Nucleopore filters which had no gas bubbles trapped between filter layers and which had been rinsed with 10 mL of methanol at 1 mL/s flow rate, 50 mL of distilled water at 5 mL/h flow rate, and 100 mL of glycine buffer at 5 mL/h flow rate.

The electrophoretic light-scattering spectra were obtained at a scattering angle of $\theta = 2.0°$ with a sample chamber of the design described previously (Haas & Ware, 1976). Palladium electrodes with platinum wire leads, pressed against the stainless-steel chamber body halves, replaced the silver–silver chloride electrodes and allowed elimination of two of the four access holes in the upper Teflon insert. Attachment of the filter holder outlet to the sample chamber inlet, and of the chamber outlet to a collection syringe with Teflon tubing, provided a continuous flow-through arrangement for filling the chamber and for retaining a portion of the sample for optical absorption spectroscopy, pH, and electrical conductivity measurements. The palladium electrodes were charged with hydrogen prior to each experiment by making the electrode in the inlet-side of the chamber negative with respect to a platinum wire inserted in the outlet tube and passing a 0.2-mA current for 10 min; then the palladium electrode in the outlet-side chamber cavity was made negative with respect to the inlet-side palladium electrode and the same current was passed for 5 min. Light from a Spectra-Physics Model 125A He–Ne laser (80 mW, 632.8-nm wavelength) was shaped to an ellipse in the scattering region by a spherical lens located at its focal length

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin.

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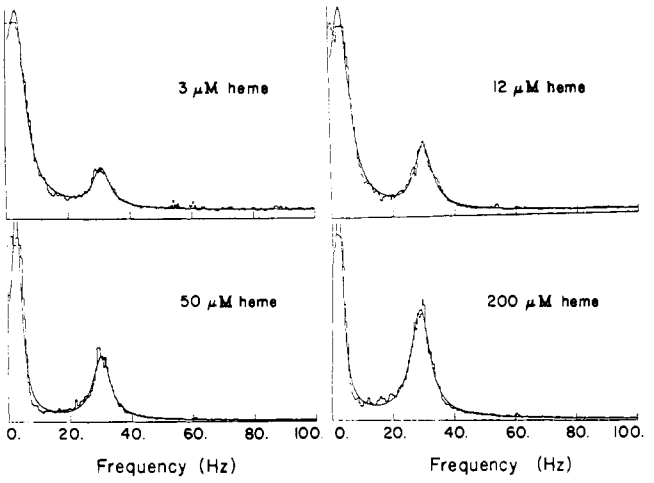

FIGURE 1: Electrophoretic light-scattering spectra for various HbCO concentrations: pH 10.2; 0.01 M ionic strength glycine–NaOH–NaCl–EDTA buffer for E = 106 V/cm; θ = 2.0°; 20.0 °C thermostating bath temperature.

in front of the chamber, preceded by a horizontally focusing cylindrical lens at a distance equal to the sum of the focal lengths of the two lenses. The focal length of the cylindrical lens was three times as long as that of the spherical lens, so that at the scattering region these illumination optics produced a focused beam which measured approximately 0.01 cm in the vertical dimension and 0.10 cm in the horizontal dimension. The greater dimension in the horizontal direction was necessary to reduce transit-time broadening of the Doppler peaks. An Electronic Measurements Model C612 constant-current power supply provided the electric field E whose value was calculated from the equation:

$$E = i/\sigma A \quad (1)$$

where i is the electrical current, σ is the electrical conductivity of the sample solution, and A is the cross-sectional area of the chamber at the scattering region. The electric field was supplied in 2-s pulses which were alternated in polarity and were separated by 15 s. The electrophoretic light-scattering frequency spectra were accumulated with a Saicor SAI-51B Real-Time Spectrum Analyzer. Spectra were digitally corrected for the slightly nonlinear frequency response of the detection electronics. Further details of our apparatus have recently been published (Smith & Ware, 1978; Haas, 1978).

Each power spectrum was least-squares fitted to a base line plus two Lorentzian line shapes, one with a center frequency at the origin to account for low-frequency intensity in the power spectrum and the other with an adjustable center frequency. The center frequency of the latter line shape is proportional to the electrophoretic mobility of the scattering centers in the sample; the half-width at half-maximum of this line shape is proportional to the diffusion coefficient D for a monodisperse sample. The electroosmotic flow in the chamber was determined from the dependence of the peak shift upon vertical position of the scattering region in the gap. All peak shifts were corrected to the point of zero electroosmotic flow. All peak widths were corrected for broadening effects such as transit-time broadening, finite aperture of the detection optics, and Joule heating of the scattering region due to the passage of electrical current.

Photon-correlation experiments were performed on hemoglobin solutions in the self-beat mode by illuminating the samples with the He–Ne laser beam, which was focused to a

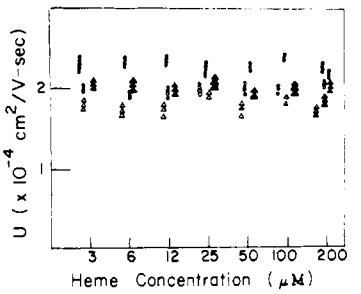

FIGURE 2: Apparent electrophoretic mobilities of single-shifted peak fitted electrophoretic light-scattering spectra of HbCO for E = 106 V/cm, θ = 2.0°, as a function of total heme concentration: (●) pH 10.6, (○) pH 10.2, (▲) pH 9.9, (△) pH 9.6.

50-μm diameter by a 3-cm focal-length spherical lens. The hemoglobin sample was contained in a scattering chamber constructed from a 0.2-cm tall section cut from a 1.0 × 0.5 cm glass fluorescence cuvette, capped above and below by Teflon squares. Approximately one coherence area of light scattered at θ = 90° was observed with an EMI 9558B photomultiplier mounted in a radiofrequency-shielded Products for Research Model TE-241-RF photomultiplier housing. An SSR Model 1120 Photon Counter discriminated and amplified the photocurrent pulses, and the autocorrelation function of these pulses was computed by a Saicor SAI-42A Correlation and Probability Analyzer operating in the pulse-counting mode. The autocorrelation functions were analyzed by the method of cumulants (Koppel, 1972) with the best least-squares base line.

Results

Electrophoretic light-scattering spectra of HbCO at pH 10.2 in 0.01 M ionic strength glycine buffer are displayed in Figure 1. These spectra were obtained with 106 V/cm electric field and 2.0° scattering angle. The stair-step line represents the actual data points, and the smooth line is the single-shifted-peak least-squares fit. The linear proportionality between the Doppler shift frequency of the shifted peak and the electric-field strength was verified for each sample. The apparent electrophoretic mobilities for hemoglobin concentrations over the range of 3 (heme) to 200 μM (heme) from pH 9.6 to 10.6, as calculated from the corrected shift of the single-shifted-peak fit, are graphed in Figure 2. The corrected spectral widths of these peaks are graphed in Figure 3. The typical value of 1 unit of standard deviation estimated from the computer-fitted line shapes is 0.1 cm^2/V-s for the electrophoretic mobilities and 0.2 Hz for the peak widths. The electrophoretic mobility of the samples increases with pH but displays no obvious dependence on hemoglobin concentration. The peak widths do not exhibit systematic dependence upon either pH or total heme concentration, although the lower signal to noise ratio for lower heme concentration results in greater scatter in the data, as expected.

An autocorrelation function obtained from a 180 μM (heme) HbCO sample (pH 10.2) is displayed in Figure 4 along with the least-squares-fitted cumulant function. The autocorrelation function decay times (half of the reciprocal of the first cumulant) from a three-cumulant fit for 90° scattering are plotted in Figure 5 for HbCo concentrations of 24 (heme) and 180 μM (heme). The decay times for pH 9.6 to 9.9 are nearly the value, $\tau_{1/2}$ (θ = 90°) = 20.5 × 10^{-6} s, which corresponds to the diffusion coefficient of the hemoglobin tetramer measured at pH 7, $D_{20,w}$ = 6.9 × 10^{-7} cm^2/s (Haas et al., 1974; Jones & Johnson, 1976). The decay times decrease between pH 9.9 and 10.6 to values more appropriate for diffusion

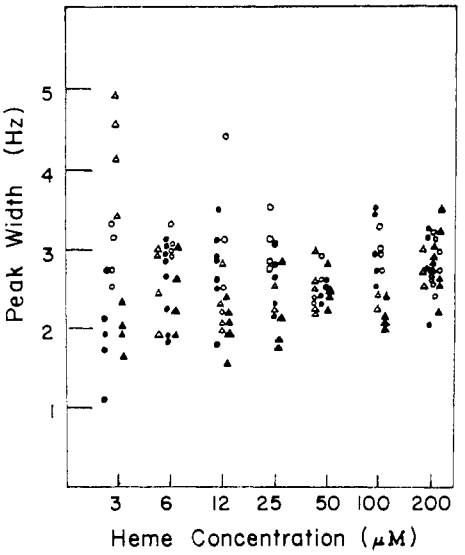

FIGURE 3: Apparent line width of single-shifted peak fitted electrophoretic light-scattering spectra of HbCO observed at $\theta = 2.0°$, $E = 106$ V/cm, as a function of total heme concentration: (●) pH 10.6, (○) pH 10.2, (▲) pH 9.9, (△) pH 9.6.

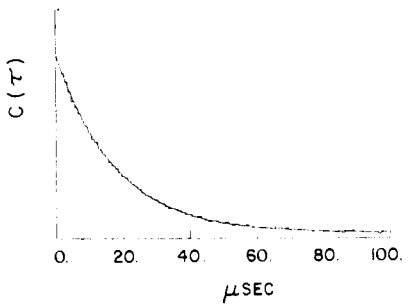

FIGURE 4: Photocount autocorrelation function obtained from 180 μM (heme) HbCO, pH 10.2, at $\theta = 90°$.

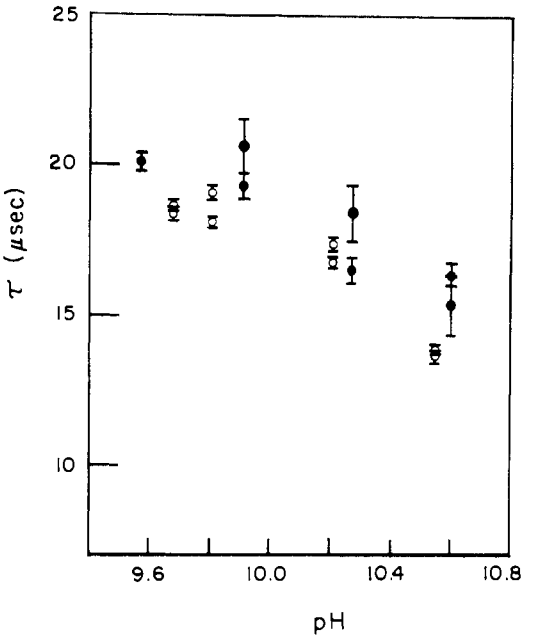

FIGURE 5: Dependence of the exponential decay time of the homodyne autocorrelation function of light scattered by HbCO upon pH: (●) 24 μM (heme), (○) 180 μM (heme).

TABLE I: First (K_1^3) and Second Cumulants (K_2^3) from Three-Cumulant Fits of Photocount Autocorrelation Functions Obtained from 180 μM (Heme) HbCO at $\theta = 90°$.

pH	1st cumulant ($K_1^3 \times 10^{-4}$ s)	2nd cumulant ($K_2^3 \times 10^{-8}$ s)
9.68	2.68 ± 0.06	0.2 ± 0.5
9.81	2.76 ± 0.05	1.2 ± 0.4
10.21	2.99 ± 0.06	1.1 ± 0.6
10.55	3.61 ± 0.08	2.2 ± 1.0

of the dimeric form. Table I lists the first and second cumulants and their estimated standard deviations from a three-cumulant fit of autocorrelation functions for 180 μM (heme) HbCO accumulated over 3 h from one coherence area. An increase of the first cumulant with pH suggests that the average size of the proteins decreases, while an increase of the second cumulant indicates increasing polydispersity of the sample; both of these trends are consistent with more extensive dissociation of HbCO tetramers at higher pH.

Electrostatic repulsion between charged macromolecules may influence their diffusional motions in low ionic strength solutions (Raj & Flygare, 1974; Phillies, 1974; Stephen, 1974; Doherty & Benedek, 1974; Weissman & Ware, 1978). It is, therefore, useful to correct our diffusion data for this effect before using these data to infer changes in the state of association of the hemoglobin. We have estimated this correction from the data of Doherty & Benedek (1974) for the increase in the diffusion coefficient due to the electrostatic repulsion observed in solutions of bovine serum albumin (BSA) in which the BSA had an electrical charge of about the same magnitude as the hemoglobin molecules in our solutions. This correction was never more than 2% for the 24 μM heme solution and never more than 15% for the 180 μM heme solution. The z-average diffusion coefficients of HbCO in Figure 6 are calculated from values of the first cumulants after correction for the electrostatic effect present in data for BSA scaled linearly by the protein concentration (Doherty & Benedek, 1974). The tetramer-dimer equilibrium constants, $K_{4,2}$, listed in Table II are inferred from the z-average diffusion coefficients by assuming the diffusion coefficient for the tetramer as 6.9×10^{-7} cm^2/s and for the dimer as 8.5×10^{-7} cm^2/s. These estimates of $K_{4,2}$ are in accord with previous measurements in this pH range (Anderson et al., 1971; Atha & Riggs, 1976).

Discussion

For the ideal case of a sample composed of a few noninteracting species of substantially different electrophoretic mobilities, each species in the sample should elicit a separate peak in the electrophoretic light-scattering spectrum. In the present work, the choice between one or more species of similar electrophoretic mobilities is decided by analyzing the envelope of the electrophoretic light-scattering spectrum as a single shifted peak and then determining whether the behavior of this spectral envelope can be the result of a single species in the sample or must be due to overlapping peaks from more than one species. If only one species is present in all of our samples, then the apparent electrophoretic mobility of the spectral envelope should be independent of HbCO concentration and should increase smoothly with pH; the corrected width of the single-shifted peak fit should be equal to the diffusion breadth expected for that species. For a sample with both tetramers and dimers present in substantial amounts, the apparent electrophoretic mobility of the single-shifted-peak fit would be a weighted average of the electrophoretic mobilities of the two hemoglobin forms; the single-shifted-peak fit width should be greater than the diffusion width for either species and should be a function of the difference in electrophoretic mobilities of the two forms.

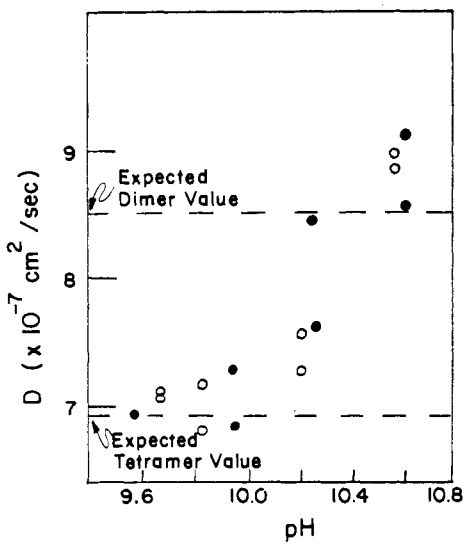

FIGURE 6: Diffusion coefficient of HbCO inferred from the exponential decay time of the autocorrelation function after correction for the electrostatic effect: (●) 24 μM (heme), (○) 180 μM (heme). The expected value of D for the tetramer was taken from previous work (Haas et al., 1974), and the expected value of D for the dimer was calculated assuming an effective radius R_0 of 23 Å and a shape correction f/f_0 of 1.006.

Lowering the total heme concentration in the sample increases the ratio of dimers to tetramers. If the electrophoretic mobility of the tetramer differs from that of the dimer, a single-shifted-peak analysis of electrophoretic light-scattering spectra collected for a series of increasing total heme concentrations at a propitious pH should display a monotonic concentration dependence of the apparent electrophoretic mobility and a fitted-peak width which increases from the dimer diffusion breadth to a maximum (when the total heme concentration is approximately 0.75 $K_{4,2}$) and then decreases toward the tetramer diffusion breadth. This rationale predicts that the apparent electrophoretic mobility and peak width at constant pH should be noticeably dependent upon hemoglobin concentration for at least one of the concentration series if the solution conditions span a sufficient range of the tetramer–dimer equilibrium. However, these anticipated trends are not evident in the data of Figures 2 and 3.

The photocount autocorrelation data for light scattered at 90° demonstrate that the solution conditions for equal concentrations of tetramers and dimers in the same sample fall within the span of HbCO concentration and pH for these experiments. The z-average diffusion coefficients in Figure 6 inferred from the autocorrelation functions confirm that sufficient amounts of the dimer form are present in our samples above pH 10 to scatter a substantial fraction of the light. Therefore, the apparent absence of concentration dependence for the peak position and width of the electrophoretic light-scattering spectra indicates that the electrophoretic mobilities of the tetramer and dimer are identical near pH 10.2 within the limits of resolution of these experiments (ca. 7% by either criterion of apparent electrophoretic-mobility dependence or peak-width dependence upon total heme concentration).

The dissociation rate for hemoglobin tetramers to dimers has been estimated to be on the order of 1 s^{-1}, depending upon solution conditions and experimental method (Gibson & Antonini, 1967; Nagel & Gibson, 1971; Anderson et al., 1971; Flamig & Parkhurst, 1977). If individual peaks from the tetrameric and dimeric forms had been resolved, the association and dissociation reaction rates might have been deduced from anomalous positions and widths of the two peaks in one electrophoretic light-scattering spectrum (Berne & Giniger, 1973; Ware, 1974). Even if the interconversion rate is so fast that the tetramer and dimer peaks are always coalesced, the apparent electrophoretic mobility produced by the single-shifted-peak analysis should still vary with total heme concentration if the electrophoretic mobility of the tetramer does not equal that of the dimer. A rapid interconversion rate might account for an absence of two resolved peaks in a single spectrum under other conditions, but only coincidence of tetramer and dimer electrophoretic mobilities can explain the lack of total heme concentration dependence for the single-shifted-peak position in these data.

TABLE II: Tetramer-Dimer Equilibrium Constant Inferred from the z-Average Diffusion Coefficient of HbCO Measured by Photon-Correlation at Various pH Values.

	$K_{4,2} = \{D\}^2/\{T\}$	
pH	24 μM (heme) sample	180 μM (heme) sample
9.6	<1 μM	<10 μM
9.9	<10 μM	<20 μM
10.2	20–1000 μM	30–150 μM
10.6	>100 μM	>500 μM

The dependence of electrophoretic mobility on particle charge and size and on solution conditions is given approximately by Henry's equation (Henry, 1931; Tanford, 1961):

$$U = \frac{Ze}{6\pi\eta R} \frac{F(\kappa R)}{(1 + \kappa R)} \quad (2)$$

where Z is the number of unit charges on the particle, e is the magnitude of a unit charge, η is the solvent viscosity, and R is the radius of a spherical particle or the effective radius of a globular particle. In eq 2, κ is the Debye–Hückel characteristic inverse screening length and $F(\kappa R)$ is called Henry's function, which varies from 1.0 for very small particles to 1.5 for very large particles. From eq 2 it can be seen that the dimer must accumulate a net negative electrical charge upon subunit dissociation in order to attain the same electrophoretic mobility as the tetramer. An increase of the electrophoretic mobility through an unexpectedly large reduction of the dimer hydrodynamic friction coefficient is ruled out by the reasonable behavior of the diffusion coefficient. The tetrameric charge of −14 inferred by eq 2 from the electrophoretic mobility at pH 9.6 agrees very well with hydrogen-ion titration values of −13 to −16 (Bucci et al., 1968; Janssen et al., 1970, 1974), but at pH 10.2 the tetrameric charge of −16 from electrophoresis falls short of the −17 to −26 charges from titration. However, a large proportion of the hemoglobin molecules is suspected to be dissociated at pH 10.2, so that titration curves may actually indicate proton-loss behavior of the dimer, lowering the estimate of the tetramer charge to between −14 and −22. This possibility was previewed by Tanford & Nozaki (1966), who found that the value of the electrostatic interaction factor w must be decreased "from a value characteristic of whole hemoglobin to a value approaching that which would be characteristic of the half-molecule" in order to fit their hydrogen-ion titration curves in 0.25 M ionic strength solution between pH 10.0 and 10.4 (Tanford & Nozaki, 1966), and other authors concur with this finding (Bucci et al., 1968). This adjustment of hydrogen-ion titration curves for dimerization brings the tetramer charge measured by electrophoretic light scattering at pH 10.2 into good agreement with the titration values. Since the resolution of our experiments requires that the free dimer possess at least 70% of the tetrameric charge in order to have indistinguishable dimer and tetramer electrophoretic mobilities, the dimers must gain an average of more

than 2.8 to 4.4 net negative charges at pH 10.2 to render the subunit dissociation imperceptible.

Comparison of the number of ionizable groups exhibiting high pK values, as measured by hydrogen-ion titration with the known chemical composition of hemoglobin, shows that the tetramer conceals some of its histidine, tyrosine, and possibly cysteine residues from the titrating solution, but many of these groups become titratable upon reaction of hemoglobin with p-mercuribenzoate (PMB) to split the tetramer into individual α and β chains or upon denaturation of the protein (Tanford & Nozaki, 1966; Bucci et al., 1968; Janssen et al., 1970, 1972, 1974; Beychok & Steinhardt, 1959). Dissociation of tetramers to dimers may be accompanied by a release of protons caused by the exposure of these residues to solution and by possible reduction of the pK values of lysine and arginine groups due to an alteration in their chemical environments. Spectrophotometric measurements which utilize the difference between the absorption coefficients at 245 nm of the protonated and unprotonated forms of tyrosine indicate titration of 1.1 tyrosines per dimer upon subunit dissociation (Anderson et al., 1971). Linked-function analysis of the dependence of $K_{4,2}$ upon pH estimates the total number of protons released at high pH upon dissociation of a tetramer to dimers as 1.6 (Atha & Riggs, 1976) or 3.6 (Anderson et al., 1971). Judging by the disparity of these estimates, proton release might actually be sufficient to account for the entire charging of the dimer.

Other ions might also bind differentially to hemoglobin tetramers and dimers and be partially responsible for the added charge of the dimer. Studies of the influence of chloride ions on the Bohr effect in hemoglobin indicate that fewer Cl^- ions are bound by HbCO at higher pH (Antonini et al., 1963; deBruin et al., 1974; Rollema et al., 1975). Scatchard & Yap (1964) found that serum albumin bound fewer chloride ions at higher pH and suggested that the small anions bind only to the acid form of the receptive sites on the protein (Scatchard & Yap, 1964). Since many residues per tetramer are candidates for ionization upon subunit dissociation but less than four protons appear to be released according to observations of the dependence of $K_{4,2}$ upon pH, we suggest that the free dimers acquire additional charge by binding chloride ions. The amount of chloride binding could be tested independently in three ways: electrochemical measurements of chloride ion activity in HbCO solutions similar to those performed on serum albumin by Scatchard & Yap (1964), membrane potential measurements employing the Donnan effect to determine the total charge of HbCO as a function of chloride ion concentration and HbCO concentration (Adair & Adair, 1940), and linked-function analysis of the dependence of $K_{4,2}$ upon chloride ion concentration at high pH. Salt concentration is already known to have a substantial effect on the tetramer-dimer equilibrium constant near pH 7, and the effect varies considerably with different ionic species (Antonini & Brunori, 1971).

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