Electrostatic Interactions in Hemoglobin from Light Scattering Experiments

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Light scattering experiments on solutions of bovine CO-hemoglobin have revealed that a substantial component of the interactions responsible for the protein stability versus tetramer-to-dimer dissociation is due to electrostatic effects. Their magnitude has been estimated to reach $\simeq 4$ kcal/mole ($\simeq 0.15$ eV) at $p\mathrm{H}=5$ compared with the nonelectrostatic term which is of the order of 7 kcal/mole ($\simeq 0.3$ eV).

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The stability of hemoglobin, a well-known globular protein, has been discussed at length and tested with a variety of experimental studies [1–4]. The data available in the literature refer to different conditions of salt concentration, cosolvents, ligation (O_2 or CO), etc., but do not appear very conclusive about the nature of the interactions that are responsible for hemoglobin stability. Hemoglobin, a tetramer which is made up of four polypeptide chains (two α chains and two β chains), is supposed to dissociate into a pair of $\alpha\beta$ dimers under a variety of conditions including low protein concentration, high ionic strength, and pH values far from the isoelectric point [3,5–8]. Different kinds of interactions occur in hemoglobin and it is generally accepted that hydrophobic contacts and electrostatic forces are the relevant interactions responsible for the protein stability. The main purpose of this research has been to elucidate the role of the electrostatic interactions in liganded hemoglobin (when bound to carbon monoxide, CO) under conditions of weak electrostatic screening at low ionic strength. These conditions have been chosen since it is known that CO binds very strongly to hemoglobin and that liganded hemoglobin dissociates more easily than nonliganded hemoglobin [8].

Since stability seems to depend upon the values of ionic strength and pH, it is assumed that some dissociation occurs when a net charge is located on the protein due to electrostatic repulsion between the dimers. By changing the pH of the solution it is possible then to vary the net charge of the protein as shown by the results on human hemoglobin [9–11], and, consequently, we expect that this may alter the fraction of dissociated hemoglobin. Attempts to correlate dissociation with electrostatic effects by means of gel filtration have not yielded reliable results [8]. At pH 5 to 8.5, from acid titration experiments, an approximate linear relationship between charge and pH is found with positive values below isoelectric pH, $p\mathrm{H}_{\mathrm{iso}}$, and negative values above $p\mathrm{H}_{\mathrm{iso}}$. Accordingly, if dissociation is dominated by electrostatic interactions it should be described by an electrostatic contribution to the free energy. This contribution must be quadratic in the net protein charge and, therefore, a parabolic dependence upon pH is expected.

Both static and dynamic (or quasielastic) light scattering have been employed here since they are a useful means for studying dissociation. This technique has two advantages over other experimental approaches previously employed: (a) It does not appreciably perturb the system and (b) estimates of the size of the scattering particles can be obtained.

When considering static light scattering it is known that the intensity of the light scattered at a distance r, per unit volume, from a solution of small particles of mass M and weight concentration c is given by [12]

$$I = I_0 \frac{4\pi^2 (dn/dc)^2}{N_A \lambda^4 r^2} cM , \tag{1}$$

where n is the solution index of refraction, I_0 the incident light intensity, N_A Avogadro's number, and λ the wavelength. If dissociation of the particles occurs then the scattered intensity is proportional to $c_T M_T + c_D M_D$, instead of cM as found for undissociated solutions, where the subscripts refer to tetramers and dimers, respectively. The fraction x of dissociated tetramers can then be expressed by

$$x = 2(I_T - I_{T,D})/I_T , \tag{2}$$

where I_T refers to the signal from a solution of undissociated tetramers and $I_{T,D}$ to the signal from the sample containing both tetramers and dimers. The value of $M_T/M_D = 2$ has been adopted when deriving Eq. (2).

In this paper, however, more emphasis has been given to dynamic light scattering which consists of measuring the autocorrelation function

$$g^{(2)}(\tau) \equiv \langle I(t) I(t+\tau) \rangle / \langle I \rangle^2 \tag{3}$$

of the scattered light intensity $I(t)$ which, for particles endowed with translational diffusion constant D, is given by [13]

$$g^{(2)}(t) = 1 + A e^{-2DK^2 t} . \tag{4}$$

In Eq. (4) A is a constant and $K = (4\pi n/\lambda)\sin(\theta/2)$ is the light scattering vector at the angle θ. For a spherical particle then, dynamic light scattering gives an estimate of its radius R since $D = k_B T/f$, where $f = 6\pi\eta R$ and η is the viscosity of the solution.

To a first approximation hemoglobin tetramers and di-

mers may be considered to possess spheroidal shape [14], with gyration radius R_T for the tetramer, and gyration radius $R_D \simeq R_T/2^{1/3}$ for the dimer. It should be pointed out, however, that with light scattering one measures the hydrated protein radius which is $\simeq 4$ Å larger [14] than the crystallographic radius (27.8 Å). The presence of a hydration layer lowers the R_T/R_D ratio from $2^{1/3}=1.26$, when not hydrated, to 1.22 when both tetramers and dimers are hydrated. Another correction factor that should be considered is due to the aspherical shape of tetramers and dimers which requires $f_T = 6\pi\eta R_T \times 1.002$ and $f_D = 6\pi\eta R_D \times 1.013$ for tetramer and dimer, respectively, since the tetramer can be described, approximately, as an oblate ellipsoid with semiaxes of 29.2, 29.2, and 25.0 Å, and the dimer as a prolate ellipsoid with semiaxes 19.4, 19.4, and 28.3 Å [14]. Accordingly, the expected ratio of the diffusion coefficients becomes $D_D/D_T = 1.207$.

Published data on tetramer $\rightleftarrows$ dimer kinetics show that the reciprocal of the rate constant, at the conditions of this work, $> 10^{-2}$ s [15], is much longer than the correlation time, 80 μs, and therefore our scattering data refer to solutions containing two kinds of particles, tetramers and dimers. Consequently the autocorrelation function is given by

$$g^{(2)}(t) = 1 + B(N_T M_T^2 e^{-D_T K^2 t} + N_D M_D^2 e^{-D_D K^2 t})^2 , \quad (5)$$

with B a constant. The values of D_T and D_D are close to each other, their expected ratio being 1.207, and therefore from the autocorrelation function we can extract only some average value of the translational diffusion coefficient, the so-called Z average [16],

$$D_Z = \frac{4N_T D_T + N_D D_D}{4N_T + N_D} , \quad (6)$$

with N_T and N_D being number concentrations. Then

$$g^{(2)}(t) = 1 + B(NM^2 e^{-D_Z K^2 t})^2 , \quad (7)$$

with $NM^2 = N_T M_T^2 + N_D M_D^2$. From scattering data we obtain D_Z and from this we can estimate the fraction x of dissociated tetramers according to

$$x = 2(D_Z - D_T)/(D_Z + D_D - 2D_T) . \quad (8)$$

Since D_Z depends upon pH then x also depends upon pH. Our data when converted to x, according to Eq. (8), are plotted in Fig. 1.

Solutions of bovine carbon monoxide hemoglobin, or CO-hemoglobin, were prepared, at 10 mM ionic concentration, employing the buffers acetate, bis-tris, tris, and borate in order to cover the pH range from 4.7 to 11.4. Values of pH outside the 5 to 10 range will not be discussed here since protein denaturation or a further dissociation into monomers may occur. (This problem will be examined elsewhere.) Concentration of hemoglobin was kept constant at about 5 mg/ml for all measurements and checked with a spectrophotometer for the presence of methemoglobin (less than a few percent). All samples were prefiltered with 0.45 μm Millipore filters and subsequently with 0.1 μm Nucleopore filters.

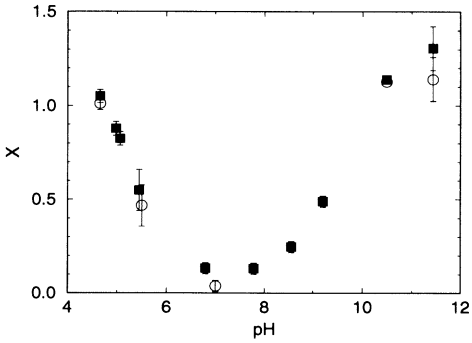

FIG. 1. Estimated fraction x of dissociated bovine CO-hemoglobin tetramers vs pH assuming that only dimers are produced. Values of x near or above unity correspond to the presence of monomers. The squares correspond to dynamic light scattering whereas the circles belong to static scattering data.

The scattering cell, 10 mm in diameter, was surrounded by a larger cell with an index-matching liquid (decahydronaphthalene) and the temperature kept at 25.5 ± 0.5 °C. The scattered light at 6328 Å (25 mW He-Ne laser) was collected at an angle of 40 deg and processed with a 4-bit unclipped Brookhaven correlator using a sample time of 3.5 μs. Local heating effects have been corrected for by extrapolating D_Z to zero laser power [17].

Single-exponential fits of the autocorrelation of the scattered signal were very successful for all solutions and yielded negligible and random residuals as shown in Fig. 2 for a typical run.

The thermodynamics of hemoglobin dissociation can be described by the free energy ΔG_{diss} which has been assumed to derive from the sum of two contributions:

$$\Delta G_{\text{diss}} = \Delta G_{\text{el}} + \Delta G_n , \quad (9)$$

where ΔG_{el} is due to the electrostatic contribution under

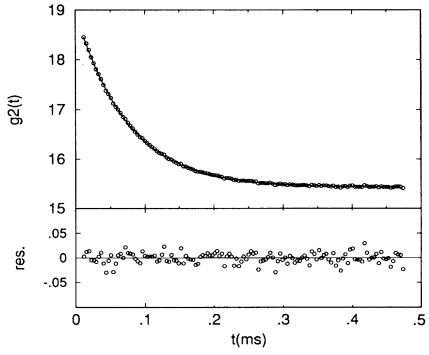

FIG. 2. Autocorrelation function $g_2(t)$ of the scattered light intensity and its residuals from a single-exponential fit. Data refer to a CO-hemoglobin solution at $x = 0.49$ dissociation.

examination in the present paper and ΔG_n is the remaining free energy term which is assumed to be independent of electrostatic interactions and pH since the polarity of the solvent does not change when different buffers are employed. When considering the interactions of a charged spherical particle of radius R with the charges in solution its electrostatic free energy is [12]

$$W_{el}=\frac{Z^2q^2}{2\varepsilon R}\left[1-\frac{\kappa R}{1+\kappa(R+a)}\right], \tag{10}$$

where Z is the number of charges q located on the particle, κ^{-1} is the Debye length, ε is the dielectric constant, and a is the radius of the counterions in solution. The electrostatic free energy changes upon $T \rightarrow 2D$ dissociation according to

$$\Delta G_{el}=2W_{el}^{D}-W_{el}^{T}. \tag{11}$$

It is assumed that $Z_T=2Z_D$ and $R_T=2^{1/3}R_D$ as discussed above with $R_T=27.8$ Å (from crystal x-ray-diffraction data). At 10 mM ionic concentration the Debye length is $\kappa^{-1}=30$ Å to be compared with an average hemoglobin-to-hemoglobin distance of $\simeq 300$ Å when its total concentration $c=5$ mg/ml. These values should correspond to very small interparticle electrostatic interactions and therefore the particles are assumed to diffuse independently of one another [18]. Hydrodynamic interactions deriving from excluded-volume effects can be considered negligible [19].

The tetramer-to-dimer equilibrium can be written in terms of the corresponding concentrations $[T]$ and $[D]$ or with the tetramer dissociated fraction x as

$$[D]^2/[T]=4cx^2/(1-x). \tag{12}$$

The dissociation free energy ΔG_{diss} can be expressed by means of x according to

$$4cx^2/(1-x)=e^{-\Delta G_{diss}/RT}, \tag{13}$$

where c is the total hemoglobin concentration (mg/ml).

In order to evaluate ΔG_{el} from Eqs. (9) and (13) we assume that ΔG_{el} vanishes at the isoelectric point which corresponds to zero net charge on hemoglobin. In Fig. 3 we plot $-\Delta G_{el}$ vs pH as obtained with the above procedure. As expected, a parabola fits the data very well, in agreement with the linear dependence of the protein charge vs $p\mathrm{H}-p\overline{\mathrm{H}}$, where $p\overline{\mathrm{H}}=7.37$, and with the quadratic dependence of W_{el} upon charge given by Eq. (10). From Fig. 3 we see that the value of $-\Delta G_{el}$ can be as high as $\simeq 3.6$ kcal/mole, at $p\mathrm{H}=5$. This value is rather relevant since ΔG_{diss} is known to fall in the 6 to 8 kcal/mole range [8,20]. From the measured value of D_Z at $p\mathrm{H}=7$ we can estimate also an average radius, $R_Z=31.8$ Å, to be compared with the reported radius of the undissociated tetramer, $R_T=32.0$–32.1 Å [14]. A comparison of the two values by means of Eq. (8), recalling that $D=k_BT/6\pi\eta R$, leads to an estimate of the dissociated tetramer fraction $x\simeq 0.06\pm 0.03$ near $p\mathrm{H}=7$, i.e., where the electrostatic interactions vanish.

From our scattering experiments, the protein charge also can be estimated since x is related to W_{el} by Eq. (10). A linear fit describes the charge of hemoglobin versus pH and a close parallel with the charge of human CO-hemoglobin, evaluated by us from published titration data [9,11], is displayed in Fig. 4.

In conclusion, by means of accurate light scattering data we have shown here that when hemoglobin is not near its isoelectric point its stability is controlled by substantial electrostatic interactions. Our approach, that has led also to very good estimates of the total charge located on CO-hemoglobin, should be appropriate for future applications also to other complex systems such as those enzymes that may dissociate, under changing environment, into subunits.

These encouraging results will be followed soon by more extended investigations both on human and bovine hemoglobin under a wider range of conditions, including those of partial binding of oxygen, in order to clarify also

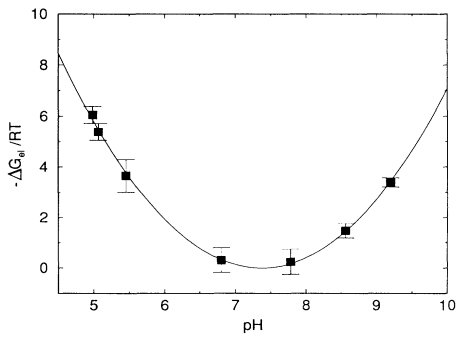

FIG. 3. Values of ΔG_{el} vs pH calculated according to Eqs. (9) and (13) from the data of Fig. 1. A parabolic fitting of the data is shown by the solid line.

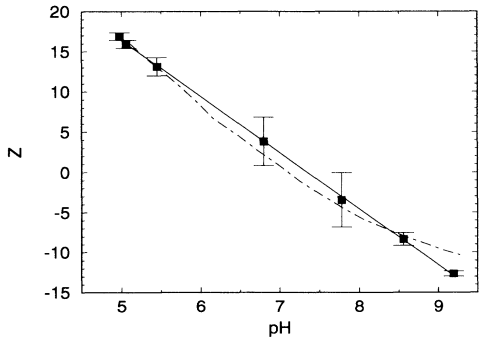

FIG. 4. Estimated number of charges Z per tetramer (squares) derived from the data of Fig. 3 by means of Eqs. (10) and (11). The straight line joining the points is a linear regression of the data. The dashed line has been constructed here from titration data on human CO-hemoglobin [11] corrected for low-ionic-strength effects derived from Ref. [9].

the role of nonelectrostatic interactions on the protein stability.

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