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A Light Scattering Investigation of the Propylurea Dissociation of Human Hemoglobin A†

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ABSTRACT: The subunit dissociation of human hemoglobin A by propylurea in several liganded and chemically modified states was investigated by light scattering molecular weight methods. The dissociation data were analyzed by means of the equation developed in our earlier studies: $\Delta F^{\circ}_{D} = \Delta F^{\circ}_{D,w} - 2N'RTK_B[D]$, where ΔF°_{D} and $\Delta F^{\circ}_{D,w}$ represent the free energy of dissociation of hemoglobin tetramers into half-molecules consisting of $\alpha\beta$ dimers in the presence and in the absence of propylurea, K_B is the binding constant of the urea to the average peptide unit, [D] is its concentration, and N' is the number of amino acid sites exposed per half-molecule on dissociation. It is found that the dissociation of oxyhemoglobin, cyanmethemoglobin, and *N*-ethylmaleimide oxyhemoglobin is characterized by essentially the same N' value of 15 to 21 ± 3, that are close to the 19 amino acid residues per surface which comprise the smaller $\alpha\beta$ contact area, seen in the X-ray crystallographic model of horse hemoglobin of Perutz and coworkers. Due to the very low degree of dissociation of deoxyhemoglobin, only a very approximate estimate of N' of about the same order of magnitude could be obtained for this form of the protein. In contrast, a significantly lower value of N' was obtained with bis(maleimidomethyl) ether modified oxyhemoglobin of 8 ± 3, that is cross-linked at cysteine residue F9 (93)β and histidine residue FG4 (97)β in the same β chains. Our results suggest that alterations caused by the presence of the cross-linking reagent reflect both the loss in amino acid residues that can interact with the urea at the blocked segments of the polypeptide chains in the dissociated state of hemoglobin and the changes in accessibility of some of the amino acid residues perturbed by the introduction of the reagent in the parent tetrameric form.

Hemoglobin is dissociated by various salts and solutes into half-molecules consisting of $\alpha\beta$ dimers of largely unaltered native conformation (Steinhardt, 1938; Benhamou et al., 1960; Rossi-Fanelli et al., 1961; Benesch et al., 1962; Kirshner and Tanford, 1964; Kawahara et al., 1965; Guidotti, 1967; Kellett, 1971; Norén et al., 1971, 1974; Thomas and Edelstein, 1972; Elbaum and Herskovits, 1974; Elbaum et al., 1974). Studies of this type have the potential value of predicting the role of the type of amino acid side chains that are responsible for the maintenance of the tertiary and quaternary structure of proteins (Kauzmann, 1959; Von Hippel and Schleich, 1969; Tanford, 1968; Elbaum et al., 1974).

In the previous publications from this laboratory the effects of increasing the hydrocarbon content of the urea and amide classes of denaturants on the conformation of single chain globular proteins (Herskovits and Jaillet, 1969; Herskovits et al., 1970a–c) and the multichain hemoglobins (Elbaum et al., 1973, 1974; Harrington et al., 1973) have been reported. The similarity of behavior of the ureas and amides as denaturants and dissociating agents has been noted (Elbaum and Herskovits, 1974). Specifically, an effective denaturant is also an effective dissociating agent; this effectiveness increases with the increase in the hydrocarbon content of the denaturant. The above study examined the effect of varying the hydrophobicity of the straight chain alkyl urea and amides on the subunit dissociation of hemoglobin. The present study deals with a detailed examination of the dissociation by a single and very effective dissociating agent, propylurea. Attention has been focused specifically on the cooperativity of the hemoglobin, the oxidation state of the iron in the heme groups, and the role of various ligands. The dissociation of the tetrameric form of human hemoglobin was followed by light scattering molecular weight measurements, and the denaturation by observing the change in optical rotation and adsorbance in the Soret region, as a function of the denaturant concentration.

Experimental Section

Hemoglobin. Hemoglobin was prepared from freshly drawn blood essentially according to Drabkin's procedure (1946) and chromatographed as previously described (Elbaum and Herskovits, 1974). *N*-Ethylmaleimide (EM)[1] and bis(maleimidomethyl) ether (BME) modified hemoglobin was prepared essentially according to the procedure of Guidotti and Konigsberg (1964) and Simon and Konigsberg (1966). Chromatographed 2–3% hemoglobin solutions,

† From the Department of Chemistry, Fordham University, Bronx, New York 10458. *Received November 18, 1974.* This investigation was largely supported by a Grant HL-14453 from the Heart and Lung Institute of the National Institutes of Health, U.S. Public Health Service.

[1] Abbreviations used are: EM, *N*-ethylmaleimide; BME, bis(maleimidomethyl) ether; Hb, hemoglobin.

$\Gamma/2 = 0.1$, pH 7, were reacted with 2 molar excess of EM per mole of β chain at 25° for 1 hr, followed by exhaustive dialysis against 0.1 *M* KCl–0.02 *M* phosphate (pH 6.9) buffer in the cold for 36 hr to remove excess EM. BME modified hemoglobin was made by converting the lysed hemoglobin solutions to the carbonmonoxy form, and then reacting the lysate with 2 molar excess of BME/mol of β chain for 24 hr at 4°, followed by chromatographic purification on 50 × 4 cm Bio-Rex 70 cation exchanger (Bio-Rad Laboratories, Calif.) columns equilibrated with pH 6.85 phosphate buffer of 0.05 *M* Na^+ content. The columns were eluted by discontinuous gradient of phosphate buffers containing 0.05 and 0.2 *M* Na^+ (Simon and Konigsberg, 1966). The leading fraction of BME-hemoglobin containing more than 2 mol of BME/mol of hemoglobin tetramer was rejected. The main second fraction eluted by 0.2 *M* Na^+ emerging at approximately 0.15 *M* Na^+ was retained for our studies. The conversion to oxy form was affected by repeated deaeration using an aspirator and reequilibration with air in the cold under bright light (using a 200-W lamp). The complete removal of CO, usually after two to three cycles of deaeration, was monitored by absorbance measurements. The complete disappearance of the 567.5- and the 537.5-nm peaks characteristic of the carboxy form of hemoglobin as reported by Sussner et al. (1974) was taken as indication of the complete conversion to oxyhemoglobin.

Concentrations of the hemoglobin solutions were based on the following per cent absorption coefficient, $A_{1\,cm}(1\%)$ 8.56 for oxyhemoglobin at 540 nm, 7.76 for cyanmethemoglobin at 540 nm, 8.4 for deoxyhemoglobin at 555 nm (Elbaum and Herskovits, 1974), and 8.56 and 8.48 for the oxy form of EM and BME modified hemoglobin at 540 nm, respectively. The latter extinction values were based on measurements on solutions of known hemoglobin concentrations following the reaction with the EM and BME reagents. The effects of added urea on these extinctions were found to be at most 5%. Where necessary corrections were made based on the changes in absorbance of solutions diluted with and without propylurea from the same stock solutions of protein.

Reagents. All the reagents were of the purest commercially available quality. The propylurea employed was once or twice recrystallized from hot ethanol and dried under vacuum. *N*-Ethylmaleimide was purchased from Eastman Kodak Co. and was used without further purification. Bis-(maleimido)methyl ether was synthesized according to the procedure of Twaney et al. (1961). The compound melted between 131 and 133° and its nuclear magnetic resonance (NMR) spectrum in trifluoroacetic acid showed two peaks of equal area which correspond to the structure of the compound containing no impurities (Arndt et al., 1971).

Light Scattering Measurements. Turbidity measurements were made at 630 nm in a light scattering spectrophotometer of Brice's design made by Wood Manufacturing Co., Newton, Pa. The use of 630-nm wavelength is warranted by the high absorption of hemoglobin solutions at lower regions of the spectrum usually employed in light scattering. Dialyzed solutions and solvent were clarified by filtration through metricel glass filters of 0.2-μ porosity and 25-mm diameter. Measurements were made in 24 × 24 × 70 mm capacity cells at a 90° angle to the incident beam. Corrections for the absorption by the hemoglobin solutions were applied by comparing the transmittances of solvent and solution at 0° angle. Correction for the depolarization was obtained by measuring the depolarization ratio (ρ) for each solution.

Special precautions had to be taken when preparing deoxyhemoglobin solutions. All solvents and solutions were deoxygenated and made 2 mg/ml in sodium dithionite. The dialysis of deoxy solutions was carried out in a closed atmosphere filled with argon. All deoxy solutions were handled inside a glove bag in an atmosphere of nitrogen. Before measuring turbidity of the deoxyhemoglobin solutions, the measuring chamber of the light scattering spectrophotometer was flushed thoroughly with argon.

The specific refractive index increment $(\partial n/\partial c)_\mu$ required for osmotic equilibrium for multicomponent systems (Casassa and Eisenberg, 1964) was measured in the light scattering instrument at 630 nm on dialyzed solutions as previously described (Elbaum and Herskovits, 1974). Table I lists the $(\partial n/\partial c)_\mu$ values, the depolarization ratios, ρ, and some of the other physical parameters obtained or used in this study.

With the protein solutions at osmotic equilibrium, the excess turbidity or rather the Rayleigh ratio, R_θ, measured in instruments of Brice's design at 90° is related to the weight average molecular weight M_w, the concentration c, and the second virial coefficient B', by the familiar relation

$$K'c/R_\theta = 1/M_w + 2B'c \qquad (1)$$

provided that the mean dimensions of the protein are less than 10% of the wavelength of the scattering light, λ employed for the measurements (Doty and Edsall, 1951). The light scattering constant K' including the Cabannes depolarization correction, $(6 + 6\rho)/(6 - 7\rho)$, is equal to $2\pi^2 n_0^2 (\partial n/\partial c)_\mu^2 / N_A \lambda^4 [(6 + 6\rho)/(6 - 7\rho)]$, where n_0 is the refractive index of the solvent, ρ is the depolarization ratio, and N_A is Avogadro's number.

The hemoglobin solution that is being studied is a mixture of tetramers and dimers. The tetramer–dimer equilibrium can be represented by an equilibrium constant, K_{Diss}, given by

$$K_{Diss} = 4\alpha^2 c/(1 - \alpha)M_4 \qquad (2)$$

where α is the degree of dissociation given by the general expression (Elbaum and Herskovits, 1974)

$$\alpha = (m/m - 1)(1 - M_w/M_4) \qquad (3)$$

M_4 is the tetramer molecular weight taken as 64,450, and m is the number of subunits formed on dissociation, namely, 2 for hemoglobin tetramer to dimer dissociation. In terms of α, M_4 and m the light scattering equation (eq 1) can be expressed as

$$\frac{K'c}{R_\theta} = \frac{1}{M_4[1 - \alpha(m - 1)/m]} + 2B'c \qquad (4)$$

Equations 2 and 4 have been used to calculate the dissociation constants and the theoretical curves required to fit the experimental data described in the results. Due to the scatter of the data based on light scattering measurements a B' value has to be assumed for fitting the data with a single K_{Diss} value. We used a value of 5×10^{-8} l. mol/g^2 based on the aqueous osmometric results of Guidotti (1967) and Elbaum and Herskovits (1974) for oxyhemoglobins and cyanmethemoglobin. This value of B' is close to the value of 4.65×10^{-8} l. mol/g^2 used by Norén et al. (1974) based on a hard sphere model (i.e., $4\bar{V}/M$). In case of deoxyhemoglobin a B' value of 15×10^{-8} gave a better fit for the urea solutions.

Table 1: Light Scattering, ORD, and Extinction Data of Human Hemoglobin A in Various States of Liganding and Subunit Dissociation in Aqueous Propylurea Solutions, 0.1 *M* KCl, 0.02 *M* phosphate (pH 6.9).

Solvent/ Propylurea Concn (*M*)	Refractive Index Increment, $(\partial n/\partial c)_\mu$	Depolarization Ratio, ρ	Cabannes Factor, $(6+6\rho)/(6-7\rho)$	M_w at c = 4 g/l.[a]	% Dissociation at c = 4 g/l. ($\alpha \times 100\%$)	Tetramer to Dimer K_{Diss} ($M \times 10^5$)	$[m']_{233}$ (deg cm^2/dmol)	$\epsilon_{\lambda,max} \times 10^{-5} b$ (412–430 nm)
				Oxyhemoglobin				
0	0.193	0.01	0.022	50,600	43	8	−8300	1.42
0.2	0.193	0.007–0.013	1.016–1.028	50,000	45	9	−8280	1.41
0.4	0.191	0.007–0.02	1.016–1.046	45,200	60	22	−7800	
0.6	0.19	0.014–0.022	1.030–1.050	43,500	65	30	−7840	1.4
0.8	0.192	0.017–0.029	1.038–1.065	41,300	72	46	−7550	1.38
1.0	0.188	0.017–0.029	1.038–1.065	38,100	82	90	−7420	1.36
				EM-Oxyhemoglobin				
0	0.189	0.013–0.022	1.028–1.049	56,400	25	2	−8180	1.21
0.4	0.19	0.014–0.039	1.030–1.09	50,000	45	9	−7850	1.26
0.6	0.189	0.022–0.028	1.05–1.063	48,700	49	12	−7510	1.22
0.8	0.19	0.034–0.057	1.077–1.113	42,300	69	38		
1.0	0.189	0.043–0.061	1.097–1.143	40,300	75	57	−7490	1.20
				BME-Oxyhemoglobin				
0	0.191	0.008–0.012	1.018–1.026	59,100	17	0.8	−7900	1.28
0.4	0.190	0.009–0.011	1.019–1.024	56,400	25	2	−7700	1.28
1.0	0.189[c]	0.008–0.011	1.018–1.024	53,900	33	4	−7100	1.29
1.4	0.187[c]	0.01–0.015	1.02–1.033	49,000	48	11	−6740	1.21
				Cyanmethemoglobin				
0	0.193	0.006–0.016	1.012–1.035	63,000	4.5	0.05	−8970	1.24
0.4	0.19	0.005–0.012	1.012–1.025	61,100	11	0.3	−8690	1.24
1.0	0.188	0.05–0.019	1.010–1.043	51,900	39	6	−7800	1.24
1.4	0.187	0.012–0.02	1.026–1.044	46,800	55	17	−7650	1.24
				Deoxyhemoglobin				
0	0.201	0.01–0.02	1.022–1.043	65,800[d]	0	0.01–0.001[d]		1.79
0.4	0.191[c]	0.011–0.039	1.022–1.088	62,400	6	~0.1		1.79
1.0	0.189[c]	0.018–0.053	1.040–1.123	60,300	13	0.5		1.78
1.4	0.187[c]	0.021–0.073	1.046–1.173	58,700	18	1		1.78

[a] Molecular weights based on smoothed $K'c/R_\theta$ vs. c curves corrected for B' plotted according to eq 4 (see Figures 1 and 2). The aqueous deoxy value is based on the extrapolation of $K'c/R_\theta$ to c = 0 by the least-squares method. [b] Extinction based on literature values for aqueous solutions given by Seamonds et al. (1971). [c] Values based on interpolation of 0 and 1.0 *M* data. [d] Molecular weight based on least-squares extrapolation shown in Figure 1A and K_{Diss} based on log K_{Diss} vs. [D] of Figure 4.

Denaturation Studies. The unfolding of hemoglobin by propylurea in the various liganded oxidation states was followed by both optical rotatory dispersion (ORD) in the ultraviolet region and absorbance measurements in the Soret region using a Cary 60 spectropolarimeter and a Cary 14 spectrophotometer. The mean residue rotation corrected for the refractive index of the solvent, $[m']_{233}$ at 233 nm, was calculated using the mean residue molecular weight, M_0, of 112.5.

Results

Subunit Dissociation of Hemoglobin. Representative data of hemoglobin dissociation due to propylurea at various concentrations studied by light scattering measurements are shown in Figures 1 and 2. The light scattering behavior of the various liganded and modified forms of hemoglobin, that is oxy-, cyanmet-, deoxy-, EM-, and BME-hemoglobin, shows increasing tendency to dissociate into dimers with increasing concentration of propylurea. Thus all the molecular weight estimates that would correspond to the experimental points are less than the tetrameric molecular weight of 64,450, except those of deoxyhemoglobin and cyamethemoglobin in aqueous solutions. The molecular weight of deoxyhemoglobin in buffered media, based on least-squares analysis of the $K'c/R_\theta$ data extrapolated to c = 0 shown in Figure 1A, was found to be 65,800. In the figure the combined data of the present study represented by the open circles, and a previous study given by closed circles (Elbaum and Herskovits, 1974) are shown. The extrapolated molecular weight is in satisfactory agreement with the value of 64,450, based on the amino acid data of human hemoglobin.

A summary of our light scattering results including the dissociation constants, K_{Diss}, for tetramer to dimer dissociation based on all our aqueous and propylurea data is given in Table I. The solid lines drawn through the data points of Figures 1 and 2 are based on some of these dissociation constants computed by use of eq 2 and 4. Estimates of the percentage of hemoglobin dissociated and the apparent molecular weights at a protein concentration of 4 g/l. are also included among the data of this table.

It is apparent from the form of eq 2 and 3, on which the estimates of the K_{Diss} are based, that these constants are subject to large experimental uncertainties where the dissociation is less than 10% or greater than 90%. The dissociation constants of deoxyhemoglobin are thus subject to greatest uncertainty. Our best estimate based on the log K_{Diss} vs. [D] extrapolation to zero propylurea concentration, dictated by eq 6 of the discussion (Figure 3), places this value between 10^{-7} and 10^{-8} which is in agreement with that given by Kellett (1971) as an upper limit, but is significantly lower than the more exact estimate of 3×10^{-12} obtained by Thomas and Edelstein (1972).

Denaturation by Propylurea. The changes in the optical

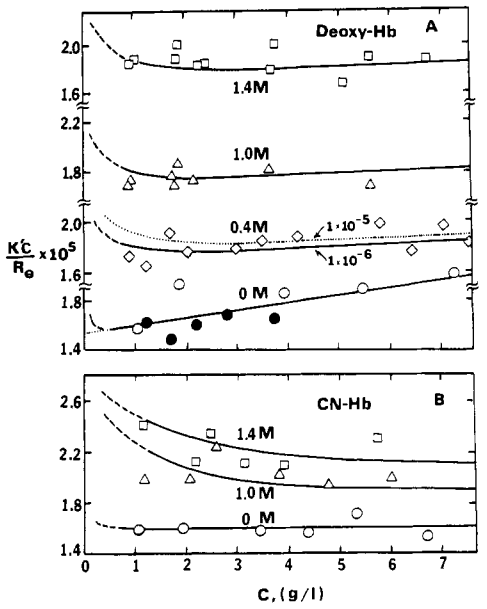

FIGURE 1: Light scattering plots of deoxyhemoglobin (A) and cyanmethemoglobin (B) in the presence of 0–1.4 *M* propylurea at 25°. All solutions contained 0.1 *M* KCl and 0.02 *M* phosphate (pH 6.9) buffer. The curves drawn are based on the best fit of the data plotted according to eq 4 with the tetramer to dimer dissociation constants, K_{Diss}, given in Table I and B' values of 15×10^{-8} and 5×10^{-8} l. mol/g^2 for deoxyhemoglobin and cyanmethemoglobin, respectively.

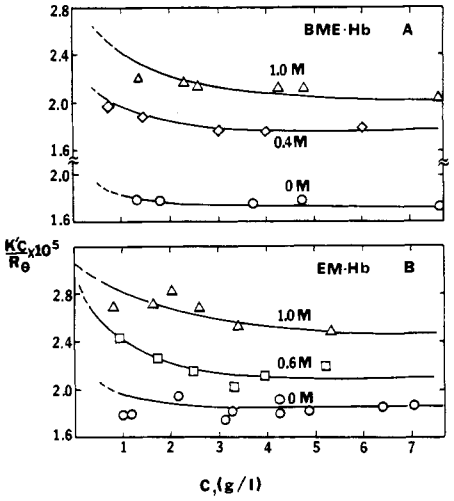

FIGURE 2: Light scattering plots of BME-modified (A) and EM-modified (B) hemoglobin in the presence of 0–1.0 *M* propylurea, and 0.1 *M* KCl, 0.02 *M* phosphate (pH 6.9), 25°. The curves drawn are based on the best fit of the data plotted according to eq 4 with tetramer/dimer dissociation constants, K_{Diss} given in Table I and B' values of 5×10^{-8} l. mol/g^2.

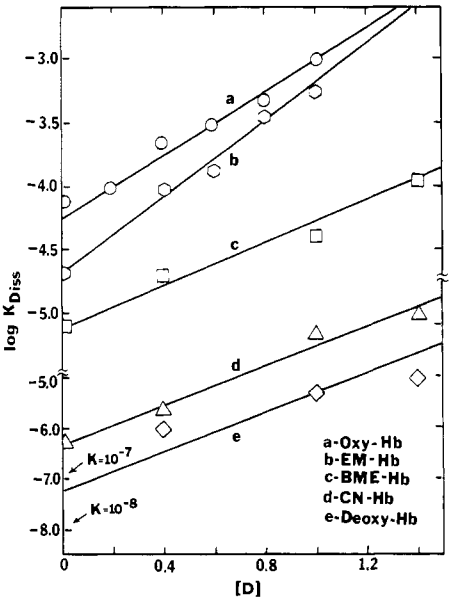

FIGURE 3: Log K_{Diss} vs. [D] plots of hemoglobin in various liganded states and deoxyhemoglobin, plotted according to eq 6. Curves: (a) oxyhemoglobin; (b) EM-hemoglobin; (c) BME-hemoglobin; (d) cyanmethemoglobin; (e) deoxyhemoglobin. Solvent conditions are described in Figure 1.

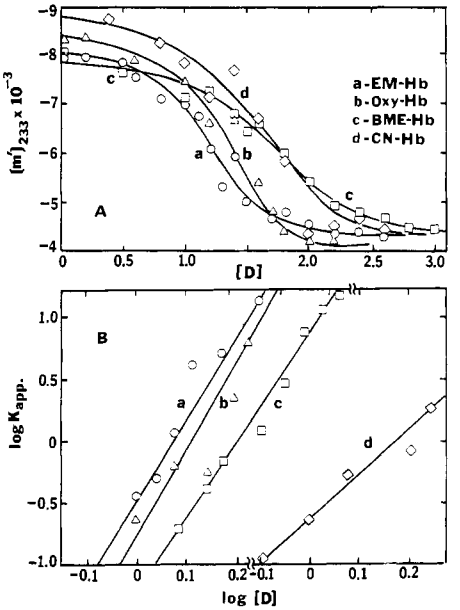

FIGURE 4: Plots of the mean residue rotation, $[m']_{233}$ of hemoglobin in various liganded state and deoxyhemoglobin as a function of propylurea concentration (A) and log K_{App} vs. log [D] plotted according to eq 5 (B). Curves: (a) EM-hemoglobin; (b) oxyhemoglobin; (c) BME-hemoglobin; (d) cyanmethemoglobin. All solvents contained 0.1 *M* KCl and 0.02 *M* phosphate (pH 6.9) buffer.

rotation at 233 nm accompanying the unfolding of the various hemoglobins by propylurea are shown in Figure 4A. The denaturation midpoints based on these measurements and similar absorption measurements at the Soret region (data not shown) are compared in Table II. Deoxyhemoglobin seems to be most stable with respect to both unfolding and subunit dissociation. The observed differences in stability of the liganded or modified hemoglobin toward propylurea denaturation seem to be less pronounced despite the much greater variation seen in the dissociation behavior of these hemoglobin forms (Table I).

The changes from native to unfolded states of hemoglobin can be analyzed by means of the empirical relation

$$K_{App} = C[D]^n \quad (5)$$

where K_{App} is the apparent equilibrium constant of the native to denatured protein transition, [D] is the concentration of the denaturant, n is the apparent order of the transition, and C is a constant characteristic of each protein-denaturant system (Kauzmann, 1959; Tanford, 1968; Sturtevant and Tsong, 1969; Brunori et al., 1972; Elbaum et al., 1974). The n values can be evaluated from a plot of log K_{App} vs. log [D]. The n parameters for the various hemo-

Table II: Denaturation Midpoints and n Parameters of Equation 5 for Various Liganded and Modified Hemoglobins Obtained with Propylurea.[a]

Hemoglobin/ Ligand	Denaturation Midpoints (M): Based on Soret Absorbance at 412–430 nm	Based on ORD Data at 233 nm	n Parameter
Hb/O_2	1.4	1.4	7.2
$EM\text{-}Hb/O_2$	1.5	1.3	6.5
$BME\text{-}Hb/O_2$	1.9	1.8	6.0
Hb/CN		1.6	4
Hb/CO		1.5[b]	4.3[b]
Hb(deoxy)	2.7		

[a] All the solutions contained 0.1 M KCl–0.02 M phosphate (pH 6.9). [b] Data of Elbaum et al. (1974).

Table III: Human Hemoglobin A Parameters Based on Equation 6.[a]

Hemoglobin/Ligand	N'	$K_{Diss} \times 10^5 M$ (tetramer–dimer)
Hb/O_2	15 ± 3	4
$EM\text{-}Hb/O_2$	16	2
Hb/CN	21	0.05
Hb(deoxy)	~20	0.01–0.001
$BME\text{-}Hb/O_2$	8	0.8

[a] The K_B value used with eq 6 was 10.8×10^{-2} (Elbaum and Herskovits, 1974)

globin forms based on such plots (Figures 4B) are also given in Table II.

Discussion

The detailed three-dimensional model of hemoglobin based on the X-ray crystallographic studies of Perutz and coworkers shows two distinct pairs of surfaces of contact between the α and β chains of the protein, consisting of 19 and 34 amino acid residues (Perutz et al., 1968; Perutz, 1970). Dissociation in neutral aqueous solutions of the hemoglobin tetramer, $(\alpha\beta)_2$, occurs symmetrically, with the molecule splitting into a pair of $\alpha\beta$ dimers. The subunit contacts that are thus abolished are probably the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ contacts of smaller area containing fewer of the amino acid side chains of the two type of contacts (Rosemeyer and Huehns, 1967; Perutz et al., 1968). In addition, two polar salt-bridge type contacts seem to be abolished upon dissociation. By considering the dissociation as a stepwise process of dissociation of the hemoglobin tetramer to dimers followed by the binding of the dissociating agent to the exposed areas of subunit contacts containing N' independent and equivalent binding sites, per dimer, Elbaum and Herskovits (1974) have obtained the expression

$$\Delta F_D^0 = \Delta F_{D,w}^0 - 2N'RTK_B[D] \quad (6)$$

which could be used to obtain estimates of the apparent number of amino acid sites exposed from determinations of the dissociation constants, K_{Diss}, as a function of the concentration of the dissociating agent [D]. With the standard free energy of dissociation of the hemoglobin tetramer to dimer defined as

$$\Delta F_D^0 = -RT \ln K_{Diss} \quad (7)$$

either in the presence or absence of dissociating reagent, designated by the subscripts D or D,w., eq 6 shows that a plot of log K_{Diss} as a function of [D] should be linear, giving a slope that is equal to $2N'K_B/2.303$ and a y intercept extrapolated to [D] = 0, equal to the log of K_{Diss} in aqueous solutions. Plots constructed on the basis of eq 6 are shown in Figure 3 for the various hemoglobins of this study. With a K_B value of 10.8×10^{-2} for propylurea used in our previous investigations of the denaturation and subunit dissociation of hemoglobin (Elbaum and Herskovits, 1974; Elbaum et al., 1974), we obtain estimates of N' of 15–21 (±3) for oxy-, EM oxy-, and cyanmethemoglobin and approximately 20 for deoxyhemoglobin[2] (Table III). It should be emphasized that our model assumes identical and noninteracting binding sites, having the same binding constants. That such assumptions are in fact reasonable is suggested by the fact that the log K_{Diss} vs. [D] curves are linear,[2] as shown in Figure 3, and that the estimated N' values are also reasonable in terms of what is known about $\alpha\beta$-contact areas of hemoglobin, based on its detailed three-dimensional structure (Perutz, 1965; Perutz et al., 1968).

The results of our analysis show that the number of amino acid sites exposed due to propylurea dissociation of human hemoglobin A is in good agreement with that predicted by Perutz' model. Moreover, the data support the arguments presented by various investigators (see, for example, Rosemeyer and Huehns, 1967; Perutz et al., 1968; Perutz, 1970) that the splitting of hemoglobin tetramers into dimers takes place symmetrically along the smaller areas of $\alpha\beta$ contacts resulting in a pair of dimers designated as $\alpha_1\beta_1$, the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimer species being equivalent (Rosemeyer and Huehns, 1967). Perhaps equally significant is the finding that the number of amino acid residues exposed per dimer in the case of deoxyhemoglobin dissociation is in the neighborhood of 20, characteristic of the same sort of symmetrical splitting along the smaller $\alpha_1\beta_2$ areas of contact. However, in view of the large experimental uncertainties involved in determining the small K_{Diss} of deoxyhemoglobin, discussed in the Results, our conclusion regarding the areas of contacts split in the dissociation of deoxyhemoglobin must be considered as tentative. Sedimentation velocity experiments with absorption optics on more dilute hemoglobin solutions employed by Edelstein et al. (1970) and Tainsky and Edelstein (1973) offer the means by which more exact values of the dissociation constants and the derived estimates of the number of amino acids in the contact areas split could be obtained.

Subtle differences in the conformation of $\alpha\beta$ dimers relative to the tetramers in the various liganded states of hemoglobin and oxyhemoglobin have been suggested by Guidotti (1967). Conformational adjustment of the side chains exposed to solvent must result from both the strain imposed

[2] Previous estimates of N' for the various ureas, based on the aqueous and 1.0 M urea and 0.2 M butylurea data, ranged from 20 to 50 for CO-hemoglobin. The disadvantage of these two point estimates, based also on eq 6, is the undue reliance on the aqueous K_{Diss} values that are difficult to estimate accurately (see Experimental Section). In addition, strong specific binding due to the presence of one or more binding sites, with K_B much higher than those based on nonspecific solvent effects (Herskovits et al., 1972a, Elbaum et al., 1974), will escape detection by this method. The presence of strong binding sites should be manifested by initial curvature of the log K_{Diss} vs. [D] curves which are absent in the case of the propylurea data of this study (Figure 3). A few such sites could easily double or triple the calculated N' values, since all that is required is that these sites should have 10 to 20 times stronger binding constants than those used for our calculations.

on the polypeptide chains by the changes in the liganding and heme iron coordination states and the effects of the solvent on the amino acid side chains exposed upon dissociation. Our present data suggest that these changes must be relatively subtle, resulting only in small alterations in the number of amino acids exposed to solvent that are not beyond the uncertainty of our estimates of N' of ± 3 groups (Table III).

The data obtained with the BME-modified oxyhemoglobin indicate that changes in the position of some of the amino acids in the contact areas of the hemoglobin subunits can significantly alter the N' values measured. Moffat's (1971) X-ray structural study of BME-hemoglobin reveals that this bifunctional reagent cross-links cysteine residue F9(93)β with histidine residue FG4(97)β in the same β chains causing alterations in their conformations that could block the entry and binding of alkylurea to some of the five amino acids involved in the formation of the closed ring structure in the dissociated form of the protein. In addition, the reagent located at the $\alpha_1\beta_2$ contact areas causes the complete displacement of tyrosine residue HC2(145)β from the pocket between helices F and H, and also the displacement of the next C-terminal residue, histidine HC3 (146)β. Small shifts or displacements are also suffered by valine FG5 (98)β, leucine B15(34)α, and proline C2(37)α, the latter resulting from the displacement of helix C of the α chain away from the β chain (Moffat, 1971). The N' value of 8 obtained with BME-oxyhemoglobin must reflect both the losses in the number of amino acid residues that can interact with urea at the blocked segments of the polypeptide chains in the dissociated state of hemoglobin, and to some extent the change in accessibility of the amino acid residues to solvent in the tetrameric state resulting from the changes in quaternary structure. In this relation it is important to recall that the experimentally determined N' parameter represents the number of newly exposed amino acid binding sites resulting from the dissociation of the hemoglobin tetramer into dimers, which are not accessible to solvent in the tetrameric state and therefore are not capable of binding urea in this state (Elbaum and Herskovits (1974)).

It is significant that in the case of monofunctional NE-modified hemoglobin the X-ray data of Perutz et al. (1969) suggest essentially no alterations in the conformation of histidine residues FG4(97)β and tyrosine HC2(145)β (Moffat, 1971; Moffat et al., 1971). The maleimide appears to be relatively unhindered and must be fairly accessible to solvent in the dissociated state of hemoglobin, not causing any significant blocking of any of the amino acids exposed as a result of the dissociation. The experimental estimates of the number of amino acid residues exposed as a result of dissociation of NE-oxyhemoglobin thus remains largely unaltered in relation to oxyhemoglobin.

Acknowledgment

We express our thanks to Dr. Steven Weinreb and Mr. Richard Comi of our department for preparing bifunctional hemoglobin reagent, bis(maleimidomethyl) ether, for us.

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Sulfhydryl Groups in Hemoglobin. A New Molecular Probe at the $\alpha_1\beta_1$ Interface Studied by Fourier Transform Infrared Spectroscopy†

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ABSTRACT: Infrared absorption bands due to sulfhydryl groups (ν_{SH}) of α-104(G11) and β-112 (G14) cysteine residues of human carboxyhemoglobin (HbCO) have been observed near 2560 cm^{-1} by use of Fourier transform infrared (FTIR) spectroscopy. The β-93 cysteine SH groups absorb infrared radiation so weakly that they are not distinguished from background. Only single SH absorption bands due to the α-104 cysteines of pig and horse hemoglobin are observed. The SH absorption bands from human HbCO disappear in alkali, are broadened by detergent or guanidinium chloride, and show a complex titration curve, and an isotopic frequency shift (ν_{SD}/ν_{SH} = 0.7267) virtually identical with that reported for methanethiol. The integrated absorption coefficient (ϵ_{mM}(area)) for 0.1 *M* ethanethiol increased with H-bond acceptor solvents in the order: CCl_4 (0.07), water (0.21), acetone (0.43), and *N,N*-dimethylacetamide (1.35 mM^{-1} cm^{-2}). Comparison of the integrated absorption coefficients for the α-104 cysteine SH (2.43), and the β-112 SH (0.80), of human HbCO with those of ethanethiol solutions suggested specifically H-bonded structures with peptide carbonyl groups 4 (or 3) residues back in the G helices. This was found to agree with a molecular model of the α-chain G helix of horse HbO_2 built to coordinates from M. F. Perutz.

Fourier transform infrared interferometry has provided a considerable enhancement of signal/noise ratio over previous infrared spectroscopic methods. This has made it possible to extend the infrared studies of small molecules to include biologically native proteins such as hemoglobin. Our earlier studies have explored the effects of local molecular structure on strongly absorbing groups such as carbon monoxide (Alben and Caughey, 1968; Caughey et al., 1969) or azide (Alben and Fager, 1972) coordinated to the heme groups in hemoglobin or myoglobin. We have now investigated the sulfhydryl groups of cysteine residues in hemoglobins from man, horse, pig, and cow, and have defined absorption bands due to sulfhydryl groups at the $\alpha_1\beta_1$ interface (α-104 and β-112 cysteine). These absorption bands provide a molecular probe into a region of hemoglobin which previously has been relatively inaccessible.

X-Ray crystallographic data indicate (Perutz, 1969) that the $\alpha_1\beta_1$ contact of hemoglobin includes 32 amino acid residues in deoxyhemoglobin and 34 residues in the oxygenated form. Most of these are nonpolar van der Waals contacts, with only four or five hydrogen bonds, all of which are probably in contact with the aqueous surface. In contrast to the $\alpha_1\beta_2$ interface, relatively small movements have been described at the $\alpha_1\beta_1$ interface as a consequence of ligand binding by hemoglobin. Many studies have stressed the importance of the $\alpha_1\beta_2$ region to the control of oxygenation, whereas the $\alpha_1\beta_1$ region of contact has been assumed to the relatively inert. Our studies of vibrational absorption bands of sulfhydryl groups at the $\alpha_1\beta_1$ interface provide a new probe of native hemoglobin structure and its conformational alterations. We will demonstrate in a later paper (Alben et al., to be published) the sensitivity of the sulfhydryl groups at the $\alpha_1\beta_1$ interface to the state of ligation of the hemes.

In this paper, we report the direct spectroscopic observation of absorption bands due to sulfhydryl vibrational transitions in aqueous solutions of native carboxyhemoglobins by Fourier transform infrared interferometry. Evidence to support the assignment of these bands includes comparison with spectra of small molecule sulfhydryls, isotopic shift in D_2O, pH titration, and comparison with animal hemoglobins which lack one or more cysteine residues. Quantitative measurements have allowed assignment of particular human hemoglobin absorption bands to SH stretching modes of the α-104 and β-112 cysteine residues, respectively. Band shapes and integrated intensities are interpreted in terms of molecular interactions present at the $\alpha_1\beta_1$ interface. In preliminary reports of some of this work, we proposed a model with intrachain H-bonding of the α-104 and β-112 sulfhydryl groups (Alben et al., 1974; Bare et al., 1974a,b).

Methods

Preparation of Carboxyhemoglobins. Fresh human, pig, horse, and cow (Holstein) blood samples (35–50 ml each)

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