Light-Scattering Investigations of the Subunit Dissociation of Human Hemoglobin A. Effects of the Aliphatic Acid Salts†

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ABSTRACT: The subunit dissociation of human hemoglobin A by the aliphatic acid salts at neutral pH has been investigated by light-scattering molecular-weight measurements at 630 nm. Dissociation of hemoglobin tetramers to $\alpha\beta$ dimers is observed in essentially all experiments at low to intermediate levels of salt concentrations, below the denaturation transitions, described in the accompanying paper (Ibanez, V. S., and Herskovits, T. T. (1976), *Biochemistry 15,* preceding paper in this issue). The effectiveness of the salts as subunit dissociating agent, reflected by the slopes, s, of the plots of $\Delta G_D°$, the standard free energy of dissociation, vs. [D], the salt concentration, is found to increase with increasing alkyl chain length or hydrocarbon content of the salt. Estimates of the apparent number of amino acid sites at the areas of contact per $\alpha\beta$ dimer formed, N', based on the slopes of the higher members of the series have been obtained using the equation, $\Delta G_D° = \Delta G_{D,w}° - 2N'RTK_B[D]$. Independent estimates of the binding constant, K_B, required for these calculations were based on free-energy transfer data of hydrophobic amino acid alkyl groups and protein denaturation data. Our estimates of N' obtained with the more reliable data of the higher members of salt series are in the ranges of 19 and 27 amino acid groups, shown by the x-ray crystallographic structure of horse and human hemoglobin of Perutz (Perutz, M. F., et al. (1968), *Nature (London) 219,* 131) and Fermi ((1975) *J. Mol. Biol. 97,* 237) for the smaller $\alpha_1\beta_2$ contact areas in the tetrameric structure. The lower estimates than 27 based on our dissociation of human hemoglobin suggest that several of the amino acid residues in the contact areas of the subunits are partially exposed to solvent. The increasing effectiveness of the higher members of acid salt series and the alkylureas suggests that hydrophobic interactions are an important source of stabilization of the tetrameric structure of hemoglobin.

The ureas and amides as well as many of the inorganic salts are known to destabilize the native conformations of proteins and nucleic acids and also to weaken protein–protein interactions at the areas of subunit contact of multichain proteins, thereby causing their dissociation to subunits (Kawahara et al., 1965; Guidotti, 1967; McCall et al., 1971; Elbaum and Herskovits, 1974; Hsu and Neet, 1975; Herskovits and Harrington, 1975). The effects of the urea and amide classes of denaturants on the tetramer–dimer equilibrium of human hemoglobin A have been investigated in some detail in our laboratory in an attempt to formulate and account for the effects of solutes on the state of association of subunit proteins in general, in terms of the number of amino acid sites at the areas of subunit contacts and the binding or interaction constants of the dissociating agent with the average amino acid (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975). We have found that the number of amino acids in the contact areas of the $\alpha\beta$ dimers of hemoglobin that are exposed as a result of dissociation of the hemoglobin tetramer into half-molecules can be correctly predicted by use of single average binding constants, K_B, which have both hydrophobic and polar group contributions depending upon the composition of the dissociating agent.

The utility of the binding parameters and the generality of the assumption of group additivity of the polar and hydrophobic group contributions to K_B were tested further by comparing the observed and predicted effects of the aliphatic carboxylate series of salts on the conformations of proteins, as reported in the accompanying paper (Ibanez and Herskovits, 1976). In this paper we report our findings with the same series on the subunit structure and dissociation of human hemoglobin A, investigated by the light-scattering molecular-weight technique.

Experimental Section

The preparation of human hemoglobin A, optical rotary dispersion, and viscosity measurements were described in related publications from our laboratory (Elbaum and Herskovits, 1974). All the reagents and salts employed were the same as those described in the accompanying paper (Ibanez and Herskovits, 1976).

Light-scattering and refractive index increment measurements were made at 630 nm in a photometer of Brice's design equipped with a differential refractometer, manufactured by Wood Mfg. Co., Newton, Pa. All measurements were made on dialyzed solutions, at constant chemical potential, clarified by filtration through metrical glass filters of 0.2-μm porosity as previously described (Harrington et al., 1973; Elbaum and Herskovits, 1974). The light-scattering data obtained as a function of concentration, c, were interpreted by means of the expression

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

where R_θ is Rayleigh's ratio measured at 90°, M_w is the weight-average molecular weight, B' is the second virial coefficient, and K' is the light-scattering constant that includes the Cabannes depolarization correction, $(6 + 6\rho)/(6 - 7\rho)$, equal to $2\pi^2 n_0^2 (\partial n/\partial c)_\mu^2 / N_A \lambda^4 \times (6 + 6\rho)/(6 - 7\rho)$, with n_0 signifying the refractive index of the solvent, $(\partial n/\partial c)_\mu$ the refractive index increment at constant chemical potential of the diffusible components (Casassa and Eisenberg, 1964), N_A Avogadro's number, λ the wavelength of the scattering light, 630 nm, and ρ the measured depolarization ratio. All our

† From the Department of Chemistry, Fordham University, New York, 10458. *Received June 17, 1976.* This investigation was supported by Grant HL 14453 from the National Institutes of Health, United States Public Health Service, and a Faculty Research Grant from Fordham University.

light-scattering data were corrected for hemoglobin absorption at 630 nm (Elbaum and Herskovits, 1974).

Hemoglobin dissociates into half molecules of $\alpha\beta$ dimers. In terms of the dissociation constant, K_D, and α, the weight fraction of protein dissociating, given by eq 2 and 3

$$K_D = 4\alpha^2 c/(1-\alpha)M_4 \quad (2)$$

and

$$\alpha = 2(1 - M_w/M_4) \quad (3)$$

the light scattering eq 1 can be expressed as

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

Equations 2 and 4 have been used to calculate the dissociation constants based on the best fit of the light-scattering data as previously described (Elbaum and Herskovits, 1974). The light-scattering data of hemoglobin is relatively insensitive to the choice of B' in the concentration region of 1 to 10 g/l. usually employed (Elbaum and Herskovits, 1974; Noren et al., 1974). For the salt dissociation data of this paper, a single B' value of 15×10^{-8} l. mol/g^2 was found to give the most satisfactory fit of the concentration dependence data (Figures 1 and 2) with a M_4 value taken as 64 450. This value is slightly more than three times the hard sphere value of 4.65×10^{-8} l. mol/g^2, based on the relationship $4\bar{V}/M_4$.

Hemoglobin concentrations were based on absorbance measurements at 539 nm of the carbon monoxy liganded form of the protein using a percent extinction coefficient, $A_{1\text{-cm}}$, of 9.04 (Elbaum and Herskovits, 1974).

Theoretical. Consider the binding of dissociating agent D to a protein of m subunits, P_m in equilibrium with its monomeric form P_1. The various species of complexes that will be in equilibrium can be represented as

$$\left.\begin{array}{l} P_m + D \rightleftarrows P_mD \\ P_mD + D \rightleftarrows P_mD_2 \\ \quad\vdots \quad\quad \vdots \\ P_mD_{n-1} + D \rightleftarrows P_mD_n \end{array}\right\} \overset{K_D}{\rightleftharpoons} m \left\{\begin{array}{l} P_1 + D \rightleftarrows P_1D \\ P_1D + D \rightleftarrows P_1D_2 \\ \quad\vdots \quad\quad \vdots \\ P_1D_{k-1} + D \rightleftarrows P_1D_k \end{array}\right.$$

Multiple equilibria formulation of the protein-dissociating agent interaction, with the results of the binomial theory of expansion (Klotz, 1953; Tanford, 1961; Van Holde, 1971) for the total concentration of protein, say in the associated state, $[P_m]$, leads to the expression of the form

$$[P_m] = [P_m^\circ](1 + K_{1,m}[D])^{N_{1,m}}(1 + K_{2,m}[D])^{N_{2,m}} \ldots\ldots (1 + K_{n,m}[D])^{N_{n,m}} \quad (5)$$

where $[P_m^\circ]$ is the concentration of the uncomplexed protein in the equilibrium mixture, and $N_{i,m}$ signifies the number of i classes of binding sites on the protein, with different equilibrium or bind constants $K_{i,m}$. In a more compact form this expression and the related expression for the concentration of the monomer form $[P_1]$ can be written as

$$[P_m] = [P_m^\circ]\prod_{i=1}^{N_m}(1 + K_{i,m}[D]) \quad (6)$$

and

$$[P_1] = [P_1^\circ]\prod_{i=1}^{N_1}(1 + K_{i,1}[D]) \quad (7)$$

where N_m and N_1 represent the maximum number of dissociating molecules that can be bound per protein molecule in the associated and the monomer forms. The dissociation constant of the subunit protein may then be expressed as

$$K_D = \frac{[P_1]^m}{[P_m]} = \frac{\left\{[P_1^\circ]\prod_{i=1}^{N_1}(1 + K_{i,1}[D])\right\}^m}{[P_m^\circ]\prod_{i=1}^{N_m}(1 + K_{i,m}[D])} \quad (8)$$

In the associated form of the protein, with m subunits, there will be m times the number of surface groups N_s capable of binding reagent, whereas in the monomer form there will be an additional set of groups, N_c, that are buried in the contact areas of the polymeric form of the protein, unable to interact with reagent. Segregation of the two sets of groups leads to the expression

$$K_D = \frac{\left\{[P_1^\circ]\prod_{i,s=1}^{N_s}(1 + K_{i,s}[D])\prod_{i,c=1}^{N_c}(1 + K_{i,c}[D])\right\}^m}{[P_m^\circ]\left\{\prod_{i,s=1}^{N_s}(1 + K_{i,s}[D])\right\}^m} \quad (9)$$

Cancellation of the identical surface terms in eq 9 leads then to the simplified expression 10.

$$K_D = [P_1^\circ]^m/[P_m^\circ]\left\{\prod_{i,c=1}^{N_c}(1 + K_{i,c}[D])\right\}^m = K_{D,w}\left\{\prod_{i=1}^{N_c}(1 + K_{i,c}[D])\right\}^m \quad (10)$$

with the products taken over all the binding sites at the contact areas from $i = 1$ to N_c. The logarithmic form of eq 10 leads to the standard-free-energy expression for dissociation of the protein into m subunits

$$\Delta G_D^\circ = \Delta G_{D,w}^\circ - RTm\sum^{i} N_i \ln(1 + K_{i,c}[D]) \quad (11)$$

where ΔG_D° and $\Delta G_{D,w}^\circ$ represent the standard free energies of dissociation in aqueous solutions in the presence and the absence of dissociating reagent of concentration [D], and N_i denotes the number of i class of binding sites at the contact areas of the subunits, with different binding constants for each class $K_{i,c}$. With independent and identical binding sites N' having the same weak binding constants, K_B, eq 11 reduces to the simpler eq 12, derived differently and used in previous investigations from our laboratory (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975; Herskovits and Harrington, 1975).

$$\Delta G_D^\circ = \Delta G_{D,w}^\circ - mN'RT\ln(1 + K_B[D]) \cong \Delta G_{D,w}^\circ - mN'RTK_B[D] \quad (12)$$

Results

Subunit Dissociation of Hemoglobin by the Aliphatic Acid Salt. Figures 1 and 2 represent some of our light-scattering results plotted according to eq 4. This form of the light-scattering equation represents the dissociation behavior of a subunit protein forming two fragments upon dissociation (m = 2), with the weight fraction α of protein that dissociates as a function of protein concentration, c, dictated by eq 2 (Elbaum and Herskovits, 1974). With the increasingly greater tendency of the hemoglobin tetramers to dissociate with increasing alkyl chain length or hydrocarbon content of the aliphatic acid-salt series, the light-scattering plots shown in Figure 1 for various 1 M salt solutions are displaced to higher $K'c/R_\theta$ values of the ordinate. Similar displacement is seen with increasing concentration of the dissociating agent, shown in Figure 2, for one of the salts of this study, sodium propionate. A summary of our data obtained with all the salts including the dissociation

TABLE I: Selected Data of the Light-Scattering Dissociation of Carboxyhemoglobin A at 25 °C and Neutral pH 7.

Solvent (M)	Refractive Index Increment, $(\partial n/\partial c)_\mu$	Refractive Index at 630 nm, n_{630}	Depolarization Ratio, ρ	Cabbanes Factor, $(6+6\rho)/(6-7\rho)$	Mol Wt, M_w[a] at c = 5 g/l.	% Dissociated at c = 5 g/l. ($\alpha \times 100\%$)	Dissociation Constant, $K_D \times 10^5$
				Sodium Formate			
1.0	0.185	1.340	0.011	1.024	57 000	23	2.1 ± 1
2.0	0.181	1.346	0.021	1.046	59 600	15	0.8
3.0	0.174	1.353	0.010	1.022	59 300	16	1
3.5	0.171	1.357	0.025	1.055	59 000	17	1.1
4.0	0.167	1.360	0.012	1.026	59 900	14	0.7
				Sodium Acetate			
1.0	0.188	1.342	0.005	1.013	53 800	33	5 ± 2
1.5	0.181	1.346	0.024	1.048	53 100	35	6
2.0	0.175	1.352	0.011	1.024	53 800	33	5
3.0	0.168	1.362	0.007	1.012	50 600	43	10
4.0	0.158	1.370	0.019	1.042	47 000	54	20
				Sodium Propionate			
0.5	0.189	1.388	0.015	1.032	56 100	26	3 ± 1
1.0	0.183	1.343	0.008	1.017	53 800	33	5
1.5	0.175	1.361	0.016	1.035	51 900	39	8
2.0	0.169	1.357	0.012	1.026	48 700	49	15
2.5	0.166	1.364	0.021	1.047	44 800	61	30
3.0	0.162	1.369	0.023	1.051	38 700	80	97
3.5	0.160	1.374	0.020	1.045	31 000	100	*b*
4.0	0.151	1.379	0.017	1.038	30 900	100	*b*
4.5	0.148	1.384	0.019	1.042	28 600	*b*	*b*
5.0	0.147	1.388	0.040	1.091	25 200	*b*	*b*
				Sodium Butyrate			
0.25	0.190	1.336	0.009	1.020	56 100	26	3 ± 1
0.5	0.187	1.340	0.014	1.031	54 800	30	4
0.75	0.185	1.344	0.014	1.031	49 300	47	13
1.0	0.184	1.347	0.015	1.033	47 400	53	18
1.25	0.181	1.352	0.015	1.033	38 300	81	110
1.5	0.180	1.356	0.031	1.070	43 200	66	40
				Sodium Valerate			
0	0.196	1.331	0.011	1.024	60 300	13	0.6 ± 0.5
0.06	0.196	1.332	0.009	1.020	58 900	17	1.1
0.13	0.197	1.334	0.008	1.018	57 700	21	1.7
0.16	0.196	1.334	0.010	1.023	59 300	16	1
0.18	0.195	1.334	0.011	1.025	57 400	22	2
0.20	0.193	1.335	0.009	1.020	54 800	30	4

[a] Calculated values based on eq 13 with α values obtained using eq 2 and the K_D data of the last column, at c = 5 g/l. These values correspond to the M_w of the smoothed $K'c/R_\theta$ vs. c data such as in Figures 1 and 2 corrected for B'. [b] Dissociates further to monomers (see Results).

constants K_D based on fitting the concentration dependence of the light-scattering plots (most of which are not shown in the figures) is given in Table I. In all cases very satisfactory fit of the data was obtained with the tetramer to dimer model of subunit dissociation.

Dissociation to hemoglobin monomers is only seen at salt concentrations where there is a significant degree of unfolding of the protein, suggested by the changes in optical rotation, absorbance, or specific viscosity of the hemoglobin solutions. Thus the molecular-weight data obtained with sodium propionate shown in Figure 3 suggest a distinct drop in M_w above the 3 M region of salt concentration, with concomitant changes in $[m']_{233}$ and η_{sp}/c. The M_w data represented by the solid line were calculated by use of eq 13 (Elbaum and Herskovits, 1974)

$$M_w = M_4(1 - \tfrac{1}{2}\alpha) \qquad (13)$$

with the required α values computed using the exponential form of eq 12

$$K_D = K_{D,w} \exp(mN'K_B[D]) \qquad (14)$$

with c = 5 g/l., m = 2, N' = 19, $K_{D,w} = 6 \times 10^{-6}$ M, and K_B = 0.039 M^{-1} for propionate based on the results of Table I and related publications from our laboratory[1] (Ibanez and Herskovits, 1976; Elbaum and Herskovits, 1974). For our calculations eq 2 and 14 have been recast into a convenient form, $\alpha^2/(1-\alpha) = K_{D,w}M_4/4c \exp(2N'K_B[D])$, giving ready estimates of α at a fixed protein concentration. Related to the further dissociation of the hemoglobin dimers above the midpoints, in 2 M caproate solutions at c = 5 g/l., a M_w = 15 900 was obtained, substantiating these results of Figure 3. With regard to the denaturation transitions seen in Figure 3, it is significant that midpoints of the transitions measured by the changes in $[m']_{233}$ and η_{sp}/c do not coincide, lending support

[1] The dissociation constant of 6×10^{-6} M is the average value based osmometric and light-scattering determination on CO-hemoglobin reported by Elbaum and Herskovits (1974) and the data of this paper.

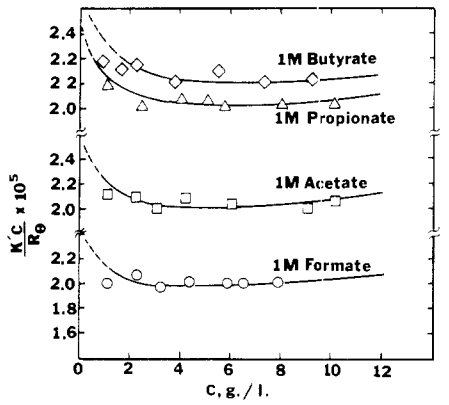

FIGURE 1: The effects of various 1 M salts on the light scattering of carboxyhemoglobin at neutral pH (7.0) and 25 ± 1 °C. All the solutions were buffered by means of 0.01 M phosphate (pH 7). The curves drawn represent the best fit values of $K'c/R_\theta$ plotted according to eq 4 with the tetramer to dimer dissociation constants, K_{diss}, listed in Table I and the B′ value of 15×10^{-8} l. mol/g^2.

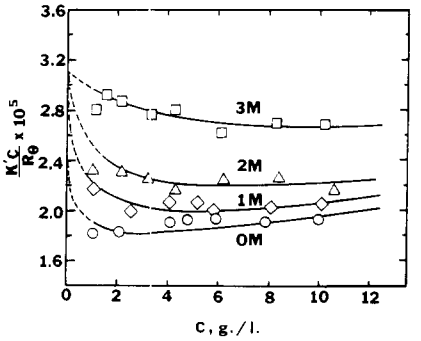

FIGURE 2: The effects of increasing propionate concentration on the light-scattering behavior of carboxyhemoglobin at neutral pH (7.0) and 25 °C. The curves drawn represent the best fit data plotted according to eq 4 with the tetramer-dimer dissociation constants listed in Table I and the B' value of 15×10^{-8} l. mol/g^2.

against the simple two-state representation of the unfolding process by the aliphatic acid salts discussed in the accompanying paper (Ibanez and Herskovits, 1976).

Analysis of the Dissociation Data. In principle one should be able to apply the logarithmic form of the basic eq 10 to the subunit dissociation data as a function of the dissociating agent concentration, and extract from the data fit best estimates of the apparent number of binding sites, N_i for various sets of sites having binding constants, $K_{i,c}$. Unfortunately the resultant expression of the form of eq 11 is too general to be of much use, without undue arbitrariness regarding the choice and number of different sets of sites and binding constants at the areas of contact of the dissociating subunits. In order to analyze our results, we have attempted to fit the log K_D vs. [D] data of Figure 4 with one or two independent sets of binding sites N_1' and N_2', with binding constants $K_{B,1}$ and $K_{B,2}$, respectively. The equation used for these calculations has the form

$$\log K_D = \log K_{D,w} + 2N_1' \log (1 + K_{B,1}[D]) + 2N_2' \log (1 + K_{B,2}[D]) \quad (15)$$

The dotted curves of the acetate and propionate data represent curve fitting with two sets of binding sites and binding constants: $N_1' = 7$, $K_{B,1} = 0.02$ M^{-1}, and $N_2' = 1$, $K_{B,2} = 0.6$ M^{-1} for acetate; and $N_1' = 18$, $K_{B,1} = 0.039$ M^{-1}, $N_2' = 1$, and $K_{B,2}' = 0.6$ M^{-1} for propionate dissociation. All the solid lines, fitted by the least-square linear regression analysis, and the

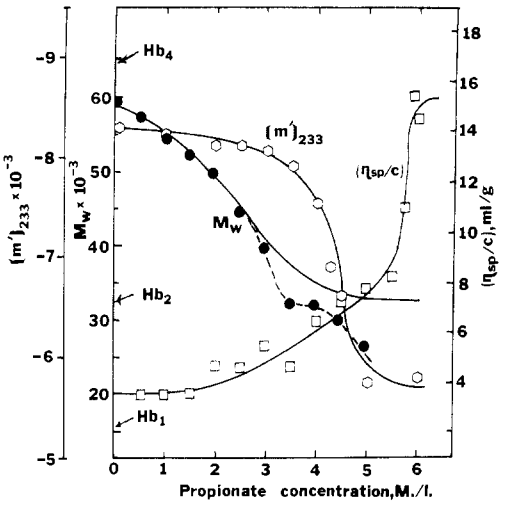

FIGURE 3: A comparison of the dissociation and denaturation effects of sodium propionate followed by the changes in the light-scattering molecular weight, M_w, the optical rotation at 233 nm, $[m']_{233}$, and the reduced specific viscosity, η_{sp}/c. The molecular weight and viscosity data correspond to protein concentrations of 0.5%, while the optical rotatory dispersion data correspond to 0.065%. As described in the text the solid lines drawn through the molecular-weight data were based on the tetramer to dimer scheme of hemoglobin dissociation.

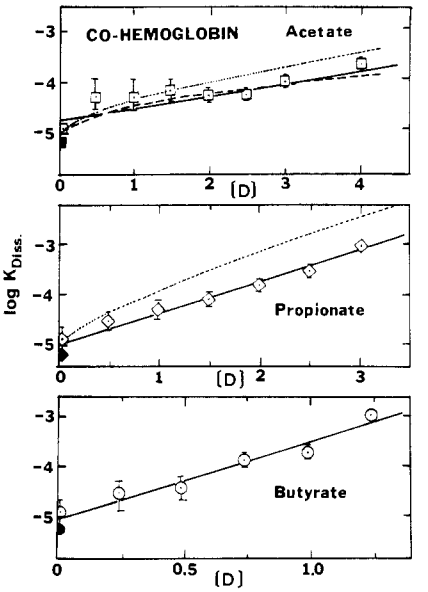

FIGURE 4: Log K_D vs. [D] plots of the light-scattering dissociation data of carboxyhemoglobin in neutral salt solutions based on eq 15 and 16. The solid lines represent least-squares fit of the data with a single set of binding sites, N', and binding constants, K_B, listed in Table II. The dotted curves of the acetate and propionate data represent fitting with two sets of binding sites and binding constants: $N_1' = 7$, $K_{B,1} = 0.02$ M^{-1} and $N_2' = 1$, $K_{B,2} = 0.6$ M^{-1} for acetate, and $N_1' = 18$, $K_{B,1} = 0.039$ M^{-1}, and $N_2' = 1$, $K_{B,2} = 0.6$ M^{-1} for propionate. The dashed line for acetate represents a single strong binding site fit, with $N_2' = 1$ and $K_{B,2}' = 0.6$ M^{-1}, suggested by the studies of Cann (1971) discussed in the text.

dashed curve for acetate are based on single sets of N' and K_B values (i.e, $N_2' = 0$), the latter with $N' = 1$ and $K_B = 0.6$ M^{-1}. For weak binding with small $K_B[D]$ values, the binding term of eq 15 is again expanded, with only the first term of the series being retained, giving

$$\log K_D = \log K_{D,w} + 2N'K_B[D] \quad (16)$$

Strong binding is probably not a property of the $\alpha_1\beta_1$ contact areas of human hemoglobin as suggested by the good linear

TABLE II: Human Hemoglobin A Parameters Based on Equation 17.

Dissociating Agent	Slope Parameter, s^a	Hydrophobic Component of K_B, $K_{H\Phi}{}^b$	Estimates of N' (with $K_B = K_{H\Phi} + K_p$)		
			$K_p = 0$	$K_p = 0.017$	$K_p = 0.01$
		Acid Salts			
Formate	-0.02 ± 0.1				
Acetate	-0.34 ± 0.1	0.011	26	10	14 ± 6
Propionate	-0.90 ± 0.1	0.029	26	17	19
Butyrate	-1.85 ± 0.1	0.076	21	17	18
Valerate	-4.1 ± 1.7	0.20	17	16	17
		Ureas			$K_p = 0.032$
Ethylurea[c]	-1.3 ± 0.1	0.029			18
Propylurea[d]	-2.2 ± 0.4	0.076	25 ± 3		17 ± 3
Butylurea[c]	-5.2 ± 0.8	0.20	22		19

[a] Slopes of $\Delta G_D°$ vs. [D] plots of data such as in Figure 5. [b] $K_{H\Phi}$ values based on free energy of transfer data of aliphatic amino acid side chains (Herskovits et al., 1970a). [c] Average estimate based on the combined osmometric and light-scattering data of Elbaum and Herskovits (1974). [d] Average values of oxy-, cyanmet-, deoxy-, and *N*-ethylmaleimide-modified oxyhemoglobin A of Bhat and Herskovits (1975).

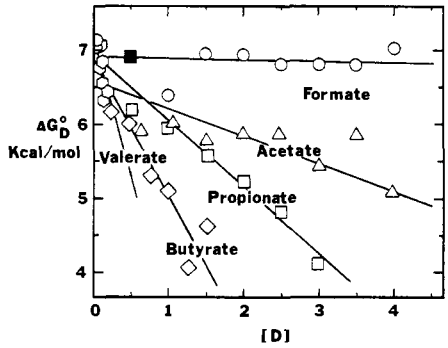

FIGURE 5: $\Delta G_D°$ vs. [D] plots for the dissociation of carboxyhemoglobin tetramer to dimers by various aliphatic acid salts based on eq 17. The lines drawn through the data points represent least-squares fit analysis, with the derived slope, s, and N' parameters listed in Table II. The data given by the filled square represent results obtained on reassociated protein in 0.5 M propionate, initially exposed and dissociated in 1.5 M propionate.

fit of the dissociation data obtained with the higher members of the acid salt series. The slight initial upswing of the acetate data, on the other hand, may be interpreted as suggesting the presence of at least one strong binding site also suggested by the studies of Cann (1971). However, the relatively large scatter of the K_D data at low degrees of protein dissociation seen with the lower members of the series, formate and acetate (Tables I and II), present really no compelling evidence for the need to consider strong and/or multiple binding sites for the whole acid salt series.

Figure 5 presents the dissociation data plotted as the standard free energy of dissociation, $\Delta G_D°$ vs. [D] based on eq 12 and 17 assuming a single set (N') independent binding sites and binding constants. The filled square represents $\Delta G_D°$ data obtained on propionate solutions initially exposed to 1.5 M concentrations that have been reversed by threefold dilution with aqueous buffer and dialysis against 0.5 M salt in the cold. Since there is little unfolding and denaturation in the salt concentration regions employed for our estimates of the slopes of the $\Delta G_D°$ vs. [D] curves, the dissociation of hemoglobin seems to be essentially reversible at low to medium concentrations of acid salt. This is also suggested by the molecular-weight data of Figure 3. Deviation from the theoretical M_w curve is only seen in the region of medium to high salt concentration above 3 M sodium propionate, where there is significant denaturation suggested by the changes in $[m']_{233}$. The slightly higher $\Delta G_D°$ obtained on the reversed protein solutions relative to other propionate data suggests the presence of some aggregated protein that is probably the product of the extra manipulations involved in the preparation of the solutions.

Table II presents a summary of the slope parameters, s, based on the data of Figure 5 and also Figure 4, related to our estimates of N' that are also listed. Because of turbidity of valerate solutions above 0.2 M (also discussed in the companion paper), the dissociation data with this salt are based on the relatively narrow range of salt concentration of 0 to 0.2 M, and are consequently subject to larger than usual experimental uncertainty. These parameters and the standard deviation of s given in this table are based on least-squares treatment of the data.

Discussion

The light-scattering results on the subunit dissociation of human hemoglobin A of this study suggest that the effectiveness of the aliphatic acid salt series as dissociating agents increases with increasing hydrophobicity or hydrocarbon content of the dissociating agent (Table I). A good measure of the effectiveness of the various salts as dissociating agents for subunit protein is the slope parameter, s, of the $\Delta G_D°$ vs. [D] curves, based on Figure 5 and listed in Table II. There is good correlation in the trend of the series as dissociating agents and their relative hydrophobicity reflected by the nonpolar component of the binding parameter $K_{H\Phi}$ also listed in column 2 of this table.

As with the alkylureas and amides (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975), we find that the dissociation of human hemoglobin A in the moderate ranges of salt concentrations below the unfolding transitions is in all cases satisfactorily described by the tetramer to dimer scheme of dissociation (Figures 1 and 2). This model of hemoglobin dissociation to $\alpha\beta$-half molecules assumes that the splitting of the functional hemoglobin tetramer occurs symmetrically along the smaller area of subunit contacts, comprising 19 to 27 amino acids per dimer surface formed and exposed to solvent, as opposed to the larger unsymmetrical cleavage of the parent protein, with 32 to 34 amino acids exposed per dimer as a result of dissociation (Perutz et al., 1968; Bolton and Perutz, 1970; Perutz and Ten Eyck, 1971; Fermi, 1975). Analysis of our

dissociation data, based on eq 16 or 17, supports this contention. For the dissociation of hemoglobin tetramers to half-molecules (with $m = 2$), the standard-free-energy expression for the process can be written as

$$\Delta G_D^\circ = \Delta G_{D,w}^\circ - 2N'RTK_B[D] \qquad (17)$$

Based on this equation, slopes of ΔG_D° or $\log K_D$ vs. [D] plots such as those of Figures 4 and 5 should provide estimates of the apparent number of amino acids exposed per dissociated half-molecule, N', provided one had independent estimates of the binding constant, K_B. For our calculations and evaluations of N' we have employed both the purely hydrophobic component, $K_{H\Phi}$ of the protein-dissociating agent interactions, and the sum of the hydrophobic and polar components of the interactions, K_P, based on our denaturation studies (Ibanez and Herskovits, 1976). The $K_{H\Phi}$ values have been calculated on the basis of free-energy-transfer data of aliphatic amino acid side chains (Herskovits et al., 1970a). The same parameters have been used in other studies from our laboratory in conjunction with the Peller and Flory equations describing the denaturation transitions of bipolymers (Herskovits et al., 1970a–c; Elbaum et al., 1974; Herskovits and Bowen, 1974; Herskovits and Harrington, 1975), including the effects of the present, aliphatic acid salt series on the conformation of human hemoglobin A, sperm whale myoglobin, and cytochrome *c* of the accompanying paper (Ibanez and Herskovits, 1976).

It is significant that our N' estimates obtained with the higher more hydrophobic members of the acid salt series are in the same ranges of estimates as those based on the alkylurea data of hemoglobin dissociation, studied by light scattering and osmometric methods (Bhat and Herskovits, 1975; Elbaum and Herskovits, 1974). The latter estimates are also included in Table II. There is a considerable uncertainty concerning the correct choice of values of the small polar contributions to K_B to be employed for such calculations. The reason for this is the fact that the K_P values are based on extrapolation of the denaturation data to zero methylene group contribution of the denaturation midpoints (Ibanez and Herskovits, 1976) and the additional assumption that the interaction of the polar carboxylate moiety with the average amino acid side chain and peptide units is the same for both surface groups found at cleavage planes of the hemoglobin subunits, and groups located at the interior regions of the subunit folds, exposed as a result of denaturation. In fact it is more reasonable to expect higher K_P values in protein denaturation because of the greater accessibility of the polar peptide portion of the average amino acid in an unfolded polypeptide chain relative to a corresponding group situated at a rigid protein surface where its accessibility to solvent from the direction of the protein is hindered. This is also suggested by the model studies of Schrier and Schrier (1967) and Nandi and Robinson (1972a,b) showing that the interactions of salts with the peptide moiety is favorable, while the interaction with nonpolar compounds representing the interactions with hydrophobic side chains is generally unfavorable. The Setschenow constants are negative in the former and positive in the latter cases. Whatever the arguments concerning the appropriate K_P values to be used may be, it is clear that the experimental estimates of N' will be most reliable with the higher, more hydrophobic, and more strongly dissociating members of the particular series. This is due to the combined effects of the lower uncertainty in the slope estimates of the steeper ΔG_D° vs. [D] curves (see Figure 5) and the more nearly negligible polar contributions to the overall K_B, required for our calculations.

Our best estimates of N' based on the dissociation data of the three higher members of the aliphatic acid salt series and the three alkylureas listed in Table II are in satisfactory accord with the 19 amino acids that comprise the smaller $\alpha_1\beta_2$ contact areas of hemoglobin, suggested by its x-ray crystallographic structure (Perutz et al., 1968). The model of Perutz and coworkers is based on 2.8-Å resolution of earlier x-ray data of horse hemoglobin. Recently Fermi (1975) reported a 2.5-Å refinement of the x-ray structure of human hemoglobin and has suggested that the $\alpha_1\beta_2$ contact areas contain several additional amino acids with a total of 27 groups instead of the approximately 20 groups suggested by the earlier data of Muirhead and Grier (1970) on deoxyhemoglobin, at 3.5-Å resolution (see Perutz and Ten Eyck, 1971). Several of the groups included in the $\alpha_1\beta_2$ cleavage area by Fermi appear to be located at the periphery of the contact regions and as a result must be at least partially exposed to solvent. Three of these groups appear to be hydrogen bonded to water molecules. Since the N′ estimates represent the difference in the number of amino acids that are exposed to solvent per $\alpha\beta$ dimer in the associated and dissociated states of hemoglobin, partially exposed groups located at the contact areas should lead to an actual underestimate of N'.[2] Consequently, the N' values derived from subunit dissociation data have been designated as an apparent number of amino acid groups exposed per contact area (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975). What is most significant in relation to our estimates of N' listed in Table II is the fact that, even if the small polar K_p contributions to the binding interactions of the protein and the dissociationg agent are ignored, the average estimate of N' will not be higher than the value of 27 suggested by Fermi (1975).

It was assumed for some time, largely on the basis of the dissociation of hemoglobin by inorganic salts (Kawahara et al., 1965), that the $\alpha_1\beta_2$ contact areas of hemoglobin are largely stabilized by polar interactions (Perutz et al., 1968). Studies from our laboratory on the dissociation of human hemoglobin by the ureas and amides have suggested that hydrophobic interactions are an important source of stabilization of the quaternary structure of this protein (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975). Our present findings, suggesting that for the higher members of the acid salt and urea series the hydrophobic component of the binding constant alone is able to account for the observed dissociation, giving the right estimate of the amino acid contacts between the $\alpha_1\beta_2$ subunits, support this observation. In a recent paper Chothia and Janin (1975) have examined the chemical nature of the contact surfaces involved in horse hemoglobin tetramer, insulin dimer, and trypsin–pancreatic trypsin inhibitor complexes and have concluded that hydrophobic interactions are the major source of stabilization in these protein–protein interactions. Their conclusions, based on the available x-ray crystallographic data of the literature, seem to support our experimental findings.

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[2] Concerning this point, see the discussion in the theoretical section related to eq 8–10.

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Intramolecular ^{1}H Nuclear Overhauser Effect Study of the Solution Conformation of Valinomycin in Dimethyl Sulfoxide†

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ABSTRACT: Determination of the mechanism of intramolecular nuclear Overhauser effects (NOE) in peptides and depsipeptides is essential to the use of this technique in conformational analysis of these and related biomolecules. Towards this end, ^{1}H NMR double-resonance studies were conducted on valinomycin in $(CD_3)_2SO$ at 90 MHz (FT mode) and 250 MHz (correlation mode). The NOE's are positive at the lower frequency and negative at the higher frequency. Consideration of the theoretical dependence of the NOE on the proton–proton internuclear correlation time and on the resonance frequency indicates that these results are explained by a predominantly dipolar relaxation mechanism. It is demonstrated that exchange modulation of scalar coupling does not contribute significantly to the NOE. A formalism for the NOE's of loosely coupled spin systems is presented which takes into account the effects of high magnetic-field strengths and long correlation times. An approximate analysis of the NOE data assuming a single correlation time for the entire molecule and ignoring cross-relaxation effects was used to evaluate various models that have been proposed for the conformation of valinomycin. The III-1 model of Patel and Tonelli (Patel, D. J., and Tonelli, A. E. (1973), *Biochemistry 12,* 486) fits the NOE and peptide N*H*C$^\alpha$*H* coupling constant data and is probably a preferred orientation in dimethyl sulfoxide. These experiments illustrate how intramolecular NOE data provide a valuable auxiliary method to other techniques for delineating the preferred solution conformation of peptides, depsipeptides, and other biomolecules.

The intramolecular nuclear Overhauser effect (NOE,[1] change in the intensity of one resonance when another resonance is irradiated) is a sensitive probe of molecular geometry which has yielded information about the detailed conformation of small molecules (Noggle and Schirmer, 1971). Few comparable ^{1}H NOE studies of peptide hormones, peptide and depsipeptide antibiotics, and proteins have been attempted because the small effects anticipated for these relatively large molecules require a precision not attainable with previously available instrumentation. The recent development of Fourier transform (reviewed by Farrar and Becker, 1971) and correlation (Dadok and Sprecher, 1974; Gupta et al., 1974) techniques for efficient signal to noise enhancement now makes possible both intramolecular (Sykes et al., 1974; Campbell et al., 1974) and intermolecular (Balaram et al., 1972a,b; Pitner et al., 1974, 1975a,b; Glickson, 1975; Glickson et al., 1976) homonuclear ^{1}H NOE conformational studies of complex

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[1] Abbreviations used are: NOE, nuclear Overhauser effect; Hyv, hydroxyvaleric acid; Lac, lactic acid; T_1, spin–lattice relaxation time; NMR, nuclear magnetic resonance; rf, radiofrequency.