

Light-Scattering Investigations of the Subunit Dissociation of Human Hemoglobin A. Effects of Various Neutral Salts[†]

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ABSTRACT: The effectiveness of various salts of the Hofmeister series as dissociating agents for human hemoglobin A tetramers has been investigated by light-scattering molecular-weight measurements. Dissociation of hemoglobin to half-molecules of $\alpha\beta$ dimers follows the order of the series dictated predominantly by the sequence of the anions $F^- < Cl^- < Br^- < ClO_4^- < SCN^-$, I^- , with the cations Na^+ and K^+ having relatively little effect on the observed dissociation. The use of equations derived for predicting the effects of dissociating reagents on the structure of subunit proteins [Herskovits, T. T., and Ibanez, V. S. (1976), *Biochemistry* 15,

5715] together with Setschenow constants based on the model amino acid data of Nandi and Robinson were found to give a satisfactory account of the dissociation behavior observed with many of the salts, giving reasonable estimates of the number of amino acids that form the smaller contact area of the $\alpha\beta$ subunits of hemoglobin shown by the Perutz crystallographic model. The analysis of the dissociation data also extends the utility of the Setschenow constants tested for the characterization of the dissociation behavior of other subunit proteins.

For the past several years in our laboratory we have examined the use of hydrophobic solutes and salts as dissociating agents for the four-subunit and the multisubunit human and earthworm hemoglobins (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975; Herskovits and Harrington, 1975; Herskovits and Ibanez, 1976). These studies have provided useful information concerning the nature of the stabilizing forces and the number of amino acids that are at the contact areas of the dissociating subunits. The use of equations based on multiple equilibria formulation of the interactions of proteins with the dissociating reagent, and binding or interaction constants, K_B , based largely on the free energy of transfer data of hydrophobic alkyl groups taken from the literature (Herskovits et al., 1970), has given us estimates of the number of amino acids at the $\alpha_1\beta_2$ contact areas of human hemoglobin A. These estimates are in reasonable agreement with the x-ray crystallographic results of Perutz et al. (1968). One has no such convenient source of interaction constants for the Hofmeister salt series which could be used to study the dissociation behavior of unknown subunit proteins and enzymes. Setschenow constants, K_s , for model peptides and hydrophobic side chains based on solubility data are available in the literature (Robinson and Jencks, 1965; Schrier and Schrier, 1967; Nandi and Robinson, 1972a,b) and have been used to estimate the effects of salts on the thermal denaturation transitions of proteins (Schrier and Schrier, 1967; Mandelkern and Stewart, 1964). The relevant literature of the latter subject has been reviewed by Von Hippel and Schleich (1969a,b).

In this paper, we report results on the light-scattering behavior of human hemoglobin in the presence of various salts of the lyotropic Hofmeister series, and discuss the relevant model data of the literature that can be used to characterize the dissociation of subunit proteins.

Experimental Section

Human hemoglobin A was prepared using Drabkin's procedure (Drabkin, 1946) and DEAE¹-Sephadex G-50 chromatography as previously described, and was stored in the cold in the carbon monoxide liganded form (Elbaum and Herskovits, 1974). The various salt solutions used were buffered with 0.01 or 0.02 M pH 6.9–7.0 phosphate buffer, with the exception of $CaCl_2$ solutions, where 0.01 M pH 7 Tris buffer had to be used. Hemoglobin concentrations were based on absorbance measurements, using the percent extinction coefficient of carboxyhemoglobin of 9.04 at 540 nm (Elbaum and Herskovits, 1974). All the salts employed were reagent grade materials. The concentrations of salt stock solutions from which dilutions for the various measurements were made were based on density determinations using the density data compiled in the "International Critical Tables" or by the use of salts that were dried under vacuum.

Light scattering and refractive index increment measurements were made in a Wood Mfg. Co. instrument of Brice's design at 630 nm. Solutions were clarified of dust and particulate matter using pairs of 25-mm filters mounted on top of one another and metrical filter disks of 0.2- μ m porosity (Gelman GA-8), as previously described (Harrington et al., 1973; Elbaum and Herskovits, 1974). Light-scattering data measured at a 90° angle were interpreted on the basis of the equation

$$K'c/R_\theta = (M_w)_{app}^{-1} = M_w^{-1} + 2B'c \quad (1)$$

where R_θ is the excess scattering ratio of protein solution over solvent, the so-called Rayleigh's ratio, M_w is the weight average molecular weight, B' is the second virial coefficient, and K' is the light-scattering constant defined in our earlier publications (Herskovits and Harrington, 1975; Herskovits and Ibanez, 1976). The refractive index increment at constant chemical potential, $(\partial n/\partial c)_\mu$, was obtained on dialyzed solutions at osmotic equilibrium (Casassa and Eisenberg, 1964).

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¹ Abbreviations used are: Gdn-HCl, guanidine hydrochloride; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ORD, optical rotatory dispersion.

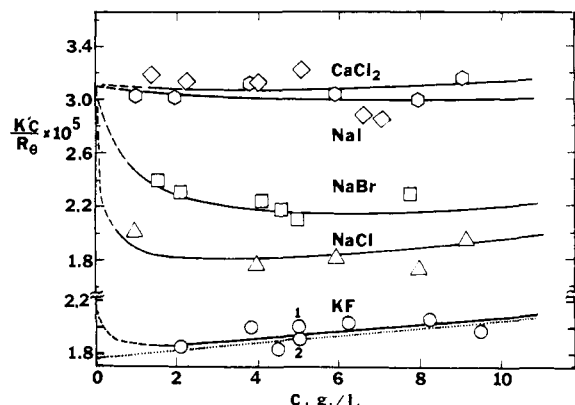


FIGURE 1: The effects of various 1 M salts on the light scattering of human hemoglobin A at neutral pH and at $25 \pm 1^\circ\text{C}$. All the solutions were buffered using 0.01–0.02 M pH 6.9–7.0 phosphate or Tris buffers. The curves drawn through the data points are based on the best-fit K_D values of Table I, plotted according to eq 1, 3, and 5.

Results

The light-scattering behavior of human carboxyhemoglobin A obtained in representative experiments of this study is shown in Figure 1. The data plotted according to eq 1 and 2 as a function of protein concentration could in all cases be fitted with the tetramer-dimer scheme of dissociation, represented by the solid lines drawn through the individual sets of data points. This scheme of fragmentation of hemoglobin tetramers to half-molecules of $\alpha\beta$ dimers is most apparent from the data of the strongest dissociating salts, 1 M CaCl_2 and 1 M NaI (Figure 1). In terms of α , the weight fraction of hemoglobin tetramers dissociating to dimers, the weight average molecular weight can be expressed as

$$M_w = M_4(1 - 0.5\alpha) \quad (2)$$

Combined with the light-scattering eq 1, our working equation in terms of α has the form

$$K'c/R_\theta = 1/[M_4(1 - 0.5\alpha)] + 2B'c \quad (3)$$

The concentration dependence of the light-scattering data, shown in Figures 1 and 2, is usually fitted with a single dissociation constant

$$K_D = m^m c^{m-1} \alpha^m / [(1 - \alpha)(M_m)^{m-1}] \quad (4)$$

where m is the number of subunits or fragments that the protein dissociates into, and M_m is the molecular weight of the subunit protein. For human hemoglobin with tetramers dissociating to dimers, $m = 2$ and

$$K_D = 4c\alpha^2 / [(1 - \alpha)M_4] \quad (5)$$

Unless high concentrations of dissociating agents are used where the hemoglobin subunits start to unfold (Elbaum and Herskovits, 1974; Herskovits and Ibanez, 1976), the dissociation data of human hemoglobin are best described by the tetramer-dimer scheme of dissociation. It is apparent from the good data fit of Figures 1 and 2A that the salt dissociation of human hemoglobin follows also the tetramer-dimer scheme of splitting along the smaller areas of contact, resulting in symmetrical products of $\alpha_1\beta_1$ type of dimers (Perutz et al., 1968).

In practice, the light-scattering data of hemoglobin in the neutral pH region, near the isoelectric point, is found to be relatively insensitive to the choice of the apparent second virial coefficient, B' (Elbaum and Herskovits, 1974; Noren et al.,

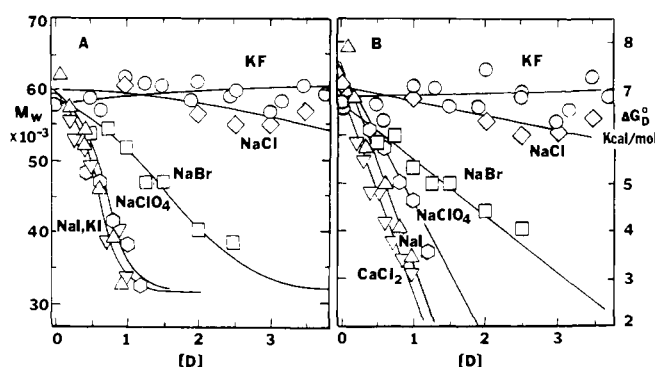


FIGURE 2: The effects of various neutral salts on the light-scattering molecular weight, M_w , of human carboxyhemoglobin (A) and on the standard free energy of dissociation, ΔG°_D , to half-molecules of $\alpha\beta$ dimers (B) plotted as a function of salt concentration, $[D]$. The solid lines drawn through the data points are based on the best-fit or least-squares parameters of eq 2 and 8, and 10, respectively, listed in Table II. Most of the data of Figure 2A were obtained at protein concentrations close to 5 g/L, or were taken from smoothed $K'c/R_\theta$ vs. c plots at $c = 5$ g/L such as Figure 1. Solvent conditions are the same in Figure 1.

1974). As with our studies of the aliphatic acid salt series on human hemoglobin A dissociation (Herskovits and Ibanez, 1976), a single B' value of $15 \times 10^{-8} \text{ L}\cdot\text{mol/g}^2$ was found to give the most satisfactory fit of the present results (some of which are shown in Figure 1). The curves represented by the solid lines were drawn according to eq 3, with the appropriate α values calculated as a function of c using eq 5, and the best fit K_D values summarized in Table I. A hemoglobin tetramer M_4 value of 64 450 was used for all our calculations. The dotted line drawn through the KF data was calculated assuming no dissociation (i.e., $K_D = 0$ and $\alpha = 0$). Least-squares regression analysis gave for the 1 M KF data an intercept and slope corresponding to $M_w = 64 100 \pm 1600$ and $B' = 16.5 \pm 7.8 \times 10^{-8} \text{ L}\cdot\text{mol/cm}^2$. The solid line for KF was calculated with a $K_B = 1 \times 10^{-6}$ and $B' = 15 \times 10^{-8} \text{ L}\cdot\text{mol/cm}^2$.

Different hemoglobin preparations gave slightly variable results that do not permit us to state with any certainty whether there is any dissociation of human CO-hemoglobin in KF solutions above 1 M. The two data points designated as 1 and 2 at a protein concentration of 5 g/L of this figure were obtained with two hemoglobin preparations giving M_w values (corrected for B') of 60 700 and 66 800. The preparation showing more association was also studied in 2 M KF (data not shown), with the least-squares results of $M_w = 69 800 \pm 1800$ and $B' = 16.9 \pm 8.1 \times 10^{-8} \text{ L}\cdot\text{mol/cm}^2$. For associating protein systems where electrostatic destabilization is unimportant due to screening effects of the electrolyte, one would expect this to occur, because of the unfavorable overall interaction of the salt with the average peptide plus side chain, represented by the interaction of a single methylene group (Table III).

In addition to the data obtained on 1 M salt solutions (Figure 1), we have also made measurements at various 0.4 M salt concentrations (concentration-dependent data not shown) and at various salt concentrations, at a single hemoglobin concentration close to 5 g/L. This approach allowed us to collect a sufficiently large number of data points for each salt using individual hemoglobin preparations, with inherently less scatter in the molecular weight data as a function of dissociating reagent concentration (Figure 2A). Analysis of our data in terms of the apparent number of amino acids at the contact areas of the dissociating subunits, N' , is described in the Discussion. For calculating the standard free energy of dissociation, $\Delta G^\circ_D = -RT \ln K_D$, one has to know α , the fraction of hemoglobin

TABLE I: Light-Scattering Data of Human Carboxyhemoglobin A in Various Neutral Salt Solutions (pH 6.9–7.0) at 25 ± 1 °C.

Salt concn (M)	$(\partial n/\partial c)_\mu$	ρ^a	$(M_w)_{app}^{-1} \times 10^5$ at $c = 5$ g/L	$K_D \times 10^5$	Salt concn (M)	$(\partial n/\partial c)_\mu$	ρ^a	$(M_w)_{app}^{-1} \times 10^5$ at $c = 5$ g/L	$K_D \times 10^5$
NaCl					NaBr				
0	0.194 ^c	0.014	1.81	0.6	0.5	0.190	0.010	2.00	4.8
1.0	0.187 ^c	0.017	1.84	1.0	0.75	0.189	0.012	1.98	4.0
2.0	0.181 ^c	0.013	1.92	2.5	1.0	0.187	0.012	2.17	12
2.5	0.178	0.011	1.97	3.8	1.25	0.185	0.013	2.30	21.5
3.0	0.175	0.010	1.96	3.5	1.5	0.183 ^c	0.012	2.29	21
3.5	0.172	0.008	1.91	2.3	2.0	0.180 ^c	0.001	2.62	66
					2.5	0.176	0.013	2.75	102
NaI					KF				
0.1	0.193	0.005	1.76	0.2	0.5	0.190	0.014	1.86	1.3
0.2	0.193	0.006	1.90	2.1	1.0	0.188 ^c	0.022	1.81	0.6
0.3	0.192	0.006	2.02	5.5	1.3	0.188	0.013	1.81	0.6
0.45	0.191	0.007	2.00	4.8	1.5	0.187	0.035	1.81	0.6
0.6	0.190	0.007	2.33	24	2.0	0.186 ^c	0.024	1.79	0.4
0.8	0.189	0.008	2.75	105	2.5	0.186	0.010	1.83	0.8
1.0	0.188 ^c	0.015	3.00	300	3.0	0.187 ^c	0.010	1.91	2.3
					3.5	0.187	0.008	1.80	0.5
					3.8	0.187	0.018	1.84	1.0
KI					NaClO ₄				
0.2	0.192	0.011	1.93	2.8	0.4	0.190	0.010	1.95	3
0.25	0.191	0.008	2.04	6.2	0.6	0.189 ^c	0.010	2.00	5
0.4	0.190	0.013	2.11	9.1	0.8	0.186	0.014	2.28	20
0.5	0.189	0.010	2.19	13	1.0	0.185 ^c	0.013	2.54	50
0.6	0.188	0.012	2.34	26	1.2	0.184	0.018	2.92	200
0.75	0.187	0.014	2.75	104					
0.8	0.186	0.016	2.65	73					
1.0	0.185 ^c	0.011	3.00	300					
NaSCN					CaCl ₂				
0.12	0.193	0.010	1.84	1.0	0.2	0.191	0.005	2.01	5
0.24	0.192	0.006	2.00	4.8	0.3	0.189	0.007	2.11	9
0.44	0.191 ^c	0.008	2.20	15	0.4	0.187 ^c	0.013	2.39	30
0.6	0.187	0.008	2.29	21	0.5	0.186	0.009	2.38	29
0.73	0.185	0.003	2.54	38	0.6	0.185	0.008	2.68	84
0.79	0.184	0.011	2.67	80	0.7	0.184	0.012	2.87	166
0.85	0.183	0.019	2.98	262	0.8	0.182	0.010	3.01	312
1.0	0.181	0.009	3.10	556	1.0	0.179 ^c	0.011	3.09	500
1.5	0.172 ^c	0.015	3.00	300					

^a ρ , depolarization ratio. ^b Experimental $(M_w)_{app}^{-1}$ values obtained near $c = 5$ g/L or taken from smoothed $(M_w)_{app}^{-1}$ vs. c curves such as in Figure 1. ^c Experimentally determined $(\partial n/\partial c)_\mu$ values, with the rest of the values given having been interpolated using the 0 and higher molar values. The aqueous value and other aqueous data are from Elbaum and Herskovits (1974), while the 2 M NaCl value is taken from Noren et al. (1974).

that dissociates, and use eq 5 to calculate K_D . In terms of the observed molecular weight changes (M_w) , α is given explicitly by the equation

$$\alpha = 2(1 - M_w/M_4) \quad (6)$$

For any fixed protein concentration at which the light-scattering experiments are made, the use of this equation requires that a nonideality correction should be applied to the apparent molecular weight, $(M_w)_{app}$. Based on eq 1, the reciprocal molecular weight is equal to $M_w^{-1} = (M_w)_{app}^{-1} - 2B'/c$. Within the uncertainties of the light-scattering technique, we have obtained essentially the same molecular weight changes using both dialyzed and undialyzed solutions, provided our data were calculated with the use of the appropriate $(\partial n/\partial c)_\mu$ values of dialyzed solutions at constant chemical potential (Casassa and Eisenberg, 1964). Hemoglobin dissociation was found to be nearly instantaneous, with little if any noticeable changes in turbidity following the mixing and filtration of the solutions. With some dissociating agents such as urea and Gdn-HCl, we have noted some increase in turbidity or aggregation on prolonged standing, presumably due to gradual unfolding of the protein also noted by Kawahara et al. (1965).

Most of the salts of the Hofmeister series are relatively in-

effective denaturing agents when compared to the effects of such reagents as urea or Gdn-HCl (Noelken, 1970; Puett et al., 1973; Harrington and Herskovits, 1975; Pace, 1975). Absorbance and ORD measurements on CO-hemoglobin solutions in the presence of 0 to 4 M NaCl have shown no significant changes in the molar extinction coefficient, ϵ_M , at the 418-nm Soret region and in the mean residue rotation, $[m']_\lambda$, of the conformationally sensitive peptide-absorbing region at 233 nm. However, with the more effective dissociating reagent NaClO₄ significant changes were noted in both of these spectroscopic parameters at salt concentrations above the dissociation transition seen in Figure 2A, with a transition midpoint of approximately 2.3 M NaClO₄. Comparable changes in absorbance at the Soret region were noted by Puett et al. (1973) using Gdn-HCl, with absorbance changes seen already in the 0.7 to 1.0 M Gdn-HCl concentration region with unliganded methemoglobin, and at higher Gdn-HCl concentrations above 1.5 M in the case of the ligand-stabilized cyanmet form of hemoglobin.

A check on the molecular weight of a 2.4 g/L carboxy-hemoglobin solution in 0.5 M Gdn-HCl gave a $M_w = 39\,000$, indicating a very extensive degree of dissociation. Removal of the dissociation reagent by dialysis gave a $M_w = 64\,000$. This

TABLE II: Dissociation Parameters of Human Hemoglobin A Based on Eq 2 and 8, and Eq 10 and 11 Obtained with Various Neutral Salts.

Salt	Eq 2 and 8				Eq 10 and 11		
	K_B	Exponential factor ($2N'K_B$) ^a	$K_{D,w} \times 10^6$ ^a	N'	Slope parameter ^a ($-2RTN'K_B$) (kcal mol ⁻¹ M ⁻¹)	$\Delta G^\circ_{D,w}$ ^a (kcal mol ⁻¹)	N'
KF	-0.005	-0.19	12		+0.072 ± 0.07	6.64 ± 0.1	
NaCl	0.014	0.49	6		-0.29 ± 0.08	7.03	
NaBr	0.053	2.0	12		-1.17 ± 0.13	6.73	
NaI	0.18 ^b	6.7	6	19	-4.15 ± 0.49	7.50	20
KI	0.17 ^b	6.1	10	18	-3.37 ± 0.18	6.83	17
NaSCN	0.16 ^b	6.1	6	19	-3.94 ± 0.28	7.14	20
NaClO ₄	0.175 ^b	5.6	6	16	-2.80 ± 0.20	7.28	15
CaCl ₂	0.18	6.7	10		-4.0 ± 0.30	6.74	
Gdn·HCl	0.20 ^b	8.4 ^c	12 ^c	21 ^c	-4.14, ^d -4.91 ^c	6.74 ^c	17 ^d , 21 ^c

^a Best fit or least-squares parameters of Figure 2A,B represented by solid lines. The Gdn·HCl data are not shown. ^b Based on K_s values of a peptide unit plus a -CH₂ methylene unit, assuming that $K_B \approx -2.303K_s$ (see footnote *b* of Table III for individual group K_s values). The rest of the values are average estimates based on the slopes ($= -2N'RTK_B$) and exponential factors ($= 2N'K_B$) of the experimental data given in columns 3 and 6, assuming $N' = 19$. ^c Unpublished results of R. C. San George. ^d Based on the ultracentrifugation data of Kawahara et al. (1965) in the 0 to 0.8 M Gdn·HCl region.

suggests that there is little if any irreversibility, despite the possibility of the dissociated hemoglobin during the longer process of removal of the reagent by dialysis.

Discussion

The dissociation of human hemoglobin A to half-molecules by the Hofmeister or lyotropic series of salts closely parallels the effects of these salts on the solubility model amino acids data, as well as their effects on the conformational stability (Mandekern and Stewart, 1964; von Hippel and Wong, 1964; Schrier and Schrier, 1967; Noelken, 1970; Kugimiya and Bigelow, 1973) and on the association-dissociation, and polymerization states of proteins (Holtzer et al., 1960; Nagy and Jencks, 1965; Dandliker et al., 1967; Guidotti, 1967; Hatefi and Hanstein, 1969; MacCall et al., 1971; Sawyer and Puckridge, 1973; Levine and Murayama, 1975; Prakash and Nandi, 1977). The effectiveness of the series as dissociating agents for hemoglobin, reflected by both the changes in molecular weight (Figure 2A), and the slope or decrement of the free energy of dissociation, $\delta\Delta G^\circ_D$, for the various salts listed in Table II, follows the order, F⁻ < Cl⁻ < Br⁻ < ClO₄⁻ < SCN⁻, I⁻ for the dominant anion effects, with little, if any, detectable difference in dissociation due to the Na⁺ or K⁺ cation contributions² beyond what is expected based on their interaction with model peptide groups (Nandi and Robinson, 1972a,b).

Various aspects of the interactions of the Hofmeister salts with biopolymers, their model compounds, and other small molecules have been interpreted either in terms of specific-site binding or nonspecific solvent effects, with varying emphasis on such details as "structure-breaking" and "structure-forming" tendencies of the added solutes (for more recent discussion of the subject, see St. Pierre and Jencks, 1969; Hatefi and Hanstein, 1969; Nandi and Robinson, 1972; von Hippel et al., 1969a,b; Roseman and Jencks, 1975). While we do not claim complete innocence regarding our own contribution to this often abused and confused subject (Herskovits and Kelly, 1973), our present efforts will be restricted strictly to "explaining" the observed changes of human hemoglobin A dissociation produced by the Hofmeister salts, and the use

of interaction or Setschenow constants, K_s , of model amino acid data taken from the literature, to characterize the nature of the contact areas of subunit proteins.

The effects of dissociating reagents, such as the ureas and the aliphatic acid salts, on the dissociation behavior of hemoglobin have been described recently (Herskovits and Ibanez, 1976), using multiple equilibria formulation of the interaction between the dissociating reagent and subunit proteins. The effect of the reagent on the dissociation constant of a subunit protein dissociating into m subunits or fragments is given by the expression

$$K_D = K_{D,w} \left\{ \prod_{i=1}^{N_c} (1 + K_{i,c}[D]) \right\}^m \quad (7)$$

where K_D and $K_{D,w}$ are the dissociation constants in the presence and the absence of dissociating reagent, $[D]$ is the activity or concentration of the dissociating reagent, and $K_{i,c}$ is the interaction or binding constant of the i th class of binding sites at the contact areas of the subunits, with the products taken over all binding sites at these areas from $i = 1$ to $i = N_c$. Assuming weak interactions with the solute that can be represented by a single value of the constant $K_{i,c}$ equal to K_B , eq 7 can be expressed in the more manageable exponential or logarithmic forms 8 and 10, used to describe the dissociation data and the dependence of the standard free energy of dissociation, ΔG°_D , on the dissociating reagent concentration $[D]$

$$\alpha^m/(1 - \alpha) = K_{D,w}(M_m)^{m-1}/m^{m-1} \exp(mN'K_B[D]) \quad (8)$$

and

$$\Delta G^\circ_D = \Delta G^\circ_{D,w} - mN'RT \ln(1 + K_B[D]) \quad (9)$$

$$\approx \Delta G^\circ_{D,w} - mN'RTK_B[D] \quad (10)$$

Figure 2A,B shows some of our light-scattering data plotted according to the combined expressions 2 and 8, and 10, using the additional approximation³ (Herskovits, et al., 1970; Herskovits and Harrington, 1972; Harrington and Herskovits, 1975)

$$K_B \approx -2.303K_s \quad (11)$$

The K_B values for analysis of the earlier data of hemoglobin dissociation obtained in our laboratory with the ureas and al-

² Similar order or ranking of the series has also been noted with regard to their effectiveness as DNA denaturants (Hamaguchi and Geiduschek, 1962) and their effects on the solubility of DNA bases and nucleosides (Robinson and Grant, 1966).

TABLE III: A Comparison of K_s and Relative K_B Estimates.

Salts	Setschenow constants, K_s				$(K_{B,X}/K_{B,Cl})$, relative	
	This study ^a	Nandi and Robinson (1972a,b) ^b	Schrier and Schrier (1967) ^c	Others ^{d,e}	This study	Norne et al. (1975) ^f
KF	+0.002	+0.023				1.0
NaCl	-0.006	-0.004	+0.02	-0.008 ^d	1.0	1.0
NaBr	-0.023	-0.012	-0.004		3.8	4.7
NaI	-0.078	-0.077	-0.024		13	10
KI	-0.067	-0.073			11	10
NaSCN	-0.073	-0.070	-0.084	-0.052 ^d	12	15
NaClO ₄	-0.060	-0.076			10	12
CaCl ₂	-0.077	-0.014	-0.030	-0.043 ^d	13	
Gdn-HCl	-0.087			-0.088 ^e	15	

^a Average values based on the experimental slopes ($= -2N'RTK_B$) and the exponential parameters ($= 2N'K_B$) listed in Table II, assuming $N' = 19$ and $K_B = -2.303K_s$. ^b Assuming that the average amino acid K_s is equal to a peptide plus a methylene group value. The K_s values listed are based respectively on the following peptide plus $-CH_2$ group values: -0.027 and +0.050 for KF; -0.037 and +0.033 for NaCl; -0.037 and +0.025 for NaBr; -0.087 and +0.01 for NaI; -0.083 and +0.01 for KI; -0.077 and +0.007 for NaSCN; -0.097 and +0.021 for NaClO₄; -0.077 and +0.063 for CaCl₂; -0.061 and -0.027 for Gdn-HCl. The Gdn-HCl values are based on the free-energy transfer data of Nozaki and Tanford (1970); the rest of the values are from Nandi and Robinson (1972a,b). Most of the $-CH_2$ group values are least-square estimates based on K_s vs. number of methylene groups per side chain of model amino acids containing 0 to 4 methylene groups. For Na and K salts, a difference of 0.004 K_s unit was assumed, based on the difference of the peptide NaCl and KCl values of Nandi and Robinson. ^c Assuming that the average amino acid K_s value is equal to an amide (CONH) plus two methylene group values. ^d Based on K_B values of Mandelkern and Stewart (1964) and assuming that their KSCN value is about the same as the NaSCN value, with $K_s \approx -K_B/2.303$. ^e Based on Δg_{tr} data of Nozaki and Tanford (1970) and Pace (1975) for a single peptide plus 0.25 of a leucine side chain value, with $K_s = \Delta g_{tr}/(2.303RT[D])$. ^f Based on ³⁵Cl NMR line-width data for the low-affinity binding sites of bovine serum albumin and neglecting the differences between K and Na ionic contributions.

iphatic acid salts were based on free energy of transfer data of the hydrophobic, alkyl portion of the dissociating reagent plus a constant polar contribution to K_B , due to the polar head-group contribution to the reagent-protein interaction (Bhat and Herskovits, 1975; Herskovits and Ibanez, 1976). In the absence of such data for the Hofmeister salts, we have used the Setschenow constants, K_s , for a peptide group plus a single methylene contribution to this constant to represent the interaction of the salt with the average amino acid. Table III presents some of the K_s values, based mainly on the model amino acid data of Nandi and Robinson (1972a,b), that we have used in conjunction with eq 2 and 8-10 to fit our experi-

mental data. It should be noted here that the K_s group values for the peptide and the methylene moiety are largely of opposite sign, and nearly canceling for the less effective members of the series, such as KF, NaCl, and NaBr. The resulting uncertainties in K_s for the average amino acid are thus too large to be of much use for our estimates of the apparent number of amino acids, N' , at the contact areas of the protein subunits. The most questionable assumption here is of course the use of a single methylene group contribution to K_s as representative of the interaction with salts of the variable side chains of the polypeptide chains in proteins. With the more effective salts, NaI, KI, NaClO₄, NaSCN, and Gdn-HCl, the methylene group contribution to the overall Setschenow constant is only of the order of 10 to 30%, making this assumption concerning the side-chain interactions of the amino acids with salts a more reasonable one.

It is gratifying that our estimates of N' (Table II) obtained with these salts are close to what one would expect for hemoglobin dissociation based on the x-ray crystallographic model of Perutz and co-workers for horse hemoglobin (Perutz et al., 1968; Perutz, 1965). As with the hydrophobic reagents, the splitting of hemoglobin tetramers to dimers seems to occur along the smaller areas of $\alpha_1\beta_2$ contacts housing some 19 amino acids in the case of the horse protein. In the case of human hemoglobin, the recent data of Fermi (1975) suggest a larger number of contacts of 27 residues, several of which are at the periphery of the subunits hydrogen bonded to water. At present, it is not clear whether the horse hemoglobin contact areas are in fact smaller than the areas in human hemoglobin, nor has the extent of water accessibility of the contact amino acids been clearly established for these two hemoglobins (Chothia et al., 1976). Such information will be clearly useful for the fuller understanding of the effects of salts and disso-

³ The relationship between K_B and K_s can be derived by assuming that the solubilizing effect of the salts is due to soluble complex formation [P·D] between the dissociating salt [D] and the average peptide group [P]. The equilibrium constant, K_B , for the reaction involved, $[P] + [D] \rightleftharpoons [P·D]$, in terms of the solubilities of the average peptide group in the presence and the absence of the dissociating salt, S and S_0 , can be expressed as $[P·D]/([P][D]) \approx (S - S_0)/(S_0[D])$ or as $(S/S_0 - 1)/(1/[D])$ (Herskovits et al., 1970). Substituting this last result for K_B into eq 9 gives for the free-energy expression

$$\Delta G^\circ_D = \Delta G^\circ_{D,w} - mN'RT \ln S/S_0 \quad (9b)$$

and recalling that solubility data in terms of the Setschenow constant, K_s , has the form

$$\ln S/S_0 = -K_s[D] \quad (9c)$$

we obtain

$$\Delta G^\circ_D = \Delta G^\circ_{D,w} + 2.303mN'RT(K_s[D]) \quad (9d)$$

Comparison of this last expression with eq 10 shows that

$$K_B \approx -2.303K_s$$

We have no conceptual problem with salts that solubilize or "salt in" the average peptide unit. It is not at all clear, however, what the nature of the presumed salt-peptide complex would be for such salts as Na₂SO₄ or KF that "salt out" the model compound. Clearly, complexes cannot form here and the $(S - S_0)$ or K_B values would be negative for such salts. We must resort here to "nonspecific solvent effects" (Robinson and Jencks, 1965; St. Pierre and Jencks, 1969) as the more encompassing explanation for the observed phenomena of salt-protein interaction and dissociation.

⁴ The actual estimates of the specific binding constant for binding of chloride and iodide ion to the low-affinity sites of this protein are much higher than our estimates and the estimates based on model amino acid data (Table III).

ciating reagents on the quaternary structure of proteins in general. The reasonable apparent estimates of contact amino acids of N' values close to 19 obtained in our present study and the previously published values based on the urea and aliphatic acid salt studies (Herskovits and Ibanez, 1976) suggest that our molecular weight data could also be used to estimate the K_s constants and compare these to other estimates of K_s of the literature. Such data summarized in Table III are especially instructive regarding the constants of the less effective members of the Hofmeister series. For these salts, the polar peptide and hydrophobic methylene group values of K_s are nearly equal in magnitude but are of opposite sign (see footnote *b* of Table III). This renders the K_s estimate for the average amino acid-salt interaction based on solubility data subject to relatively large experimental uncertainty. Save perhaps for the K_s estimate obtained with the divalent CaCl_2 salt, our estimates of K_s are in relatively good agreement with the most direct estimates based on model solubility data obtained by Nandi and Robinson (1972a,b), and halide ion quadrupole relaxation estimates of Norne et al. (1975) obtained on bovine serum albumin and reported as relative ion binding affinities, $K_{B,X}/K_{B,Cl}^4$.

Initially, the pronounced effects of salt on the stability of the quaternary structure of such proteins as hemoglobin were viewed as evidence for the presence of strong polar interactions at the contact areas of the subunits, as opposed to primarily hydrophobic interactions and stabilization of the quaternary structure (Kawahara et al., 1965; Perutz, 1965; Thomas and Edelstein, 1973). More recently, however, perhaps as a result of the studies of Hatefi and Hanstein (1969) on the effects of salts on a variety of macromolecular phenomena where hydrophobic interactions are thought to be the dominant sources of stabilization energy, the dissociation and depolymerization effects of the Hofmeister series of salts are taken as evidence for hydrophobic stabilization (MacCall et al., 1971; Levine and Murayama, 1975; Prakash and Nandi, 1977). Based on other evidence from the literature, hydrogen bonding has also been invoked as the dominant source of stabilization energy in the case of proteins such as hemoglobin and β -lactoglobulin (Sawyer and Puckridge, 1973). With regard to the effects of these salts on the peptide constituents and the hydrophobic side chains found in proteins, it is important to note that the dominant effects of the salts originate from their favorable interactions with the polar peptide group (represented by negative K_s values in Table III) and the decreasingly less favorable interactions with the hydrophobic groups (based on the positive K_s values of the methylene group), in going from the less effective to the more effective members of the series. The peptide and methylene group data of Nandi and Robinson (1973a,b) suggest that salts such as KF should tend to promote association, as is found in this study (Figure 2A,B). This is due to the large unfavorable hydrophobic component of the salt-protein interactions, reflected by the large positive K_s of the methylene group value. On the other hand, salts such as KI or NaSCN should produce pronounced dissociation or depolymerization, because of the appreciably more favorable interaction with the peptide group and the much diminished unfavorable interactions with hydrophobic groups. The methylene group interactions of these two salts appear to be only of the order of 10 to 15% of the overall interaction and the related K_s of the average amino acid. These salts will thus be clearly strong dissociating agents for subunit proteins, irrespective of the presence or absence of hydrophobic amino acid side chains at the contact areas of their subunits and the dominance of hydrophobic or polar interactions of the contact energy. The observed

order or ranking of the Hofmeister series or misplacement of certain members of the series relative to what one would expect cannot be used to differentiate between the various forces that stabilize protein-protein interactions. With regard to hydrophobic effects, the ureas and aliphatic acid salt series of increasing hydrocarbon content have been suggested as possible probes (Herskovits and Ibanez, 1976). These studies and the model calculations of Chothia and Janin (1975) concerning the contact areas of the hemoglobin subunits buried in the tetrameric structure have established the importance of hydrophobic stabilization in the case of human hemoglobin.

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Partial Amino Acid Sequence of Brain Actin and Its Homology with Muscle Actin[†]

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ABSTRACT: Actin was purified from calf brains by chromatography on DEAE-Sephadex and hydroxylapatite. The protein was then subjected to amino acid sequence analysis by isolating and sequencing its cyanogen bromide peptides. CB-1, 3, 4, 5, 6, 9, 10, and 12 correspond to equivalent segments of rabbit skeletal muscle actin, while substitutions involving methionines give rise to some new peptides. The region that corresponds to CB-13 in muscle actin becomes two peptides in the brain protein because of a Leu → Met replacement at

position 16, while Met → Leu substitutions at positions 176 and 298 give rise to two larger peptides, CB-15+7 and CB-8+2, which correspond to muscle actin CB-15 fused with CB-7 and CB-8 fused with CB-2, respectively. The peptides that have been isolated from brain actin contain 267 of the 374 residues in actin, of which 157 have been unequivocally identified. When the data are compared with those for rabbit skeletal muscle actin, 11 replacements are seen; thus the two actins differ at about 7% of the positions examined.

The basic elements of the muscle contractile apparatus exist in many types of cells and seem to be responsible for the generation of force in a variety of loci; this fact justifies the use of data on the muscle system as a frame of reference for characterization of analogous proteins in cells from tissues other than muscles. During the past few years, the body of information about the basic structure and organization of the myofibrillar proteins has developed rapidly; in particular the amino acid sequences of all of the major proteins, except the myosin heavy

chain, have been determined, and the functional roles of the various components have in general been identified. Thus, the contractile apparatus of a nonmuscle cell can be dissected, and rigorous comparisons of the components with corresponding proteins from muscle, carried out at several levels, including organizational, functional, structural, and genetic are instructive in identifying relationships between muscle and nonmuscle contraction.

At this time, the protein that provides the greatest degree of continuity among the various contractile systems is actin. Its role in the thin filament of muscle is well understood, and it is now clear that most eukaryotic cells, and possibly prokaryotic cells as well, contain actin. Actin probably functions in force generation only in combination with myosin, and actin-myosin interaction seems to be controlled by proteins that respond to variable levels of intracellular calcium; myosin and control proteins have been identified in some nonmyofibrillar contractile systems, and a reasonable assumption is that these proteins exist in some form wherever actin is found. Thus, when actin is identified in a given cell, it seems likely that a

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