The Effects of Salts on the Subunit Structure and Dissociation of *Lumbricus terrestris* Hemoglobin†

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ABSTRACT: The effects of the neutral salts of the Hofmeister series, NaCl, $NaClO_4$, $MgCl_2$, NaI, and also guanidine hydrochloride (Gdn·HCl), on the subunit organization and the state of association of *Lumbricus terrestris* hemoglobin were examined by light scattering molecular weight measurements. The subunit dissociation of the parent duodecameric structure of 3×10^6 molecular weight by various salts is similar in pattern to the sequential splitting of the associated protein to half-molecules of hexamers of 1.5×10^6 molecular weight, followed by further dissociation at higher reagent concentration to monomers of 250000 molecular weight. Duodecamer to hexamer dissociation is observed in 0.4 *M* $MgCl_2$, 1–2 *M* NaCl, and 1 *M* Gdn·HCl, while hexamer to monomer dissociation is seen in the presence of 1 *M* $MgCl_2$. All three species of duodecamers, hexamers, and monomers seem to be present in 1 *M* $NaClO_4$. Further splitting of the monomers or A subunits to smaller B fragments of one-third to one-quarter molecular weight is observed in 1 M NaI solutions. Optical rotation in the peptide region and absorption measurements in the Soret region indicate the salt dissociation of *Lumbricus terrestris* hemoglobin is not accompanied by major changes in the folding of the subunits, except in the case of the strong protein denaturant, Gdn·HCl. Relative to the dissociation effects of the urea series of compounds reported in the preceding paper (Herskovits and Harrington, 1975), the neutral salts appear to be much more effective dissociating agents for *L. terrestris* hemoglobin. This suggests that polar and ionic interactions are relatively more important for the maintenance of the associated form of the protein than hydrophobic interactions. This conclusion is also supported by calculations of the possible effects of binding of $NaClO_4$, based on the Setschenow constants of the literature describing the interaction of salts with the peptide and hydrophobic alkyl group of the average amino acid found in proteins, on the standard free energy of dissociation of the duodecamer to hexamer.

The light scattering study of the preceding paper on *Lumbricus terrestris* oxyhemoglobin (Herskovits and Harrington, 1975) has established that the duodecameric form of this protein found in aqueous solutions at neutral pH can be dissociated sequentially by various ureas to half-molecules of hexamers, followed by further dissociation at higher concentrations of the dissociating agent to monomers. In solvents such as 2 *M* propylurea all three species of duodecamers, hexamers, and monomers were found to be present. However, as a class of dissociating agents the ureas were found to be relatively ineffective. Analysis of our data based on the equations developed and tested in previous studies from our laboratory (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975) has shown that there are relatively few hydrophobic binding sites at the areas of contact of the pair of hexamers forming the parent duodecameric structure. This suggests that the forces which stabilize the parent duodecameric structure of the protein in neutral aqueous solutions must be largely nonhydrophobic in origin.

From what has been learned from the effects of the ureas and the effects of pH on the subunit organization of the protein (Chiancone et al., 1972; Harrington et al., 1973), ionic and polar interactions should dominate the contact energy between the pairs of hexamers forming the duodecameric structure. Thus one would anticipate that the effects of salts of the Hofmeister series on the subunit organization and the state of association of this hemoglobin should be much more pronounced than the corresponding effects of the ureas. This paper reports the effects of such salts on the subunit organization and the conformation of *Lumbricus terrestris* hemoglobin, investigated also by light scattering molecular weight methods, optical rotatory dispersion, and absorbance measurements.

Materials and Methods

L. terrestris oxyhemoglobin was prepared essentially according to the method of Rossi-Fanelli et al. (1970) as described in the accompanying paper (Herskovits and Harrington, 1975). All the salts and solvents employed were reagent or spectral grade. The guanidine hydrochloride (Gdn·HCl)[1] used was Ultra-Pure grade purchased from Schwarz/Mann.

Light scattering measurements were made in a modified Brice photometer at 630 nm on dialyzed protein solutions at constant chemical potential (Elbaum and Herskovits, 1974; Herskovits and Harrington, 1975). Optical rotatory dispersion (ORD) and absorption measurements were made in Cary 60 and Cary 14 recording instruments. Protein concentrations were based on percent extinction coefficients of 5.95 at 540 nm for the oxy form of hemoglobin that corresponds to a molar value of 13.7×10^3 per mole of heme (Rossi-Fanelli et al., 1970).

Results

A summary of the light scattering data obtained with the various neutral salt solutions listed, and the related refrac-

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[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

Table I: Light Scattering, ORD, and Absorbance Data of *Lumbricus terrestris* Oxyhemoglobin in Various Neutral Salt Solutions.

Solvent	Refractive Index Increment, $(\partial n/\partial c)_\mu$	Refractive Index at 630 nm, n_{630}	Depolarization Ratio, ρ	Cabannes Factor, $(6+6\rho)/(6-7\rho)$	Molecular Wt, M_w[a]	$[m']_{233}$	$\epsilon_M \times 10^{-5}$ at 417 nm
1.0 *M* NaCl	0.193	1.337	0.00869	1.019	2.94×10^6	−5700 ± 300	1.14
2.0 *M* NaCl	0.187	1.347	0.00886	1.019	2.78×10^6	−5430	1.05
1.0 *M* $NaClO_4$	0.188	1.335	0.01060	1.023	1.50×10^6[b]	−5340	1.06
0.4 *M* $MgCl_2$	0.193	1.332	0.00935	1.020	2.98×10^6	−5760	1.12
1.0 *M* $MgCl_2$	0.187	1.351	0.02050	1.045	0.71×10^6[b]		0.97
1.0 *M* NaI	0.188	1.349	0.01650	1.037	0.17×10^6		1.04
1.0 *M* Gdn·HCl	0.188	1.343	0.00972	1.021	2.38×10^6	−5355	1.08
2.0 *M* Gdn·HCl	0.150	1.362	0.04250	1.097	0.66×10^6	−3330	0.56
2.0 *M* Gdn·HCl + 0.1 *M* mercaptoethanol	0.150	1.359	0.08840	1.213	0.18×10^6	−2800	0.40
6.0 *M* Gdn·HCl	0.147	1.428	0.02530	1.056	0.45×10^6	−2150	0.30

[a] Extrapolated values from $K'c/R_\theta$ vs. c plots of Figures 1 and 3 to $c \rightarrow 0$ based on the light scattering eq 1 of the preceding paper (Herskovits and Harrington, 1975). [b] Single concentration value at $c = 0.8$ g/l.

tive index and depolarization data, is given in Table I. Except for the 1 *M* $NaClO_4$ and 1 *M* $MgCl_2$ data where curvature in the usual $K'c/R_\theta$ vs. protein concentration, c, plots did not permit extrapolation to zero concentration, the molecular weights listed are extrapolated values. The 1 *M* $NaClO_4$ and $MgCl_2$ values given refer to a protein concentration of 0.8 g/l. Figure 1 shows the data obtained in various dialyzed salt solutions at 25 ± 1°.

Molecular weights of less than 3×10^6 suggest that the salts have caused dissociation and splitting of the parent duodecameric structure to half and one-twelfth molecular weight fragments, shown by both electron micrographic (Levine, 1963; Guerritore et al., 1965) and light scattering studies (Harrington, et al., 1973; Goss et al., 1975; Herskovits and Harrington, 1975). The light scattering data shown in Figure 1 have been analyzed and fitted based on duodecamer to hexamer and hexamer to monomer dissociation, described by:

$$K'c/R_\theta = [(1-\alpha_1)M_{12} + \alpha_1(1-\alpha_2)M_6 + \alpha_1\alpha_2M_1]^{-1} + 2B'c \quad (1)$$

$$K_{12,6} = \frac{4c[\alpha_1(1-\alpha_2)]^2}{(1-\alpha_1)M_{12}} \quad (2)$$

$$K_{6,1} = \frac{4.67 \times 10^4 c^5(\alpha_1\alpha_2)^6}{[\alpha_1(1-\alpha_2)](M_6)^5} \quad (3)$$

where α_1 and α_2 are the weight fractions of duodecamer dissociating to hexamer and hexamer to monomer, respectively, and M_{12}, M_6, and M_1 are the molecular weights of the duodecameric, hexameric, and monomeric species present in solution taken at 3×10^6, 1.5×10^6, and 0.25×10^6, and B' is the second virial coefficient. Details concerning the fitting of our data are given in the preceding paper (Herskovits and Harrington, 1975), using the same B' value of 5×10^{-9} l. mol/g^2 as that of this paper. In relation to the form of eq 2 and 3 it is worth noting that for $\alpha_2 = 0$ or for $\alpha_1 = 1$ these equations are reduced to the general form

$$K_{Diss} = \frac{m^m \alpha^m c^{m-1}}{(1-\alpha)(M_1)^{m-1}} \quad (4)$$

given also as eq 4 in the preceding paper, where M_1 is the molecular weight of the protein dissociating initially or undergoing further dissociation, that is M_{12}, M_6 or M_1, and

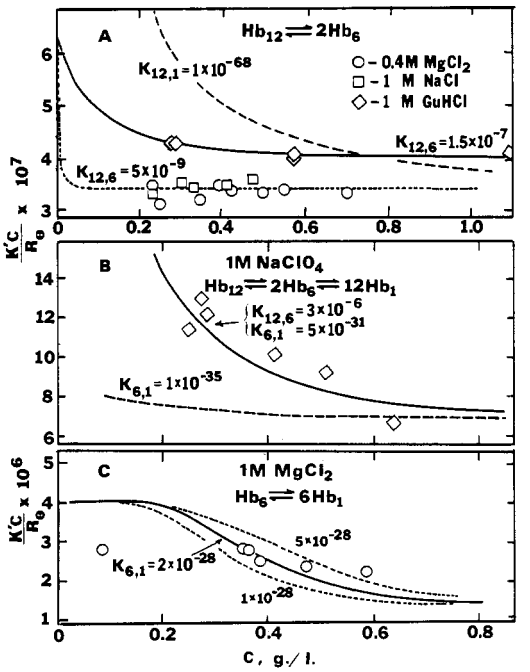

FIGURE 1: The effects of neutral salts (pH 7.0) on the light scattering of *L. terrestris* oxyhemoglobin fitted according to eq 1 of the text. (A) Duodecamer to hexamer dissociation data in 0.4 *M* $MgCl_2$, 1 *M* NaCl, and 1 *M* Gdn·HCl, fitted with dissociation constants, $K_{12,6} = 1.5 \times 10^{-7}$ to 5×10^{-9}. For comparison the duodecamer to monomer scheme of dissociation with a $K_{12,1} = 1 \times 10^{-68}$, represented by the dashed line, is also included in this part of the figure. (B) Duodecamer-hexamer-monomer scheme of dissociation in 1 *M* $NaClO_4$ fitted with $K_{12,6} = 3 \times 10^{-6}$, $K_{6,1} = 5 \times 10^{-31}$. For comparison the hexamer-monomer scheme of dissociation curve with $K_{6,1} = 1 \times 10^{-35}$ is also included in this part of the figure. (C) Hexamer to monomer dissociation in 1 *M* $MgCl_2$ fitted with $K_{6,1} = 2\text{-}5 \times 10^{-28}$. All the solutions were buffered using 0.02 *M* phosphate (pH 7.0) and studied at 25 ± 2°.

m is the number of subunits or fragments formed upon dissociation.

Figure 1A presents examples of the data fit for duodecamer to hexamer dissociation, with equilibrium constants $K_{12,6} = 1.50 \times 10^{-7}$ to 5×10^{-9} based on eq 4 giving best account of the 0.4 *M* $MgCl_2$, 1 *M* NaCl, and 1 *M* guanidine hydrochloride (Gdn·HCl) data. As with the alkylureas, the more powerful dissociating solvent systems such as 1 *M* $NaClO_4$ and 1 *M* $MgCl_2$ cause further splitting of *L. ter-*

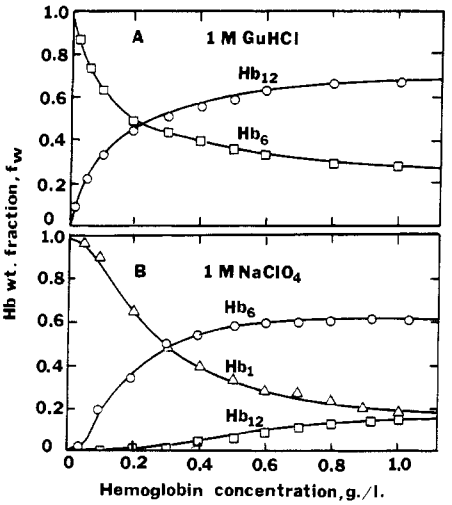

FIGURE 2: Species distribution plots of *L. terrestris* oxyhemoglobin as a function of protein concentration in neutral 1 *M* Gdn·HCl and 1 *M* $NaClO_4$ based on the data of Figure 1A and B. (A) Duodecamer–hexamer distribution in 1 *M* Gdn·HCl. (B) Duodecamer–hexamer–monomer distribution in 1 *M* $NaClO_4$. (See text concerning the calculations of the weight fraction of species present.)

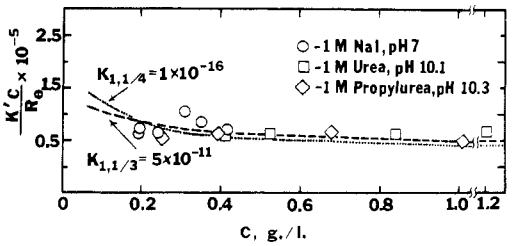

FIGURE 3: A comparison of the light scattering data of *L. terrestris* hemoglobin dissociation by neutral 1 *M* NaI (pH 7) and alkaline 1 *M* urea and 1 *M* propylurea (pH 7.1–7.3). The data were fitted by assuming dissociation of the monomer to fragments of ⅓ of its molecular weight with $K_{1,1/3} = 5 \times 10^{-11}$ (curve represented by dashed lines), and by dissociation to ¼ of its molecular weight with $K_{1,1/4} = 1 \times 10^{-16}$ (curve represented by dotted line). The NaI solutions were buffered by 0.02 *M*, pH 7 phosphate, temperature, 25 ± 2°, whereas the urea solutions were buffered by 0.05 *M* borate, pH 10.1–10.3, temperature, 10 ± 2°.

restris hemoglobin to monomers. This is suggested by the fitting of the data of Figure 1B and C, with the former $NaClO_4$ data requiring the presence of all three species of duodecamers, hexamers, and monomers in equilibrium with equilibrium constants $K_{12,6} = 3 \times 10^{-6}$ and $K_{6,1} = 5 \times 10^{-31}$. For 1 *M* $MgCl_2$, the hexamer to monomer equilibrium with $K_{6,1} = 2 \times 10^{-28}$ gives a moderately satisfactory fit of the data. It is possible that at the high degree of subunit dissociation seen with this solvent, some species of lower molecular weight resulting from the further breakdown of the monomeric units to B type of subunits (Guerritore et al., 1965) of one-third to one-quarter of the monomer molecular weight of 250000 are also produced. This is suggested by the somewhat flatter curve required than the theoretical curves drawn with the latter equilibrium constants, in order to accommodate all the experimental data points of Figure 1C, including the single low point below 0.1 g/l. of hemoglobin. Further dissociation of this hemoglobin is clearly indicated in 1 *M* NaI the most strongly dissociating electrolyte system investigated in this study. However, we have not attempted a further refinement of the data fit of Figure 1C in terms of mixtures of hexamers and monomers and further fragments of the monomeric species.

The distribution of hemoglobin species in two of the dissociating solvents, 1 *M* Gdn·HCl and 1 *M* $NaClO_4$, is shown in Figure 2A and B. The weight fractions of species present, f_w, are equal to $f_{12} = (1 - \alpha_1)$, $f_6 = \alpha_1(1 - \alpha_2)$, and $f_1 = \alpha_1\alpha_2$, with $\alpha_2 = 0$ for the duodecamer to hexamer dissociation found in the presence of 1 *M* Gdn·HCl. The dissociation constants used to generate the α_1 and α_2 values as a function of protein concentration, based on eq 2–4, are $K_{12,6} = 1.50 \times 10^{-7}$ for 1 *M* Gdn·HCl, and $K_{12,6} = 3 \times 10^{-6}$ and $K_{6,1} = 5 \times 10^{-31}$ for 1 *M* $NaClO_4$. The latter constants are based on the data fit of Figure 1A and B.

We have also attempted to fit the data of the splitting of the monomeric species to smaller fragments. Figure 3 shows fits of the data obtained in neutral 1 *M* NaI assuming that the fragments have molecular weights of one-third and one-quarter of the monomers of 250000. We have also included in this figure our 1 *M* urea and 1 *M* propylurea data obtained at higher pH's of 10.1 and 10.3, that seem to fall in the same patterns of fragmentation having the same weight average molecular weights as the neutral 1 *M* NaI solutions.

Discussion

Analysis of the light scattering data shown in Figures 1–3 suggests that the pattern of dissociation of *L. terrestris* duodecamers by the Hofmeister series of salts is similar to the dissociation patterns produced by the alkylurea series of the preceding paper (Herskovits and Harrington, 1975). Dissociation of the parent duodecameric structure in neutral aqueous solutions is found with the less effective members of the series, such as 1–2 *M* NaCl, and in the presence of a lower concentration of the more effective salts such as 0.4 *M* $MgCl_2$ and 1 *M* Gdn·HCl. As shown in Figure 1B and C the dissociation of half-molecules or hexamers to monomers is suggested by the light scattering in 1 *M* $MgCl_2$, while in 1 *M* $NaClO_4$ all three species of parent duodecamers, hexamers, and monomers seem to be present. As shown by the dashed lines in Figure 1A and B we could not account for the concentration dependence of the light scattering in terms of duodecamer to monomer dissociation of the 1 *M* NaCl, 1 *M* Gdn·HCl, and 0.4 *M* $MgCl_2$ data, nor in terms of hexamer to monomer dissociation of the 1 *M* $NaClO_4$ data, fitted with dissociation constants $K_{12,1} = 1 \times 10^{-68}$ and $K_{6,1} = 1 \times 10^{-35}$, respectively. The most effective dissociating electrolyte, NaI, seems to dissociate the *L. terrestris* hemoglobin already at the 1 *M* level of concentration to fragments smaller than the basic monomeric unit of 250000 visualized in the electron microscope (Levine, 1963; Guerritore et al., 1965). Unfortunately, due to appreciable scatter of our data of Figure 3 little information is provided concerning the nature of the B subunits that is part of the organization of the monomers, designated as A subunits (Guerritore et al., 1965; Rossi-Fanelli et al., 1970). Our data seem to be equally well accommodated by dissociation to either one-third or to one-quarter molecular weight B subunits, lending no real support to either the three or four B subunit model (Rossi-Fanelli et al., 1970; Wiechelman and Parkhurst, 1972). It is interesting that the dissociation in 1 *M* urea and 1 *M* alkylureas at alkaline pH's of 10.1–10.3 seems to fall also into this pattern of splitting of the A subunits (see Table I of the preceding paper). For this reason we have included in this figure the light scattering data obtained in 1 *M* urea and 1 *M* propylurea at pH 10.1–10.3.

In one respect the effects of the salts differ from that of the usual denaturing reagents such as the urea series, and

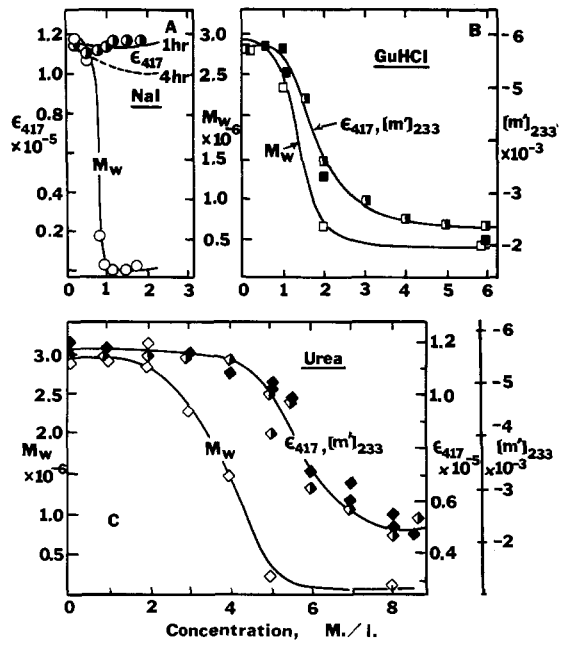

FIGURE 4: A comparison of the dissociation and denaturing effects of NaI, Gdn·HCl, and urea followed by the changes in light scattering molecular weight, M_w (data represented by open symbols), absorbance changes, ϵ_{417}, in the Soret region (data represented by half-filled symbols), and optical rotation, $[m']_{233}$, at 233 nm (data represented by filled symbols). All the solutions were buffered by 0.02 M pH 7 phosphate, 25 ± 2°.

that is that neither the Soret absorbance nor the optical rotation in the conformationally sensitive peptide absorbing region at 233 nm is significantly altered in the 1–2 M ranges (Table I), Gdn·HCl being an exception which is a commonly used strong denaturing salt (Tanford, 1970). Extensive dissociation of parent duodecameric structure of *L. terrestris* hemoglobin can be achieved by the salts without substantial alterations in the folding of the individual subunits. This is demonstrated by the ORD and ϵ_{417} data of Table I and Figure 4. In Figure 4 the effects of NaI, Gdn·HCl, and urea concentration on the molecular weight, ORD, and the Soret absorbance are compared. In regard to the further splitting of the 250000 monomers to B subunits, it is significant that the ϵ_{417} of the protein is only marginally affected in 1 M NaI solutions. Unfortunately the mean residue rotation could not be measured in this medium, because of the strong absorption of NaI solutions in the far-ultraviolet region. In 1 M urea solutions at pH 10.1, with the same sort of splitting present, the $[m']_{233}$ is found to be only slightly altered from −5430 to −5200 deg cm²/dmol (Herskovits and Harrington, 1975).

The denaturing effects of the alkylureas and their increasing effectiveness with increasing chain length or hydrocarbon content was used to demonstrate that the folding of the *L. terrestris* hemoglobin subunits is stabilized by hydrophobic forces. Our findings that the same ureas have much less pronounced effects on the subunit dissociation have suggested that hydrophobic interactions are much less important for the maintenance of quaternary structure or state of subunit organization of this hemoglobin (Herskovits and Harrington, 1975). In our studies we have used the free energy expression

$$\Delta G°_D = \Delta G°_{D,w} - mN'RTK_B[D] \qquad (5)$$

to describe the dissociation of the protein to m subunits (m = 2 for duodecamer to hexamer dissociation with N' representing the number of hydrophobic binding sites exposed as a result of dissociation). In this equation $\Delta G°_D$ and $\Delta G°_{D,w}$ represent the standard free energy of dissociation in the presence and absence of dissociating reagent of concentration [D], and K_B is the binding constant of the reagent assuming independent and equal binding sites (Elbaum and Herskovits, 1974). In relation to the effects of salts on the conformation and temperature transition of globular proteins, the studies of Schrier and Schrier (1967) and Nandi and Robinson (1972a,b) have established that to a fair degree of accuracy group additivity of interaction for the peptide moiety and the hydrophobic portion of the average amino acid with salts can be assumed. The peptide group which contributes favorably to the interactions is characterized by negative Setschenow constants, K_S, while the hydrocarbon portion of the average amino acid contributes unfavorably to the interactions with a positive K_S. For purposes of calculation Schrier and Schrier have found that a good account of the denaturation data for ribonuclease and ichtyocol gelatin could be obtained by assuming that the nonpolar contribution of the average amino acid to K_S is equivalent to 1.1 and 0.9 $-CH_3$ units. The same assumptions of group additivity can be used to see what effects some of the salts should have on the dissociation of *L. terrestris* hemoglobin. Based on the K_S values of −0.097 for the peptide interaction with $NaClO_4$ and an approximate value of 0.05 per methylene group (Nandi and Robinson, 1972a,b), we obtain an estimate for K_B = 0.1 per average amino acid consisting of a peptide and a methylene unit.[2] With this K_B value and $\Delta G°_{D,w}$ and $\Delta G°_D$ values of 11.6 and 7.5 kcal/mol for the dissociation of *L. terrestris* hemoglobin in water and 1 M $NaClO_4$ (Table II) we obtain an N' value of 35 per hexamer formed. With the ureas an N' value of 10 ± 4 was obtained in the preceding paper.

Table II: A Summary of the Dissociation Constants and Free-Energy Changes for the Subunit Dissociation of *L. terrestris* Oxyhemoglobin.

Dissociating Medium	$K_{12,6}$[a] (M^{-1})	$K_{6,1}$[a] (M^{-6})	$\Delta G°_{6,1}$ (kcal/mol)
0.1 M KCl, 0.02 M pH 7.0 phosphate	3 × 10⁻⁹[b]		11.6
2 M NaCl, 0.02 M pH 7.0 phosphate	3 × 10⁻⁸		10.3
1 M Gdn·HCl, 0.02 M pH 7.0 phosphate	1.5 × 10⁻⁷		9.3
1 M $NaClO_4$, 0.02 M pH 7.0 phosphate	3 × 10⁻⁶		7.5
1 M $MgCl_2$, 0.02 M pH 7.0 phosphate		5 × 10⁻²⁸	

[a] Based on the best fit of the data, with examples shown in Figure 1 utilizing eq 1–4 of the text. [b] Extrapolated value based on Figure 7A of the preceding paper (Herskovits and Harrington, 1975).

Two explanations can be offered for the much higher estimate of amino acid binding sites with this salt relative to

[2] This estimate of K_B is based on the further assumption that $K_B = -2.303K_s$ dictated by earlier studies from our laboratory (Herskovits et al., 1970; Herskovits and Harrington, 1972), with K_s taken as equal to K_s (peptide) + $K_s(-CH_2)$. Other estimates of K_B have not been reported in the literature. However, K_B values based on denaturation experiments using the Peller–Flory equation have been reported for KSCN and $CaCl_2$, that have comparable K_s values to $NaClO_4$. These K_B values are respectively 0.12 and 0.1 (Mandelkern and Stewart, 1964).

the urea estimate. (1) The polar and ionic character of contact areas of the hexamers is such that a greater number of polar ions can be bound relative to the hydrophobic reagents. (2) The number of binding sites is about the same, but is is necessary to take into account the weakening effects of salts on the ion pairs or salt bridges formed between charged carboxylate and charged lysyl and/or arginine side chains, and other electrostatic and ionic interaction. The latter effects represent a $\Delta G°_D$ of about 3 kcal/mol of the observed $\Delta G°_D$ of dissociation of 4.1 kcal/mol due to 1 *M* $NaClO_4$.

It is instructive to compare the above estimates of N' to similar estimates for human hemoglobin A, which also dissociates to half-molecules with the exposure of 19 amino acid sites per surface contact based on X-ray information (Perutz, 1965; Perutz et al., 1968) and the potential disruption of two salt bridges formed per half-molecule. Based on the dissociation constants, $K_{4,2} = 8 \times 10^{-5}$ for oxyhemoglobin in aqueous solutions (Bhat and Herskovits, 1975) and $K_{4,2} = 1 \times 10^{-2}$ in 1 *M* $NaClO_4$ (Guidotti, 1967), $\Delta G°_{D,w} = 5.6$ and $\Delta G°_D = 2.7$, giving us an estimate of N' equal to 24. This value should be compared to $N' = 15$–21 based on the dissociation by the ureas (Bhat and Herskovits, 1975; Herskovits and Harrington, 1975). The higher estimate of 24 based on the $NaClO_4$ data suggests that the binding interactions of salts with the polar and ionic portions of such groups as arginine, glutamine, asparagine, glutamic, and aspartic acid cannot be ignored,[3] as has been done by assuming that the contributions of the peptide moiety and the hydrophobic portions of the amino acids, approximated by the contribution to K_B of a single methylene unit, are all that needs to be considered explicitly. As described in the Discussion of the preceding paper with the two arginine and a charged histidine groups there are eight such potential sites of interaction per $\alpha\beta$-contact site exposed upon dissociation of human hemoglobin. Moreover, the precise contribution of the salt bridges in salt solutions to the stability has not been fully established (Perutz et al., 1968; Thomas and Edelstein, 1973).

Thus, at our present state of knowledge only qualitative conclusions can be really drawn based on the above analysis, and also based on our other data obtained with the much more strongly dissociating salts, $MgCl_2$ and NaI, relative to the marginal effects of the ureas. What seems to be apparently established is that polar and ionic interactions must be invoked as the main sources of stabilization energy that holds the pairs of hexamers of *L. terrestris* hemoglobin together in its duodecameric form found at physiological pH and ionic conditions.

[3] In order to take proper account of the additional contributions of ionic and electrostatic interactions to $\Delta G°_D$ the simple form of eq 5 would have to be altered to include $\Delta G°$ terms due to polar, electrostatic ionic, or pH components

$$\Delta G°_D = \Delta G°_{D,w} - mNRTK_B[D] + \Delta G°_{pol.} + \Delta G°_{el.} + \Delta G°_{pH}$$

The last ionic term would arise because of the differences in pK's of the ionizable side chains in the associated and dissociated states (Tanford, 1970; Puett, 1973) with

$$\Delta G°_{pH} = -RT \ln \frac{[\prod^{i} (1 + K_{i,D}/[H^+])]^m}{\prod^{i} (1 + K_{i,A}/[H^+])^m}$$

where $K_{i,D}$ is the dissociation constant of the ith ionizable group of the contact area in the dissociated state of the protein, and $K_{i,A}$ is the dissociation constant of the same groups in the associated state, and m is the number of subunits formed upon dissociation. The type and number of amino acids ($\Delta\nu$) that are involved, including the groups participating in ion pair formation, may be estimated by studying the pH dependence of the association process at constant ionic strength since the theory of linked functions defines $\Delta\nu$ in the transition region as the slope, $\partial(\log K)/\partial(\text{pH})$.

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