The Dissociation of Proteins by Chaotropic Salts*

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SUMMARY

Chaotropic salts were examined with a view to their use as protein-dissociating agents. The rank order of their ability to dissociate proteins followed the Hofmeister series for proteins which self-interact mainly via the formation of intermolecular hydrogen bonds (β-lactoglobulin A, hemoglobin). For hydrophobic associations (β-casein, concanavalin A, chymotrypsin), misplacements in the Hofmeister series were noted. Chaotropic salts do not necessarily bring about complete dissociation of protein polymer to protein monomer. However, dissociation was induced at salt concentrations which did not cause major shifts in protein conformation. Thus, chaotropic salts should prove most useful in dissociating multienzyme complexes and in improving the solubility of membrane-bound proteins.

Dissociation of a protein is frequently necessary for the determination of its minimum molecular weight. High concentrations of urea, guanidine hydrochloride, or detergents are often employed for this purpose. However, when used in conjunction with ultracentrifuge techniques, these denaturants lead to problems associated with nonideality, the analysis of three-component mixtures, and the selective binding of solvent components to the macromolecule (1–4). To circumvent these problems, we have investigated the use of chaotropic salts (i.e. salts whose anions favor the transfer of apolar groups to water (5)) for dissociating proteins. Such salts are known to increase the solubility of nonpolar molecules in water, and thermodynamic evidence suggests that they act by making water more "disordered" or lipophilic (5, 6). Previous studies have shown that relatively low concentrations of chaotropic salts cause the dissociation of actin, hemoglobin, and antigen-antibody complexes (7–9).

This communication examines the following questions. Can chaotropic salts be used as general reagents for dissociating proteins? Is destruction of tertiary structure concomitant with dissociation? Do chaotropic salts act with equal effect on proteins which associate hydrophobically and on those which associate via the establishment of intermolecular hydrogen bonds? For the latter question we have chosen several representative proteins. The dimer-octamer association of β-lactoglobulin A is considered to occur via intermolecular hydrogen bond formation involving Asp 64 (10–12); the degree of association decreases with increasing temperature. On the other hand, the temperature and ionic strength dependence of the association of β-casein (13, 14) and of concanavalin A (15) suggests that hydrophobic interactions predominate in the association mechanism. We do not wish to imply that these associations are due entirely to one type of interaction or another; rather the thermodynamic evidence suggests that one particular bonding interaction predominates. Although our study is restricted to the action of anions, one should recall that cations can also be arranged in a Hofmeister series according to their salting-in and salting-out properties.

EXPERIMENTAL PROCEDURE

Materials—β-Lactoglobulin A and β-casein A were prepared by the methods of Armstrong et al. (16) and Aschaffenburg (17), respectively. In each case, milk was obtained from cows typed homozygous for the A genetic variant.1 The purity of the β-casein A preparation was checked by disc acrylamide gel electrophoresis, and densitometric scanning of the gels revealed the presence of less than 2% α-casein. Concanavalin A was prepared by the method of Agrawal and Goldstein (18). Inorganic salts were of analytical grade. Sodium salts of the haloacetates were prepared by neutralizing the acids with NaOH at ice bath temperature immediately before use.

Methods—Sedimentation velocity and optical rotatory dispersion methods have been described previously (19, 20). The weight average sedimentation coefficient (s_20,w) for the β-lactoglobulin A system was calculated from the rate of movement of the square root of the second moment of the entire reaction boundary at pH 4.6, 4°C. Schlieren patterns were measured with a two-dimensional comparator (Nikon Shadowgraph model 6C), and the profiles were redrawn on graph paper with a larger scale. Peak areas were measured by trapezoidal integration and were corrected for radial dilution. Most experiments were conducted in single sector cells. At high salt concentrations, some baselines were checked by running a sample of the solvent at the same speed and bar angle for equivalent time. Sedimentation coefficients were corrected to 20°C in water. The relative viscosities of solvents containing neutral salts were measured with a capillary viscometer, and solvent densities were measured pycnometrically. Ion pair activities were calculated from data compiled by Robinson and Stokes (21).

McKenzie et al. (19) have shown that the association of β-

1 We thank Dr. Lindsay Bailey, of the South Australian Department of Agriculture, for typing and supplying this milk.
The salt concentration was varied by the addition of NaCl (O) or NaSCN (●). The ionic strength of the acetate buffer was 0.05 and the pH was 4.6,4".

Fig. 1 (left). The effect of salt concentration on the weight average sedimentation coefficient (S_{20,w}) of β-lactoglobulin A at pH 4.65, 4". The ionic strength of the acetate buffer was 0.05 and the salt concentration was varied by the addition of NaCl (○) or NaSCN (●). The right-hand ordinate expresses the equilibrium constant (K_e) for the dimer-octamer association.

Fig. 2 (center). The natural logarithm of the association constant (K_e) plotted against the natural logarithm of the ion pair NaSCN. The data; the method has been described in detail in an earlier paper (20).

TABLE I

<table>
<thead>
<tr>
<th>Salt</th>
<th>ln K_e</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>3.17</td>
</tr>
<tr>
<td>NaBr</td>
<td>4.00</td>
</tr>
<tr>
<td>NaClO₄</td>
<td>3.96</td>
</tr>
<tr>
<td>CF₃COONa</td>
<td>3.54</td>
</tr>
<tr>
<td>NaSCN</td>
<td>3.47</td>
</tr>
<tr>
<td>CCl₃COONa</td>
<td>2.88</td>
</tr>
</tbody>
</table>

*pH 4.6, 4", I = 0.175 (0.05 M acetate, 0.125 M salt).

β-Lactoglobulin A can be followed conveniently by measuring the a_0 parameter of the Moffitt-Yang equation. Equilibrium constants so obtained agree with those found with the use of sedimentation equilibrium and light-scattering methods. The technique was used here to determine thermodynamic parameters pertaining to the effect of thiocyanate on the association of β-lactoglobulin A. Equilibrium constants for this association were calculated also from weight average sedimentation velocity data; the method has been described in detail in an earlier paper (20).

Protein concentrations were measured spectrophotometrically with a Zeiss PMQ II instrument. Extinction coefficients (E_{1%}) used were 9.6 for β-lactoglobulin A (22), 12.4 for concanavalin A (23), and 4.6 for β-casein A (24).

Results

β-Lactoglobulin A

Sedimentation Velocity—The comparative effects of NaCl and NaSCN on the dimer-octamer association of β-lactoglobulin A are shown in Fig. 1. With increasing ionic strength, the weight average sedimentation coefficient decreases from that characteristic of the octamer (7.23 S) to that characteristic of the dimer (2.86 S). The right-hand ordinate of Fig. 1 expresses the change in association constant K_a (1^base mole^-7). In NaSCN the dissociation to dimer is practically complete at an ionic strength of 0.4, whereas in NaCl an ionic strength of 1.0 is required to cause complete dissociation. Within the concentration range studied, NaSCN did not bring about dissociation of dimer to monomer.

A variety of neutral salts was tested by sedimentation velocity at an ionic strength of 0.175. The results presented in Table I show that the order of effectiveness of these salts in causing dissociation follows the Hofmeister series: CCl₃COO^- > SCN^- > CF₃COO^- > Br^- > NO₃^- > Cl^-.

The plot of ln K versus ln α, (Fig. 2) is a direct measure of z pref, ref. This plot for NaCl is linear with z pref, ref equal to -0.4. However, the plot for NaSCN is linear only in the higher concentration range studied (0.10 to 0.25 M NaSCN), where the slope is -1.8.

The above analysis utilizes ion pair activities of the salts involved, and therefore z pref, ref refers to the interactions of both anion and cation with the protein. Individual ion contributions could be assessed only if independent activities could be measured. However, the individual ion contributions can be described by Equation 6 of Aune et al. (20), and it then becomes clear that nonintegral values of z pref, ref are possible. For example, if only one ion of a uni-univalent salt becomes preferentially bound to the dimer on dissociation, then z pref, ref would be -0.5. Alternatively, nonintegral values of z pref, ref can occur when the dissociation involves a change in hydration as well as a change in the binding of salt. Tanford (27) has shown that Equation 1 then becomes

\[ \Delta \bar{V}_{x, \text{pref}} = \Delta \bar{V}_{x} - \frac{m_x}{m_{H_2O}} \Delta \bar{V}_{H_2O} \]
and water, respectively. However, it is unlikely that the hydration term in Equation 2 makes a significant contribution to the case under study. The dissociation of β-lactoglobulin A occurs at relatively low salt concentrations such that $m_2/m_{H_2O} \approx 6 \times 10^{-4}$. Thus, unrealistically high values of $\Delta_{2,H_2O}$ would be required to affect $\Delta_{2,prot}$ significantly. However, a small degree of preferential hydration to the dimer is probable for the following reason. If no major change in conformation occurs on dissociation of octamer to dimer, then carboxyl groups known to be situated at contact or intersection sites (11, 12) would be in exposed or accessible situations. Kunitz (28) has estimated that ionized glutamate and aspartate residues in a polypeptide chain bind 6 or 7 molecules of water, whereas the protonated ionized glutamate and aspartate residues bind only 2 molecules of water. Thus the small increase in hydration upon dissociation will depend not only on the number of carboxyl groups which become exposed to solvent but also on their individual $pK$ values.

Optical Rotatory Dispersion—Thermodynamic analysis of the dissociation of β-lactoglobulin A in NaSCN was carried out in the following manner. At $3^\circ$ and at $I = 0.075$ (0.05 M acetate, 0.025 M NaSCN) the protein exists predominantly as the octamer, and optical rotatory dispersion analysis of this solution as a function of temperature (19, 20) yields values of the association constant. The enthalpy of association may then be obtained with increasing thiocyanate concentration, the enthalpy becomes less negative, increasing from $-73$ Cal per mole at $I = 0.075$ to $-57$ Cal per mole at $I = 0.15$ (0.03 M acetate, 0.10 M NaSCN). The entropy of association remained constant with temperature but increased from $-200$ e.u. at $I = 0.075$ to $-160$ e.u. at $I = 0.15$. Clearly, increasing the concentration of thiocyanate causes both enthalpy and entropy to become more positive.

β-Casein A

At 4°C, near neutrality β-casein exists as a monomer of molecular weight 25,000 but associates to high molecular weight polymers as the temperature is raised (13). At 14°C (pH 7.5, phosphate buffer), $I = 0.1$, two major peaks occur, the slower being monomer (1.5 S) and representing approximately 80% of the material present, and the faster peak being polymer (5.4 S). The sedimentation coefficient of the faster peak is strongly concentration-dependent due to nonideality of the polymer forms (13). Area analysis of our sedimentation velocity patterns revealed the presence of small amounts of intermediate sedimenting between the two major peaks; at no point did the refractive index gradient between the peaks return to zero. The three peaks were resolved graphically, and Fig. 4 shows the change in the relative areas of the peaks as the ionic strength of the buffer is increased by the addition of NaCl. Up to $I = 0.3$ there is an increase in the area of the intermediate peak with a concomitant loss in the proportion of monomer present; the proportion of polymer remains unchanged. At $I > 0.3$, the proportion of polymer increases and the proportions of both intermediate and monomer decline. Thus, the extent of association varies with ionic strength. At $I < 0.3$, the monomer associates only to the intermediate form. At $I > 0.3$, the proportion of intermediate declines and the equilibrium favors the high molecular weight forms. The presence of an intermediate in this association has not been recognized previously. Fig. 5 shows the total amount of polymer (intermediate plus higher molecular weight forms) as a function of ionic strength for several neutral salts. NaCl promotes the association, whereas NaSCN prevents it and in fact causes detectable dissociation. The results for this largely hydrophobic association agree with the finding that NaCl decreases the solubility of small nonpolar molecules in water, whereas NaSCN increases their solubility (5, 30). Fig. 5 also shows the effect of a variety of salts on the association at $I = 0.5$. There are some notable misplacements when these results are compared with the classical Hofmeister series. Perchlorate promotes the association,
whereas its salting-in effect is usually closer to that of thiocyanate. Nitrate and bromide have negligible effects on the association, whereas their action is usually closer to that of NaCl. The order of effectiveness of these ions does not parallel the Hofmeister series.

**Concanavalin A**

At pH 7, concanavalin A associates from a dimer to a tetramer. The association is favored by high ionic strength and temperature (15). Sedimentation velocity experiments were carried out at I = 0.5 (phosphate buffer, pH 7.0) to determine the effectiveness of inorganic salts in dissociating the tetramer. Thiocyanate, perchlorate, and dibromocetate caused complete dissociation of the tetramer to the dimer. The order of effectiveness in causing dissociation was as follows: SCN\(^-\), ClO\(_4\)^-, CBr\(_2\)COO\(^-\) > CCl\(_3\)COO\(^-\) > NO\(_3\)^- > Cl\(^-\) > CF\(_3\)COO\(^-\). In comparing this order with the Hofmeister series, CCl\(_3\)COO\(^-\) is a notable misplacement; its salting-in effect is usually greater than that of SCN\(^-\) or ClO\(_4\)\(^-\).

**DISCUSSION**

A complete mechanistic explanation of the Hofmeister series in terms of structural changes to both water and macromolecule has proved difficult due to the complexity of multicomponent interactions. Nevertheless, it is useful here to enumerate several of the factors involved. The elegant chromatographic experiments of von Hippel et al. (31) have shown directly that inorganic salts are capable of binding to the amide dipole with relative affinities that follow the Hofmeister series. It is reasoned that vicinal methyl groups can modify such binding by inducing partial chaotrope structure in adjacent water (32). Differences in the binding of salts increase as the number of vicinal methyl groups is increased. Other evidence (33) suggests that the relative binding of salts may be directed by the degree of solvation of individual anions, the less solvated anion being more strongly bound. Finally, there is the view expressed earlier that the Hofmeister series is primarily a reflection of the degree to which individual ions disrupt the hydrogen-bonded structure of water (5, 30). It is likely that all of these factors act in concert to affect the Hofmeister series, and attempts to understand the process have not always been satisfactory (see Ref. 34). A detailed discussion is available elsewhere (30).

Certainly, in the case of \(\beta\)-lactoglobulin A, the dissociating actions of NaCl and NaSCN can be accounted for on the basis of their preferential binding to the dimer species. Preferential hydration is unlikely to affect the equilibrium constant significantly, but it would appear that no more than 0.5 molecule of NaCl and 2 molecules of NaSCN are preferentially bound to the dimer. The nonlinearity in Fig. 2 at low concentrations of NaSCN (<0.15 M) suggests a cooperative interaction involving exposure of additional binding sites as binding proceeds. For example, such cooperativity occurs during the denaturation transition of lysozyme induced by guanidine hydrochloride (35). However, no evidence of conformational changes in \(\beta\)-lactoglobulin A at low concentrations of NaSCN was obtained from optical rotatory dispersion analysis. Indeed, changes in optical rotation were observed only at NaSCN concentrations greater than 3 M. Similarly, the dissociation of concanavalin A occurred at NaSCN concentrations less than those required to perturb the secondary and tertiary structures to any great degree.

The figures stated above for preferential binding to the \(\beta\)-lactoglobulin dimer should be treated as maximum limits since the available theory (25, 27) attributes the total change in association constant to the preferential binding of solvent components. However, the direct effect of chaotropic salts on water structure may also contribute to observed changes in the association constant. The proposition that chaotropic salts increase the solubility of nonpolar molecules in water by destroying or minimizing the hydrogen-bonded structure of water (5, 30) has important implications in associating protein systems. If such association occurs through hydrogen bonds either directly or via water bridging (30) we might expect that the effectiveness of neutral salts in preventing the association would parallel their effectiveness in increasing the solubility of nonpolar molecules in water, as expressed by the Hofmeister series. This parallel is realized in the case of the dimer-octamer association of \(\beta\)-lactoglobulin A, an association in which hydrogen bonds predominate in the interaction mechanism (11, 12, 20). Certainly, the positive shift in enthalpy is consistent with the failure of intermolecular hydrogen bonds to form in this solvent. This behavior is in contrast to the effect of deuterium oxide, which promotes the association to octamer and causes a slight negative shift in enthalpy (20).

Hemoglobin offers another example of a hydrogen-bonded association. The increased dissociation of genetic variants of hemoglobin which have amino acid substitutions at intersubunit sites (Hb Philly, Hb Kansas) stresses the importance of hydrogen bondings involved in the dissociation of proteins by chaotropic salts. The results suggest that in the case of this conclusion.

In contrast to these results, notable exceptions to the Hofmeister series exist in those associations which are favored by increasing temperature. Such associations are largely entropically driven, suggesting that hydrophobic interactions are the main contributor to the interaction energy. Mismatches in the Hofmeister series include perchlorate, nitrate, and bromide in the case of \(\beta\)-casein A, and trichloroacetate in the case of concanavalin A. Another example comes from the work of Aune et al. (26), who carried out an extensive study of the dimerization of chymotrypsin. They concluded that the observed enhancement of dimerization on increasing the salt concentration could be attributed to the removal of structured water from the contact region during association. However, they found that perchlorate and sulfate had an effect on the association opposite to that predicted on the basis of their salting-in and salting-out properties.

Aune et al. (26) noted that a Hofmeister series established by solubility measurements was a consequence of the interaction of the solvent with the entire protein surface and that the intercesion site between subunits need not be representative of the protein surface in general. Thus, the salting-in and salting-out effects of neutral salts on proteins need not parallel their effects on subunit interactions. The results suggest that in the case of hydrophobically associating proteins some anions interact very specifically with the subunit contact areas. Further associations will have to be examined to determine the generality of this conclusion.

The foregoing discussion suggests that several factors are involved in the dissociation of proteins by chaotropic salts. First, preferential binding of salt to the lower molecular weight species may be sufficient to induce dissociation. The disso-
associated species may possess extra binding sites for salt which are inaccessible in the polymer unit. An increase in the strength of binding to the dissociated species is another, but less attractive, possibility. Second, chaotropic salts may disrupt the hydrogen-bonded structure of water; and so increase the "solubility" of nonpolar areas of the protein surface; this effect is of particular importance in hydrophobically associating proteins. Finally, chaotropic salts may act directly by disrupting intersubunit hydrogen bonds. We may expect all of these mechanisms to operate in proteins to varying degrees, depending on the nature and balance of intersubunit forces.

We turn now to a more practical matter. The dissociation induced by chaotropic salts need not be complete. For example, dissociation of the octamer of $\beta$-lactoglobulin A and the tetramer of hemoglobin and concanavalin A proceeds to the dimer but not to the monomer. Although 1 M thiocyanate is sufficient to cause complete dissociation of $\beta$-lactoglobulin A and concanavalin A to dimer, individual proteins are likely to vary in their susceptibility to dissociation by chaotropic salts. Temperature, pH, and protein concentration are additional factors which will determine the concentration of salt required for a specific degree of dissociation. Thus, it is unlikely that chaotropic salts will be used as general reagents for dissociating proteins in the same manner as is guanidine hydrochloride (39). However, chaotropic salts have the advantage that dissociation may be induced without causing major changes in the secondary and tertiary structures of the protein. Moreover, this dissociation may occur at relatively low salt concentrations, so minimizing nonideal effects which arise in three-component systems (40).

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