Protein Stabilization and Destabilization by Guanidinium Salts†

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ABSTRACT: Preferential interactions of bovine serum albumin were measured with guanidine sulfate, guanidine acetate, and guanidine hydrochloride. The results showed an increasing preferential hydration with increasing salt concentration for the sulfate, positive preferential salt binding for the hydrochloride, and an intermediate situation for the acetate. These results correlate well with the known effects of the three salts on protein stability, namely, the stabilizing effect of guanidine sulfate and the denaturing effect of guanidine hydrochloride. Comparison of guanidinium and magnesium salts indicated that the substitution of guanidinium ion for Mg^{2+} decreases the preferential hydration and increases the preferential salt binding, suggesting that the perturbation by guanidinium ion binding of the surface free energy is greater than that by Mg^{2+} ion. It was concluded that guanidine salts are not a special class, but their activity toward proteins is modulated by the same fine balance between hydration and salt binding to protein as in the case of other salts, with the second factor being stronger in guanidine salts.

Guanidinium salts are among the most interesting protein perturbants because of the strong denaturing activity usually associated with the guanidinium ion. As is well-known, the effectiveness of salts on the solubility and stability of proteins follows generally the Hofmeister series of anion (Hofmeister, 1888), in particular when the cations are monovalent (von Hippel & Schleich, 1969). In a study of the effect of guanidinium salts (Gdn salts)[1] on the thermal denaturation of ribonuclease A, von Hippel & Wong (1965) have shown that these salts also obey the Hofmeister series; i.e., GdnSCN was most effective in decreasing the transition temperature just as NaSCN among the Na salts, whereas $(Gdn)_2SO_4$ was not effective at all but rather increased it like Na_2SO_4. Gdn·HCl and GdnOAc were close to GdnSCN and $(Gdn)_2SO_4$, respectively, in a manner parallel to NaCl and $NaCH_3COO$. A striking feature is that the nature of the cationic species can make a major difference in effectiveness: thus, Gdn·HCl is a strong denaturant, while NaCl is rather a stabilizer. These characteristics of the Gdn salts are similar to those of the divalent cation salts; for example, $MgCl_2$ is a protein destabilizer, while $MgSO_4$ has no such effect (von Hippel & Schleich, 1969). What are the factors which cause the difference between the various Gdn salts and between the Na and Gdn salts of the same anionic species?

In previous papers (Arakawa & Timasheff, 1982a, 1984), describing the preferential interactions of monovalent and divalent cation salts with proteins, the established correlation between the nature of these properties and protein stability and solubility was shown to hold for salts, as well. Measurements of the interactions of proteins with Gdn·HCl and $(Gdn)_2SO_4$ (Gordon, 1972; Bull & Breese, 1970, 1976) have resulted in observations of essentially large total binding of additive for the Gdn·HCl systems and strong hydration for the $(Gdn)_2SO_4$ systems. Following Tanford's (1968, 1970) proposal [see also Pace & Vanderburg (1979)], Gordon (1972) pointed out the importance of the direct binding of Gdn·HCl to proteins to the mechanism of their denaturation. No explanation was offered, however, for the mechanism of stabilization by $(Gdn)_2SO_4$. In order to elucidate further the mechanisms by which the guanidine salts act on proteins, we have undertaken a densimetric study of the preferential interaction of $(Gdn)_2SO_4$, GdnOAc, and Gdn·HCl with bovine serum albumin (BSA), interpreting the results in terms of rigorous thermodynamic expressions for multicomponent systems and the Wyman (1964) linkage theory.

In the case of monovalent cation salts (Arakawa & Timasheff, 1982a), the preferential hydration of proteins is related primarily to the effect of these salts on the surface free energy of proteins in aqueous solution, as is also the case with such solvent systems as sugars (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982b) and amino acids (Arakawa & Timasheff, 1983). While no such correlation was found for divalent cation salts (Arakawa & Timasheff, 1982a, 1984; T. Arakawa and S. N. Timasheff, unpublished results), their preferential interactions with proteins could be explained in terms of two opposed factors, namely, the unfavorable interaction due to the increased surface free energy by the salts and the favorable interaction due to the binding of their ionic species to peptide bonds and to some side chains. It is the purpose of this paper to demonstrate that the mechanism of the Gdn salt interactions with BSA is the same as that of the divalent cation salts.

Materials and Methods

Materials. Bovine serum albumin (BSA) was purchased from Sigma (lot 89C-9300). Ultrapure Gdn·HCl was obtained from Heico, and GdnOAc and $(Gdn)_2SO_4$ were from Eastman. Gdn·HCl and GdnOAc solutions were filtered through a sintered glass filter. The $(Gdn)_2SO_4$ solution was passed through an activated charcoal column prior to filtering. Gdn·HCl and $(Gdn)_2SO_4$ solutions were made in 0.02 M acetate buffer, pH 4.5, and the GdnOAc solution was adjusted to pH 5.6 with acetic acid. The protein was dissolved in distilled, deionized water, dialyzed thoroughly against it, passed through a sintered glass filter, and lyophilized.

Preferential Interaction Measurements. Preferential interactions were determined by densimetry using an Anton Paar DMA-02 precision densimeter at 20 °C according to the method described previously (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981; Arakawa & Timasheff, 1982b; Arakawa & Timasheff, 1984).

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[1] Abbreviations: Gdn salt, guanidinium salt; Gdn·HCl, guanidine hydrochloride; GdnOAc, guanidine acetate; $(Gdn)_2SO_4$, guanidine sulfate; GdnSCN, guanidine thiocyanate; Gdm^+, guanidinium ion; BSA, bovine serum albumin; ATGEE, acetyltetraglycine ethyl ester.

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The preferential interaction parameter was calculated from (Casassa & Eisenberg, 1961)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = \rho_0(\phi_2^0 - \phi_2'^0)/(1 - \rho_0\bar{v}_3) \quad (1)$$

where g_i is the concentration of component i in grams per gram of water, μ_i is its chemical potential, ρ_0 is the density of the solvent, and ϕ_2^0 and $\phi_2'^0$ are the partial specific volumes of protein obtained at conditions at which the molalities of the solvent components and their chemical potentials were, in turn, kept identical in the reference solvent and in the protein solution. In these studies, components are designated according to the conventional notation; i.e., component 1 is water, component 2 is protein, and component 3 is additive (Scatchard, 1946; Stockmayer, 1950). The preferential hydration parameter is related to preferential interaction with component 3 by

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -(1/g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (2)$$

Preferential interaction can be expressed in molal unit, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, by multiplying by a molecular weight factor, where m_i is the molality of component i. In thermodynamic terms, preferential interaction is given by

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = (\partial\mu_3/\partial m_2)_{T,P,m_3} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,P,m_2} \quad (3)$$

where $\mu_i = \mu_i^0 + RT \ln a_i$, μ_i^0 and a_i are the standard chemical potential and activity of component i, respectively, and R is the universal gas constant. The self-interaction term, $(\partial\mu_3/\partial m_3)_{T,P,m_2}$, was calculated from the mean ionic activity coefficient, $\gamma_\pm$, of the salts (Schrier & Schrier, 1977; Barone et al., 1976) by

$$(\partial\mu_3/\partial m_3)_{T,P,m_2} = nRT/m_3 + nRT(\partial \ln \gamma_\pm/\partial m_3)_{T,P,m_2} \quad (4)$$

where $n = 2$ for Gdn·HCl and GdnOAc and $n = 3$ for $(Gdn)_2SO_4$. For GdnOAc, the second term was neglected since no activity data are available. Total bindings of salt and water are related to the preferential interaction parameter by (Inoue & Timasheff, 1972)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - g_3A_1 \quad (5)$$

where A_1 and A_3 are the amounts of water and salt in g_i per gram of protein in the protein domain.

Protein Concentration Measurements. Protein concentrations were measured spectrophotometrically, except where indicated otherwise, by using an absorbance value of 6.58 dL/(g cm) at 278 nm (Noelken & Timasheff, 1967). Possible variation in absorptivity in concentrated salt solutions or in the denatured state should not affect the preferential interaction results, since they are obtained from the difference between ϕ_2^0 and $\phi_2'^0$, both being influenced to the same extent by the variation of the absorptivity.

The partial specific volumes of Gdn·HCl, GdnOAc, and $(Gdn)_2SO_4$ were determined by densimetry. The Gdn salts were dried in a vacuum oven at room temperature over P_2O_5 for more than 8 h. The dried salt and the solvent were weighed into small test tubes in various ratios, and the mixtures were kept at 20 °C. After dissolution, the density measurements were carried out at the same temperature. From the densities of the mixture and the solvent the apparent partial specific volume, ϕ_{app}, was calculated, and $\bar{v}_3$ was obtained by extrapolating ϕ_{app}, determined at salt concentrations between 20 and 100 mg of salt/mL of solvent, to zero concentration of the added salt.

Results

The measured partial specific volumes are summarized in Table I. The value of ϕ_2^0 ranged between 0.732 and 0.739 mL/g except in 3 M Gdn·HCl. The scattering is due probably to the variation in the absorptivity of BSA, as well as to a possible change in the partial specific volume itself in the concentrated salts. The low value in 3 M Gdn·HCl reflects protein denaturation (Lee & Timasheff, 1974). The preferential interaction parameter was calculated from the listed values of ϕ_2^0, $\phi_2'^0$, and $\bar{v}_3$. For Gdn·HCl, it had been reported to be 0.763 mL/g at 6 M (Lee & Timasheff, 1974), which is consistent with the value expected by extrapolation to 6 M of the data at 1 and 3 M. The preferential interaction parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, displays a dramatic variation for the three Gdn salts. There is a large preferential binding of Gdn·HCl to BSA, i.e., an excess of Gdn·HCl molecules in the protein domain over its concentration in the bulk solvent, while the value for the $(Gdn)_2SO_4$ solutions indicates that, except in 0.5 M salt, the salt is preferentially excluded from the protein domain; i.e., BSA is preferentially hydrated. For GdnOAc the value of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was intermediate between those of the other two salts and not much different from zero within experimental error. The resultant preferential hydration parameter $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_2}$ is given in the seventh column of Table I. For $(Gdn)_2SO_4$, it increased gradually from a value not significantly different from zero at 0.5 M to a large positive value at 2 M. Since this is preferential and not total interaction, this result does not necessarily mean an actual increase in protein hydration, as will be shown later. Setting the salt binding, A_3, equal to zero in eq 6 results in a total hydration, A_1, of 0.316 g/g in 2 M $(Gdn)_2SO_4$, which is close to the usually observed hydration of proteins, 0.2–0.4 g/g (Kuntz, 1971; Kuntz & Kauzmann, 1974; Bull & Breese, 1968). This suggests that $(Gdn)_2SO_4$ is indeed largely excluded from the domain of the protein. The preferential interaction parameter in molal units, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, given in the eighth column of Table I, brings out vividly the difference between the preferential interactions of Gdn·HCl and $(Gdn)_2SO_4$. This difference is further demonstrated in Figure 1, which shows a sharp decrease with increasing salt concentration above 0.5 M for $(Gdn)_2SO_4$, while Gdn·HCl displays a monotone increase in preferential binding.

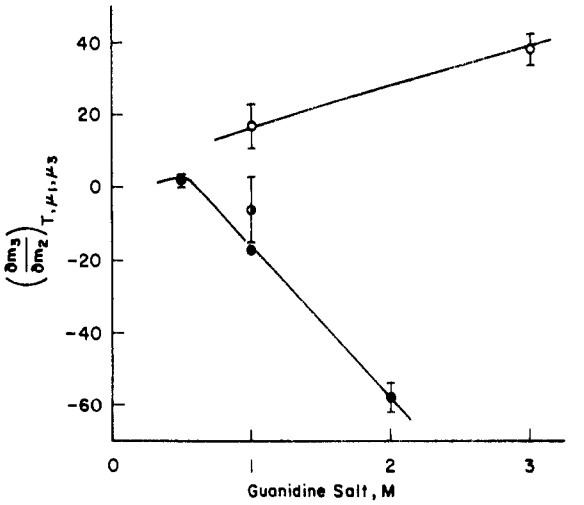

FIGURE 1: Preferential interactions of guanidine salts with BSA in aqueous solution: Gdn·HCl (○), GdnOAc (◑), and $(Gdn)_2SO_4$ (●).

The change in the chemical potential of the protein induced by addition of the salt, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, was calculated by using the values of $(\partial \ln \gamma_\pm/\partial m_3)_{T,P,m_2}$ listed in column 11 of Table I, and the results are given in column 9 of the table. For Gdn·HCl, addition of the salt decreases the activity of BSA and hence increases its solubility, whereas in the case of $(Gdn)_2SO_4$ at 1 and 2 M, the salt increases the activity of the

Table I: Interaction Parameters of BSA with Guanidine Salts

concn (M)	$\bar{v}_3$ (mL/g)	ϕ_2^0 (mL/g)	ϕ_2^{*0} (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	g_3 (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial\mu_2/\partial\mu_3)_{T,P,m_2}$ [(cal/mol of protein)/mol of salt]	$(\partial\mu_3/\partial m_3)_{T,P,m_2}$ [(cal/mol salt)/mol of salt]	$nRT(\partial \ln \gamma_\pm/\partial m_3)^a$ [(cal/mol of salt)/mol of salt]	A_3^b (g/g)	ν_3^b (mol/mol)
						Gdn·HCl						
1.0	0.732	0.735 ± 0.001	0.729 ± 0.001	0.0246 ± 0.0082	0.103	−0.239 ± 0.080	17.5 ± 5.8	−13800 ± 4600	790	−290	0.056	39.5
3.0[c]	0.747	0.728	0.718 ± 0.001	0.0536 ± 0.0054	0.365	−0.147 ± 0.015	38.2 ± 3.8	−7300 ± 9100	190	−110	0.163	116
						GdnOAc						
1.0	0.778	0.739 ± 0.001	0.741 ± 0.002	−0.0102 ± 0.0153	0.133	0.077 ± 0.115	−5.84 ± 8.73	6100 ± 9100	1043		0.030	17.0
						$(Gdn)_2SO_4$						
0.5	0.588	0.732 ± 0.001	0.729 ± 0.001	0.0081 ± 0.0054	0.115	−0.071 ± 0.047	2.55 ± 1.70	−4600 ± 3000	1780	−1500	0.043	13.4
1.0	0.609	0.734 ± 0.001	0.750 ± 0.002	−0.0521 ± 0.0098	0.247	0.211 ± 0.040	−16.4 ± 3.1	8700 ± 1600	530	−1000	0.022	6.9
2.0	0.631	0.734 ± 0.002	0.775 ± 0.001	−0.184 ± 0.013	0.585	0.316 ± 0.022	−58.0 ± 4.1	15700 ± 1100	270	−380		

[a] n = 2 for Gdn·HCl and GdnOAc; n = 3 for $(Gdn)_2SO_4$. Data taken from Schrier & Schrier (1977) for Gdn·HCl and from Barone et al. (1976) for $(Gdn)_2SO_4$. [b] For calculation, A_1 = 0.3 g/g was assumed. [c] Calculated with the absorbance value of 6.25 dL/(g cm) at 278 nm.

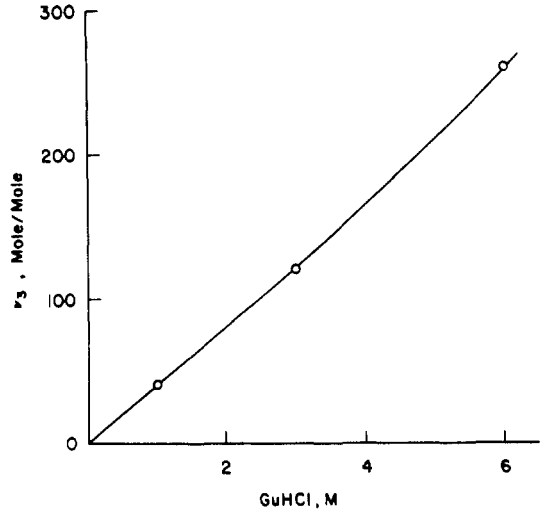

FIGURE 2: Total Gdn·HCl binding to BSA. The hydration value, A_1, was assumed to be 0.3 g/g. The point at 6 M is taken from the data of Lee & Timasheff (1974) recalculated with A_1 = 0.3 g/g.

protein; i.e., the system is thermodynamically destabilized. It should be noted that for Gdn·HCl and $(Gdn)_2SO_4$, the term, $nRT(\partial \ln \gamma_\pm/\partial m_3)_{T,P,m_2}$ (n = 2 and 3, respectively) is very large relative to nRT/m_3, indicating that neglect of the activity coefficient term can lead to totally incorrect conclusions. Since for GdnOAc $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ was close to zero, the value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is also not different from zero regardless of the value of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ used; i.e., correction with $(\partial \ln \gamma_\pm/\partial m_3)_{T,P,m_2}$ is not important in this case. The total bindings of the salts, calculated by assuming a hydration value of BSA, A_1, equal to 0.3 g/g, are given in the last two columns of Table I. Figure 2 shows that the total binding of Gdn·HCl increases almost linearly with salt concentration, just as is true of lysozyme (Lee & Timasheff, 1974), indicating that the binding is not saturating. The binding is smaller for GdnOAc and very small for $(Gdn)_2SO_4$.

Our results were compared with those of Gordon (1972) and Bull & Breese (1976) for $(Gdn)_2SO_4$, although their values were obtained at relatively high protein concentrations without extrapolation to infinite dilution; i.e., their values might be influenced by the protein concentration dependence of the interaction. Gordon obtained a value of A_1 = 0.28 g/g at 2 M salt with the assumption that no salt binds to the protein, which is comparable to our results (0.32 g/g). Bull and Breese analyzed their experimental results with the assumption that the bindings of water and salt at different salt concentration are constant and obtained A_1 = 0.45 g/g and ν_3 (salt binding in moles per mole) = 20. Our results were recalculated for the $(Gdn)_2SO_4$ system by using their hydration value (A_1 = 0.45 g/g). This gave ν_3 = 19, 19, and 25 mol of $(Gdn)_2SO_4$/mol of BSA at 0.5, 1.0, and 2.0 M salt, respectively, i.e., values similar to that of Bull & Breese (1976). These consistent results establish clearly that BSA is preferentially hydrated in $(Gdn)_2SO_4$ solutions at salt concentrations above 1 M.

Discussion

The present results support again the general observation that, in three-component systems, the preferential interactions of proteins with solvent components are directly related to the effects of additives on protein stability and solubility, whatever the mechanism of the interactions (Timasheff et al., 1976; Pittz & Timasheff, 1978; Lee & Lee, 1979; Arakawa & Timasheff, 1982a). The large positive value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ found for $(Gdn)_2SO_4$ indicates that this salt should decrease the solubility

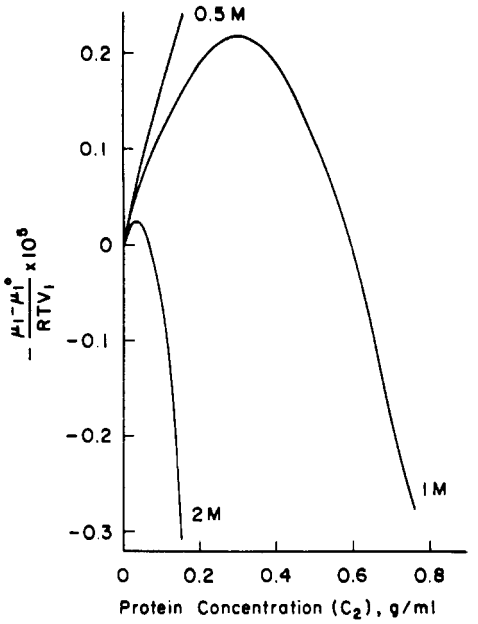

FIGURE 3: Phase isotherm for BSA in the aqueous $(Gdn)_2SO_4$ system.

of proteins, while the opposite should be true of Gdn·HCl. The value close to zero for GdnOAc indicates that this salt should have no effect on protein solubility. Thus, the effectiveness of the Gdn salts as protein solubilizers should increase in the order of $Cl^- > OAc^- > SO_2^{2-}$, just as in the case of Na and K salts, i.e., in a manner fully consistent with the Hofmeister series of anions. In other words, in this respect, the Gdn salts behave in a normal manner.

The effect of preferential interactions on protein solubility may be described more quantitatively in terms of the phase isotherm (Flory, 1953; Nord et al., 1951; Pittz & Timasheff, 1978)

$$(\mu_1 - \mu_1^0)/(RTV_1) = -(C_2/M_2)[1 + [V_m/(2RTM_2)]C_2[(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3} + (\partial\mu_2/\partial m_3)_{T,P,m_2}(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}] + O(C_2^2)] \quad (6)$$

where V_1 is the molar volume of the water, V_m is the volume of solution containing 1 kg of water, and $(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3}$ is the excess chemical potential of the protein. This was applied to $(Gdn)_2SO_4$, and the results are given in Figure 3. In the calculation, the value of $(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3}$ was assumed to be due solely to the excluded volume of the protein. As seen, at 1 and 2 M $(Gdn)_2SO_4$, $\mu_1 - \mu_1^0$ is first negative, and then after passing a minimum, it starts increasing and becomes positive at protein concentrations of 600 and 70 mg/mL, respectively. Negative values indicate that the solution is thermodynamically favorable, while positive values correspond to unstable solutions and phase separation. Thus, $(Gdn)_2SO_4$ can be classified as a salting-out salt, although a weak one, much weaker than such salts as Na_2SO_4, since it is effective significantly only at the 2 M level. No protein solubility data being available for the Gdn salts, this conclusion cannot be compared with actual data.

The effects of the various Gdn salts on protein stability deduced from the preferential interactions can be compared best in terms of the Wyman linkage relations (1964) for the denaturation reaction

$$N \rightleftharpoons D$$

$$(\partial \ln K/\partial \ln a_3)_{T,P,m_2} = (\partial m_3/\partial m_2)^D_{T,\mu_1,\mu_3} - (\partial m_3/\partial m_2)^N_{T,\mu_1,\mu_3} = \Delta\nu_3 - (m_3/55.5)\Delta\nu_1 = \Delta\nu \quad (7)$$

where K is the equilibrium constant of the denaturation reaction, D and N refer to the denatured and native proteins, respectively, $(\partial m_3/\partial m_2)^D_{T,\mu_1,\mu_3}$ and $(\partial m_3/\partial m_2)^N_{T,\mu_1,\mu_3}$ are the corresponding preferential interaction parameters, and $\Delta\nu_3$ and $\Delta\nu_1$ are the differences in the total binding of salt and water expressed in moles of component i per mole of protein between the denatured and native forms of the protein, respectively. Taking first the Gdn·HCl system, Kuntz (1971) has shown that the total binding of water, i.e., hydration, does not change significantly during urea or Gdn·HCl denaturation, i.e., $\Delta\nu_1 = 0$. On the other hand, the binding of Gdn·HCl increases with increasing salt concentration without saturation, meaning that additional binding sites become available at higher Gdn·HCl concentrations due to protein unfolding. With identical affinity of Gdn·HCl to sites on the native and denatured proteins, more salt should be bound to the latter than to the former, i.e., $\Delta\nu_3 > 0$. As a consequence, $\Delta\nu$ is positive, addition of GuHCl should increase the equilibrium constant K, and protein denaturation should be favored.

For the $(Gdn)_2SO_4$ system, it is reasonable to assume that the protein was in the native form at the salt concentrations used in this study. Then

$$(\partial m_3/\partial m_2)^{obsd}_{T,\mu_1,\mu_3} = (\partial m_3/\partial m_2)^N_{T,\mu_1,\mu_3} = \nu_3^N - (m_3/55.5)\nu_1^N \quad (8)$$

When ν_3 is set equal to 0, the hydration is 0.316 g/g at 2 M salt. Positive salt binding, as proposed by Bull & Breese (1976), would render hydration even higher (0.45 g/g). Thus, the assumption that the hydration is higher than that used with Gdn·HCl (0.3 g/g) still gives a lower total binding of $(Gdn)_2SO_4$ than of Gdn·HCl; i.e., the binding affinity of $(Gdn)_2SO_4$ is lower than that of Gdn·HCl. Therefore, the value of $\Delta\nu_3$ for $(Gdn)_2SO_4$ should be less positive. The assumption that $\Delta\nu_1 = 0$ for $(Gdn)_2SO_4$ just as for Gdn·HCl leads to a positive value of $\Delta\nu$ but smaller than that for Gdn·HCl, indicating that $(Gdn)_2SO_4$ is at least less effective in inducing denaturation. The assumption that ν_1 does not change during denaturation in the presence of $(Gdn)_2SO_4$ is unrealistic. It is known that, for systems in which there is little binding of the additive, protein hydration increases with the surface area of the protein (Arakawa & Timasheff, 1982b). The surface tension increment clearly predicts this.[2] If this is true also for the $(Gdn)_2SO_4$ system, $\Delta\nu_1$ should be positive, leading to a compensation of the positive value of $\Delta\nu_3$ and contributing to an overall decrease of $\Delta\nu$.

Examination of eq 7 reveals not only that the value and sign of $\Delta\nu$ depend on the changes in hydration ($\Delta\nu_1$) and interaction with the additive ($\Delta\nu_3$) but that they are also functions of the total concentration of the additive, m_3, in the system; i.e., a change in hydration at a high value of m_3 may overcome a positive $\Delta\nu_3$, which would be dominant at lower m_3. When the native protein is used, the assumption of $A_1 = 0.45$ g/g results in a large total binding already at 0.5 M salt with little increase at higher salt concentrations. Thus, the observed positive (or near zero) value of $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ at 0.5 M and negative values at higher salt concentrations can be just a reflection of the variation of m_3 (see eq 8). The same may be true for the denatured form as well; i.e., ν_3^D may be nearly independent of salt concentration. If both ν_3^N and ν_3^D are independent of salt concentration, $\Delta\nu_3$ should be constant. Now, if the protein hydration depends only on the surface area of the protein and both ν_1^N and ν_1^D are independent of m_3, $\Delta\nu_1$ will also be constant. Constant values of $\Delta\nu_1$ and $\Delta\nu_3$ mean that variations in $\Delta\nu$ depend only on m_3; i.e., the first term

[2] Recently, it has been observed that $(Gdn)_2SO_4$ increases the surface tension of water as does Na_2SO_4 (Y. Kita, T. Arakawa, and S. N. Timasheff, unpublished results).

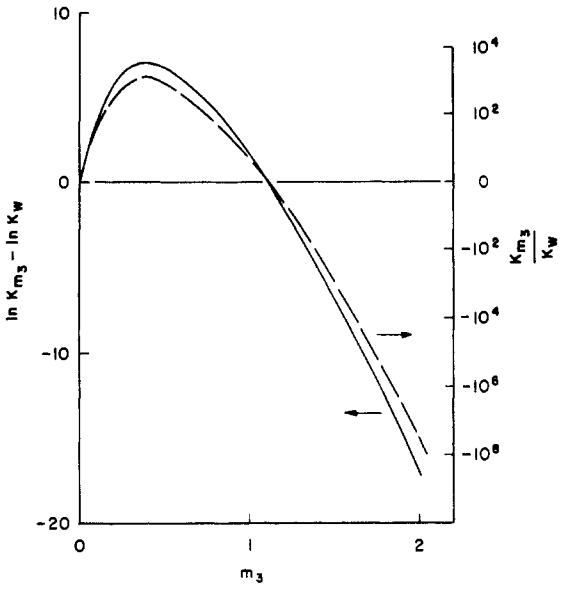

FIGURE 4: Equilibrium constant for BSA denaturation as a function of $(Gdn)_2SO_4$ concentration: solid line, $\ln K_{m_3} - \ln K_w$ (left ordinate); broken line, K_{m_3}/K_w (right ordinate). The curve was calculated as described in the text.

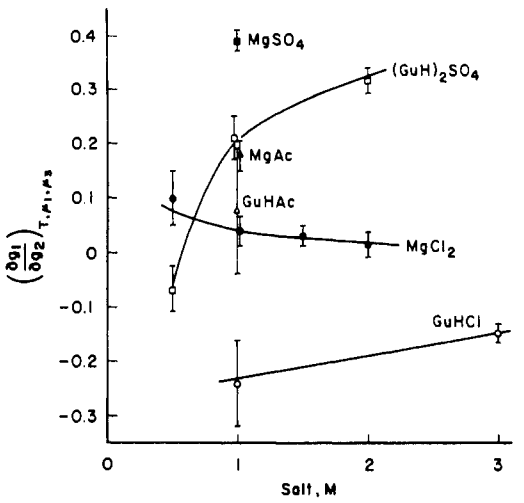

FIGURE 5: Comparison of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ between Gdn and Mg^{2+} salts. Open symbols, Gdn salts; closed symbols, Mg salts. Chloride salts (circles); acetate salts (triangles); sulfate salts (squares).

of eq 7 is more significant at low values of m_3, and the second term becomes progressively predominant with increasing m_3. This progression is shown vividly by the calculation depicted in Figure 4, in which the equilibrium constant of denaturation in the presence of $(Gdn)_2SO_4$, K_{m_3}, is calculated as a function of m_3 at constant $\Delta\nu_1$ and $\Delta\nu_3$ and expressed relative to that in water, K_w. In this calculation, $\Delta\nu_1$ was set equal to 0.45 g/g at all salt concentrations, and $\Delta\nu_3$ was calculated from a simple binding equation, $\Delta\nu_3 = \Delta n k m_3/(1 + k m_3)$, where k and Δn are the salt binding constant and the difference in the number of available binding sites between the denatured and native proteins. These parameters were set equal to 4 and 20, respectively, resulting in a nearly constant value of $\Delta\nu_3$ at $m_3 \geq 0.5$. As can be seen, addition of the salt first increases the denaturation equilibrium constant, K_{m_3}. Then, after passing through a maximum, K_{m_3} decreases, and finally, above 1 M salt, it assumes values lower than K_w; i.e., the salt now acts as a protein stabilizer rather than a denaturant, even though binding has not varied. It should be pointed out that, for this equation to be rigorously correct, the activity of salt should be used instead of m_3. This, however, is not important for the present calculation which was strictly a demonstration of how preferential interactions can affect the equilibrium constant of protein denaturation, even when binding does not change. This analysis in terms of the balance between hydration and salt binding explains the difference between Gdn·HCl and $(Gdn)_2SO_4$ in their effectiveness as denaturants. The same arguments lead to the conclusion that the effectiveness of GdnOAc as a protein destabilizer is intermediate between the other two salts, resulting in the same order of their effectiveness as on the thermal stability of RNase A (von Hippel & Wong, 1965).

At this point, it would seem of interest to compare the Gdn salts to the Mg^{2+} family. This is done in Figure 5 in which the preferential hydration parameter is used as an indicator of whether an additive is a protein denaturant or stabilizer. As seen, guanidinium showed generally lower values of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ than Mg^{2+} when forming a salt with the same anion, indicating a lower effectiveness of Gdn salts as stabilizers or a higher effectiveness as denaturants. When we look more closely at $(Gdn)_2SO_4$, its value of the preferential hydration is lower than that of $MgCl_2$ at 0.5 M salt, close to that of $Mg(OAc)_2$ at 1.0 M and approaches to the level of $MgSO_4$ at 2 M, suggesting that $(Gdn)_2SO_4$ is more effective in protein stabilization at its higher concentrations. The effectiveness of the salts may be compared also by the parameter $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$. Such a comparison is made in Table II for the Na^+, divalent cation and Gdn salts at 1 M with BSA as the protein. It is seen that for all the anionic species, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ increases in the order of Na^+ < divalent cation < Gdm^+, meaning that ability to denature proteins increases and the stabilizing action decreases in the above order. By use of the lysozyme results, Gdn·HCl was found to be comparable to $MgCl_2$, suggesting that the effectiveness is somewhat dependent on the nature of the protein, but with no change in the order. For the anions, the effectiveness as stabilizers is consistently SO_4^{2-} > OAc^- > Cl^-, i.e., in the order of the Hofmeister series. In the above discussion it was assumed that the negative preferential binding of $(Gdn)_2SO_4$ to BSA is due to an exclusion of the salt, just like Na_2SO_4.

Table II: Comparison between $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ of 1 M Na, Divalent Cation, and Gdn Salts

cation	anion (mol/mol)		
	Cl^-	OAc^-	SO_4^{2-}
Na^+	−17	−22	−35
Mg^{2+}, Ca^{2+}, Ba^{2+}	−3∼3	−8 ∼ −13	−27[a]
Gdn^+	18	−6	−16

[a] The value is for $MgSO_4$.

Solubility measurements of nonpolar amino acids in Gdn·HCl (Nozaki & Tanford, 1963, 1970) have shown that Gdn·HCl interacts favorably not only with the peptide units but also with the side chains both nonpolar and polar and, in particular, with that of tryptophan. On the other hand, $MgCl_2$, which has been proposed to bind to the peptide groups by the same mechanism as Gdn·HCl (Robinson & Jencks, 1965a,b), showed a significant unfavorable free energy change in its interaction with the nonpolar side chains (Arakawa & Timasheff, 1984). Since both Gdn·HCl and $MgCl_2$ contain Cl^- as the anion, this difference in their interactions with nonpolar side chains must be ascribed to their cationic species. Gordon (1972) has observed an increase in the solubility of the model peptide, ATGEE, in Gdn·HCl solutions and a decrease in $(Gdn)_2SO_4$ solutions. Since the solubility of this model peptide is generally decreased in salting-out salts and increased in salting-in salts (Robinson & Jencks, 1965), these observations

support our assignment of $(Gdn)_2SO_4$ to the class of salting-out salts, while Gdn·HCl should be classified as a salting-in salt. It may be concluded, therefore, that the guanidinium salts are not special in their interactions with proteins and that their observed preferential interactions should be regarded in the same light as those of other salts. It must be emphasized, however, that a significant difference does exist between Na^+, divalent cation, and Gdn salts in the values of the interaction parameters, i.e., the last salts bind more strongly. This arises obviously from the higher affinity of Gdm^+ for the peptide bonds (Robinson & Jencks, 1965), as well as for side chains, such as that of tryptophan (Nozaki & Tanford, 1970; Lee & Timasheff, 1974).

Registry No. Gdn·HCl, 50-01-1; GdnOAc, 34771-62-5; $(Gdn)_2SO_4$, 594-14-9; $MgCl_2$, 7786-30-3; $Mg(OAc)_2$, 142-72-3; $MgSO_4$, 7487-88-9; NaCl, 7647-14-5; NaOAc, 127-09-3; Na_2SO_4, 7757-82-6.

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