pH Corrections and Protein Ionization in Water/Guanidinium Chloride

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ABSTRACT More than 30 years ago, Nozaki and Tanford reported that the pK values for several amino acids and simple substances in 6 M guanidinium chloride differed little from the corresponding values in low salt (Nozaki, Y., and C. Tanford. 1967. *J. Am. Chem. Soc.* 89:736–742). This puzzling and counter-intuitive result hinders attempts to understand and predict the proton uptake/release behavior of proteins in guanidinium chloride solutions, behavior which may determine whether the $\Delta G^{\circ}_{N\text{-}D}$ values obtained from guanidinium chloride-induced denaturation data can actually be interpreted as the Gibbs energy difference between the native and denatured states (Bolen, D. W., and M. Yang. 2000. *Biochemistry.* 39:15208–15216). We show in this work that the Nozaki-Tanford result can be traced back to the fact that glass-electrode pH meter readings in water/guanidinium chloride do not equal true pH values. We determine the correction factors required to convert pH meter readings in water/guanidinium chloride into true pH values and show that, when these corrections are applied, the effect of guanidinium chloride on the pK values of simple substances is found to be significant and similar to that of NaCl. The results reported here allow us to propose plausible guanidinium chloride concentration dependencies for the pK values of carboxylic acids in proteins and, on their basis, to reproduce qualitatively the proton uptake/release behavior for the native and denatured states of several proteins (ribonuclease A, α-chymotrypsin, staphylococcal nuclease) in guanidinium chloride solutions. Finally, the implications of the pH correction for the experimental characterization of protein folding energetics are briefly discussed.

INTRODUCTION

The thermodynamic stability of proteins may be described by the protein stability curve: the profile of unfolding Gibbs energy versus temperature (Becktel and Schellman, 1987). Unfolding Gibbs energy values are usually determined from urea-induced or guanidinium chloride (GdnHCl)-induced denaturation profiles by assuming that the linear dependence of ΔG with denaturant concentration (C) observed within the narrow transition range can be extrapolated down to $C = 0$ (the so-called "linear extrapolation method," or LEM). Often, however, significant differences between $\Delta G^{\circ}_{N\text{-}D}$ values obtained from urea denaturation and GdnHCl denaturation (or between GdnHCl denaturation and thermal denaturation) are found. This fact, together with the obvious extrathermodynamic nature of the LEM, cast doubts about the reliability of the $\Delta G^{\circ}_{N\text{-}D}$ values determined from chemical denaturation experiments (for discussions on chemical denaturation see Bolen and Yang, 2000; Courtenay et al., 2000; Makhatadze, 1999; Ibarra-Molero and Sanchez-Ruiz, 1996). $\Delta G^{\circ}_{N\text{-}D}$ values determined from GdnHCl denaturation appear particularly suspect because this denaturant is a salt, and high salt concentrations are expected to efficiently screen the contributions from charge-charge interactions to protein stability; as a result, these contributions might not be present in the $\Delta G^{\circ}_{N\text{-}D}$ values determined from GdnHCl denaturation using the LEM (Santoro and Bolen, 1992; Monera et al., 1994; Ibarra-Molero and Sanchez-Ruiz, 1996; Makhatadze et al., 1998; Ibarra-Molero et al., 1999); furthermore, it is also possible that specific binding of guanidinium or chloride ions to the native state can have effects on stability and distort the LEM value (Greene and Pace, 1974; Santoro and Bolen, 1988; Pace et al., 1990; Hagihara et al., 1993; Mayr and Schmid, 1993; Yao and Bolen, 1995).

Recently, Bolen and Yang (2000) had the insight to use the number of protons bound to the protein as an experimental thermodynamic parameter to follow GdnHCl-induced denaturation. This parameter is particularly informative in this case because it should be highly sensitive to the effect of GdnHCl on electrostatic interactions. Bolen and Yang (2000) used it, in fact, to characterize the denaturant effect on the nature of native and denatured proteins and found that, in some cases, the number of protons bound to a given protein state does not change with denaturant concentration ("fixed thermodynamic character"), while in other cases a significant effect is found ("variable thermodynamic character"). On this basis, they could define three classes of proteins: 1) fixed thermodynamic character of native and denatured states (or ensembles); 2) variable thermodynamic character in the native state and fixed thermodynamic character in the denatured state; 3) variable thermodynamic character in both the native and denatured states. Bolen and Yang (2000) suggested that the interpretation of LEM-determined $\Delta G^{\circ}_{N\text{-}D}$ values as the Gibbs energy difference between the N and D states at the limit of zero denaturant concentration is only possible for those proteins that exhibit fixed thermodynamic character in their native and denatured states.

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In view of the above, it appears of considerable interest to be able to understand in molecular terms the origin of the proton uptake/release behavior of protein states in GdnHCl and to develop procedures to predict this behavior. However, before this goal can be addressed (what we eventually do in this work), it is essential to find an explanation to a surprising experimental result reported by Nozaki and Tanford more than 30 years ago (Nozaki and Tanford, 1967a). These authors measured the pK values for several amino acids and simple substances in 6 M GdnHCl and found values that differed little from the corresponding pK values in low-salt solutions; as a result the hydrogen titration curves for denatured lysozyme and ribonuclease A in 6 M GdnHCl have been rationalized on the basis of low-salt, model pK values (Nozaki and Tanford, 1967b; Yao and Bolen, 1995; Whitten and Garcia-Moreno, 2000). The Nozaki-Tanford result is puzzling and counter-intuitive because high salt concentrations are expected to strongly affect ionization processes; in fact, classical studies using hydrogen-electrode cells (King, 1945; Harned and Owen, 1958) showed that molar concentrations of NaCl and other salts have a significant effect on the pK values for carboxylic acids, and it is not at all clear why GdnHCl should behave in a radically different way.

We show in this work that the Nozaki-Tanford result can be traced back to the fact that glass-electrode, pH meter readings in water-cosolvent mixtures (such as water/GdnHCl) do not equal the true pH values. Furthermore, we report here the correction factors required to convert the glass-electrode, pH meter readings in water/GdnHCl to true pH values and show that, when this correction is applied, pK values for carboxylic acids in 6 M GdnHCl nicely fit the trend defined by the published sodium chloride effect on those pK values (determined using a hydrogen-electrode cell, which does not require pH correction). These results will allow us to suggest plausible GdnHCl concentration dependencies for the pK values of carboxylic acids in proteins and we will show that, on the basis of these dependencies, the effect of GdnHCl on the proton uptake/release behavior of several proteins can be qualitatively reproduced.

MATERIALS AND METHODS

pH measurements

pH measurements were performed at 25°C with a Crison (Alella, Spain) 52-09 glass electrode connected to a Crison Digit-501 pH meter that can detect 0.01 units of pH. The scale of the pH meter was adjusted with 4.00 and 7.02 aqueous standard buffers from Crison.

Materials

Hydrochloric acid and sodium acetate were analytical grade from Panreac (Barcelona, Spain) and Sigma-Aldrich (Madrid, Spain), respectively. Guanidinium chloride was ultrapure grade from Pierce. The concentration of GdnHCl in water/GdnHCl mixtures was determined from refraction index measurements (Pace et al., 1989) using an Atago R500 hand refractometer. Concentrations of sodium acetate in stock solutions were calculated from the weight used of the solid product. Concentrations of hydrochloric acid were calculated from the pH of the aqueous solutions, as is explained further below.

THEORETICAL BACKGROUND

Activity and ionization constants

The activity of a species (i) in a given state of a system is defined in terms of its chemical potential (partial Gibbs energy) in that particular state (μ_i) and in a selected standard state (μ_i°):

$$\mu_i = \mu_i^\circ + RT \ln a_i \tag{1}$$

where, by definition, activity is unity in the standard state. Activity is usually interpreted as a "corrected concentration":

$$a_i = \gamma_i \cdot [i] \tag{2}$$

where $[i]$ is the molar concentration (see Note 1 at end of text) and γ_i is the activity coefficient. However, care must be exercised in the choice of the standard state if we expect the activity to be close to the concentration in the conditions of interest. Thus, the standard state for aqueous solutions is customarily chosen in such a way that the activity approaches the concentration as the species becomes very diluted in water:

$$a_i^W = \gamma_i^W \cdot [i] \quad \text{with } \gamma_i^W \to 1 \quad \text{when } [i] \to 0 \text{ in water} \tag{3}$$

However, this choice of standard state may not be convenient when working with mixed solvents (such as water/alcohol, water/dioxane or, the case of interest here, water/GdnHCl) because the solvent effect on the activity coefficient γ_i^W would lead to activities that differ profoundly from the concentrations in the water/cosolvent mixtures (see below and chapter 8 of Bates, 1973). In fact, when working with mixed solvents, it is convenient to choose a different standard state for each mixture composition in such a way that activity is close to the concentration regardless of the mixture composition. That is, the standard state for species i in a given mixture (i.e., a given mixture composition) is chosen so that,

$$a_i^* = \gamma_i^* \cdot [i] \quad \text{with } \gamma_i^* \to 1 \quad \text{when } [i] \to 0 \text{ in the mixture under study} \tag{4}$$

From a molecular viewpoint, γ_i^* coefficients for diluted solutes in a given solvent (or solvent mixture) reflect the solute-solute interactions in that solvent; such interactions do not exist at infinite dilution and, thus, $\gamma_i^* \to 1$ when $[i] \to 0$, as shown in Eq. 4. However, γ_i^W coefficients reflect solute-solute *and* solute-solvent interactions and, consequently, they are not expected to become unity when $[i] \to 0$ in a given solvent (unless, of course, the solvent is pure water: see Eq. 3). Actually, the infinite dilution limit of a γ_i^W coefficient in a given solvent mixture is a measure of the solute-solvent interactions in that mixture as compared with the solute-solvent interactions in water. For this reason, infinite dilution γ_i^W coefficients are often referred to as "primary medium effects" or simply "medium effects," and they are obviously related to the transfer Gibbs energy of the solute from water to the solvent mixture.

The difference between the two activity scales we have defined (Eqs. 3 and 4) can be readily seen if we consider a simple ionization equilibrium:

$$AH \leftrightarrow A + H^+ \tag{5}$$

for which we can write two "thermodynamic" equilibrium constants (in terms of activities):

$$K^{W} = \frac{a_{A}^{W} \cdot a_{H}^{W}}{a_{AH}^{W}} \quad (6)$$

$$K^{*} = \frac{a_{A}^{*} \cdot a_{H}^{*}}{a_{AH}^{*}} \quad (7)$$

and a "practical" equilibrium constant in terms of concentrations:

$$K^{C} = \frac{[A] \cdot [H^{+}]}{[AH]} \quad (8)$$

It can be easily shown that K^{W} is simply the ionization equilibrium constant at infinite dilution of the acid (and any other ions present) in water; as such, its value is expected to differ significantly from that of the practical constant, K^{C}, in a water/cosolvent mixture.

However, for a solvent mixture of given composition, the value of K^{C} will approach the value of K^{*} as the mixture becomes diluted in the species AH, A, and H^{+}. In fact, K^{*} is the infinite dilution limit of K^{C} *in the mixture*. Values of K^{C} for acid ionization in water-cosolvent mixtures can be rigorously obtained using an electrochemical cell consisting of a hydrogen electrode and an AgCl:Ag electrode as reference (the Nernst equation can be posed rigorously for this cell since a single liquid phase is present and there are no liquid junctions). The values of K^{C} determined at different acid concentrations in a given mixture can then be extrapolated to infinite dilution to obtain the value of K^{*} in that mixture. pK* values thus obtained for acetic acid ionization and for the first ionization of glycine in concentrated salt solutions had already been reported in the literature ~50 years ago (King, 1945; Harned and Owen, 1958).

The definition of pH

pH is defined as the minus logarithm of hydrogen ion activity:

$$-\log_{10} a_{H} = -\log_{10}(\gamma_{H} \cdot [H^{+}]) \quad (9)$$

Three important points about this definition must be noted:

1. The activity for an individual ion cannot be measured. As a result, the currently used pH scale for aqueous solutions (the operational or conventional pH scale) relies upon the pH values assigned to certain standard buffers on the basis of a physically reasonable convention about the activity coefficient of an individual ion. For instance, a method of assigning $-\log_{10} a_{H}$ values to standard buffers (which has been used by the U.S. National Bureau of Standards: see chapter 4 in Bates, 1973) involves determining $-\log_{10}(a_{H} \cdot \gamma_{Cl})$ from the electromotive force (e.m.f.) of a hydrogen-silver chloride cell without liquid junction and using an expression derived from the Debye-Hückel law (known as the Bates-Guggenheim convention) to evaluate the activity coefficient of the chloride ion. The issue of the evaluation of the activity coefficient of an individual ion and its relation with the definition of the pH scale will be discussed in more detail in the next section ("The definition of the pH* scale in water/GdnHCl").
2. The hydrogen ion activity involved in the definition of pH is expected to be a "corrected" hydrogen ion concentration (i.e., not very different from $[H^{+}]$ under the conditions of interest). This means that the appropriate pH scale in mixed solvents is actually a pH* scale:

$$pH^{*} = -\log a_{H}^{*} = -\log_{10}(\gamma_{H}^{*} \cdot [H^{+}]) \quad (10)$$

in such a way that γ_{H}^{*} approaches unity (Eq. 4) as the solution becomes very diluted in the ionic solutes (note that, in water/GdnHCl mixtures, GdnHCl is taken to be the cosolvent and not a "solute").

3. pH* values in mixed solvents could, in principle, be determined from the e.m.f. of a hydrogen-silver chloride cell (and using some reasonable convention for the activity coefficient of an individual ion). The hydrogen electrode, however, is not practical for routine use and cells based on the glass electrode are used instead. Consider a pH meter connected to a glass-electrode cell that is calibrated using standard aqueous buffers and, subsequently, immersed in a water-cosolvent mixture: *the pH meter reading obtained (pH_r) does NOT equal the true pH of the mixture (pH*)* (see Note 2):

$$pH^{*} = pH_{r} + \delta pH^{*} \quad (11)$$

and the correction factor, δpH*, can be expressed as (see chapter 8 in Bates, 1973),

$$\delta pH^{*} = \log_{10} \gamma_{H}^{W} - E_{j} \quad (12)$$

where E_{j} is the liquid junction potential (in units of pH) and γ_{H}^{W} is the infinite-dilution activity coefficient of the proton taking the solution in water as standard state (see Eq. 3); γ_{H}^{W} is related to the Gibbs energy associated to the transfer of the proton from water to the mixture and is known as the "medium effect of the proton." In any case, the independent calculation of the two terms of Eq. 12 is difficult and requires empirical extrathermodynamic methods (see chapter 8 in Bates, 1973); therefore, only δpH* is usually evaluated from experimental data, as we explain further below.

The fact that corrections are required to convert pH meter readings in mixed solvents into true pH values is, of course, well-known and values of the correction factors (δpH*) for the more commonly used water-organic solvent mixtures (such as water/alcohol or water/dioxane) were published in the literature long ago (Gutbezahl and Grunwald, 1953; Van Uitert and Haas, 1953; Van Uitert and Frenelius, 1954; Bates, 1973; Sanchez-Ruiz et al., 1984; Cortijo et al., 1988) and found to be significant (in some cases, of the order of 1 pH unit).

The definition of the pH* scale in water/GdnHCl

A pH* scale in a given mixed solvent is operationally defined through the assignment of pH* values to certain solutions in that solvent. Once this has been achieved, these solutions can be used to calculate the pH correction factors as the difference between the pH* value and the pH meter reading obtained for the solutions using a glass-electrode–pH meter system which has been calibrated with standard aqueous buffers.

In previous work with mixed solvents, it was found convenient to use diluted solutions of strong acids (ClH, for instance) to define the pH* scale. Thus, for solutions of strong acids the hydrogen ion concentration may be easily known and the assignment of the pH* value (Eq. 10) only requires that we make a reasonable assumption about the γ_{H}^{*} coefficient of the hydrogen ion. The usual procedure is to take γ_{H}^{*} as equal to the mean ionic coefficient of a 1:1 electrolyte in a solution of the same ionic strength, an assumption which is consistent with the simple electrostatic theory of ion-ion interactions in solutions. Note that, since γ* coefficients for dilute electrolytes reflect the interactions between the solute ions and these interactions depend mainly on charge, we may expect that the assigned pH* value will not depend significantly on the 1:1 electrolyte chosen to estimate γ_{H}^{*}. Thus, for instance, at 25°C and a salt concentration of 0.01 M, the $\gamma_{\pm}^{*}$ coefficients for ClH, ClNa, and ClK are, respectively, 0.9048, 0.9032, and 0.902 (see chapter 12 in Harned and Owen, 1958). In fact, all 1:1 electrolytes show very similar values for the $\gamma_{\pm}^{*}$ coefficient in aqueous solution at ionic strengths on the order of 10^{-2} and lower, as it is clearly shown in Fig. 12-5-3 (page 513) of Harned and Owen (1958), where data for a large number of electrolytes are displayed. It is interesting that the simple Debye-Hückel limiting law gives 0.889 for the individual activity coefficient of a monovalent ion at 25°C and ionic strength 0.01 M, and that extensions of the Debye-Hückel law that include a size parameter give values that range between 0.898, for Rb^{+}, Cs^{+}, and NH_{4}^{+}, and 0.914 for

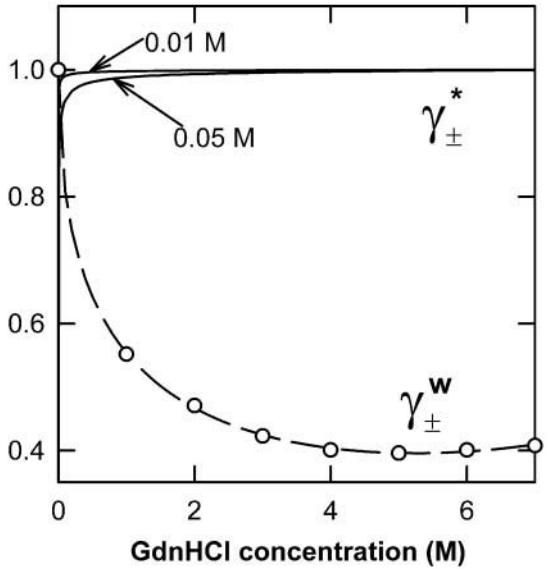

FIGURE 1 Mean ionic activity coefficients in water/GdnHCl mixtures at 25°C. (○) Experimental values of $\gamma^W_\pm$ for GdnHCl taken from Makhatadze et al. (1993). The continuous lines are the values of the $\gamma^*_\pm$ coefficients for a hypothetical solute calculated from the $\gamma^W_\pm$ values as explained in the Appendix; the numbers alongside the lines stand for the solute concentrations.

H^+ itself (see Table 3-3 on page 49 of Bates, 1973). Also, the Bates-Guggenheim convention (page 75 in Bates, 1973) gives 0.903 for the activity coefficient of the chloride ion at 0.01 M ionic strength. From the point of view of pH* assignation, all these activity coefficient values are essentially equivalent, as their $\log_{10}$ values differ in 0.01 pH units or less; thus, we could use any of them as an estimate of γ^*_H in Eq. 10 and we would calculate the same (to all practical purposes) pH* value for a 0.01 M salt solution.

The activity coefficient values given above correspond to a purely aqueous solution (not a mixed solvent) and are just meant to show that γ^*_H estimation in Eq. 10 is *not* a critical step in the definition of a pH scale, and that the mean ionic activity of *any* 1:1 electrolyte can be safely used as an estimate of γ^*_H, provided that the solute concentration is not too high. To the best of our knowledge, however, values of $\gamma^*_\pm$ for electrolytes in water/GdnHCl are not available in the literature. Of course, mean ionic activity coefficients of GdnHCl in water/GdnHCl mixtures have been published (Pace, 1986; Makhatadze et al., 1993), but they are, obviously, cosolvent $\gamma^W_\pm$ coefficients (i.e., they approach unity as GdnHCl becomes infinitely diluted in water; see Eq. 3) and not the required solute $\gamma^*_\pm$ values (which would approach unity as the solute becomes infinitely diluted in a given water-cosolvent mixture). As we show in the Appendix, nevertheless, statistical thermodynamics arguments can be used to derive, from the experimentally available cosolvent $\gamma^W_\pm$ data, the $\gamma^*_\pm$ values for a hypothetical solute that behaves as the cosolvent in terms of intermolecular interactions, but which is distinguishable from the cosolvent. This calculation (described in some detail in the Appendix) yields a physically reasonable and intuitive result (Fig. 1): for guanidinium chloride concentrations of 1 M and higher and solute concentrations of the order of 0.01 M, the $\gamma^*_\pm$ coefficient is practically equal to 1. This result was to be expected: γ^* coefficients for diluted electrolytes in a given solvent reflect the ion-ion interactions in that solvent (or solvent mixture) and those interactions should be screened out in water/GdnHCl mixtures because the cosolvent is a salt. In fact, a simple calculation based on the Debye-Hückel law supports this view (see Eqs. A33–A39); of course, we do not mean that the Debye-Hückel law holds at molar concentrations of GdnHCl but simply that, according to the Debye-Hückel law, the screening of interactions between solute ions caused by the ionic cosolvent becomes efficient at very low cosolvent concentrations. Hence, we may expect solute $\gamma^*_\pm$ values to become unity at comparatively low concentrations of the ionic cosolvent and, once $\gamma^*_\pm \cong 1$, it will remain in that value upon further increases in cosolvent concentration. This is, in fact, the behavior observed in Fig. 1 for the $\gamma^*_\pm$ value of the hypothetical solute.

We conclude, therefore, that we may safely take $\gamma^*_\pm$ (and, consequently, γ^*_H) as equal to unity for GdnHCl concentrations of 1 M and higher. As a result, for the GdnHCl concentrations at which the pH correction is significant, the pH* scale may be defined through,

$$pH^* = -\log_{10}[H^+] \quad (13)$$

That is, in water/GdnHCl mixtures, Sorensen's original definition of pH in terms of hydrogen ion concentration holds and the theoretical calculation of pH*, as well as the determination of the pH-correction factors, becomes straightforward. It must be noted that the correctness of Eq. 13 in water-GdnHCl is supported by the fact that the calculated pH correction factors are found to be independent of hydrogen ion concentration (see first section in Results and Discussion).

RESULTS AND DISCUSSION

Corrections for the measurement of pH in water/GdnHCl

The pH correction factor for a given water/cosolvent mixture can be easily calculated (as pH*-pH_r, see Eq. 11) from the pH meter reading corresponding to a solution of known hydrogen ion concentration in the mixture and the pH* value calculated using Eq. 13. The specific procedure we have used for the determination of δpH* (similar to that we used for water-dioxane in Sanchez-Ruiz et al., 1984 and Cortijo et al., 1988) is briefly described below.

To determine the pH correction factor for a given water-GdnHCl mixture, we measured the pH meter reading for two ClH solutions, in water and in the water/GdnHCl mixture, prepared volumetrically in such a way that they had the same analytical concentration of ClH. The "water solution" is used to determine the concentration of hydrogen ion (the same in both solutions) that subsequently will allow us to calculate theoretically the pH* value for the water/GdnHCl solution. Thus, the pH meter reading for the ClH solution in water equals the true pH value:

$$pH^*(\text{water solution}) = -\log_{10}(C_{ClH} \cdot \gamma_{ClH}) \quad (14)$$

where we have used the fact that ClH is a strong electrolyte (and, therefore, the hydrogen ion concentration equals the analytical concentration of ClH: C_{ClH}) and have assumed that the activity coefficient of the hydrogen ion in water equals the mean activity coefficient of hydrochloric acid (γ_{ClH}); the validity of this approximation has been discussed above (see last section in "Theoretical background"). γ_{ClH} Values in water solution as a function of ClH concentration were tabulated by Harned and Owen (see appendix A in Harned and Owen, 1958) and

can be adequately described by the following empirical equation (Sanchez-Ruiz, 1983),

$$\log_{10}\gamma_{ClH} = -0.5125 \cdot C_{ClH}^{1/2} + 0.7672 \cdot C_{ClH} - 1.874 \cdot C_{ClH}^{2} \quad (15)$$

which is valid at 25°C and within the 0–0.05 M ClH concentration range. Equation 14, together with Eq. 15, allows us to obtain C_{ClH} from the pH measurement in water by using a simple iterative procedure (we begin by assuming $\gamma_{ClH} = 1$ and we solve Eq. 14 to obtain a first estimate of C_{ClH}, which is used—Eq. 15—to derive a first estimate of γ_{ClH}, which allows us—Eq. 14—to arrive at a second, and better, estimate of C_{ClH} and so on; this procedure is continued until the values of [ClH] and γ_{ClH} no longer change). Note again that the C_{ClH} value obtained in this way is the ClH concentration in the water solution and, also, in the matching ClH solution in water-GdnHCl. For this latter solution the pH meter reading (pH_r) does not equal the true pH value (pH*), but pH* is easily calculated as $-\log_{10}[H^+] = -\log_{10}C_{ClH}$ (Eq. 13) and the correction factor is simply given by pH* − pH_r.

The above procedure was performed for several water-GdnHCl mixtures of GdnHCl concentration within the 0.5–6.4 M range. Actually, for each GdnHCl concentration, we carried out three determinations of δpH* using three different ClH concentrations ($4.69 \cdot 10^{-3}$ M, $9.62 \cdot 10^{-3}$ M, $2.08 \cdot 10^{-2}$ M) and found no significant differences (<0.02 units of pH in all cases); the values of δpH* given in Fig. 2 are the average of the three determinations. For the highest GdnHCl concentration used (6.4 M), we carried out five determinations of δpH* using ClH concentrations within the $1.3 \cdot 10^{-3}$–$4.3 \cdot 10^{-2}$ M range; again, no significant ClH concentration effect on the δpH* values was detected (see Fig. 2, *inset*). These results support the correctness of the pH* scale defined through Eq. 13 and that our determinations of δpH* are not distorted by any possible ionizable impurities present in GdnHCl.

It is of interest that Nozaki and Tanford defined in their 1967 paper (Nozaki and Tanford, 1967a) an apparent activity coefficient for the hydrogen ion as the difference between the pH meter reading (which they called simply "pH") and $-\log_{10}[H^+]$. It should be clear from the preceding discussions that this apparent activity coefficient should equal our pH correction factor. In fact, there is excellent agreement between the apparent coefficients reported by Nozaki and Tanford and the δpH* values determined in this work (see Fig. 2).

The δpH* versus C dependence shown in Fig. 2 can be qualitatively understood if we assume (see Eq. 12) that the value of the correction is mainly determined by the medium effect of the proton, that is, by the activity coefficient γ_H^W (whose value in a given water/GdnHCl mixture is related to the Gibbs energy change associated with the transfer of the hydrogen ion from water to the mixture). Thus, plots of γ^W

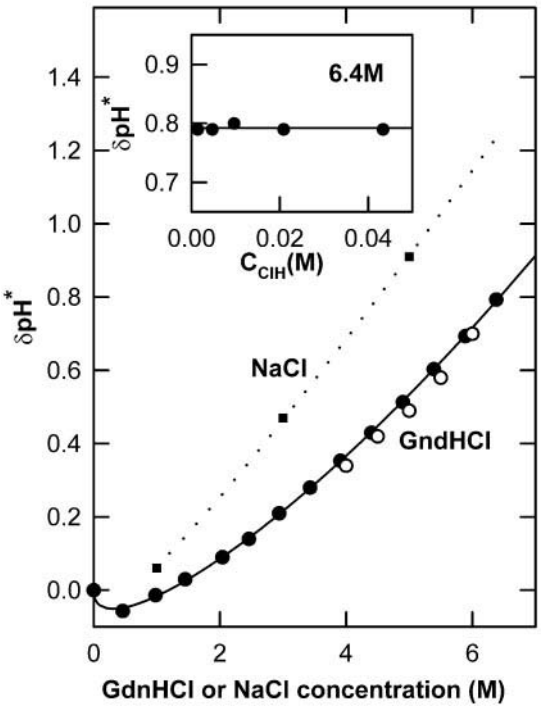

FIGURE 2 Correction factors for the pH measurement in water/GdnHCl and water/NaCl. (●), experimental values of the correction factor in water/GdnHCl determined in this work; each value is the average of three measurements (which differed in <0.02 in all cases). The continuous line is the dependence of δpH* with GdnHCl concentration given by Eq. 16. (○), values of the "apparent" activity coefficient for the hydrogen ion in water/GdnHCl reported by Nozaki and Tanford (1967a). *Inset*: values of the correction factor determined in 6.4 M GdnHCl using solutions of different ClH concentration (C_{ClH}). (■), experimental values of the correction factor in water/NaCl determined in this work; each value is the average of three measurements (which differed in <0.02 in all cases); the dotted line is shown to guide the eye.

coefficients for electrolytes versus salt concentration often show a minimum at a concentration of the order of molar [see chapters 12 and 14 in Harned and Owen (1958) and chapter 3 in Brockris and Reddy (1970)]; that is, the coefficient decreases with salt concentration at low salt and increases at high salt, which is precisely the behavior observed for δpH* in Fig. 2 (it is also the behavior observed for the $\gamma_{\pm}^W$ coefficient of GdnHCl in Fig. 1, although the minimum there is less pronounced). The low-salt decrease is explained by the Debye-Hückel stabilization of ions. The high-salt increase has been attributed to the ion-solvent interactions: ions are hydrated and, therefore, their transfer to mixtures of high cosolvent concentration (i.e., low water concentration) is unfavorable. A simple model that describes the γ^W values for electrolytes in terms of the Debye-Hückel stabilization and the hydration index of the ions can be found in chapter 3 of Brockris and Reddy (1970); this model is capable, in fact, of fitting the γ^W values for electrolytes in a wide salt concentration range.

Regardless of molecular interpretations, the dependence of the pH correction factor with GdnHCl concentration shown in Fig. 2 can be adequately described by the following empirical equation,

$$\delta pH^* = -0.182 \cdot C^{1/2} + 0.161 \cdot C + 5.5 \cdot 10^{-3} \cdot C^2 \quad (16)$$

which can be useful for interpolation purposes.

For comparison, we also show in Fig. 2 some values of the pH correction in water/NaCl mixtures, determined using the same procedure we have described above for water/GdnHCl and assuming that γ_H^* can be taken as unity (Eq. 13). The δpH* values in water/NaCl are of the same order, but somewhat higher than those determined in water/GdnHCl.

Finally, it must be noted that the pH correction factors in water-GdnHCl and water/NaCl are significant, reaching ~0.8 units of pH for 6.4 M GdnHCl and ~0.9 units of pH for 5 M NaCl.

The explanation of the Nozaki-Tanford result

Fig. 3 shows pK* values for acetic acid ionization and for the first ionization of glycine in water/NaCl mixtures of NaCl concentration within the 0–3 M range. It is important to note that these pK* values were published ~50 years ago (King, 1945; Harned and Owen, 1958) and obtained by extrapolating to infinite dilution in each water/NaCl mixture the K^C values determined rigorously using a hydrogen-silver chloride cell; that is, *no pH corrections needed to be applied*.

We want to emphasize here that pH corrections are needed in mixed solvents when working with a glass-electrode cell. δpH* values are certainly large in water/NaCl (Fig. 2), but the pK* values in water/NaCl shown in Fig. 3 were determined using a hydrogen-electrode cell: they are the correct values and no pH correction is required. However, Nozaki and Tanford (1967a) determined the pK values in 6 M GdnHCl using a cell based on the glass electrode and they did not apply any correction to the pH measurements (unknown at the time). As a result, the pK values they reported (shown in Fig. 3) are "apparent" values (pK_{app}) that differ from the true pK values (i.e., pK* values) by the same amount that the pH meter readings differ from the true pH values (pH*):

$$pK^* = pK_{app} + \delta pH^* \quad (17)$$

The values of pK* for acetic acid and glycine ionization derived from the Nozaki-Tanford data using Eq. 17, and our values of δpH* (Fig. 2) are also given in Fig. 3. It is clear that the "uncorrected" pK_{app} values in 6 M GdnHCl do not fit the general trend defined by the pK* values in water/NaCl mixtures with an NaCl concentration in the 0–3 M concentration range; however, *the corrected pK* values do!*

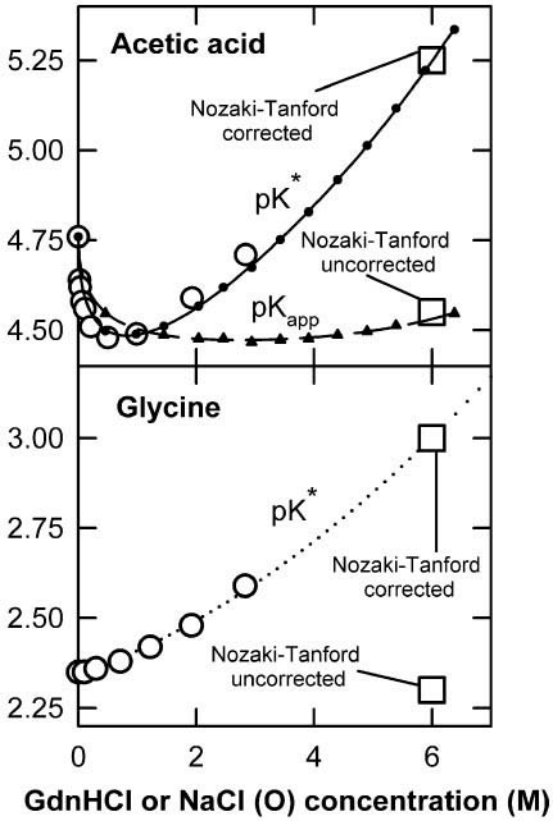

FIGURE 3 Effect of GdnHCl and NaCl concentration on the pK values for carboxylic acid ionization. *Top:* (○), pK* values for acetic acid ionization in NaCl solutions determined using a hydrogen-silver chloride cell (Harned and Owen, 1958). (●), values of pK* for acetic acid ionization determined in this work using a glass-electrode cell and applying the pH corrections given in Fig. 2. Each value shown is the average of three determinations, which differed in <0.02 in all cases. The continuous line is the dependence of pK* with GdnHCl concentration given by Eq. 22. (▲), apparent pK_{app} values for acetic acid ionization that are obtained if the pH correction is neglected. The dashed line is the dependence of pK_{app} with GdnHCl concentration given by Eq. 23. The pK value for acetic acid reported by Nozaki and Tanford (1967a) is labeled "Nozaki-Tanford uncorrected." The Nozaki-Tanford value corrected using the δpH* value at 6 M GdnHCl concentration reported in this work (Fig. 2) is labeled "Nozaki-Tanford corrected." *Bottom:* (○), pK* values for the first ionization of glycine in NaCl solutions determined using a hydrogen-silver chloride cell (King, 1945). The pK value for the first ionization of glycine reported by Nozaki and Tanford (1967a) is labeled "Nozaki-Tanford uncorrected." The Nozaki-Tanford value corrected using the δpH* value at 6 M GdnHCl concentration reported in this work (Fig. 2) is labeled "Nozaki-Tanford corrected." The dotted line is shown to guide the eye.

Thus, the data shown in Fig. 3 indicate that the apparent agreement between pK values in the 6 M GdnHCl and low-salt in water reported by Nozaki and Tanford was the result of having neglected the pH corrections and that, in fact, there is a significant GdnHCl concentration effect on the pK values.

pK* values for acetic acid ionization in water/GdnHCl

The interpretation of the Nozaki-Tanford result given above is based on the (reasonable) assumption that the NaCl effect on pK* is similar to the GdnHCl effect and that, as a result, pK* values in 6 M GdnHCl should fit the trend defined by the pK* values in 0–3 M NaCl. To test this assumption, we have obtained the pK* values for acetic acid ionization in water/GdnHCl mixtures with a GdnHCl concentration range of 0.5–6.4 M (these pK* values will also be useful for subsequent calculations reported in this work). The procedure for pK* determination is briefly described below.

Using Eq. 7 together with Eqs. 4, 10, and 11, the following expression for the acetic acid pK* can be easily derived,

$$pK^* = -\alpha - \log_{10}(\gamma^*_A/\gamma^*_{AH}) + pH_r + \delta pH^* \quad (18)$$

where α is,

$$\alpha = \log_{10}([A^-]/[AH]) \quad (19)$$

To determine the pK* value for acetic acid ionization in a given water/GdnHCl mixture, we measured the pH meter reading for two solutions of ClH and sodium acetate (AcNa), in water and in the water/GdnHCl mixture, prepared in such a way that they had the same analytical concentrations of ClH and sodium acetate (C_{ClH} and C_{AcNa}). Also, these concentrations were chosen so that $C_{ClH} \approx C_{AcNa}/2$. Using mass-balance and electroneutrality conditions, it is straightforward to write α in terms of these analytical concentrations:

$$\alpha = \log_{10}\left(\frac{C_{ClH} - [H^+] + [OH^-]}{C_{AcNa} - C_{ClH} + [H^+] - [OH^-]}\right) \quad (20)$$

The pH* values of the ClH/AcNa solutions are such, however, that $[H^+]$ and $[OH^-]$ are much smaller than C_{ClH} and C_{AcNa}, and can be safely neglected in Eq. 20. Hence,

$$\alpha = \log_{10}\left(\frac{C_{ClH}}{C_{AcNa} - C_{ClH}}\right) \quad (21)$$

which means that α is given by the analytical concentrations and, therefore, its value is the same for the two matched solutions (in water and in the water/GdnHCl mixture). Accordingly, the α value can be calculated from the pH meter reading in the aqueous solution by using Eq. 18 together with the following: 1) the pK* value for acetic acid ionization in water, which is known and equal to 4.756; 2) the activity coefficient for undissociated acetic acid (γ^*_{AH} in Eq. 18), which can be taken as unity in diluted aqueous solution because the net charge of this species is zero; 3) the activity coefficient for the acetate ion (γ^*_A in Eq. 18) in aqueous solution, which can be estimated as the mean ionic coefficient of hydrochloric acid (Eq. 15) at a C_{ClH} value equal to the ionic strength of the AcNa/ClH solutions (easily shown to be equal to the analytical concentration of sodium acetate); and 4) $\delta pH^* = 0$ in water.

Once the α value is known, Eq. 18 can be used to calculate the pK* value in the water/GdnHCl mixture from the pH meter reading corresponding to the AcNa/ClH solution in the mixture (the required δpH^* value is given by Eq. 16 and both activity coefficients, γ^*_A and γ^*_{AH}, can now be taken as unity).

The above procedure was performed for several water/GdnHCl mixtures with GdnHCl concentration within the 0.5–6.4 M range. In fact, for each GdnHCl concentration, we carried out three determinations of pK* using different concentrations of sodium acetate ($1.98 \cdot 10^{-2}$ M, $1.00 \cdot 10^{-2}$ M, and $4.95 \cdot 10^{-3}$ M) and found no significant differences (<0.02 units in all cases). The values given in Fig. 3 are the average of the three determinations and can be adequately described by the following empirical equation,

$$pK^* = 4.76 - 0.618 \cdot C^{1/2} + 0.356 \cdot C - 1.12 \cdot 10^{-2} \cdot C^2 + 1.30 \cdot 10^{-3} \cdot C^3 \quad (22)$$

Note that the pK* values we have determined for acetic acid in water/GdnHCl are in fact close to those reported in the literature for water/NaCl mixtures with NaCl concentration within the 0–3 M range and, in addition, the *corrected* Nozaki-Tanford value at 6 M GdnHCl is in good agreement with our values.

In Fig. 3 we have also plotted the apparent pK values for acetic acid ionization in water/GdnHCl; that is, the pK values that would have been obtained if the pH correction had not been applied (these apparent pK values are given by $pK_{app} = pK^* - \delta pH^*$; see Eq. 17). The pK_{app} versus GdnHCl concentration dependence can be described by the following empirical equation:

$$pK_{app} = 4.76 - 0.436 \cdot C^{1/2} + 0.195 \cdot C - 1.67 \cdot 10^{-2} \cdot C^2 + 1.30 \cdot 10^{-3} \cdot C^3 \quad (23)$$

Note that, as was to be expected, the *uncorrected* Nozaki-Tanford value at 6 M GdnHCl is in good agreement with our pK_{app} values (Fig. 3).

The effect of GdnHCl on carboxylic acid ionization

It is clear from the data shown in Fig. 3 that there is a significant GdnHCl (salt) concentration effect on the ionization of carboxylic acids. This effect can be interpreted as follows:

1. For an ionization that involves charge separation in different molecular species, the pK* value decreases in the approximate 0–1 M GdnHCl (salt) concentration range due to a Debye-Hückel stabilization of the charged spe-

cies created upon ionization. This decrease is observed for acetic acid ionization,

$$CH_3 - COOH \leftrightarrow CH_3 - COO^- + H^+ \quad (24)$$

but not for the first ionization of glycine because, in this latter case, ionization does not involve charge separation in different molecular species:

$$^+NH_3 - CH_2 - COOH \leftrightarrow {}^+NH_3 - CH_2 - COO^- + H^+ \quad (25)$$

where the net charge of the zwitterion is zero and we have 1 positive charge on the reactants side and 1 positive charge on the products side.

2. In addition, there is a smooth increase of pK* with GdnHCl (salt) concentration, which can be interpreted as a general solvent effect. In the case of the first ionization of glycine, this is the only effect observed. In the case of acetic acid ionization, the combination of this smooth increase with the much sharper decrease caused by Debye-Hückel stabilization produces a minimum in the plot of pK* versus GdnHCl (salt) concentration at ~1 M salt. Further insight into the origin of this solvent effect can be obtained by writing pK* as,

$$pK^* = pK^W - \log_{10}\gamma^W_{AH} + \log_{10}\gamma^W_A + \log_{10}\gamma^W_H \quad (26)$$

which can be easily obtained from Eqs. 3 and 6, using the fact that γ^* coefficients can be taken as unity in water/GdnHCl and, therefore, $K^* \cong K^C$. Because pK^W is strictly a constant (i.e., independent of solvent composition), the dependence of pK* with GdnHCl concentration is determined by the medium effects of the three species involved (that is, by the coefficients γ^W_{AH}, γ^W_A, and γ^W_H). Actually, it appears plausible that the gradual pK* increase observed at high GdnHCl concentration mainly reflects the medium effect of the proton ($\log\gamma^W_H$ in Eq. 26); at least, this assumption has the advantage of rationalizing the two following observations: 1) the gradual pK* increases observed for acetic acid and first ionization of glycine (Fig. 3) are similar; this is easily explained, as the $\log_{10}\gamma^W_H$ term is the same in both cases; 2) the plots of apparent pK_{app} versus GdnHCl concentration (Fig. 3) do not show a significant "gradual-increase effect" at high cosolvent concentration; if the pH correction factors are mainly determined by the medium effect of the proton (as we have discussed above), then "neglecting" the pH correction is expected to cancel the gradual increase in pK*, since $pK_{app} = pK^* - \delta pH^*$ (Eq. 17).

The effect of GdnHCl on the number of protons bound to denatured and native proteins

We have obtained estimates of the effect of GdnHCl on the number of protons bound (ν) to denatured α-chymotrypsin, ribonuclease A, and staphylococcal nuclease in the acidic pH range ($pH_r \leq 4.5$) on the basis of the three following assumptions: 1) At the acidic pH values under consideration only carboxylic acid groups contribute to the effect of GdnHCl on ν; 2) carboxylic acid groups at high GdnHCl concentration in denatured proteins behave independently (charge-charge interactions are assumed to be screened out in concentrated GdnHCl). Accordingly, ν may be expressed as,

$$\nu = \nu_0 + \sum_j \frac{1}{10^{-(pK^*_j - pH^*)}} \quad (27)$$

where the sum is over the carboxylic acid groups and ν_0 is the number of protons bound to all other ionizable groups (lysines, histidines, tyrosines, amino terminal) which are taken as constant (i.e., independent of GdnHCl concentration) in the acidic pH range.

It must be noted that experimental studies on GdnHCl-induced denaturation have been carried out so far at constant pH meter reading (constant pH_r) rather than at a constant value of "true" pH (constant pH*). However, from Eqs. 11 and 17 we have that,

$$pK^* - pH^* = pK_{app} - pH_r \quad (28)$$

and Eq. 27 can then be written as,

$$\nu = \nu_0 + \sum_j \frac{1}{10^{-(pK_{appij} - pH_r)}} \quad (29)$$

Therefore, the calculation of the effect of GdnHCl concentration on ν can be performed for a given (constant) value of pH_r, provided that expressions for the GdnHCl dependence of the apparent pK values are available; 3) the effect of GdnHCl on the pK_{app} values for carboxylic acid groups is assumed to be the same as that found for acetic acid (Fig. 4 and Eq. 23), although the constant term in Eq. 23 is modified, so that the generally accepted "model" values for glutamate, aspartate, and carboxyl terminal (4.5, 4.0, and 3.6, respectively) are obtained at zero GdnHCl concentration:

$$pK_{app}(Glu) = 4.50 - 0.436 \cdot C^{1/2} + 0.195 \cdot C - 1.67 \cdot 10^{-2} \cdot C^2 + 1.30 \cdot 10^{-3} \cdot C^3 \quad (30)$$

$$pK_{app}(Asp) = 4.00 - 0.436 \cdot C^{1/2} + 0.195 \cdot C - 1.67 \cdot 10^{-2} \cdot C^2 + 1.30 \cdot 10^{-3} \cdot C^3 \quad (31)$$

$$pK_{app}(\text{carboxyl term.}) = 3.60 - 0.436 \cdot C^{1/2} + 0.195 \cdot C - 1.67 \cdot 10^{-2} \cdot C^2 + 1.30 \cdot 10^{-3} \cdot C^3 \quad (32)$$

In Fig. 4 we show the effect of GdnHCl on the number of protons bound to the denatured states of α-chymotrypsin, ribonuclease A, and staphylococcal nuclease, as calculated

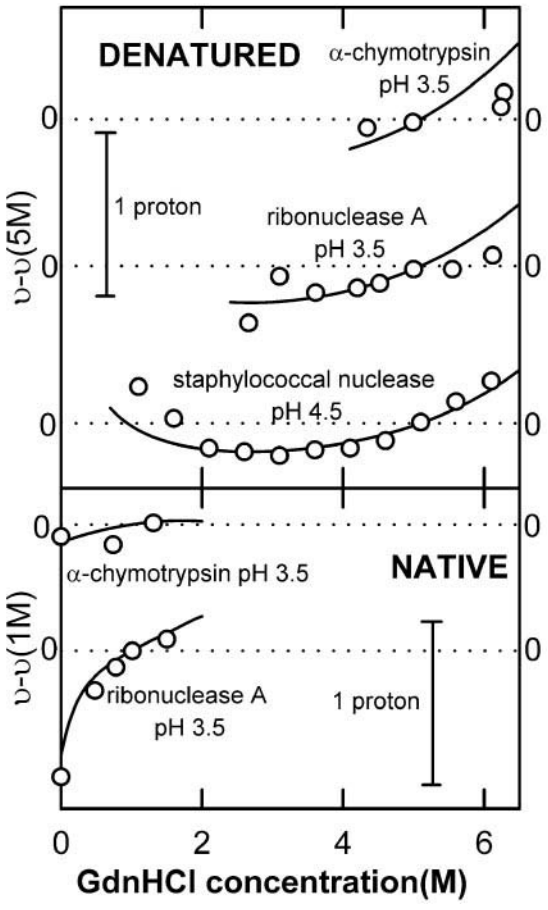

FIGURE 4 Effect of GdnHCl concentration on the number of protons bound to the denatured (*top*) and native (*bottom*) states of the indicated proteins. (○), experimental values reported by Bolen and Yang (2000). The continuous lines are the dependencies calculated theoretically as described in the text. Note that the values corresponding to different proteins have been shifted in the vertical axis for the sake of clarity. The pH values shown are those reported by Bolen and Yang (2000); note that, according to the views expressed in this work, they should be interpreted as pH meter readings.

on the basis of the above procedure. Note that what we plot in Fig. 4 is actually $\nu - \nu$(5 M GdnHCl).

In the case of native proteins, we may expect the ionization behavior of carboxylic acid groups to differ significantly from that predicted using "model" pK values, even in the absence of GdnHCl. We will assume that this difference is mainly due to the effect of charge-charge interactions, since most ionizable groups in proteins are exposed to the solvent. Note also that experimental data on the number of protons bound to native proteins (Bolen and Yang, 2000) correspond to comparatively low GdnHCl concentrations at which charge-charge interactions are not expected to be completely screened out. We have used our implementation of the Tanford-Kirkwood model (Tanford and Kirkwood, 1957) to estimate the effect of charge-charge interactions. The calculation was carried out as previously described (Ibarra-Molero et al., 1999) with the only difference that the intrinsic pK values for carboxylic acid groups at a given GdnHCl concentration (input of the Tanford-Kirkwood calculation) were taken to be equal to the values given by Eqs. 30–32. The results obtained for the native states of ribonuclease A and α-chymotrypsin are shown in Fig. 4 as the difference $\nu - \nu$(1 M guanidine).

In Fig. 4 we also show the experimental values reported by Bolen and Yang (2000) for the GdnHCl concentration effect on the number protons bound to the native and denatured states of ribonuclease A, α-chymotrypsin, and staphylococcal nuclease. It is clear that our (admittedly over-simplistic) calculations successfully reproduce some of the experimentally observed trends. Thus, the broad minimum in the plot of ν versus GdnHCl concentration for denatured staphylococcal nuclease is also found in our calculated values (as was to be expected because the GdnHCl concentration dependence of the pK_{app} values used in the calculation—Eqs. 30–32 and Fig. 3—also show a broad minimum). Also, the increase in ν with GdnHCl concentration found experimentally for native ribonuclease A is well-reproduced by our calculations. This increase in the calculated values is due to the fact that, at low GdnHCl concentration, the effective pK values (pK_{eff}) for carboxylic acid groups in ribonuclease A (given by the Tanford-Kirkwood calculation) are significantly lower than the corresponding pK_{app} values (used as input of the Tanford-Kirkwood calculation), because charge-charge interactions with other (predominantly positive) charged groups stabilize the carboxylate anion. Guanidinium chloride, however, screens charge-charge interactions and pK_{eff} values increase with GdnHCl concentration, with the concomitant increase in the number of protons bound (Fig. 4). In the case of native α-chymotrypsin the increase of pK_{eff} with GdnHCl concentration is found to be less pronounced and, as a result, the profile of calculated ν versus GdnHCl concentration is almost flat, in agreement with the experimental result (Fig. 4).

CONCLUDING REMARKS

We have shown that, when the appropriate pH corrections are applied, pK values for simple substances in water/GdnHCl are found to depend significantly on GdnHCl concentration. The fact that apparent (i.e., uncorrected) pK values in 6 M GdnHCl are similar to the low-salt values is possibly due to the fact that both the general solvent effect on pK and the pH correction factor are mainly determined by the medium effect of the proton (as a result, "neglecting" the pH correction cancels the general solvent effect, which does not appear in the uncorrected apparent pK values).

This work also implies that GdnHCl-induced denaturation experiments reported so far in the literature were carried at constant value of the pH meter reading (because pH corrections were unknown and, therefore, not applied).

We want to emphasize, however, that we do *not* suggest at this stage that the experimental procedure for GdnHCl denaturation studies be modified, so that a constant value of "true" pH is achieved. In fact, it could be argued that, in GdnHCl denaturation studies, both pH_r and pH* should be changed with GdnHCl concentration in such a way that the protonation state of the ionizable groups relevant for stability does not change, although this kind of experiment may be difficult or impossible to carry out in practice. Also, analyses given in this work indicate that consistent calculations can be carried out on the basis of uncorrected pH meter readings and apparent pK values (see Eqs. 29–32).

The above points notwithstanding, however, it is clear that denaturation Gibbs energy values reported in the literature for the same "pH" value (actually pH meter reading) and determined using different types of denaturation experiments (thermal, urea-induced, and GdnHCl-induced denaturation) may correspond to different values of pH*, a fact that might contribute to the discrepancies often found. In connection with this, it would be of interest to determine the pH correction factors for water-urea mixtures (work in progress).

By using simple models we have been able to qualitatively reproduce some of the general features of the GdnHCl-induced proton uptake/release behavior for the native and denatured states of several proteins. Although this result is certainly encouraging, it must be taken with due caution, because our calculations are based upon some very simplistic assumptions: 1) assuming that ionizable groups in denatured proteins behave in an independent manner; 2) modeling the pK_{app} for Glu and Asp residues in denatured proteins on the basis of the pK_{app} values for acetic acid ionization; 3) assuming that deviations of the pK values in the native state from the model values are exclusively due to charge-charge interactions; 4) modeling charge-charge interactions in the native state on the basis of the Tanford-Kirkwood sphere model; 5) using the Tanford-Kirkwood model (which is based upon the linearized Poisson-Boltzmann equation) at rather high salt concentrations. It appears conceivable, in fact, that other models could also explain the observed trends and, consequently, we do not feel that we can draw reliable conclusions regarding the role of the possible residual structure in the denatured states and the binding of GdnHCl ions from the comparisons shown in Fig. 4.

The above points should not obscure, however, the main suggestion of the agreement shown in Fig. 4, namely that some overall electrostatic features of native and denatured proteins (such as the proton uptake/release behavior) can be predicted on the basis of very simple models.

NOTES

1. We will use the molar concentration scale throughout this work. Accurate thermodynamic pK values for simple substances are often reported in the molal concentration scale [see, for instance, King (1945); Harned and Owen (1958)]. When required, we have converted the molal-scale literature values to the molar scale using the known densities of the solutions. In any case, the differences we found between the molal-scale pK values and the corresponding molar-scale ones were always very small, and often negligible.

2. The terminology we are using (Eq. 11) differs from that commonly found in the literature. Workers on water/alcohol systems have used pH* = pH − δ, where the pH meter reading is simply "pH" and the correction factor is −δ. Workers on water/dioxane mixtures have written Eq. 11 as $pH = B + \log U^{\circ}_{H}$, where pH is now the "true pH" (i.e., pH* in our terminology), the pH meter reading is B and the cumbersome notation $\log U^{\circ}_{H}$ is used for the correction factor. We believe that the terminology we have chosen (Eq. 11) will be more acceptable to the readers.

APPENDIX: THE CALCULATION OF γ^* VALUES FOR A HYPOTHETICAL SOLUTE IN A MIXED SOLVENT FROM THE COSOLVENT γ^W VALUES

Values of a mean ionic activity coefficient for a 1:1 electrolyte in water/GdnHCl are required, as estimates of γ^*_H, for the definition of the pH* scale. In principle, we may take advantage of the fact that, in this case, the cosolvent is a salt and use the published values for $\gamma_{\pm}$ of GdnHCl in water/GdnHCl mixtures (Pace, 1986; Makhatadze et al., 1993). However, the published activity coefficients are cosolvent $\gamma^W_{\pm}$ values which approach unity as GdnHCl becomes infinite by diluted in water. What we need to define the pH* scale are, in fact, solute $\gamma^*_{\pm}$ values that can be associated to an electrolyte that is present at low concentration in a given water/GdnHCl mixture. Here, we will use a statistical-mechanical approach to derive a procedure that allows the experimental $\gamma^W_{\pm}$ values to be transformed into the required $\gamma^*_{\pm}$ values.

The plan of this appendix is the following. First, we will briefly describe the statistical-mechanical expression for the chemical potential advocated by Ben-Naim (1992). Second, for the sake of clarity, we will proceed to derive the relationship between the two activity coefficients assuming that all species in solution (cosolvent and solute) are neutral. Then, we will demonstrate that the same relationship applies when the cosolvent and the solute are electrolytes. Finally, we will show that the results obtained for $\gamma^*_{\pm}$ are qualitatively consistent with the Debye-Hückel law.

The chemical potential of a species i in a liquid or a liquid mixture can be written as (see pages 320–321 and 422–423 in Ben-Naim, 1992):

$$\mu_i^M = \mu_i^{\#} + kT \cdot \ln(\rho_i \cdot \Lambda_i^3) \qquad \text{(A1)}$$

where we conform to common statistical-mechanical usage and define the chemical potential per molecule (μ_i^M) rather than per mole (μ_i), which is the usual convention in macroscopic thermodynamical treatments. Of course, both chemical potentials are, in fact, identical, except for a factor of scale which is equal to Avogadro's number (N_0):

$$\mu_i = N_0 \cdot \mu_i^M \qquad \text{(A2)}$$

Defining the chemical potential per molecule leads naturally to its interpretation as the work associated to the addition of a molecule to a macroscopically large system. According to the right-hand side of Eq. A1, this work is split into two contributions:

1. The pseudo-chemical potential ($\mu_i^{\#}$); that is, the work associated with the addition of a given molecule (the *test molecule*) at a fixed position in the system:

$$\mu_i^{\#} = W(i|S) - kT \cdot \ln q_i \qquad \text{(A3)}$$

where q_i is the internal partition function of the molecule i (including the rotational, vibrational, and electronic degrees of freedom) and $W(i|S)$ is the coupling work of the test molecule to the rest of the system. It is

important to note that this coupling work may be interpreted as the average Gibbs energy of interaction of the molecule i with the system (see pages 423–424 in Ben-Naim, 1992).

2. The liberation term; that is, the work associated with the removal of the constraint imposed by fixing the position of the test molecule: $kT\ln(\rho_i \cdot \Lambda_i^3)$, ρ_i being the number density (number of molecules of type i per unit volume) and Λ_i the one-dimensional momentum partition function of the molecule i ($\Lambda_i = h/(2\pi m_i kT)^{1/2}$, where m_i is the molecular mass).

Consider now a water-cosolvent mixture. In this appendix we will follow Scatchard notation and use subscripts 1 and 3 to designate the main solvent (water) and the cosolvent, respectively (subscript 2 will be reserved for the solute). From Eq. A1 the chemical potential of the cosolvent in a mixture of a given molar cosolvent concentration (C_3) can be written as:

$$\begin{aligned}\mu_3^M\{C_3\} &= \mu_3^{\#}\{C_3\} + kT\cdot\ln(\rho_3\cdot\Lambda_3^3)\\ &= W(3|C_3) - kT\cdot\ln q_3 + kT\cdot\ln C_3\\ &\quad + kT\cdot\ln(N_0\cdot\Lambda_3^3)\end{aligned} \tag{A4}$$

where we have used Eq. A3 and the relationship between number density and molar concentration: $\rho_i = C_i \cdot N_0$. $W(3|C_3)$ in the equation above represents the Gibbs energy of interaction of a test molecule of cosolvent with a water-cosolvent mixture of C_3 cosolvent concentration.

The classical thermodynamics expression for the chemical potential of the cosolvent is:

$$\mu_3\{C_3\} = \mu_3^W + RT\cdot\ln C_3 + RT\cdot\ln\gamma_3^W\{C_3\} \tag{A5}$$

Using $\mu_3 = N_0\cdot\mu_3^M$ (Eq. A2) and solving for the activity coefficient, we get:

$$\begin{aligned}RT\cdot\ln\gamma_3^W\{C_3\} &= N_0\cdot W(3|C_3) - RT\\ &\quad\cdot\ln q_3 - \mu_3^W + RT\cdot\ln(N_0\cdot\Lambda_3^3)\end{aligned} \tag{A6}$$

The activity coefficient in Eq. A5 is defined in such a way that it approaches unity as the mixture becomes infinitely dilute in the cosolvent and, consequently, the standard chemical potential is defined as:

$$\begin{aligned}\mu_3^W &= \lim_{C_3\to0}(\mu_3\{C_3\} - RT\cdot\ln C_3)\\ &= \lim_{C_3\to0}(N_0\cdot\mu_3^M\{C_3\} - RT\cdot\ln C_3)\end{aligned} \tag{A7}$$

Using Eq. A4, we now have:

$$\begin{aligned}\mu_3^W &= \lim_{C_3\to0}(N_0\cdot W(3|C_3) - RT\cdot\ln q_3\\ &\quad + RT\cdot\ln(N_0\cdot\Lambda_3^3))\end{aligned} \tag{A8}$$

and

$$\mu_3^W = N_0\cdot W(3|C_3=0) - RT\cdot\ln q_3 + RT\cdot\ln(N_0\cdot\Lambda_3^3) \tag{A9}$$

where $W(3|C_3 = 0)$ is the Gibbs energy of interaction of a test molecule of cosolvent with the pure main solvent (water). Equation A9 can also be obtained by setting $C_3 = 0$ and $\gamma_3^W = 1$ in Eq. A6 and solving for μ_3^W. Equation A9 confirms that the μ_3^W standard chemical potential is independent of solvent composition.

We assume now that a solute (component 2) is present in the water/cosolvent mixture. Its chemical potential in a solution of given solute and cosolvent concentrations (C_2 and C_3) is given by,

$$\begin{aligned}\mu_2^M\{C_2, C_3\} &= W(2|C_2, C_3) - kT\cdot\ln q_2 + kT\cdot\ln C_2\\ &\quad + kT\cdot\ln(N_0\cdot\Lambda_2^3)\end{aligned} \tag{A10}$$

which is analogous to Eq. A4. Note $W(2|C_2, C_3)$ is the Gibbs energy of interaction of a test molecule of the solute with a system that contains water and solute and cosolvent at concentrations C_2 and C_3.

The macroscopic thermodynamics expression for the chemical potential of the solute is:

$$\mu_2\{C_2, C_3\} = \mu_2^*\{C_3\} + RT\cdot\ln C_2 + RT\cdot\ln\gamma_2^*\{C_2, C_3\} \tag{A11}$$

and using $\mu_2 = N_0\cdot\mu_2^M$ (Eq. A2) and solving for the activity coefficient, we get

$$\begin{aligned}RT\cdot\ln\gamma_2^*\{C_2, C_3\} &= N_0\cdot W(2|C_2, C_3)\\ &\quad - RT\cdot\ln q_2 - \mu_2^*\{C_3\}\\ &\quad + RT\cdot\ln(N_0\cdot\Lambda_2^3)\end{aligned} \tag{A12}$$

which is analogous to Eq. A6.

The activity coefficient in Eq. A11 is defined in such a way that it approaches unity as the solute becomes infinitely diluted in a water-cosolvent mixture of given cosolvent concentration (C_3). Accordingly, the μ_2^* standard chemical potential is defined as,

$$\begin{aligned}\mu_2^*\{C_3\} &= \lim_{C_2\to0}(\mu_2\{C_2, C_3\} - RT\cdot\ln C_2)\\ &= \lim_{C_2\to0}(N_0\cdot\mu_2^M\{C_2, C_3\} - RT\cdot\ln C_2)\end{aligned} \tag{A3}$$

and using Eq. A10:

$$\mu_2^*\{C_3\} = N_0\cdot W(2|C_3) - RT\cdot\ln q_2 + RT\cdot\ln(N_0\cdot\Lambda_2^3) \tag{A14}$$

where $W(2|C_3)$ is the interaction Gibbs energy of a test molecule of the solute with a water-cosolvent mixture of given composition (C_3) in which no other solute molecule is present. Equation A14 can also be obtained by setting $C_2 = 0$ and $\gamma_2^* = 1$ in Eq. A12 and solving for μ_2^*. Note that the presence of $W(2|C_3)$ in the right-hand side of Eq. A14 confirms that the μ_2^* standard chemical potential depends on cosolvent concentration.

Substituting A14 into A12, we arrive at the following molecular interpretation of the solute activity coefficient;

$$RT\cdot\ln\gamma_2^*\{C_2, C_3\} = N_0\cdot[W(2|C_2, C_3) - W(2|C_3)] \tag{A15}$$

that is, the activity coefficient is related to the difference between the Gibbs energy of interaction of the solute test molecule with the system at a solute concentration C_2 [$W(2|C_2, C_3)$] and the solute-infinite-dilution limit of that interaction Gibbs energy [$W(2|C_3)$]. For low C_2 the contribution of solute-solvent interactions is expected to be approximately the same in $W(2|C_2, C_3)$ and $W(2|C_3)$, and the difference between the two W terms in the right-hand side of Eq. A15 will mainly reflect the solute-solute interactions, which is consistent with the usual molecular interpretation of γ^* coefficients.

So far we have not made any statement about the nature of the solute. We assume now that, from the point of view of the molecular interactions in solution, the solute behaves like the cosolvent and, consequently, we can substitute cosolvent for solute in the interaction Gibbs energy terms (note, however, that the solute molecules are distinguishable from the cosolvent molecules and we still have different liberation terms for the solute and the cosolvent). For a solute thus defined, the interaction Gibbs energy of the solute test molecule with a solution of given solute and cosolvent concentrations (C_2 and C_3) equals the Gibbs energy of interaction of a cosolvent

test molecule with a solution of cosolvent concentration $C_2' = C_2 + C_3$: $W(2|C_2, C_3) = W(3|C_3')$. Likewise: $W(2|C_3) = W(3|C_3)$. Hence Eq. A15 can be rewritten as:

$$RT \cdot \ln \gamma_2^*\{C_2, C_3\} = N_0 \cdot [W(3|C_3') - W(3|C_3)] \quad \text{(A16)}$$

We now apply Eq. A6 for the cosolvent activity coefficient to the concentrations C_3 and C_3', solve for the Gibbs interaction terms, $W(3|C_3')$ and $W(3|C_3)$, and substitute them into A16 to obtain:

$$\ln \gamma_2^*\{C_2, C_3\} = \ln \gamma_3^W\{C_3'\} - \ln \gamma_3^W\{C_3\} \quad \text{(A17)}$$

with $C_3' = C_3 + C_2$.

Equation A17 allows the calculation of γ_2^* from the experimentally available γ_3^W values. However, so far we have overlooked the fact that, in the case of interest here, both the cosolvent and the "hypothetical" solute are 1:1 electrolytes. We show below that Eq. A17 still holds in this case.

For a cosolvent that is a 1:1 electrolyte we can formally write equations analogous to A4 for the chemical potential of the cation and the anion. These, however, must then be combined to give the chemical potential of the electrolyte:

$$\mu_3^M\{C_3\} = \mu_{3+}^M\{C_3\} + \mu_{3-}^M\{C_3\} \quad \text{(A18)}$$

which yields,

$$\mu_3^M\{C_3\} = W(3 + |C_3) + W(3 - |C_3) - kT \cdot \ln(q_{3+}q_{3-}) + 2kT \cdot \ln C_3 + kT \cdot \ln(N_0^2\Lambda_{3+}^3\Lambda_{3-}^3) \quad \text{(A19)}$$

where we are using 3+ and 3− to denote the cation and the anion of the cosolvent, respectively. Thus, for instance, $W(3 + |C_3)$ is the Gibbs energy of interaction of a test cosolvent cation with a water/cosolvent mixture of C_3 cosolvent concentration.

The macroscopic thermodynamics equation for the chemical potential of the cosolvent is now:

$$\mu_3\{C_3\} = \mu_3^W + 2RT \cdot \ln C_3 + 2RT \ln \gamma_{3\pm}^W\{C_3\} \quad \text{(A20)}$$

and using Eq. A2 we obtain the following expression for the mean ionic activity of the cosolvent:

$$2RT \cdot \ln \gamma_{3\pm}^W\{C_3\} = N_0W(3 + |C_3) + N_0W(3 - |C_3) - RT \cdot \ln(q_{3+}q_{3-}) - \mu_3^W + RT \cdot \ln(N_0^2\Lambda_{3+}^3\Lambda_{3-}^3) \quad \text{(A21)}$$

Likewise, for the chemical potential of a solute that is a 1:1 electrolyte, we can write the following statistical and classical thermodynamics expressions:

$$\mu_2^M\{C_2, C_3\} = W(2 + |C_2, C_3) + W(2 - |C_2, C_3) - kT \cdot \ln(q_{2+}q_{2-}) + 2kT \cdot \ln C_2 + kT \cdot \ln(N_0^2\Lambda_{2+}^3\Lambda_{2-}^3) \quad \text{(A22)}$$

$$\mu_2\{C_2, C_3\} = \mu_2^*\{C_3\} + 2RT \cdot \ln C_2 + 2RT \cdot \ln \gamma_{2\pm}^*\{C_2, C_3\} \quad \text{(A23)}$$

and the following expression for the mean ionic activity coefficient of the solute is obtained using Eq. A2:

$$2RT \cdot \ln \gamma_{2\pm}^*\{C_2, C_3\} = N_0W(2 + |C_2, C_3) + N_0W(2 - |C_2, C_3) - RT \cdot \ln(q_{2+}q_{2-}) - \mu_2^*\{C_3\} + RT \cdot \ln(N_0^2\Lambda_{2+}^3\Lambda_{2-}^3) \quad \text{(A24)}$$

The standard chemical potential in Eq. A23 is defined as the $C_2 \to 0$ limit of the difference $\mu_2 - 2RT \cdot \ln C_2$. Using this definition, the equality $\mu_2 = N_0 \cdot \mu_2^M$ (Eq. A2) and Eq. A22, we get,

$$\mu_2^*\{C_3\} = N_0W(2 + |C_3) + N_0W(2 - |C_3) - RT \cdot \ln(q_{2+}q_{2-}) + RT \cdot \ln(N_0^2\Lambda_{2+}^3\Lambda_{2-}^3) \quad \text{(A25)}$$

which upon substitution into Eq. A24 leads to,

$$2RT \cdot \ln \gamma_{2\pm}^*\{C_2, C_3\} = N_0[W(2 + |C_2, C_3) + W(2 - |C_2, C_3)] - N_0[W(2 + |C_3) + W(2 - |C_3)] \quad \text{(A26)}$$

which is entirely analogous to Eq. A15. We assume now that the solute ions, although distinguishable from the corresponding cosolvent ions, behave like the cosolvent ions in terms of the molecular interactions in solution and, therefore, that we are allowed to substitute 3 by 2 in the Gibbs energy interaction terms (as we did above to transform Eq. A15 into Eq. A16):

$$2RT \cdot \ln \gamma_{2\pm}^*\{C_2, C_3\} = N_0[W(3 + |C_3') + W(3 - |C_3')] - N_0[W(3 + |C_3) + W(3 - |C_3)] \quad \text{(A27)}$$

with $C_3' = C_2 + C_3$. Finally, we apply Eq. A21 to the concentrations C_3' and C_3, solve for $[W(2 + |C_3') + W(2 - |C_3')]$ and $[W(2 + |C_3) + W(2 - |C_3)]$, and substitute into A27 and arrive at

$$\ln \gamma_{2\pm}^*\{C_2, C_3\} = \ln \gamma_{3\pm}^W\{C_3'\} - \ln \gamma_{3\pm}^W\{C_3\} \quad \text{(A28)}$$

which is essentially equivalent to Eq. A17, although Eq. A28 explicitly recognizes that the cosolvent and the solute are 1:1 electrolytes.

The published $\gamma_{3\pm}^W$ coefficients for GdnHCl in water/GdnHCl (Makhatadze et al., 1993) can be described by the following equation (obtained through nonlinear, least-squares fitting):

$$\ln \gamma_{3\pm}^W = -0.699 \cdot C^{1/2} + 0.104 \cdot C + 4.692 \cdot 10^{-3} \cdot C^2 \quad \text{(A29)}$$

where we use C for the GdnHCl concentration but keep the symbol C_3 for the specific cosolvent concentration at which the solute activity coefficient is calculated, and C_3' for $C_3 + C_2$. Actually, Eq. A29 can be used with $C = C_3$ and $C = C_3'$, and the resulting expressions for $\ln \gamma_{3\pm}^W\{C_3'\}$ and $\ln \gamma_{3\pm}^W\{C_3\}$ can be substituted into A28 to yield,

$$\ln \gamma_{2\pm}^*\{C_2, C_3\} = -0.699 \cdot [(C_3 + C_2)^{1/2} - C_3^{1/2}]$$

$$+ 0.104 \cdot C_2 + 4.692 \cdot 10^{-3} \cdot [(C_3 + C_2)^2 - C_3^2] \quad \text{(A30)}$$

Equation A30 can indeed be used to calculate the activity coefficient of the hypothetical solute. Nevertheless, a more compact expression for $\ln \gamma^*_{2\pm}\{C_2, C_3\}$ can be derived if we take into account that the values of C_2 of interest here are rather low (of the order of 0.01 M) and, therefore, we can safely assume that the dependence of $\ln \gamma^W_{3\pm}$ with GdnHCl concentration is linear within the $C_3 - C_3'$ concentration range; accordingly, Eq. A28 can be written as,

$$\ln \gamma^*_{2\pm}\{C_2, C_3\} = \left(\frac{\partial \ln \gamma^W_{3\pm}}{\partial C}\right)_{C_3} \cdot (C_3' - C_3) = \left(\frac{\partial \ln \gamma^W_{3\pm}}{\partial C}\right)_{C_3} \cdot C_2 \quad \text{(A31)}$$

Note that Eq. A31 is simply a Taylor expansion truncated in the linear term (an excellent approximation in this case, due to the smallness of C_2). Equation A29 can now be differentiated and substituted into Eq. A31 to yield:

$$\ln \gamma^*_{2\pm}\{C_2, C_3\} = C_2 \cdot [-0.350 \cdot C_3^{-1/2} + 0.104 + 9.384 \cdot 10^{-3} \cdot C_3] \quad \text{(A32)}$$

For the solute concentrations of interest here, Eqs. A30 and A32 yield identical results, for all practical purposes (for instance, for $C_3 = 3$ M and $C_2 = 0.01$ M, Eq. A30 gives $\gamma^*_{2\pm} = 0.999301$, while Eq. A32 gives $\gamma^*_{2\pm} = 0.999306$). In particular, both equations indicate that $\gamma^*_{2\pm} \cong 1$ for GdnHCl concentrations of the order of 1 M and higher and for electrolyte (solute) concentrations of the order of 0.01 M (see Fig. 1). It is important to note that this result is entirely consistent with the simple electrostatic theory of ion-ion interactions in solution, as we show below:

The chemical potential of the solute can also be written as,

$$\mu_2\{C_2, C_3\} = \mu_2^W + 2RT \cdot \ln C_2 + 2RT \cdot \ln \gamma^W_{2\pm}\{C_2, C_3\} \quad \text{(A33)}$$

where the activity coefficient approaches unity as the solution becomes infinitely diluted in both the solute and the cosolvent. Accordingly, the standard chemical potential in Eq. A33 is defined through,

$$\mu_2^W = \lim_{C_2, C_3 \to 0}[\mu_2\{C_2, C_3\} - 2RT \cdot \ln C_2] \quad \text{(A34)}$$

Clearly, the $\gamma^W_{2\pm}$ coefficient in Eq. A33 measures solute-solute and solute-cosolvent interactions which are, in fact, ion-ion interactions because both the solute and the cosolvent are electrolytes. It follows that, if the solute and cosolvent concentrations are sufficiently low, we can use Debye-Hückel law to estimate $\gamma^W_{2\pm}$ and write Eq. A33 as,

$$\mu_2\{C_2, C_3\} = \mu_2^W + 2RT \cdot \ln C_2 - 2RT\mathrm{A}\sqrt{C_3 + C_2} \quad \text{(A35)}$$

where A is the Debye-Hückel constant (1.172 for aqueous solution and 25°C), and we have used that because the solute and the cosolvent are 1:1 electrolytes; the molar ionic strength is $I = C_2 + C_3$.

The chemical potential in Eq. A35 is, of course, the same as the one in Eq. A23. Equating the right-hand sides of Eqs. A35 and A23 and solving for the $\gamma^*_{2\pm}$ coefficient, we get

$$2RT \ln \gamma^*_{2\pm}\{C_2, C_3\} = \mu_2^W - \mu_2^*\{C_3\} - 2RT\mathrm{A}\sqrt{C_3 + C_2} \quad \text{(A36)}$$

The $\gamma^*_{2\pm}$ coefficient approaches unity as the solute becomes infinitely diluted for a given cosolvent concentration and, therefore, the standard chemical potential in Eq. A23 is defined by,

$$\mu_2^*\{C_3\} = \lim_{C_2 \to 0}[\mu_2\{C_2, C_3\} - 2RT \cdot \ln C_2] \quad \text{(A37)}$$

and using Eq. A35 for μ_2 in A37 we get,

$$\mu_2^*\{C_3\} = \mu_2^W - 2RT\mathrm{A}\sqrt{C_3} \quad \text{(A38)}$$

which can be substituted into A36 to yield,

$$\ln \gamma^*_{2\pm}\{C_2, C_3\} = -\mathrm{A}[\sqrt{C_3 + C_2} - \sqrt{C_3}] \quad \text{(A39)}$$

Eq. A39 can be used for some quick "back-of-the-envelope" calculations. Thus, assume that the solute concentration is $C_2 = 0.01$ M; then, for cosolvent concentrations $C_3 = 0$ M, $C_3 = 0.02$ M, $C_3 = 0.05$ M, and $C_3 = 0.1$ M, Eq. A39 gives the following values for the $\gamma^*_{2\pm}$ coefficient: 0.889, 0.963, 0.974, and 0.982, respectively. For molar cosolvent concentrations, Eq. A39 gives values of the activity coefficient that are essentially unity. Of course, the approximations behind the Debye-Hückel law only hold for very low salt concentrations. The point of the calculation is, however, to show qualitatively the following: 1) the $\gamma^*_{2\pm}$ coefficient measures the interactions between the solute ions; 2) these interactions are efficiently screened out by a cosolvent, which is itself a salt; 3) hence, the $\gamma^*_{2\pm}$ coefficient is expected to become unity at rather low cosolvent concentrations (of course, once the coefficient is unity, further increases in cosolvent concentration are not expected to change that value). These qualitative conclusions are in agreement with the $\gamma^*_{2\pm}$ values calculated for the hypothetical solute using Eqs. A30 or A32 (Fig. 1).

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