Urea, but not guanidinium, destabilizes proteins by forming hydrogen bonds to the peptide group

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The mechanism by which urea and guanidinium destabilize protein structure is controversial. We tested the possibility that these denaturants form hydrogen bonds with peptide groups by measuring their ability to block acid- and base-catalyzed peptide hydrogen exchange. The peptide hydrogen bonding found appears sufficient to explain the thermodynamic denaturing effect of urea. Results for guanidinium, however, are contrary to the expectation that it might H-bond. Evidently, urea and guanidinium, although structurally similar, denature proteins by different mechanisms.

denaturation | hydrogen exchange | osmolyte | solvation

Because of its fundamental importance, the study of protein molecular stability has engaged the attention of protein chemists for the last 100 years (1, 2). Experimental and theoretical investigations of protein stability have often focused on small-molecule osmolytes that can be used to modulate stability in vitro (3–6) and are used by virtually all organisms to counter biochemical stress in vivo (7). The mechanism of osmolyte action continues to be controversial. Opposing positions favor either a direct interaction between protein and osmolyte (8–11), such as hydrogen bonding, or an indirect effect mediated by the alteration of water structure (12, 13), or a mixture of both (14–19). It has been difficult to distinguish between these direct and indirect models because the osmolyte–protein interaction is so weak.

It is a thermodynamic truism (20–22) that the concentration of destabilizing osmolytes must be enriched relative to water molecules in the vicinity of the protein surface that becomes newly exposed upon unfolding (preferential interaction). In a thermodynamic sense, denaturing osmolytes "bind" selectively to the increased surface of unfolded proteins and thus bias the folded/unfolded equilibrium toward denaturation. Similarly, stabilizing osmolytes must be preferentially excluded from the protein surface. This is true independently of the physical mechanism of the "binding" or "antibinding" interaction. Thermodynamically based studies directed at the binding/antibinding problem have been designed to measure the transfer free energy of the various component groups of proteins between water and osmolyte solutions. Results show that the main-chain peptide group makes the largest contribution to the energetics, accounting for ≈80% of the effect of urea and of strong stabilizers such as trimethyl amine *N*-oxide (TMAO) (23). Similar determinations for guanidinium are not yet available, but it seems clear that guanidinium also favorably interacts with the peptide group (8, 11, 24).

To search for the mechanistic basis of thermodynamic destabilization due to urea and guanidinium, we tested the possibility that they exert their denaturing effect through peptide group hydrogen bonding. This can be done experimentally by the straightforward measurement of acid- and base-catalyzed hydrogen exchange (HX) in a small-molecule peptide model. Osmolyte that is H-bonded to the peptide NH site will block access of OH^- ion and thus inhibit base-catalyzed HX. H-bonding to the peptide carbonyl will inhibit carbonyl protonation and therefore interfere with acid-catalyzed HX.

Results

Hydrogen–deuterium exchange was measured by 1D NMR, as a function of pD in D_2O, for an alanine-based model compound containing 2 peptide groups (dialanine; Fig. 1). The HX rate follows the typical V-shaped curve (see Fig. 2), where the low and high pD branches represent specific acid-catalyzed and specific base-catalyzed exchange, respectively. Log (rate) vs. pD curves were fitted as explained in *Materials and Methods* to obtain acid- and base-catalyzed rate constants. Measured effects of added osmolytes, shown in the figures, are listed in Table 1.

Among the tested compounds, urea is the only one that conspicuously changes HX rate (see Figs. 2 and 3 and Table 1). Fitting of the data in Fig. 2 to Eq. **3** yields rate constants that increase (acid-catalyzed) or decrease (base-catalyzed) upon addition of urea. The changes in rate constant relative to buffer alone are shown in Fig. 2 *C* and *D*.

Urea Blocks Amide HX. We first consider the base-catalyzed HX rate, which strongly decreases upon addition of urea. In base-catalyzed HX, the peptide NH group transfers its proton to a transiently H-bonded hydroxide. If urea is H-bonded to the peptide NH, hydroxide cannot access the proton and HX is blocked. The degree of slowing is thus a direct measure of the degree of H-bonding of urea to the peptide NH. For example the relative rate in 3.8 M urea is 0.55, which corresponds to a time-averaged accessibility for the peptide NH of 55% exposed and 45% H-bonded.

Fig. 2*C* compares the relative rate with expectations for different urea interaction models. The dotted line corresponds to random urea–peptide encounter. Schellman suggested that volume fraction is a good measure of random interaction (25), and the line shown in Fig. 2*C* quantifies this suggestion. The HX data show that urea blocks the peptide NH to a higher degree than either random encounter or the thermodynamically neutral limit at which structure destabilizing peptide–urea binding would compensate for the stabilizing mutually excluded volume because of nonselective peptide group–urea steric interaction (often referred to as a crowding effect; see also *Discussion*).

Fig. 2*C* shows that the competition between urea and water for H-bonding to the peptide NH is well expressed in terms of Schellman's solvent exchange concept (20, 25). Urea that binds to the peptide group displaces water, and vice versa. Because urea is ≈3 times larger than water, 1 urea molecule should replace ≈3 water molecules. We take this number into account explicitly, extending the simplified Schellman treatment that 1

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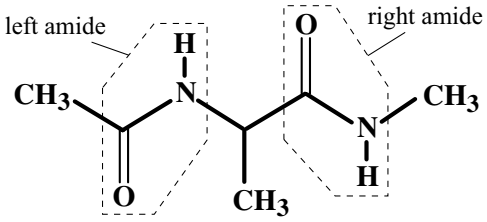

Fig. 1. Dialanine (*N*-acetyl-L-alanine *N*′-methylamide). H-D exchange of the 2 peptide groups, measured by NMR, was used to assess peptide H-bonding by urea and guanidinium.

urea replaces 1 water molecule (20). The expected fractional degree of binding of urea to a single peptide NH site is then given by Eq. **1**.

$$\bar{X}_{\text{urea}} = 1 - \frac{1}{1 + K\phi_{\text{urea}}/\phi^3_{\text{water}}}. \qquad [1]$$

Here, ϕ_{urea} and ϕ_{water} are the volume fractions of urea and water, respectively, and K is the exchange constant. Expected HX rate relative to 0 M urea is then given by Eq. **2**.

$$1 - \bar{X}_{\text{urea}} = \frac{1}{1 + K\phi_{\text{urea}}/\phi^3_{\text{water}}}. \qquad [2]$$

The experimental data are well represented by Eq. **2** using the single fitting parameter $K = 2.8$ (Fig. 2*C*).

Schellman estimated a smaller value for K_{average} of 1.2 based on the unfolding transitions of 5 proteins (25). However, this value refers to the average interaction site, not just the peptide NH, when 6–8 interaction sites per amino acid are assumed. In fact, side chains have slightly unfavorable urea interaction energetics (26), corresponding to $K < 1$. The backbone, however, has an energetically favorable urea interaction (26), which is reflected in the elevated K observed here. The value of $K = 2.8$ also fits well to the exchange constant obtained from the action of urea in denaturing alanine helices (27) when the different concentration scales (volume fraction vs. number fraction) and stoichiometries (3:1 vs. 1:1) used are taken into account. Similarly, the partition coefficient of urea between bulk solvent, and the peptide NH can be calculated to be ≈3 times larger than the value reported for the entire peptide group (28). This is understandable because the H-bonding site corresponds to only a small fraction of the peptide surface. These considerations favor the conclusion that the peptide NH is a major interaction site.

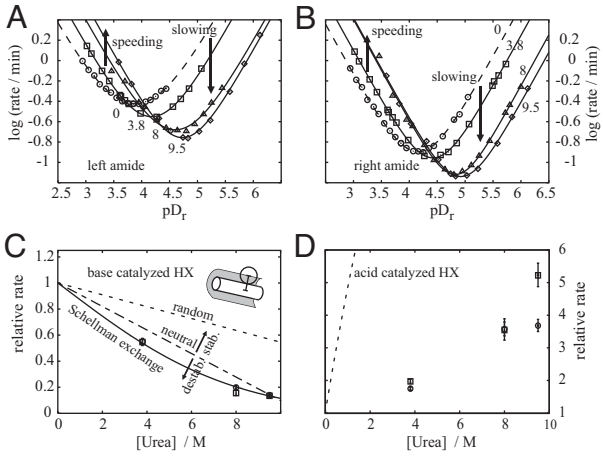

Fig. 2. Effect of urea on hydrogen exchange. (*A* and *B*) pD-dependent exchange rates for the left and right amide of dialanine at 0, 3.8, 8, and 9.5 M urea. Urea increases the acid-catalyzed rate and decreases the base-catalyzed rate. Lines are fits according to Eq. **3**. (*C*) Relative decrease of base-catalyzed rates in urea obtained from the fits in *A* and *B*. The rates fit well to Schellman's exchange model with an exchange constant of $K = 2.8$ (solid line, Eq. **2**). The broken lines show the effect on HX expected for random peptide–osmolyte encounter, and for thermodynamically neutral peptide–osmolyte interaction (zero urea excess or depletion). Thermodynamic neutrality occurs when the added occupancy due to selective binding becomes just equal to the random occupancy lost because of the volume exclusion illustrated in the *Inset*. (*D*) Relative increase in acid catalyzed HX rate in urea obtained from the fits in *A* and *B*. The dotted line shows the expected increase in acid-catalyzed rate based on the protonation pK_a difference between water and urea (see *Discussion*).

Urea May H-Bond to the Peptide Carbonyl. The acid-catalyzed HX rate in urea solution is higher than in the absence of urea (Fig. 2 and Table 1). This can be expected when it is understood that acid-catalyzed HX proceeds predominantly through transient protonation of the peptide carbonyl group (29) and deprotonation of the peptide NH by water or another general base like urea. In fact, calculations based on proton-transfer theory predict a larger acceleration of HX rate due to urea than is found. The shortfall could be for several reasons, one of which is urea binding to and blocking the protonation of the peptide carbonyl. Because of the complexity of the situation, it is difficult to reach a definite conclusion on this question (see *Discussion*).

Guanidinium Does Not H-Bond to the Peptide Group. Surprisingly GdmCl slows HX to only a minor degree (Table 1), even less than expected for random encounter (volume fraction). Furthermore, even the small effect seen for the GdmCl salt is accurately matched by NaCl (Fig. 3 *A* and *B*). In the case of specific base-catalyzed exchange, this is expected because the guanidinium cation cannot H-bond to and block the NH group. However, acid-catalyzed HX is also largely unaffected even though guanidinium is able to form a blocking H-bond to the peptide carbonyl. Evidently this does not occur.

Protecting Osmolytes Have Minor Effects. Polyols affect HX to only a small degree (Table 1). This is understandable because polyols are excluded from the peptide backbone (30). Consistent with a random effect equal to the volume fraction of osmolyte, 21% vol/vol of glycerol leads to 21–26% reduction in HX rate. Sorbitol, which is more excluded from the peptide than glycerol (30), has negligible effect.

The same data show that bulk viscosity does not play a noticeable role in osmolyte-dependent HX. The viscosity of the urea solutions used here ranges from 1.1 to 1.5 mPa·s (1 Pa·s = 10 Poise) (31). It is 2.1 mPa·s in the case of glycerol (32), and a little higher for sorbitol (33). The negligible viscosity effect is not surprising. Diffusion of the HX catalysts, H^+ and OH^- in water, occurs by structural diffusion (the redefinition of H to O bonds) rather than by molecular transport (34, 35) and so will be insensitive to bulk viscosity.

Water-Catalyzed Rate. We observe a significant decrease in water-catalyzed rate in all osmolyte solutions but the results are inconclusive. There is no obvious pattern, in part because the rates are not well determined, and the mechanism of water catalysis is not clear.

Discussion

Guanidinium Does Not H-Bond to the Peptide Group. Our data show that guanidinium affects HX rate no more than expected by random encounter. Evidently it does not selectively H-bond to the peptide group. This is understandable for base catalysis because guanidinium cannot H-bond to the peptide NH.

Acid-catalyzed HX can be blocked by compounds that H-bond

Table 1. Rate of acid-, base-, and water-catalyzed HX in various osmolyte solutions

	Concentrations		Relative rate*			Activity corrections	
	C/M	ϕ_V	Acid	Base	Water	a_W	ΔpOD^-
Buffer	–	–	1	1	1	1	0
Urea	3.8	0.17	1.9	0.55	0.6 ± 0.2	0.93	0.03
	8.0	0.36	3.6	0.18	0.5 ± 0.2	0.83	0.08
	9.5	0.43	4.5	0.14	0.35 ± 0.2	0.79	0.10
GdmCl	4.0	0.28	0.77	0.80	0.3 ± 0.1	0.87	0.06
NaCl	4.0	0.08	0.74	0.95	0.25 ± 0.1	0.84	0.08
Glycerol	2.9	0.21	0.74	0.79	0.7 ± 0.25	0.93	0.03
Sorbitol	1.5	0.18	0.96	1.1	0.5 ± 0.2	0.97	0.01

ϕ_V is volume fraction.
*Corrected for small activity changes (see *Methods*).

to either the peptide NH or carbonyl. Guanidinium could H-bond to the peptide carbonyl and thus block the carbonyl protonation step of acid-catalyzed HX, but this does not occur beyond the level of random encounter, again ruling out H-bonding. This is surprising because guanidinium is thought to H-bond to the peptide carbonyl (10, 11, 36–38). On the other hand, the absence of guanidinium to peptide H-bonding is consistent with the behavior of guanidinium in solution. It can, in principle, form H-bonds with water (39) but neutron diffraction experiments find that it has no recognizable hydration shell, presumably because of geometric constraints (18). Rather than forming an extensive H-bonding network, guanidinium tends to engage in transient stacking interactions with itself (39) and with other planar groups (10, 36, 39). Also, its low activity coefficient is indicative of guanidinium–guanidinium association (40). Crystallographic structures show the guanidinium group of arginine to be stacked against protein aromatic groups (41, 42) and asparagine side-chain amides (43) much more frequently than expected by chance. In a cocrystal structure of diglycine with guanidinium chloride, the guanidinium does not H-bond to the peptide group, but interacts with the carboxylic acid (44).

Guanidinium is often found to be approximately twice as efficient as urea in denaturing proteins but this varies with the protein target. Helical peptide studies show that guanidinium can be up to 4-fold more efficient than urea when planar amino acid side chains are major contributors to helical stability (45). In contrast, guanidinium is barely more efficient than urea if stabilization is due mainly to salt bridges, where urea plus NaCl can be used to simulate the guanidinium effect (46).

The finding that guanidinium promotes polyproline II (PPII) structure was originally taken to indicate that guanidinium H-bonds to the peptide group (38). In light of our finding that such H-bonding is absent, it is plausible that the mode of interaction that leads to the maximally exposed PPII main chain conformation is a space-demanding stacking of guanidinium against the peptide group.

These results together with the absence of H-bonding indicated by HX consistently argue that stacking rather than H-bonding is the most likely mode for guanidinium interaction with the peptide group.

The finding that guanidinium chloride and sodium chloride have comparable effects on HX is consistent with findings that they have similar propensities to accumulate close to peptide groups (47).

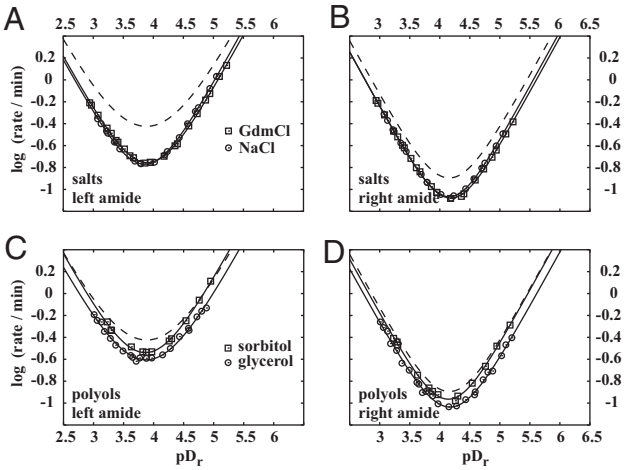

Fig. 3. Effect of salts and polyols on hydrogen exchange. (*A* and *B*) pD-dependent exchange rates for the left and right peptide groups of dialanine at 4 M GdmCl and NaCl. (*C* and *D*) pD-dependent exchange rates for the left and right peptide group of dialanine at 1.46 M sorbitol and 2.92 M glycerol. Solid lines are fits according to Eq. **3**. The dashed line is the reference curve in the absence of additive. Osmolyte concentrations are in Table 1.

Urea H-Bonds to the Peptide Group. The base-catalyzed HX results indicate that urea H-bonds to the peptide NH in excess of random and even thermodynamically neutral encounter (Fig. 2*C*). Interpretation of the acid-catalyzed HX result is more complex.

Acid-catalyzed HX proceeds predominantly through transient protonation of the peptide carbonyl group (29) and concomitant deprotonation of the peptide NH by water ($pK_a = -1.7$), or another general base like urea ($pK_a = -0.3$) (48). Normally, the leaving proton equilibrates rapidly within the peptide–catalyst encounter complex, before the complex separates. Therefore, on a per-molecule basis, urea should be a more efficient acceptor than water by a factor of $10^{1.7-0.3} = 25$. The base-catalyzed HX results indicate that the peptide NH group is occupied by urea, able to accept the NH proton, ≈45–86% of the time at 3.8 M to 9.5 M urea (Table 1). Thus, urea can be expected to accelerate acid-catalyzed HX by at least 10- to 20-fold, as obtained by weighting the factor of 25 according to the urea fractional occupancy (45–86%). However, the observed HX rate increases by only a factor of 2–5 (Fig. 2*D*), short of expectations by ≈75%. One possible reason is that urea also H-bonds to and blocks the peptide carbonyl. If both peptide carbonyl sites have to be urea-bound to block protonation, the degree of urea H-bonding to each carbonyl site ($0.75^{0.5} = 0.87$) would be similar to the urea occupancy of the peptide NH as determined by base-catalyzed HX. Similar H-bonding at both peptide NH and carbonyl sites is reasonable because urea is a very good H-bond donor and acceptor. Urea readily incorporates into the H-bonded network of water and H-bonds equally well with other urea molecules (49–53). Some MD simulations indicate that urea aggregates in preference to urea–water interaction, but this appears to be an

BIOPHYSICS

artifact of the OPLS force field (53, 54). Similarly, there are conflicting results with the CHARMM force field, sometimes indicating that urea does H-bond to the peptide carbonyl (10) and sometimes that it does not (55). Overall, urea is recognized to be a good H-bond donor and acceptor in aqueous solutions (unlike guanidinium), consistent with the supposition that urea may H-bond to both sites on the peptide group.

However, the shortfall in the observed rate of acid-catalyzed HX might also originate from other sources. The primary uncertainty lies in the relative lifetimes of peptide-bound protonated urea and water, which depends on their rates of dissociation. The diffusion-controlled separation of reactants depends on several potentially canceling contributions (diffusion coefficients, reaction distances, electrostatic interactions) (56). One cannot exclude the possibility that the above estimate for the expected rate should be increased or lowered based on those effects. Also, the expected fast rate of proton transfer within the encounter complex, necessary for the 25-fold factor, may be slowed if transfer is associated with changes in electronic or molecular structure (56). The most obvious example is peptide group tautomerization upon protonation, although this should not affect the relative rate of urea vs. water. Further, a combined Raman scattering and quantum mechanics study indicates that urea planarity is maintained (48). Therefore, it is unlikely that changes in electronic or molecular structure contribute significantly to the difference in acid-catalyzed HX between buffer and urea solutions.

Urea-Peptide H-Bonding Seems Sufficient to Account for the Thermodynamic Effect. Urea denatures proteins because it is enriched in the neighborhood of exposed peptide groups (26). The enrichment is relative to random urea distribution. So, in the process of converting the urea occupancy at the peptide NH to thermodynamic numbers, we must first subtract the effect of random occupancy from the HX data. As noted before, the volume fraction of an osmolyte may be used as a measure for random occupancy of the peptide H-bonding sites. Fig. 2*C* compares the expected effect of random occupancy of urea on peptide HX rate to the observed effect. The enrichment of urea at the peptide surface relative to bulk concentration is then given by the actual degree of occupancy of each H-bonding site minus the random occupancy (volume fraction).

Furthermore, the thermodynamically relevant enrichment is determined by the difference between depletion due to the mutually excluded volume between peptide and urea (stabilizing; see *Inset* in Fig. 2*C*) (25) and enrichment by direct or indirect interaction (destabilizing), perhaps because of H-bonding. Following Schellman (25), the number of volume-excluded urea molecules per peptide can be calculated as cV_{ex}, where c is urea concentration and V_{ex} is the overall excluded urea-to-peptide contact volume, ≈135 mL/(mol peptide) (30). Because a peptide group has 3 H-bonding sites, 1 at the NH and 2 at the CO, we assign a value of $cV_{ex}/3$ to each site. To compensate for the negative excluded volume contribution per site, $cV_{ex}/3$, urea has to exceed this excluded volume and the random effect, indicated in Fig. 2*C*, by selective interaction. On the neutral line in Fig. 2*C*, urea exclusion and enrichment by binding thermodynamically cancel. The difference between the neutral line ($1 - \phi_{urea} - cV_{ex}/3$) and the experimental data represents the contribution of H-bonding at the NH site to the thermodynamic excess of urea at the peptide group. The number calculated from the HX data are 0–0.1 urea molecules.

Representing the excluded volume by the contact volume implies that packing effects do not have to be taken into account explicitly. Packing may increase the urea concentration at the peptide surface, primarily in the first solvation shell. Because the HX data give information on urea molecules that are in direct contact with the peptide group, such effect should already be accounted for. Thus, the contact volume may but slightly overestimate the excluded volume because of packing.

How much does the peptide carbonyl contribute to the urea accumulation? One cannot give a definite answer solely based on the acid-catalyzed HX data. However, given the general behavior of urea in solution it is reasonable to assume that the 2 peptide carbonyl sites behave similarly to the peptide NH. The HX results are consistent with this assumption. One may then expect a total thermodynamic excess of approximately 3 times the effect at the NH site, 0–0.3 urea molecules per peptide group, depending on the urea concentration. The number determined by thermodynamic experiment in the concentration range studied here is 0.08–0.12 (30). The subtlety of the urea effect is demonstrated by the closeness to zero of both HX-derived and thermodynamically derived numbers. The HX results require substantial H-bonding of urea to the peptide group, which just overcompensates the excluded volume effect to an extent that can account for the thermodynamic effect. Thus, the peptide–urea interaction thermodynamics can be fully explained if urea H-bonds equally well to peptide NH and carbonyl. Less strong H-bonding at the carbonyl would be sufficient if we have overestimated the excluded volume because of packing.

In summary, whereas the present work demonstrates H-bonding of urea to the peptide NH, a similar degree of H-bonding also to the peptide carbonyl seems highly plausible, and can account for the observed thermodynamic effect.

End-Group Effects. Unlike the case for most protein peptide groups, it is possible that our results are affected by end-group effects because our model compound is small (57). End-group effects for peptide models are large in the presence of charged N and C termini (58). Our model does not have such charges. A previous HX study of charged glycine peptides in water and 6 M urea show a clear effect of the end-group charges on HX (59), but the general trend of a strong reduction of base-catalyzed HX rate upon addition of urea remains.

Conclusions

HX results show that urea H-bonds to peptide NH groups and probably also to peptide CO groups to a degree that is able to explain the thermodynamic effect of urea on the protein backbone. The HX results also demonstrate that guanidinium does not H-bond to the peptide group. Previous reports favor a modified direct interaction model that depends on stacking.

Materials and Methods

N-acetyl-L-alanine *N*′-methylamide was purchased from Bachem Bioscience. The NMR chemical shift of the peptide group on the right of the alanine side chain resonates relatively upfield (60).

Kinetic HX data were collected by 1D ^{1}H-NMR using a Varian 500-MHz spectrometer with cold probe. H-D exchange reaction was initiated by diluting 20 μL of the peptide (1.8 M in H_2O) into 640 μL of cosolute solution, buffered with 50 mM deuterated succinate in D_2O. The cosolutes were deuterated, except for glycerol and sorbitol. Samples were then pipetted into NMR tubes and loaded into the spectrometer, and sequential 1D spectra were recorded to follow the time-dependent loss of the NH resonances as H to D exchange proceeded. All sample handling and NMR experiments were at 20 °C.

HX rates were obtained from the time-dependent, monoexponential decrease in amplitude of NH resonance peaks. Data were plotted to produce V-shaped curves of $\log(k_{ex})$ vs. pD (see figures), and these were fit by Eq. **3**

$$k_{ex} = k_A\, 10^{-pD} + k_B\, 10^{-pOD} + k_W a_{D_2O} \qquad [3]$$

to obtain second-order rate constants for catalysis by acid (k_A), base (k_B), and water (k_W). The pD_r values in the figures are directly read from the pH electrode, thus requiring the usual correction by 0.4 units for use in Eq. **3** (61).

From pK_D-pD we obtain pOD. K_D was taken as $10^{-15.05}$ (62). Solution nonideality effects in water dissociation (pK_D) and water chemical activity (a_{D20}) were taken into account as follows.

The fit equation for the pD-dependent rate constant profiles (Eq. **3**) requires knowledge of pD, pK_D, and a_{D20}. High-precision osmotic coefficient data ϕ_{osm} are available for all of the additives used here, and thus we can calculate the chemical activity of water

$$a_{D_2O} = \mathrm{Exp}(-\phi_{osm} M_W m_{osm}/1000\ \mathrm{g/kg}), \quad [4]$$

where M_W is the molecular weight of water, m_{osm} is the total molality of additives, and ϕ_{osm} is the osmotic coefficient.

The pH meter reading with the usual correction of 0.4 (61) is assumed to directly represent the chemical activity of D^+, because changes in electrode junction potential may be neglected in osmolyte solution (63, 64). Calculation of pOD is based on the thermodynamic condition for water dissociation:

$$\mu_{D^+} + \mu_{OD^-} = \mu_{D_2O}. \quad [5]$$

The chemical potentials μ are formulated with infinite dilution as a standard state for the ionic species, and the pure substance for water:

$$\mu^0_{D^+} + RT\ln(a_{D^+}) + \mu^0_{OD^-} + RT\ln(a_{OD^-}) = \mu^0_{D_2O} + RT\ln(a_{D_2O}), \quad [6]$$

or

$$\mu^0_{D^+} - \mathrm{pD}\cdot RT\ln 10 + \mu^0_{OD^-} - \mathrm{pOD}\cdot RT\ln 10 = \mu^0_{D_2O} + RT\ln(a_{D_2O}). \quad [7]$$

The standard chemical potentials represent pK_D, and so we get

$$\mathrm{pOD} = \mathrm{p}K_D - \mathrm{pD} - \frac{\ln(a_{D_2O})}{\ln 10} = \mathrm{p}K_D - \mathrm{pD} + \frac{\phi_{osm} M_W m_{osm}}{\ln 10\cdot 1000\ \mathrm{g/kg}}. \quad [8]$$

Note that all nonideal contributions are taken into account in the chemical activities a_i, and thus pK_D is defined as constant at infinite dilution of osmolyte. The numbers are given in Table 1, as obtained from previously published data (40, 65).

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