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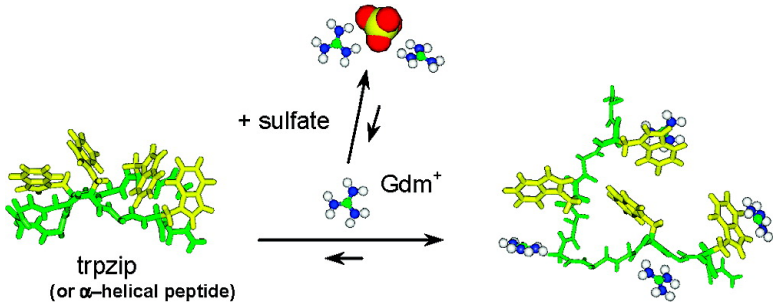

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The Reversal by Sulfate of the Denaturant Activity of Guanidinium

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Abstract: Guanidinium (Gdm^+) chloride is a powerful protein denaturant, whereas the sulfate dianion (SO_4^{2-}) is a strong stabilizer of folded protein states; Gdm_2SO_4 is effectively neutral in its effects on protein stability. While the "neutralizing" effects of protein-stabilizing solutes on the activity of denaturants can be broadly interpreted in terms of additive effects of the solutes, recent experimental and simulation studies support a role for hetero-ion interactions in the effect of sulfate on Gdm^+ denaturation [Mason, P. E.; et al. *J. Phys. Chem. B* **2005**, *109*, 24185−24196]. Here we describe an experimental strategy for testing this mechanism that involves spectroscopic analysis of the separate effects of alkali metal sulfates (Na_2SO_4, Rb_2SO_4), GdmCl, and Gdm_2SO_4 on the folded populations of several peptides chosen to dissect specific noncovalent contributions to the conformational stability of proteins [alanine-based helical peptides stabilized by hydrogen bonds, tryptophan zipper (trpzip) peptides stabilized largely by cross-strand indole−indole interactions]. While the trpzip peptides are highly sensitive to GdmCl denaturation, they are unaffected by NaCl, Na_2SO_4, or Gdm_2SO_4, indicating that the reversal of the denaturant activity of Gdm^+ by sulfate in this case is not due to competing stabilizing (sulfate) and destabilizing (Gdm^+) interactions. Gdm_2SO_4 was found to retain considerable denaturant activity against alanine-based α-helical peptides. The differences in the effects of Gdm_2SO_4 on the two peptide types can be understood in terms of the different mechanisms of Gdm^+ denaturation of trpzip peptides and helical peptides, respectively, and the specific nature of Gdm^+ and SO_4^{2-} ionic "clustering" that differentially affects the ability of Gdm^+ to make the molecular interactions with the peptides that underlie its denaturant activity.

1. Introduction

High concentrations of neutral and charged solutes can have profound effects on the conformational stabilities of proteins in solution.[1,2] Protein denaturants, urea and guanidinium (Gdm^+) in particular, have been used for more than 50 years to modulate the equilibria between the unfolded and folded states of proteins. These phenomena have been utilized to great effect in advancing our understanding of the thermodynamic stabilities of proteins in their native conformations and the pathways by which these native states are attained.[3] In addition, natural systems have evolved for the protection of native protein states in some cells that are subject to high concentrations of denaturating solutes.[2] A classical example is the production of trimethylamine-*N*-oxide (TMAO) as a protecting solute to counter the effects of urea in several cell types.[4]

At first sight, the ability of protein-stabilizing solutes to counteract the effects of protein denaturants seems simple to understand. One of the more reliable correlates of solute effects on protein conformational stability is the extent to which solutes are either excluded from the protein surface (stabilizing solutes),[1c,2,5] or concentrated at the protein surface (protein denaturants)[6,7] relative to the bulk solute concentration. Proteins adopt conformations that either minimize their exposed surface (folded states) in the presence of excluded solutes or maximize their exposed surface (unfolded states) in the presence of solutes that preferentially partition at protein surfaces. A combination of excluded and surface-partitioning solutes might be expected to have roughly additive effects on protein stability, and such an additive contribution has been described for combinations of uncharged solutes (urea and TMAO,[8] or urea and sarcosine[9]) to the stabilities of α-helical peptides and a small protein.

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The situation is likely to be more complex for charged solutes since the combination of protein-stabilizing (excluded) ions with protein-destabilizing (preferentially partitioning) ions would seem to require a degree of charge separation in solution in order for additive contributions to be manifest within the paradigm of preferential partitioning. On the other hand, ionic solute pairs do exhibit what approximates to additive effects on protein stability. Thus, guanidinium chloride is a powerful protein denaturant,[1,3,6] sodium sulfate stabilizes folded protein states,[1c,1d,10] and Gdm_2SO_4 is effectively neutral in its effects on protein stability.[11]

We have recently proposed an alternative explanation for the reversal of guanidinium denaturant activity by sulfate, based on neutron diffraction measurements of solutions of guanidinium salts combined with molecular dynamics simulations.[12,13] Highly denaturing salts (GdmCl and GdmSCN) exhibit characteristic Gdm^+-Gdm^+ self-interactions in solution in which the poorly hydrated surfaces above and below the guanidinium molecular "plane" result in "stacking" in solution, rather similar to that expected from planar hydrophobic groups like aromatic rings. The hydration properties of Gdm^+ (see ref 14) can account for some of the preferential interaction of this denaturing cation with the protein surface since multiple weak interactions can be made with a variety of side-chain groups in proteins.[14−16] Simulations of Gdm_2SO_4, on the other hand, exhibit a destruction of Gdm^+ self-stacking as a result of strong ion-pairing with sulfate to produce long-range clustering of sulfate and Gdm^+ ions.[13] It is easy to see how such a phenomenon would attenuate the denaturing activity of Gdm^+. Rather than approximately additive effects of a strongly denaturing cation (Gdm^+) and a strongly stabilizing anion (sulfate), sulfate in this model acts directly to limit access of Gdm^+ to the protein surface. In this case, arguments based on preferential partitioning do not require charge separation in solution (sulfate exclusion and Gdm^+ preferential partitioning with respect to the protein surface).

Several further experimental expectations are implicit in this model for the effect of sulfate on Gdm^+ denaturing activity, one of which is explored in this study. This relies on the possibility of identifying stabilizing interactions that contribute to the thermodynamic stability of protein folded states, which are susceptible to the denaturing effects of Gdm^+ but are not enhanced by sulfate. If the effects of sulfate (stabilizing) and Gdm^+ (denaturing) are approximately additive, then sulfate should be relatively ineffective in attenuating the effects of Gdm^+ on these interactions. Alternatively, in the ion-pairing scenario, sulfate should retain the ability to attenuate the denaturing activity of Gdm^+. In the study reported here, we have assessed the effects of stabilizing sulfate salts (sodium and rubidium sulfate), the denaturing chloride salt of Gdm^+, and the relatively "neutral" salt Gdm_2SO_4 on several partially folded polypeptides chosen to represent different contributions to the conformational stability of proteins. We find that one class of peptides fulfils exactly the criteria of high Gdm^+ sensitivity and insensitivity to sulfate (and Gdm_2SO_4) and that solute effects on each of the peptides studied can be interpreted in terms of a significant role for ion association in concentrated aqueous solution.

Table 1. Peptide Sequences[a]

alahel	Ac-AAQAAAEQAAAAQAAY-NH_2
alahel-E2	Ac-AEQAAAAQAAAAQAAY-NH_2
alahel-21-E2	Ac-AEQAAAAQAAAAQAAAAQAAY-NH_2
trpzip1	S**W**T**W**EGNK**W**T**W**K-NH_2
trpzip2	S**W**T**W**ENGK**W**T**W**K-NH_2
MrH4a	KK**L**T**V**SINGKK**I**T**V**SA

[a] The amino acid side chains involved in stabilizing interactions are highlighted for the β-hairpin peptides.

2. Methods

2.1. Peptide Synthesis, Purification, and Characterization. The peptides (see Table 1) were synthesized by Dr. G. Bloomberg of the Bristol Centre for Molecular Recognition using Fmoc chemistry. Using methods previously described for peptide purification and characterization by our group,[15] each peptide was purified by HPLC and confirmed to be at least 96% pure by analytical HPLC and to have the predicted *m*/*e* ratio by mass spectrometry.

2.2. Spectroscopic Measurements. Spectroscopic measurements were made in a buffer composed of either 10 mM potassium phosphate (pH 7.0 or 3.0) or 10 mM Tris-HCl, pH 7.5. Peptide concentrations of stock solutions were measured using either the Tyr absorbance of alahel peptides (ϵ_{275} = 1450 $M^{-1}\cdot cm^{-1}$) or the Trp absorbance of trpzip peptides (ϵ_{280} = 5600 $M^{-1}\cdot cm^{-1}$ per mole of Trp). The concentration of MrH4a was estimated as described previously.[17] Note that we use the shorthand "alahel" for the class of alanine-based helical peptides designed by Scholtz and Baldwin[18] (see Table 1). Circular dichroism (CD) spectra were obtained in quartz cuvettes having path lengths of 0.1 mm, 1 mm, 2 mm, or 1 cm, using a Jobin-Yvon CD6 spectrapolarimeter with the temperature of the cuvette holder maintained using a Haake circulating waterbath. Accurate temperatures were obtained by direct measurement within samples using a Hanna H198801 thermocouple thermometer. All solute stock solutions were made by dissolving the required weight of solute into a minimal volume of buffer, readjusting the pH (with orthophosphoric acid or potassium hydroxide for phosphate solutions, and with HCl for Tris-HCl solutions), and finally adjusting the solution to the correct volume with the appropriate buffer.

2.3. Analysis of Spectroscopic Data. 2.3.1. β-Hairpin Peptides. The CD data for trpzip peptides were collected in molecular ellipticity mode and converted to mean residue ellipticity, θ, after subtracting the appropriate blank spectra. The mean residue ellipticity (θ_{227}) at 227 nm was used as a measure of the folded state for trpzip peptides. We used the data of Cochran et al.[19] and estimated f_B for trpzip1 as 0.60 at 42 °C, where f_B is the fraction of folded (β-hairpin) peptide. Using this estimate, we calculated f_B of trpzip2 at 42 °C to be 0.85, in excellent agreement with the data of Cochran et al.[19] A value for θ_u, the mean residue ellipticity at 227 nm for the unfolded peptide, of 2450 $deg\cdot cm^2\cdot mol^{-1}$ was determined from the spectrum of KWTWK-NH_2. Values for f_B at each addition of solute were determined for both trpzip1 and trpzip2, using eq 1, in which θ_{max} = 76 750 $deg\cdot cm^2\cdot dmol^{-1}$ and

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$$f_B = \frac{\theta - \theta_u}{\theta_{max} - \theta_u} \quad (1)$$

$\theta_u = 2450$ deg·cm^2·dmol^{-1}. Equivalent data for MrH4a, based on mean residue ellipticities at 217 nm, were used in eq 1 as follows: $\theta_u = 835$ deg·cm^2·dmol^{-1} (measured from the C-terminal octapeptide, GKKITVSA) and $\theta_{max} = 14\,000$ deg·cm^2·dmol^{-1}, which assumes that the maximum sulfate-induced signal (see Results) corresponds to 100% β-structure.

2.3.2. Helical Peptides. Data were collected in molecular ellipticity mode and were converted to mean residue ellipticity, θ, after subtracting the appropriate blank spectra. The fraction of helix (f_H) was determined from the θ_{222} value using eq 2,[20] where T is the temperature in °C, θ_r

$$f_H = \frac{(\theta_{222} - \theta_r)}{(1 - 3/N_r)(-44000 + 250T) - \theta_r} \quad (2)$$

is the temperature-dependent value of θ_{222} for the random coil form of the peptide, and the constant $(1 - 3/N_r)$ corrects for the three non-hydrogen-bonded amide carbonyls in a C-terminally carboxamidated peptide having N_r residues. We estimated that θ_r has a value of $(2200 - 53T)$ from that data plotted in Figure 2 of ref 20. We analyzed the denaturant dependence of helix content using the Zimm−Bragg theory,[21] in which the value of f_H is fitted to eq 3, to determine a value for s, the

$$f_H = \frac{\sigma s}{(s-1)^3}\left(\frac{(ns^{n+2} - (n+2)s^{n+1} + (n+2)s - n)}{n\{1 + [\sigma s/(s-1)^2][s^{n+1} + n - (n+1)s]\}}\right) \quad (3)$$

helix propagation parameter. The analysis essentially followed Baldwin and colleagues,[22] in which the helix nucleation constant, σ, was fixed at 0.003, and n is the number of amide bonds in the peptide. We used the linear extrapolation method (LEM)[23] for analyzing the denaturation concentration dependence of s according to eq 4, where s_0 is s in the

$$\ln s = \ln s_0 - \frac{m[\text{denaturant}]}{RT} \quad (4)$$

absence of denaturant and R is the gas constant (1.987 cal·mol^{-1}·K^{-1}); m is the *per-residue* Gibbs energy of helix propagation as a function of the molar denaturant concentration.

3. Results

Since solute-induced conformational stabilization *and* destabilization were of interest, spectroscopic analyses were made at temperatures where appreciable amounts of both folded (30−50%) and unfolded peptide conformations coexist. Chosen temperatures were 15 °C for alahel-E2, 25 °C for Alahel-21-E2, 42 °C for trpzip1, and 50 °C for trpzip2. MrH4a has marginal folded state stability (no more than 20%) as a monomer at low pH at its temperature of maximum stability (31 °C), and *destabilizing* solute effects were not considered reliable for quantitative analysis of MrH4a.

3.1. Tryptophan Zipper Peptides. Trpzip1 is very sensitive to the denaturing activity of guanidinium chloride.[15] Since rubidium is a weak denaturant of trpzip1[17] and potassium sulfate has low aqueous solubility, we used the sodium salt of sulfate to assess the effect of the latter anion on the conformational stability of trpzip1. In addition, we measured all data on trpzip1 and trpzip2 at pH 3.0 to eliminate any contributions to stability

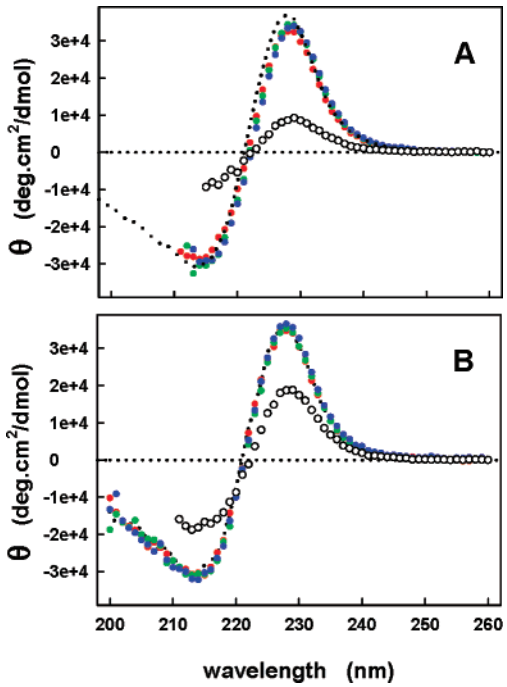

Figure 1. Circular dichroism spectrum of trpzip1 (40 μM) in 10 mM potassium phosphate buffer, pH 3.0, at 42 °C. Panel A: dotted line, no additional solute; open circles, plus 6 M GdmCl; colored circles, plus Gdm_2SO_4 at 1 M (red), 2 M (green), and 3 M (blue) concentrations. Panel B: dotted line, no additional solute; open circles, plus 1.5 M GdmCl; colored circles, plus Na_2SO_4 at 0.5 M (red), 1 M (green), and 1.5 M (blue) concentrations.

from salt bridge interactions involving the E5 residue of the peptide. Figure 1 illustrates the effects of GdmCl, Gdm_2SO_4, and Na_2SO_4 on the conformational stability of trpzip1, as determined by the intensity of the CD spectral maximum at 227 nm resulting from exciton coupling between tryptophan indole side chains in close proximity.[19] Since the interacting indole groups are on opposing peptide strands of the β-hairpin structure,[19] this signal provides a clear signature of the folded state structure as described in detail previously.[15,19]

At 42 °C, pH 3.0, the intensity of the positive CD signal at 227 nm corresponds to 45% of the native state signal for trpzip1 (Figure 1). In the presence of 6 M GdmCl, trpzip1 is 11% folded under these conditions (Figure 1a). Gdm_2SO_4 at concentrations up to 3 M (corresponding to 6 M Gdm^+) causes a small shift in the position of the spectral maximum by 1−2 nm but has only a very small effect on the intensity of the maximum. At 3 M Gdm_2SO_4, trpzip1 is still 42% folded under these conditions. This is consistent with previous observations that sulfate effectively reverses the denaturant activity of Gdm^+.[1d,11]

Despite the reversal of the denaturant activity of Gdm^+ on trpzip1, Na_2SO_4 itself has a negligible effect on the folding equilibrium of trpzip1 at 42 °C, pH 3.0 (Figure 1b). Although the effects can only be measured up to a concentration of 1.5 M Na_2SO_4, due to the limited solubility of this salt and a tendency to precipitate trpzip1 at high concentrations, this is sufficient to illustrate only very limited folded state stabilization (around 45% folded to 47% folded at 1.5 M Na_2SO_4). GdmCl at this concentration (1.5 M) considerably shifts the folding equilibrium toward the unfolded state (from 45% folded to 27% folded). These results demonstrate that the direct stabilization of the folded state structure of trpzip1 at 42 °C, pH 3.0, is not

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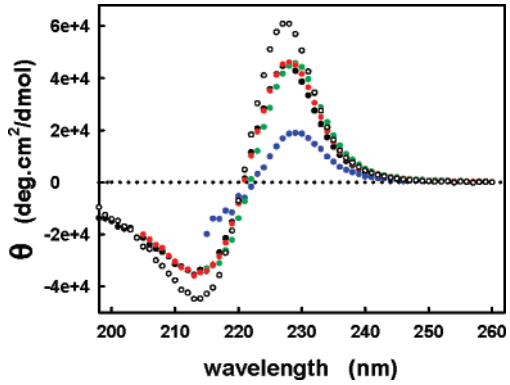

Figure 2. Circular dichroism spectrum of trpzip2 (40 μM) in 10 mM potassium phosphate buffer, pH 3.0, at 50 °C (●) and with the addition of either 2 M Na_2SO_4 (red), 3 M Gdm_2SO_4 (green), or 6 M GdmCl (blue). The spectrum of trpzip2 in potassium phosphate buffer, pH 3.0, at 20 °C is also shown (○).

a factor in the reversal of the denaturant activity of Gdm^+ in the mixed salt (Gdm_2SO_4).

We explored the generality of these observations for the indole−indole interaction (trpzip peptides) with a similar series of experiments on trpzip2, a peptide having considerably higher native state stabilization due to a more thermodynamically favorable β-turn tetrapeptide sequence.[19] At 20 °C, pH 3.0, trpzip2 is 85% folded. The folding equilibrium is shifted toward the unfolded state upon warming, so that at 50 °C (pH 3.0), trpzip2 is 56% folded (Figure 2). As with trpzip1, 6 M GdmCl greatly reduces the proportion of the folded state to around 20% (50 °C, pH 3.0), whereas the folded state equilibrium is essentially unaffected by 3 M Gdm_2SO_4 (equivalent to 6 M Gdm^+) and 2 M Na_2SO_4 (Figure 2). These results are entirely consistent with those observed for trpzip1.

3.2. Alanine-Based α-Helical Peptides. The effects of GdmCl on the attenuation of hydrogen-bonded structure in monomeric alanine-based α-helices have been described before.[15,24] To assess the effects of sulfate salts on helical peptide stability, we chose to study a 16-residue polypeptide, alahel-E2 (peptide "E2" in the designation of Scholtz et al.[18]), for two reasons. Initial studies on the effects of sulfate salts of alkali metal cations on alahel (see sequence in Table 1) indicated that the stabilizing effect of sulfate tends to saturate at concentrations above around 1 M solute for this peptide; indeed, the solute effects on helical stability could be fitted to a weak binding equilibrium (not shown), and similar nonlinear effects of sulfate salts on helical peptide stability have been described before.[25] We reasoned that any contributions to helical peptide stability due to sulfate binding would likely involve the N-terminal helix dipole charge (positive) and the non-hydrogen-bonded peptide amide NH groups at the N-terminus. Moving the glutamic acid residue from residue 7 in alahel to residue 2 in alahel-E2 should neutralize the N-terminal helix dipole charge and attenuate potential sulfate binding through charge repulsion. Second, the E2 residue might be expected to reduce charge-screening effects on helical stability that act independently of the Hofmeister effects of interest in our study. In an N-terminally acetylated and C-terminally amidated peptide, the helix dipole charges at both the N- and C-termini suppress helix stability, resulting in

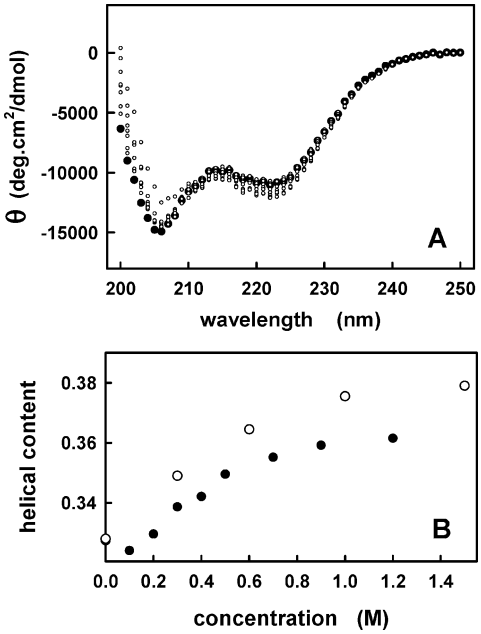

Figure 3. (A) Circular dichroism spectrum (mean residue ellipticity, θ) of alahel-E2 (40 μM) in 10 mM potassium phosphate buffer, pH 7.0, at 15 °C (●), and in the presence of Rb_2SO_4 (○) at the concentrations indicated by the filled circle data points in panel B. (B) Helical content of alahel-E2 (40 μM) in 10 mM potassium phosphate buffer, pH 7.0, as a function of the concentration of Rb_2SO_4 (●) or Na_2SO_4 (○).

significant charge-screening (stabilizing) effects of electrolytes.[25,26] The E2 residue of alahel-E2 stabilizes the α helix by neutralizing the N-terminal helix dipole charge, and the effect of salt is to attenuate this stabilizing interaction (destabilizing the N-terminal region of the helix), while stabilizing the C-terminus. This compensating effect of N-terminal helix destabilisation and C-terminal stabilization by salts renders the peptide overall less affected by electrolyte effects on helix stability arising from helix dipole charge interactions. In accordance with the expected effect of a glutamic acid at residue 2, alahel-E2 (33% helical) is more stable than alahel (27% helical) at 15 °C in water at pH 7.

Despite using a peptide chosen to suppress potential sulfate binding near the N-terminus, and generalized electrolyte effects resulting from interaction with helix dipole charges, the effect of alkali metal sulfate salts on the helical stability of alahel-E2 remain nonlinear and saturate at solute concentrations approaching the maximum solubility of these salts (Figure 3). The stabilizing effects of Na_2SO_4 and Rb_2SO_4 are small (33−38% helix content at saturating concentrations of Na_2SO_4; 33−36% helix at saturating concentrations of Rb_2SO_4). Although these stabilizing effects of sulfate on helical stability (and their nonlinear nature) are small, they are reproducible, being observed with all of the helical peptides studied, as well as being reported in an earlier study.[25] We suspect that nonlinear effects of Na_2SO_4 relate to concentration-dependent association of Na^+ and SO_4^{2-} ions for several reasons described more fully in the Discussion. First, the concentration dependence of the number density (average number of nuclei per Å^3) of Na_2SO_4 is nonlinear, tailing off at high solute concentrations, consistent

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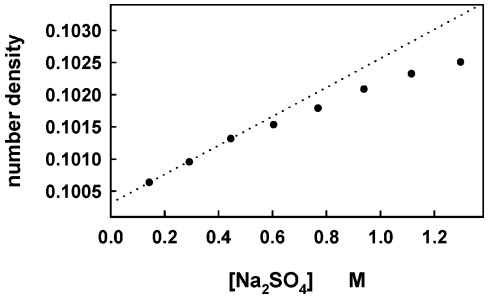

Figure 4. Number density (atomic nuclei per Å^3) of Na_2SO_4 solutions as a function of molar solute concentration in water. The dotted line is an extrapolation of a linear regression of the first three data points. The number densities were calculated from the physical densities of Na_2SO_4 solutions (grams per cm^3) tabulated in ref 27, knowing the atomic composition of the solution at each solute concentration.

with concentration-dependent weak ionic association (Figure 4). This curvature must be due to a second-order effect such as ion-pairing. There are two factors that affect the number density of a solution at infinite dilution: the volume of the ion and the packing of water around that ion. Both of these factors should yield a linear dependence of number density on concentration. However, as the ions start to associate at higher concentrations, second-order effects (release of some hydration shell waters into bulk solution) will be observed, as seen in Figure 4. The effects are relatively small, since even in a 1.5 M Na_2SO_4 solution, only about 6 atom % of the solution is ions, and so any second-order effects on the number density are expected to be of the order of a few percent. Interestingly, the number density plots tail off toward the concentrations where these salts precipitate (around 1.7 M Na_2SO_4 at 15 °C and 1.9 M Na_2SO_4 at 25 °C, in our studies).

As expected, alahel-E2 is denatured by GdmCl (Figure 5a). Suprisingly, Gdm_2SO_4 also considerably destabilizes alahel-E2 (Figure 5b), and a very similar attenuation of α-helical structure in alahel by Gdm_2SO_4 was also observed (not shown). The effects of GdmCl and Gdm_2SO_4 are characteristic of Hofmeister effects in which a linear contribution (destabilizing) to the free energy of the folded state is maintained over a large solute concentration. This can be expressed as an *m* value describing the per-molar contribution of the solute to the free energy of the folded state (eq 4). The *m* value is the gradient of the linear relationship between the free energy of the folded state (expressed, for an α-helical peptide, through the Zimm−Bragg analysis) and the solute concentration (Figure 6a). The *m* values for denaturation of alahel-E2 by GdmCl and Gdm_2SO_4 are 34 and 17 kcal·mol^{-1}·M^{-1}, respectively, *expressed per mole of* Gdm^+. In fact, when expressed in terms of *absolute* molarity (comparing the per-mole effects of GdmCl and Gdm_2SO_4), Gdm_2SO_4 is virtually indistinguishable from GdmCl as a denaturant of alanine-based helical peptides.

Analyzing the data for Na_2SO_4 and Rb_2SO_4 using the Zimm−Bragg analysis and plotting this with the data for GdmCl and Gdm_2SO_4 (Figure 6a) reinforces the fact that the effects of the alkali metal sulfate salts on helical stability are highly nonlinear and are small, especially at concentrations above ∼0.5−0.8 M. We made a separate series of measurements on a peptide equivalent to alahel-E2 but extended by one 5-amino acid repeating unit (alahel-21-E2) in an attempt to minimize "end effects" that might relate to sulfate binding or helix dipole charge effects. However, the data were very similar to those with alahel-

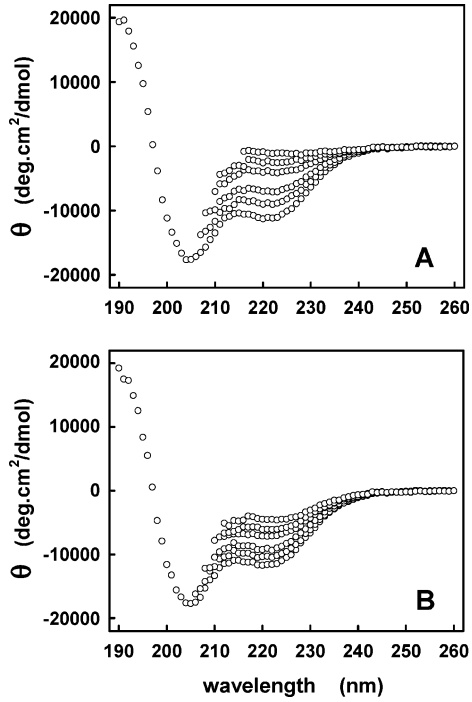

Figure 5. (A) Circular dichroism spectrum (mean residue ellipticity, θ) of alahel-E2 (40 μM) in 10 mM potassium phosphate buffer, pH 7.0, at 15 °C in the presence of GdmCl at the following molar concentrations (bottom to top): 0, 0.5, 1, 2, 3, and 4 M. (B) As in panel A, but the solute is Gdm_2SO_4 at the following molar concentrations (bottom to top): 0, 0.2, 0.5, 1, 1.5, and 2 M.

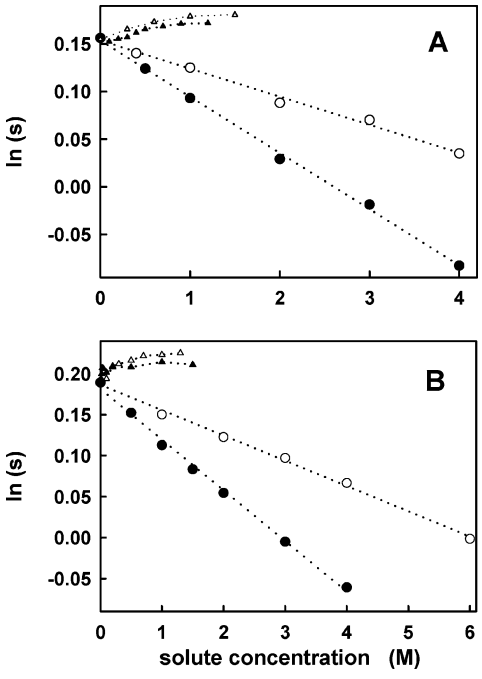

Figure 6. Denaturant dependence of ln(*s*) for alahel-E2 (A) and alahel-21-E2 (B) at 15 °C, pH 7.0, in the presence of GdmCl (●) and Gdm_2SO_4 (○). Note that the solute concentration for Gdm_2SO_4 is per mole of Gdm^+ (i.e., absolute concentrations of Gdm_2SO_4 are 0.5 times those indicated). Dotted lines for the GdmCl and Gdm_2SO_4 are linear regression fits to the data. Also shown are equivalent data for the effects of Rb_2SO_4 (▲) and Na_2SO_4 (△) on peptide stability, calculated from the data in Figure 3 for alahel-E2, and from equivalent data (not shown) for alahel-21-E2. For these data, the dotted lines are drawn to guide the eye.

E2, with small, nonlinear effects on helical stability induced by alkali metal sulfate salts and *m* values for GdmCl denatur-

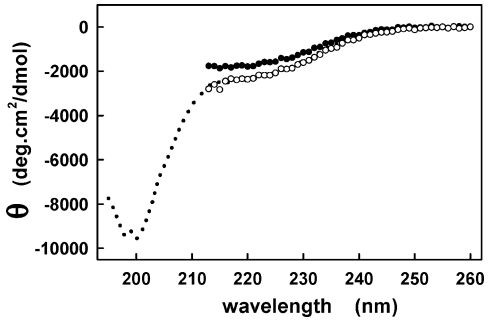

Figure 7. Circular dichroism spectrum (mean residue ellipticity, θ) of MrH4a (160 μM) in 10 mM potassium phosphate buffer, pH 3.0, at 31 °C (dotted line) and in the presence of 3 M Gdm_2SO_4 (○) or 4 M GdmCl (●).

ation (34 kcal·mol^{-1}·M^{-1}) and Gdm_2SO_4 denaturation (17 kcal·mol^{-1}·M^{-1}) very similar to those for alahel-E2 (Figure 6b). These observations reinforce the conclusion that the nonlinear effects of alkali metal sulfate salts on helical peptide stability are not due to end effects (such as sulfate binding to the peptide N-termini) but arise from weak ionic association between alkali metal ion and sulfate that eventually leads to precipitation of the salt. The retention of significant denaturant activity in Gdm_2SO_4 against alanine-based peptides is not the first example of anomalous solute effects on this class of peptides. For example, Courtenay et al. calculated denaturant sensitivities (*m* values) of similar peptides that were significantly larger than expected on the basis of calculated surface area exposed upon unfolding.[28]

3.3. MrH4a. This peptide,[29] one of a series of small β-hairpin peptides based on a design to mimic the DNA-biding domain of the *met* repressor,[30] is stabilized by a cluster of aliphatic amino acid side chains and potentially provides a model for exploring solute effects on polypeptide structure stabilized by the "classical" hydrophobic effect. Four aliphatic amino acid side chains (L3, V5, I12, V14), two from each of the antiparallel peptide strands of the β-hairpin, constitute the stabilizing cluster, and the peptide shows a mild cold denaturation, with a maximum proportion folded at 31 °C.[29] The burial of hydrophobic surface in the folded state is small, however, and the peptide is no more than 20% folded at 31 °C at low pH in the absence of a stabilizing salt bridge.[17,29] The peptide is unaffected by 3 M Gdm_2SO_4 and retains ~75% of θ_{217} in the presence of 4 M GdmCl (Figure 7). The latter observation cannot reliably be interpreted in terms of resistance of cross-strand interactions to GdmCl in MrH4a, since the folded state population is already small, some of the ellipticity might arise from the inherent stability of the optimized turn sequence in MrH4a,[30] and even unfolded peptides can show some negative ellipticity at 217 nm. However, the peptide undergoes a marked (and reversible) sulfate-induced folding transition (Figure 8a) that is highly temperature sensitive, the structured state showing cold denaturation with a maximum folded population near 22 °C (Figure 8b), which is characteristic of polypeptide structure having a significant stabilizing contribution from the hydrophobic effect. Sulfate-induced structuring of MrH4a is linked to peptide self-association, as indicated by a strong peptide concentration dependence for folding and structure formation (not shown).

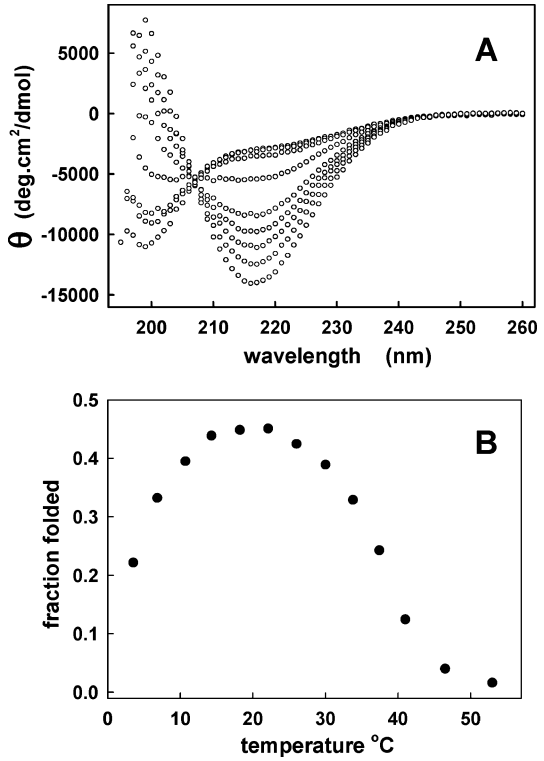

Figure 8. (A) Circular dichroism spectrum (mean residue ellipticity, θ) of MrH4a (80 μM) in 10 mM potassium phosphate, pH 3.0, at 31 °C (upper spectrum) and in the presence of Na_2SO_4 at the following molar concentrations (remaining spectra, top to bottom): 0.5, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, and 1.6 M. (B) Temperature dependence of the folded population of MrH4a (80 μM) in potassium phosphate buffer, pH 3.0, containing 1.0 M Na_2SO_4.

Table 2. Summary of Salt Effects on Peptide Conformational Stability

	salt		
peptide/primary stabilization	GdmCl	Gdm_2SO_4	Na_2SO_4
trpzip/cross-strand indole interactions	strong denaturant	no effect	no effect
alahel/H-bond-stabilized α-helix	strong denaturant	50% of Gdm^+ activity retained	weak nonlinear stabilization
MrH4a/cross-strand aliphatic side-chain interactions	denaturant[a]	no effect	stabilization of structure via peptide self-association

[a] MrH4a shows marginal monomeric stability in water at pH 3.0, precluding detailed interpretation of destabilizing solute effects.

For the purposes of the present study, a comparison of the effects of Na_2SO_4 (Figure 8) and Gdm_2SO_4 (Figure 7) on this folding transition indicates that Gdm^+ effectively abolishes the structure-stabilizing effect of sulfate on the associated state of MrH4a, much in the same way that sulfate abolishes the denaturant activity of Gdm^+ on the trpzip peptides.

4. Discussion

The observations described here (Table 2) can be understood in relation to the ionic associations of cations with sulfate in aqueous solution. The data support a mechanism for the well-known sulfate reversal of the denaturant activity of Gdm^+ in which direct association between Gdm^+ and sulfate ions in solution suppresses the interactions between Gdm^+ and protein that result in denaturation.

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This conclusion is most strongly supported by the effects of salts on the stability of the trpzip peptides. We previously showed that trpzip peptides are highly sensitive to the denaturant activity of Gdm^+ (as the GdmCl salt).[15] This denaturant sensitivity most likely results from the complementary nature of the planar aromatic indole side chains of tryptophan and the planar guanidinium ion,[14] as observed in molecular dynamics simulations of a tryptophan-containing peptide in GdmCl, in which the Gdm^+ ion and indole side chain form weak (cation−π) "stacking" interactions.[16] Trpzip peptides are unaffected by high concentrations of Gdm_2SO_4, consistent with previous observations of the lack of denaturant activity of the Gdm^+ sulfate salt.[11] Studies with non-ionic solutes (e.g., TMAO and urea,[8] or sarcosine and urea[9]) support additive effects of protein-stabilizing and denaturing solutes on polypeptide stability. However, this cannot be the case with the reversal of the denaturant activity of Gdm^+ on trpzip peptides by sulfate, since sulfate has no stabilizing effect on trpzip peptides. Rather, the sulfate directly reduces the effectiveness of Gdm^+ as a denaturant. Two other conclusions arise from the lack of effect of alkali metal sulfate salts on the stability of trpzip peptides. First, the observation reinforces the conclusion that the indole−indole interaction differs substantially from a "hydrophobic interaction",[17,19,31] since the latter is expected to be stabilized by sulfate. Second, the lack of stabilization of trpzip peptides by sulfate indicates that the ion does not indiscriminately stabilize compact structured polypeptide states in solution, at least on the scale of a 12-amino-acid residue peptide.

Support for an ion association mechanism for the sulfate-induced reversal of the indole−Gdm^+ interactions that underlie the powerful denaturant activity of Gdm^+ on trpzip peptides comes from MD simulations of Gdm^+ salts in concentrated solution. Much in the same way that Gdm^+ forms stacking interactions with indole groups in MD simulations,[16] Gdm^+ ions make pronounced self-"stacking" interactions in MD simulations of the chloride salt,[12] a consequence of the unique hydration properties of the Gdm^+ ion in which the molecular surfaces above and below the molecular plane are poorly hydrated (hydrophobic).[14] In MD simulations of Gdm_2SO_4, strong hetero-ion association results in the formation of clusters in which Gdm^+ "stacking" is destroyed.[13] This strong interaction of sulfate with Gdm^+ that attenuates the self-stacking interactions, resulting in ion clustering, is likely to similarly attenuate the stacking interactions between the planar indole and Gdm^+ groups that underlies the strong denaturant sensitivity of trpzip peptides to Gdm^+. Thus, sulfate reverses the denaturant activity of Gdm^+ without itself being a stabilizer of the folded state of trpzip peptides.

Solute effects on the stability of alanine-based helical peptides are more complex, although the observations are also compatible with ionic associations in concentrated electrolyte solution. The nonlinear effects of sulfate on the stability of helical peptides, which appear to saturate at high concentrations, has been observed before[25] and might arise from either saturatable effects involving direct interaction of sulfate and peptide (weak binding) or indirect effects that relate to the concentration-dependent properties of the solute. Three pieces of evidence support the latter (indirect) effect, rather than sulfate binding, as an explanation of the nonlinear contributions of sulfate to helical peptide stability. First, the use of a peptide with a negatively charged side chain at the N-terminus should suppress sulfate binding to the N-terminus, the only realistic site for potential sulfate binding to a helical peptide lacking side-chain positively charged groups. Second, extension of the alahel-E2 peptide by an additional pentapeptide sequence should additionally reduce "end-effects" that might involve sulfate binding. In neither case was the small and nonlinear stabilization of the helical peptides greatly affected. Finally, the concentration dependence of the number density of Na_2SO_4 is nonlinear, directly supporting weak concentration-dependent ionic association for nonlinear effects on helical peptide stability.

More interesting is the unexpected retention of considerable denaturant activity on the helical peptides in Gdm_2SO_4. Gdm_2SO_4 retains very close to 50% of the denaturant activity of GdmCl when compared with equivalent molar Gdm^+ concentrations and acts like a classical Hofmeister salt with linear relationships between solute concentration and the free energy of the folded state relative to the unfolded state, up to high solute concentrations. Can these observations be rationalized within the same framework of hetero-ion association that accounts for the loss of denaturant activity against trpzip peptides of Gdm^+ as its sulfate salt? In answering this question, it is important to recognize the different mechanisms of Gdm^+ denaturation of trpzip peptides and the alanine-based helical peptides. As described above, the dominant contribution to Gdm^+ denaturation of trpzip peptides is weak stacking interactions involving Gdm^+ and the indole side chains of the four Trp residues whose cross-strand interactions dominate the stability of these small β-hairpin peptides.[19] The denaturation of helical peptides by Gdm^+ is dominated by hydrogen-bonding interactions involving Gdm^+ and the peptide backbone amide groups.[15] In a polyalanine α-helix with a small number of solubilizing side chains, there is negligible contribution to stability from the hydrophobic effect.[32] Analysis of the hetero-ion clustering in MD simulations of Gdm_2SO_4[13] indicates that, while clustering destroys the ability of Gdm^+ to make self-"stacking" interactions, the clusters "tie up" only around 50% of the hydrogen-bonding Gdm^+ N−H groups in SO_4^{2-} hydrogen bonds. More specifically, whereas Gdm^+ in solutions of denaturant salts (MD simulations of GdmCl or GdmSCN) makes around 4.9 hydrogen bonds to water per Gdm^+, in MD simulations of Gdm_2SO_4, Gdm^+ forms on average 2.7 hydrogen bonds to water and 3.3 hydrogen bonds to sulfate oxygen atoms.[13] Thus, while Gdm^+ hetero-ion interactions with sulfate in solution significantly reduce the hydrogen-bonding capabilities of Gdm^+, the ion retains a reduced ability to make hydrogen bonds with the polypeptide backbone that compete with the intramolecular hydrogen bonds that dominate alanine-based α-helical peptide stability. Although we cannot rule out more complex interpretations involving the solution properties of these ionic solutes, the ionic clustering observed in MD simulations provides a parsimonious explanation of the retention of denaturant activity against alanine-based helical peptides in Gdm_2SO_4.

The data on trpzip and α-helical peptides indicate that the stabilization of proteins by sulfate has only small contributions from effects on aromatic−aromatic interactions or hydrogen bonds. The dominant effect of sulfate is expected to arise from

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promotion of the hydrophobic effect,[1c,d,10] and this is consistent with the sulfate-induced, self-associated structured state in MrH4a that exhibits the cold denaturation characteristic of structure stabilized by sequestering nonpolar amino acid side chains from water. MrH4a undergoes only small solute-induced structural stabilization (or destabilization) as a monomer, due to limited burial of nonpolar surface, and to our knowledge there is no small model peptide available with a significant *monomeric* folded state population in water, whose structuring is dominated by the classical hydrophobic effect. We have therefore been unable to assess reliably the separate effects on folding of the individual components of the ionic solutes, as was done with the trpzip and helical peptides. However, Gdm^+ was shown to abolish the sulfate-induced self-association and structuring of MrH4a. These observations are consistent with expectations based on sulfate and Gdm_2SO_4 effects on protein stability,[10,11] and the reversal by Gdm^+ of sulfate-induced self-association that is driven by the clustering of hydrophobic side chains may also be understood in terms of sulfate$-Gdm^+$ hetero-ion interactions in solution, although in this case additive effects cannot be discounted.

5. Conclusions

Recent experimental and computational analyses of the solution structures of Gdm^+ salts[12−14] inform our interpretation of the effects of these salts on the conformational stabilities of peptides chosen to represent different contributions to the conformational stability of proteins. These interpretations fit readily into a general experimental framework for solute effects on proteins, namely the extent to which solutes are concentrated at the protein surface relative to the bulk concentration (denaturants) or are excluded (protein stabilizers).[1e,2,5−7] This framework can be further generalized in terms of the hydration properties of the solutes[33,34] and, especially for ionic pairs, their interactions in solution. Gdm^+, a complex weakly hydrated cation,[14] makes multiple interactions with proteins, involving hydrogen bonding with the peptide backbone and "stacking" interactions with weakly hydrated groups, especially aromatic side chains, and also the planar π-bonded systems of Gln, Asn, and Arg.[15,16] Hetero-ion-pairing between Gdm^+ and SO_4^{2-} that destroys (Gdm^+−indole) or attenuates (Gdm^+−peptide hydrogen bonds) these interactions underlies the reversal of the denaturant activity of Gdm^+ as its sulfate salt.

While there are multiple ways in which denaturants may interact with protein groups, there is only one way in which protein-stabilizing solutes can be excluded from the protein surface—these solutes are preferentially hydrated, maintaining their hydration shells in preference to shedding waters to make solute−protein interactions. Protein stabilization by strongly hydrated, excluded ionic solutes results from either unfavorable interactions of the buried protein groups (nonpolar side chains and peptide backbone) with the ordered waters in the solute hydration shells or the decrease in bulk water as solvent is recruited into ion solvation as the ion concentration increases, or a combination of both (e.g., ref 34). Hetero interactions between anion (sulfate in this study) and cation can displace solvation shell waters to reverse these effects, and this occurs weakly in the case of $Na^+-SO_4^{2-}$ interactions, as observed in the downward curvature in the number density plot (Figure 4) and nonlinear effects on helical peptide stability, and more strongly in the case of the particularly favorable $Gdm^+-SO_4^{2-}$ interactions[13] that effectively reverse the protein-stabilizing properties of sulfate. While the ions SO_4^{2-} (stabilizing) and Gdm^+ (destabilizing) lie at the extreme ends of the respective Hofmeister series for anions and cations and have complementary molecular structures that enhance hetero interactions in solution, hetero interactions in other ionic pairs (e.g., Na_2SO_4) may also contribute to their solution properties, particularly at the high concentrations used to assess solute effects on protein stability. These conclusions may not be restricted to ionic solutions, since recent simulations have indicated that interactions between non-ionic solutes, TMAO and urea, make a contribution to the properties of the ternary mixture (TMAO−urea−water), including the reversal by TMAO of the protein denaturant activity of urea.[35]

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