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Role of cations in stability of acidic protein *Desulfovibrio desulfuricans* apoflavodoxin

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ABSTRACT

Apoflavodoxin from the sulfate reducing bacteria *Desulfovibrio desulfuricans* is a small, acidic protein with a net charge of −19 at neutral pH. Here, we show that monovalent cations in biologically relevant amounts have dramatic effects on apoflavodoxin stability. The effect is largest for Gdm^+ and decreases as a function of increased cation charge density ($Gdm^+ > NH_4^+ \geqslant K^+ \sim Cs^+ \sim Na^+ > Li^+$). A linear correlation of stabilizing effects with cation hydration properties suggests an important role of dehydration in efficient cation interaction with the protein. The effects on stability are due to preferential binding of one cation to native apoflavodoxin and results in an increase in thermal midpoint of 20 °C and the free energy of unfolding (at 20 °C) increases fivefold. Tuning of biophysical properties (such as folding and ligand/cofactor binding) of acidic proteins by cation binding may be important *in vivo*.

The stability of folded proteins in aqueous solution depends strongly on solvent conditions as many proteins exhibit salt- and pH-dependent stability parameters. Salts influence protein stability directly by preferential binding to the native or unfolded state or indirectly by changing the properties of solvent water [1–4]. The indirect effects of salts are similar for different proteins and are usually additive. They tend to follow the Hofmeister series of cations and anions [5,6]. Hofmeister discovered that a series of salts have consistent effects on protein solubility, and the series was subsequently found applicable to many other protein processes in which water-accessible surface area changes. The ranking for monoatomic cations correlates with surface charge density, but for complex, polyatomic cations, the underlying mechanism of the Hofmeister ordering is not entirely clear [7–9]. While the current literature contains multiple examples of correlations between anionic effects on protein structure and enzyme activity and anion position in the Hofmeister series [e.g. 2,10–14; for reviews: 15,16], correlations between the effects of cations on protein properties and the Hofmeister series are less explored [17–20]. This is likely the result of the fact that anions are more efficient than cations in affecting the properties of polypeptide chains and many studied proteins are basic (i.e., net positive charge at neutral pH). The anion–water interaction is stronger than the cation–water interaction due to the asymmetry of the dipoles in water [3,21]; therefore, anions have a greater effect on water ordering than cations. Obviously, interactions between ions and proteins will depend on the electrostatics of the protein surface: cation–protein interactions will be more pronounced if the net charge of the protein is negative (i.e., for acidic proteins).

Flavodoxin from the sulfate-reducing bacterium *Desulfovibrio desulfuricans* (ATCC 29577) is a short-chain flavodoxin that is an excellent model system for characterization of the effects of cations on protein folding and stability. Both thermal and chemical processes have been reported at different conditions and are all reversible [22–24]. *Desulfovibrio desulfuricans* flavodoxin is a small (15.7 kDa), single-domain, highly-acidic protein. It contains a net negative charge of −19 at neutral pH. Although effects of salts (especially phosphate and sulfate salts) on the stability of various flavodoxins were previously noted by our lab and others [23,25,26], cationic effects were addressed only later for the apoflavodoxin from *Anabaena* PCC7119 [23]. Whereas anionic effects on apoflavodoxin may be due to anions binding specifically in the binding site for the flavin mononucleotide (FMN) phosphate moiety, cationic effects on apoflavodoxin stability should be general to the group of acidic proteins. *Desulfovibrio desulfuricans* and some other sulfate reducing bacteria are halophilic and can grow in salt concentrations greater than 4 M [28]. Preliminary studies of osmo-

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regulation in these species indicate that they accumulate salts internally.

Our interest in cation interactions was triggered by the observation of a surprising stabilizing effect of the bulky cation, guanidinium (Gdm^+; chemical formula $C(NH_2)_3^+$), on *D. desulfuricans* apoflavodoxin. Guanidinium chloride (GdmCl) is one of the most powerful destabilizing salts and is widely used to denature proteins in the molar range. The Gdm^+ cation is the effective unfolding agent. Its unfolding potency can be weakened by combination with strongly stabilizing anions, such as sulfate in $(Gdm)_2SO_4$, or increased by combination with destabilizing anions, such as thiocyanate in GdmSCN [29–31]. Because of its ionic character, GdmCl might not act only as a denaturant. At low concentrations (< ~0.5 M), the ionic character of Gdm^+ can prevail and has been found to lead to modest increases in protein stability [32–35]. It has been shown that Ribonuclease T1 is stabilized by 0.4 °C at pH 5 in 0.3 M Gdm^+ [32]. Moreover, for the protein FprA, 0.25 M Gdm^+ stabilized a compact form [35]. In addition, ubiquitin is stabilized by GdmCl concentrations up to 2 M although the effect was proposed to originate in chloride binding [36].

Here, we have systematically characterized the concentration dependent (in the 0–0.3 M range) effects of guanidinium ions, and five smaller monovalent cations (i.e., Cs^+, NH_4^+, Na^+, K^+, and Li^+), on *D. desulfuricans* apoflavodoxin thermal and chemical stability at neutral pH. We find a direct correlation between the efficacy of the cations in increasing protein stability and the intrinsic hydration properties of the cations. We discovered that the effect stems from specific binding of one cation per protein. Implications of the results in terms of the biological environment and earlier *in vitro* flavodoxin data are discussed.

Materials and methods

Protein preparation

Apoflavodoxin from *D. desulfuricans* (ATCC 29577) was expressed in *Escherichia coli* and purified as previously described [22,37] with slight modification. Apoflavodoxin was first isolated from a Q-Sepharose ion exchange column and further purified using a Superdex-75 gel filtration column on an AKTA FPLC system (Amersham-Amersham Pharmacia) [38].

Thermally-induced unfolding

Apoflavodoxin thermal unfolding was monitored using circular dichroism (CD) performed using a 1-mm cell on a Jasco-810 spectropolarimeter and fluorescence using a 1-cm reduced-volume cell on a Cary Eclipse (Varian) spectrophotometer. In all experiments, a final concentration of 20 μM protein was used. All samples were allowed to equilibrate at 20 °C for ~30 min prior to heating experiments. All samples contained a final concentration of 10 mM Hepes (pH 7) and a range of concentrations of salts of various types (i.e., CsCl, GdmCl, NaCl, KCl, NH_4Cl, LiCl, $NaClO_4$, Na_2SO_4). All salts used were of the highest purity and all stock solutions were filtered using 0.22 μm syringe filters (Fisher) prior to usage. For both CD and fluorescence thermal unfolding, a rate of 1.5 °C/min was chosen. No scan rate dependence in apoflavodoxin unfolding was evident in the range of 0.1 °C to 2.5 °C/min. Thermal unfolding experiments proceeded from 20 to 90 °C. Reversibility of transitions were more than 90% in all cases (data not shown). CD at 222 nm and fluorescence (excitation at 285 nm) at 320 nm and 355 nm were monitored as a function of temperature. Dependences of the thermal transition temperatures, T_{trs}[3], on cation concentrations were fitted according to the following equation, which assumes one-to-one binding:

$$S = \Delta S \frac{[\text{cation}]}{K_{d,app} + [\text{cation}]}, \quad (1)$$

where S is the measured parameter (here, T_{trs}), ΔS is a fitted parameter indicating the difference between the measured parameter at saturated salt concentrations and in buffer, and $K_{d,app}$ is the apparent dissociation constant for a given cation.

[3] *Abbreviations used:* T_{trs}, temperature of thermal transition; CD, circular dichroism; ANS, anilino-8-naphthalene sulfonate; DSC, differential scanning calorimetry.

Urea-induced unfolding

Urea-induced unfolding of apoflavodoxin in 10 mM Hepes (pH 7) as well as in 10 mM Hepes (pH 7) with 250 mM salt (CsCl, GdmCl, KCl, NaCl, NH_4Cl, LiCl, $NaClO_4$, Na_2SO_4) was monitored using both CD and fluorescence at 20 °C. A final apoflavodoxin concentration of 20 μM was used in all experiments and samples were allowed to equilibrate for ~1 h prior to CD or fluorescence measurements. Solutions were made at 0.25 M increments of urea between 0 and 6 M urea. Highest quality urea (Sigma–Aldrich) was made fresh to a stock of 10 M and filtered prior to use using 0.22 μm syringe filters (Fisher). CD was monitored from 260 to 200 nm and fluorescence monitored (excitation at 285 nm) from 300 to 550 nm.

Circular dichroism

CD measurements in the far-UV region were performed with 20 μM apoflavodoxin at 20 and 90 °C in 10 mM Hepes buffer (pH 7) with a 1-mm path length. Each spectrum is the result of the averaging of nine consecutive scans. Spectra in the near-UV region were performed with 40 μM apoflavodoxin at 20 °C in 10 mM Hepes buffer (pH 7) with a 1-cm path length. Each spectrum is the result of the averaging of 20 consecutive scans. The temperature of the sample was controlled with a PTC-348 WI Peltier block.

ANS fluorescence

Starting solutions contained 20 μM apoflavodoxin and 200 μM 1-anilino-8-naphthalene sulfonate (ANS) in 10 mM Hepes (pH 7); the temperature was maintained at 20 °C. In all experiments, incremental amounts of salts (CsCl, GdmCl, NaCl, KCl, NH_4Cl, LiCl, $NaClO_4$, Na_2SO_4 in separate experiments) were titrated into the starting solution to final concentrations between 0 and 400 mM salt. Fluorescence was monitored using excitation at 395 nm and emission from 405 to 700 nm; all signals were corrected for dilution effects. An average of 10 scans was taken at each concentration of salt and all solutions were allowed to equilibrate until repeated sets of 10 scans overlapped (on average, about 10 min). Dependences of the wavelength of maximum ANS fluorescence, λ_{max}, on cation concentrations were fitted into Eq. (1). Control experiments of salt added to ANS solutions without any protein were also performed and revealed that there was no effect on ANS emission wavelength by the salts in the absence of protein.

Differential scanning calorimetry (DSC)

DSC measurements were performed on a VP-DSC instrument. Before and after thermal transition measurements, several buffer baselines were recorded to examine the signal reproducibility. Buffer and chemical baselines were subtracted from the calorimetric scans of proteins. Buffer and samples were degassed and stirred for 10 min at 15 °C. DSC samples contained 40–100 μM protein in 10 mM Hepes (pH 7) containing appropriate concentrations of GdmCl. Samples were loaded into the DSC cells at room temperature. Scan rates were 1.5 °C/min (except in tests of scan rate dependence). Calorimetric enthalpies of thermal transitions, ΔH_{cal}, were obtained after numerical integration of excess heat capacity. The van't Hoff enthalpy, ΔH_{vH}, was calculated simultaneously from the same calorimetric curve. The ratio of $\Delta H_{vH}/\Delta H_{cal}$ provides information about the character of the thermal transition. If the transition is two-state without intermediates or protein–protein interactions, the ratio should equal one [39].

Results

Electrostatic surface of native flavodoxin

Based on similarities in the primary structure of flavodoxins from *D. desulfuricans* and *D. vulgaris* (2fx2.pdb) proteins (47% sequence identity), a model of *D. desulfuricans* flavodoxin was constructed using the web service, 3D jigsaw [40,41]. The model of *D. desulfuricans* flavodoxin showed high similarity to the *D. vulgaris* flavodoxin structure. The electrostatic potential map of the surface of *D. desulfuricans* apoflavodoxin reveals a negatively-charged surface with only a few patches of positive charges (Fig. 1A). This is in good agreement with the net negative charge of −19 at neutral pH (28 negatively-charged and 9 positively-charged residues at pH 7). Thus, it appears that the electrostatic surface of apoflavodoxin involves unfavorable charge–charge repulsions. This was also concluded for *Anabaena* flavodoxin using a range of structure-based electrostatic calculations [27]. Alignment of several *Desulfovibrio*

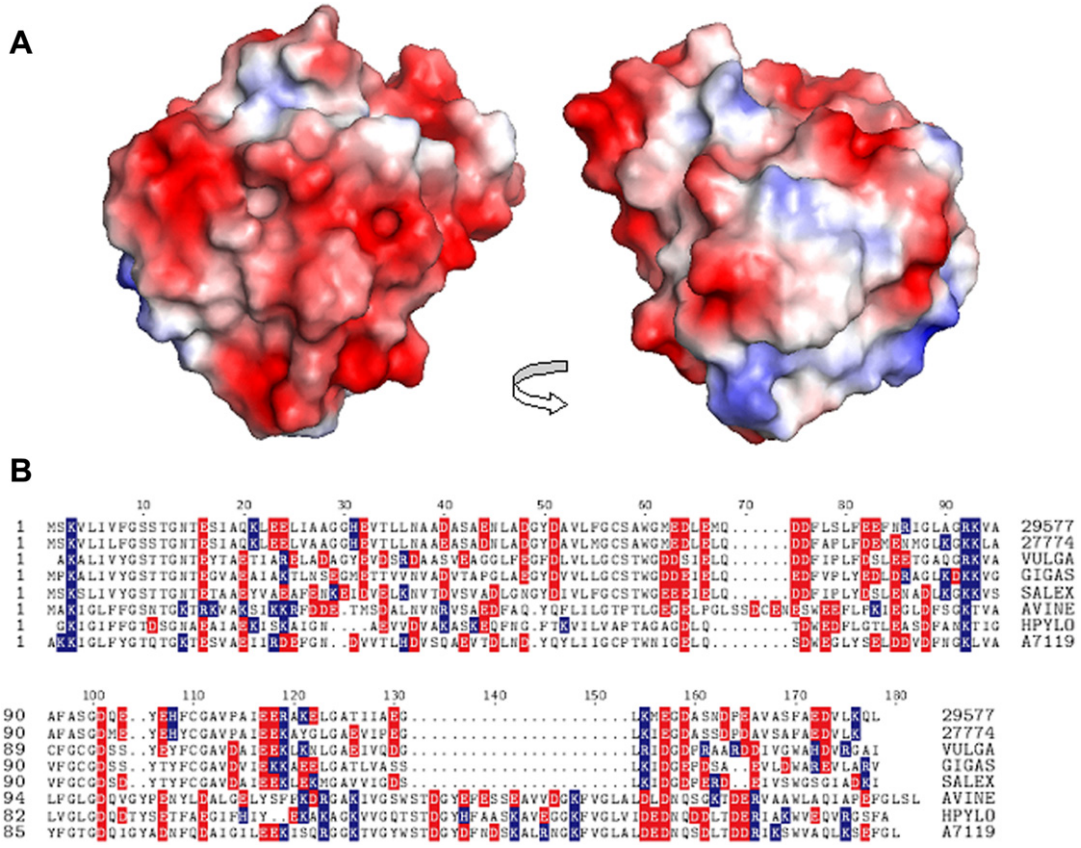

Fig. 1. (A) Electrostatic map of model of apoflavodoxin from *D. desulfuricans* made using the program 3D jigsaw based on similarity with *D. vulgaris* flavodoxin. The potentials (in vacuum) are color coded from blue (positive) to red (negative). (B) Sequence alignment of various flavodoxins showing all charged residues (acidic Asp and Glu residues in red and basic Arg and Lys residues in blue) aligned with Clustal W. Short-chain flavodoxins included in the alignment are from the following host organisms: *Desulfovibrio desulfuricans* ATCC 29577 (29577), *Desulfovibrio desulfuricans* ATCC 27774 (27774), *Desulfovibrio vulgaris* (VULGA), *Desulfovibrio gigas* (GIGAS), and *Desulfovibrio salexigens* (SALEX). Long-chain flavodoxins included are from the following host organisms: *Azotobacter vinelandii* (AVINE), *Helicobacter pylori* (HPYLO), and *Anabaena* PCC7119 (A7119).

and other flavodoxin sequences (Fig. 1B) reveals that all have large amounts of charged residues, although sequence identity and clustering of negative charges appear highest in the *Desulfovibrio* variants.

Thermal denaturation of apoflavodoxin in presence of cations

We find that thermal unfolding of apoflavodoxin in Hepes, pH 7 is an apparent two-state process: CD- and fluorescence-detected thermal curves are sigmoidal and overlap with each other. Also, DSC-derived thermograms agree with the CD and fluorescence data (Fig. 2). At this condition, apoflavodoxin displays a low thermal stability with a T_{trs} of about 46 °C. Upon addition of Gdm^+ up to 0.25 M, the T_{trs} for apoflavodoxin gradually increases by ~20 °C, to 66 °C. Still, at all conditions, the reaction appears two-state as CD, fluorescence and DSC all give the same result. Further additions of GdmCl above 0.25 M led to destabilization of the protein, in accordance with the denaturant properties of Gdm^+. The similarity of the T_{trs} values obtained by the three independent detection methods, as well as a ratio of van't Hoff and calorimetric enthalpy close to 1 at all Gdm^+ conditions (Table 1), indicate a two-state thermal transition of apoflavodoxin. We note that the enthalpic ratio deviates from 1 in the absence of Gdm^+. This points to possible dimer formation at higher temperatures. Attempts to detect dimers by glutaraldehyde crosslinking were, however, unsuccessful. Alternatively, the deviating ratio may indicate that apoflavodoxin unfolding in the absence of salts involves intermediate species or that the starting population is heterogeneous [42].

To better understand the mechanism by which Gdm^+ stabilizes apoflavodoxin, we extended our measurements to five other monovalent cations: cesium, ammonium, sodium, potassium, and lithium (all as chloride salts to keep the anion constant). These particular cations were chosen because of their different positions in the Hofmeister series [3,43]. The thermal experiments were repeated as a function of these cations and we found that all the sampled salts stabilized apoflavodoxin similarly to Gdm^+. For each salt, the increase in protein T_{trs} with increasing salt concentration has the appearance of a binding curve (Fig. 3). Close inspection of the dependences of T_{trs} versus [cation] reveals that the cations have different efficiencies of protein stabilization at non-saturating conditions. Apparent dissociation constants of protein–cation complexes obtained from these "binding curves" (using Eq. (1) in Materials and methods) correlate with the position of the cations in the Hofmeister series. The chaotropic, weakly-hydrated Gdm^+ is most efficient in stabilizing apoflavodoxin, whereas the kosmotropic, strongly-hydrated Li^+ is least efficient (inset, Fig. 3).

Although the protein may have many weak, non-specific binding sites for the cations, the binding events important for improving thermal stability may account for only a subset of sites involving specific binding. For two-state reactions, the cation dependence on T_{trs} can be used to estimate the number of cations ($\Delta\nu$) dissociated upon protein unfolding [44]:

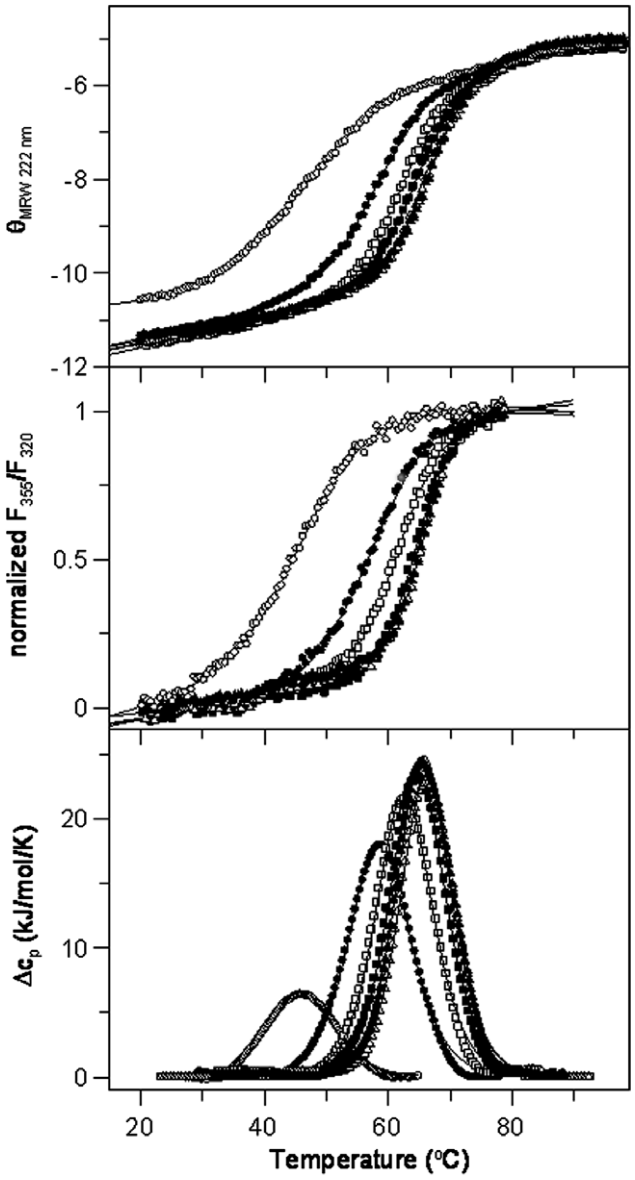

Fig. 2. Thermal denaturation (1.5 °C/min) of apoflavodoxin in the presence of various concentrations of GdmCl measured by far-UV CD (upper), fluorescence (middle), and DSC (lower panel). Protein concentration in CD and fluorescence were 20 μM in all cases. Concentration of GdmCl is (from left): 0, 10, 25, 50, 100, 250 mM.

$$\Delta v = -\frac{\Delta H_{\mathrm{cal}}(T_{\mathrm{trs}})}{RT_{\mathrm{trs}}^2}\cdot\frac{\mathrm{d}T_{\mathrm{trs}}}{\mathrm{d}\ \ln[\mathrm{cation}]}, \qquad (2)$$

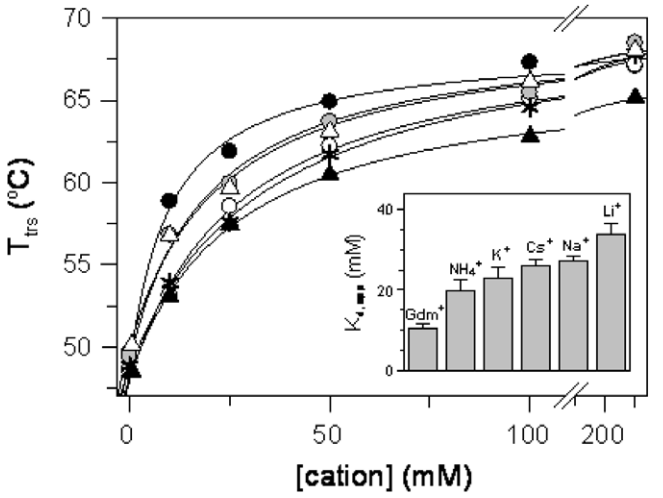

Fig. 3. Dependences of T_{trs} on cation (Gdm^+ (black circles), NH_4^+ (grey circles), Cs^+ (black triangles), K^+ (white triangles), Na^+ (white circles), Li^+ (asterisks)) concentration. Inset: Apparent dissociation constants of cations, obtained from fitting of experimental values to Eq. (1), are shown in the order of on cation position in the Hofmeister series ($K_d(Gdm^+)$ = 10.2 ± 1.2 mM, $K_d(NH_4^+)$ = 19.7 ± 2.7 mM, $K_d(K^+)$ = 22.9 ± 3.2 mM, $K_d(Cs^+)$ = 26.1 ± 1.5 mM, $K_d(Na^+)$ = 27.0 ± 3.2 mM, $K_d(Li^+)$ = 33.7 ± 2.6 mM).

where $\Delta H_{cal}(T_{trs})$ is calorimetric enthalpy, and R is the universal gas constant (8.3145 J/mol/K). The Δν values obtained for Gdm^+ (using the data in the 10–100 mM range) indicate that the thermal effects on apoflavodoxin are a result of preferential binding of ∼1 cation to the folded state (Table 1). Similarly, Δν values obtained for the other cations, using the ΔH_{vH} values instead of ΔH_{cal} also indicate preferential binding of ∼1 cation to the folded state of apoflavodoxin (not shown).

Apoflavodoxin native-state effects due to cation binding

The effects of cations on apoflavodoxin native state structure were assessed by far- and near-UV CD. We found a minor, ∼5%, increase in negative CD signal at 222 nm upon additions of cations but no change in the spectral shape (Fig. 4). This change is small in comparison to the ∼15% increase in negative CD signal at 222 nm observed for apoflavodoxin in the presence of crowding agents [45]. The near-UV CD loses some intensity but, again, there is no change in spectral shape upon cation addition. There was no effect of cations on the unfolded-state random-coil-like CD signal.

We also probed the effects of the six cations on the native state of apoflavodoxin using ANS emission changes. ANS is a hydrophobic probe with a fluorescence emission that depends on its surrounding environment and is often used to probe changes in

Table 1
Thermodynamic parameters of *D. desulfuricans* apoflavodoxin in the presence of various concentrations of Gdm^+ obtained from thermal denaturation monitored by far-UV CD, fluorescence (F) and DSC methods at pH 7.0

[Gdm^+] (mM)	$T_{trs,CD}$ (°C)	$T_{trs,F}$ (°C)	$T_{trs,DSC}$ (°C)	ΔH_{cal} (kJ/mol)	r[a]	Δν[b]
0	45.9	46.2	46.2	102	2.57	–
10	58.5	58.1	58.7	240	1.15	1.48
25	62.4	61.7	62.7	266	1.15	1.23
50	64.5	64.6	64.9	295	1.10	0.90
100	66.4	66.2	66.2	330	1.01	0.44
250	66.9	66.5	65.8	302	1.08	–
500	65.4	64.4	64.3	252	1.13	–
1000	58.9	58.7	57.6	146	1.76	–

Errors in all parameters were less than 5%.
[a] $r = \Delta H_{vH}/\Delta H_{cal}$. Both values are obtained from the DSC experiments (only ΔH_{cal} is shown; ΔH_{vH} can be obtained from the ratio).
[b] Number of Gdm^+ dissociated upon unfolding (Eq. (2)).

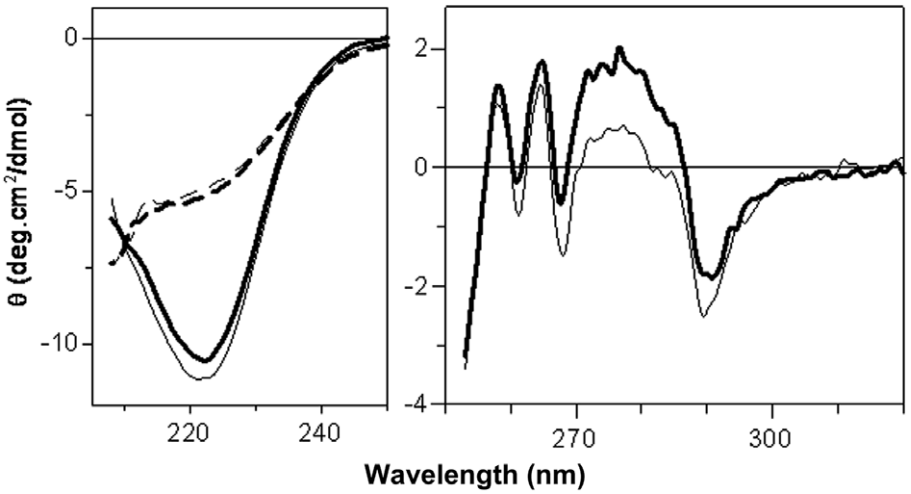

Fig. 4. CD of apoflavodoxin in the far-UV (left), and near-UV region (right): in 10 mM Hepes, 20 °C (solid thick line), 250 mM GdmCl, 20 °C (solid thin line), in 10 mM Hepes, 90 °C (dashed thick line), and 250 mM GdmCl, 90 °C (dashed thin line). Peptide: deg cm^2/dmol 10^{-3} of amide bonds; Near-UV: deg cm^2/dmol 10^{-4} of protein.

protein tertiary structure and to identify intermediate species. Upon binding to partially-folded proteins, the emission from ANS increases significantly and also shifts to the blue [46]. We found that ANS emission in the presence of apoflavodoxin exhibited cation-dependent shifts of the emission maximum (Fig. 5). In the presence of cations, ANS emission shifts to shorter wavelengths with slight intensity change; this indicates cation-induced interactions of ANS with apoflavodoxin. The magnitude of the shift is the largest, and the required amount of cation needed is smallest, for the Gdm^+ cation. On the other hand, the magnitude of the shift is the smallest for the Cs^+ cation. However, there is no apparent correlation between the magnitude of the shift and the required amount of cation for inducing the shift. Although the nature of ANS interaction with apoflavodoxin in the presence of cations is not clear, the relative changes in the ANS emission position can be used to assess apparent cation interactions with apoflavodoxin (analogous to the analysis of T_{trs} values above). Examination of the ANS emission maxima shift derived "binding curves" of cations allowed for estimations of apparent dissociation constants of the protein–cation complexes via Eq. (1) (inset, Fig. 5). The relative trend of "affinity" values obtained from the ANS experiments correlates with the trend observed via the T_{trs} values.

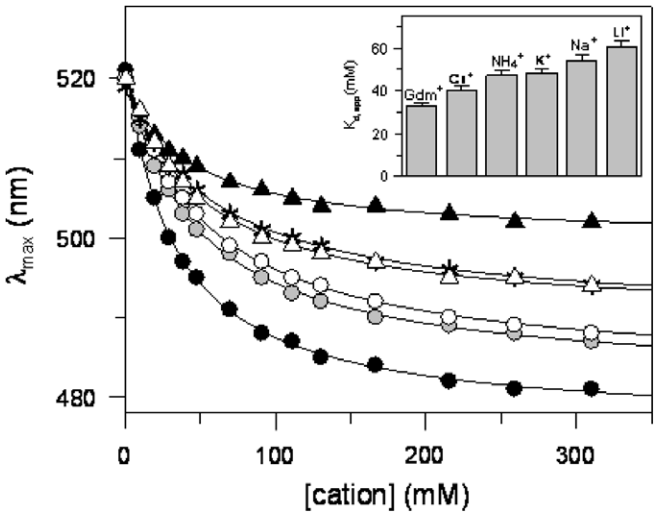

Fig. 5. ANS fluorescence maxima as function of cations (Gdm^+ (black circles), NH_4^+ (grey circles), Cs^+ (black triangles), K^+ (white triangles), Na^+ (white circles), Li^+ (asterisks)). Inset: Apparent dissociation constants of cations, obtained from fitting of experimental values to Eq. (1), are shown in the order of position in the Hofmeister series ($K_d(Gdm^+)$ = 32.7 ± 1.5 mM, $K_d(Cs^+)$ = 40.1 ± 2.0 mM, $K_d(NH_4^+)$ = 47.2 ± 2.3 mM, $K_d(K^+)$ = 48.2 ± 2.4 mM, $K_d(Na^+)$ = 54.2 ± 2.6 mM, $K_d(Li^+)$ = 60.7 ± 2.9 mM).

The order of ions in the Hofmeister series is often correlated with their interaction preferences with water molecules which may be described by the entropies of aqueous ions, hydration entropies, Jones–Dole coefficients or apparent hydration number [47]. In fact, plots of apparent cation–apoflavodoxin dissociation constants (from both T_{trs} and ANS data) and entropies of aqueous cations [48] (excluding Gdm^+ as no value has been reported) reveal a linear correlation between these parameters: the higher the cation's hydration entropy, the stronger the apparent effect on protein properties (Fig. 6A). Large cation hydration entropy correlates with less favorable water–cation interactions and, apparently, that favors cation–apoflavodoxin interactions. The correlation between apparent K_d values and the known hydration entropies for NH_4^+, Na^+, K^+, Cs^+, and Li^+ enabled us to predict the hydration entropy for Gdm^+ to be 220 ± 40 J/K/mol. We also found that the apparent K_d values for the cations correlate with cation hydration number [47] (Fig. 6B).

Isothermal unfolding of apoflavodoxin in the presence of cations

The effect of cations on chemical stability of apoflavodoxin at 20 °C (pH 7) was studied by urea-induced denaturation experiments monitored by CD at 222 nm and fluorescence ratio (320/355 nm) (Fig. 7). Both detection methods give identical curves, in accord with apparent two-state reactions. Also earlier work has reported apparent two-state equilibrium-unfolding reactions for apoflavodoxin in urea [24]. In the absence of cations (i.e., in Hepes), the free energy of unfolding of apoflavodoxin is only ~5.3 kJ/mol. This is a very low value for a protein this size and suggests that the structure is frustrated due to unfavorable interactions. However, in the presence of 250 mM Gdm^+, the stability of the protein is increased by 19 kJ/mol, to 24 kJ/mol, and the unfolding midpoint shifts from ~2 to ~4 M urea (Table 2). Similar stabilizations of apoflavodoxin towards urea perturbation were observed in the presence of 250 mM of the other cations (Table 2). Thus, cations (including Gdm^+) can improve protein thermodynamic stability fivefold (i.e., by 400%) in this particular case.

It is clear from the data in Table 2 that the cations induce higher relative change in exposed surface area upon unfolding (i.e., higher m values). This is in accord with cations binding to the surface neu-

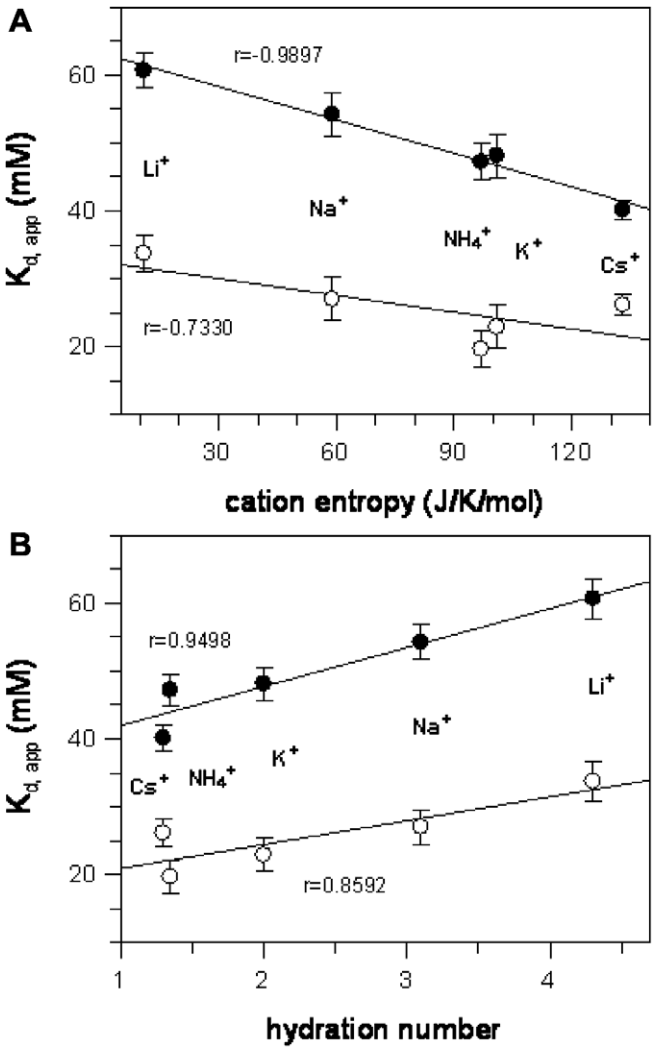

Fig. 6. Correlations of apparent dissociation constants of cations, obtained from Fig. 3 (white) and Fig. 5 (black), with entropies of aqueous cations (A) and hydration numbers of cations (B). The *r* values show correlation coefficients for corresponding curves. Value of standard molar entropy and hydration number for Gdm^+ is not available.

Table 2
Thermodynamic parameters (ΔG_U, *m*, and $[urea]_{1/2}$) for apoflavodoxin in the absence (10 mM Hepes, pH 7.0, 20 °C) and in the presence of 250 mM Gdm^+, NH_4^+, Cs^+, K^+, Na^+, and Li^+ obtained from urea-induced denaturation probed by far-UV CD and fluorescence (F)

Cation	Method	ΔG_U (kJ/mol)	*m* (kJ/mol/M)	$[urea]_{1/2}$ (M)
–	CD	4.4 ± 0.7	2.5 ± 0.9	1.9
	F	6.2 ± 0.4	2.8 ± 0.1	2.3
Gdm^+	CD	25.2 ± 1.9	6.2 ± 0.2	4.0
	F	22.7 ± 0.7	5.8 ± 0.2	3.9
NH_4^+	CD	24.4 ± 2.3	6.0 ± 0.6	4.0
	F	21.8 ± 1.8	5.3 ± 0.5	4.1
Cs^+	CD	23.5 ± 1.2	5.3 ± 0.3	4.4
	F	24.7 ± 1.8	5.5 ± 0.4	4.5
K^+	CD	24.0 ± 0.8	5.5 ± 0.2	4.5
	F	23.5 ± 1.5	5.2 ± 0.3	4.5
Na^+	CD	26.9 ± 0.8	5.9 ± 0.2	4.5
	F	26.8 ± 1.3	5.9 ± 0.3	4.6
Li^+	CD	22.4 ± 0.8	5.4 ± 0.2	4.1
	F	29.0 ± 1.6	7.1 ± 0.4	4.1

tralizing negative charges and thereby creating a more rigid fold. This argument can also explain the observed ANS data: with cations bound, the more rigid apoflavodoxin fold may have distinct hydrophobic pockets for ANS to bind in; in contrast, the protein without cations may be too dynamic to interact with ANS. Thus, the cation-induced interactions between apoflavodoxin and ANS may be a direct effect of the cations on the surface structure and/or dynamics.

Effect of anions on thermal stability of apoflavodoxin

In an effort to differentiate between the effects of cations and anions, a set of anions including kozmotropic (sulfate), neutral (chloride), and chaotropic (perchlorate) were used in combination with Na^+ and the effects on apoflavodoxin T_{trs} (Fig. 8) and maximum of ANS fluorescence (not shown) were compared. To take into account the stabilizing effect of sodium cations, the dependences were scaled to the concentration of cation. The data in Fig. 8 shows that there are distinct effects of the anions at >100 mM but that all effects overlay at <100 mM. Thus, the effect of Na^+ dominates at low salt concentrations; at higher concentrations, stabilization and destabilization effects (in comparison with chloride anions) of sulfate and perchlorate anions, respectively, are evident. The trend of anionic effects on apoflavodoxin stability at

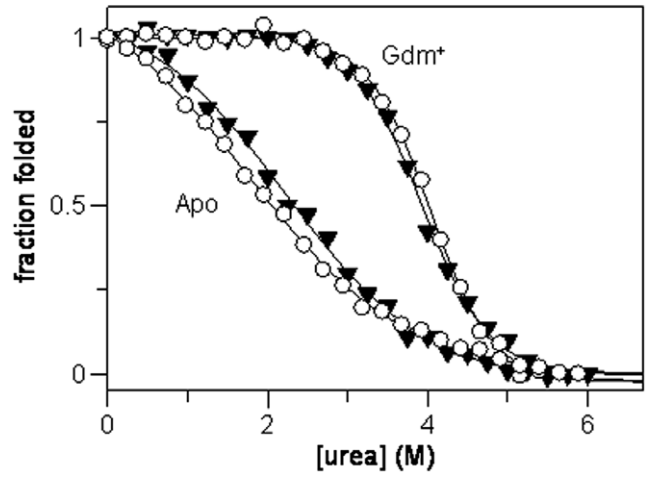

Fig. 7. Urea-induced denaturation of apoflavodoxin in Hepes with and without 250 mM GdmCl monitored by fluorescence (circles) and CD at 222 nm (triangles). CD and fluorescence changes were normalized for comparison purposes. Values from two-state fits are listed in Table 2 (including data on all other cations).

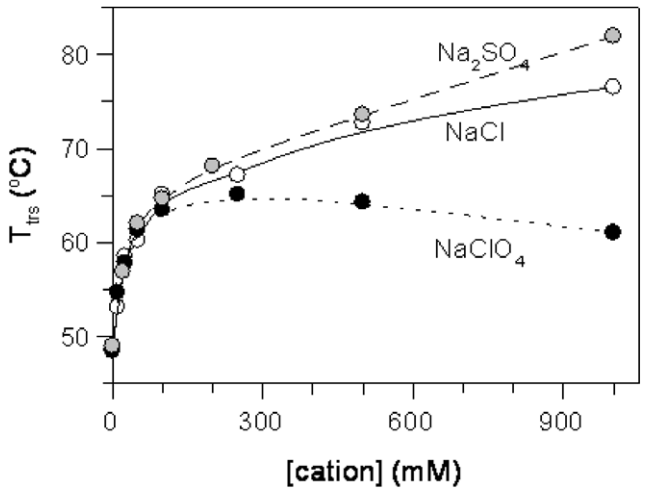

Fig. 8. T_{trs} of apoflavodoxin (pH 7) as a function of Na_2SO_4, NaCl, $NaClO_4$ additions. Salt concentrations are described in terms of the concentration of cations.

high-salt concentrations follows the Hofmeister series for anions: sulfate is strongly hydrated, chloride is less hydrated, and perchlorate is only weakly hydrated [3]. Note that this is in reverse order, as compared to the cations, in terms of hydration preferences and stabilization effect on apoflavodoxin. However, it has been shown earlier that sulfate can specifically bind in the FMN-binding site (at the position of the FMN phosphate group) as well as to the surface [49]. Thus, the trend of anionic effects should not be analyzed in general terms.

Discussion

The stability of the native state of proteins is often affected by salts. Monovalent salts affect the stability of mesophilic proteins by modifying the ionic strength of the solution, which can be stabilizing or destabilizing depending on the specific charge distribution within the protein [28]. On the other hand, the electrostatic interactions on thermophilic protein surfaces are often optimized [50], and high ionic strength leads to protein destabilization [51]. In contrast, proteins from halophilic and psychrophilic organisms are usually stabilized by the presence of salts [52] due to neutralization of repulsive forces of negative charges on their surfaces [53]. It was recently reported that screening of repulsive long-range Coulombic interactions among negatively-charged residues on the surface of *Anabaena* apoflavodoxin leads to its stabilization [27]. We observe the same phenomenon here, although to a much larger extent, for the halophilic *D. desulfuricans* apoflavodoxin at neutral pH using a different set of cations, including the chaotropic denaturant cation, Gdm^+. We found that both the thermal and chemical stability of apoflavodoxin can be improved dramatically (T_{trs} increases by 20 °C; thermodynamic stability at 20 °C increases fivefold, pH 7) by the presence of monovalent cations in the 100–250 mM range.

Based on the electrostatic surface map of *D. desulfuricans* apoflavodoxin (Fig. 1), electrostatic repulsion of negative charges on the surface, like in *Anabaena* apoflavodoxin, is likely the major force causing low protein stability under the conditions of low salt. Cations can reduce the repulsion by (i) the Debye–Hückel screening effect, (ii) the interaction with negative charges by direct ion-pair formation, and (iii) indirectly by affecting the water structure and consequently modulating hydrophobic interactions of proteins [4,10,11]. If the Debye–Hückel screening is the major contributing factor, the effects of various cations will be determined by the ionic strength of the solvent, not by the type of cation. The efficacy of affecting water structure will play a role in the case the effectiveness of various cations follows the Hofmeister series [54]. Our results show that for cation-induced stabilization of apoflavodoxin, all the above factors are involved to some degree. In accord with Debye–Hückel screening, the stabilization effect of cations at saturation concentrations (>250 mM of salt) is type-independent. This is also what was reported for *Anabaena* apoflavodoxin previously [27]. Analysis of the data in terms of differential ion binding in folded versus unfolded states indicates preferential binding of ~1 cation in the folded state. This supports direct cation interactions with surface groups on the protein forming ion pairs. In earlier studies, this was also suggested for *Anabaena* flavodoxin [26,27]. Finally, the efficiency of cationic effects on apoflavodoxin stability (in the 0–200 mM range) correlates with the position of the ion in the Hofmeister series, implying an important role of cation–water interactions.

The observed correlation between cation affinity for the protein and cation-hydration properties (Fig. 6) suggest that the effects on apoflavodoxin can be explained by cation water properties [54,55]. For cations with high hydration entropies, water interactions are weak (i.e., Gdm^+) and such ions are only weakly hydrated. To interact with the protein surface, the cations need to overcome an energy barrier, the so-called "hydration force" [56] related to removal of water molecules interacting with the cation. Thus, strongly-hydrated kozmotropic cations, such as Na^+ and Li^+, have a higher energy barrier than less hydrated chaotropic cations, such as Gdm^+ and Cs^+. Our data suggests that this hydration barrier plays a role in defining the cation's effect on protein stability.

According to Collins [3], interactions between chaotropic cations, such as Gdm^+, and kozmotropic anions, e.g., carboxylate groups of aspartate and glutamate sidechains which provide apoflavodoxin's negative charges, are unfavorable. The reason is that chaotropic cations, with low charge density, should be unable to perturb the strong hydration sphere of kozmotropic anions due to unfavorable energetics. This hypothesis contradicts our findings but can be reconciled with the idea that the unfavorable energy (step 1, $\Delta G_1 > 0$ kJ/mol) of formation of a chaotropic cation/kozmotropic anion pair is compensated for by a decrease in unfavorable electrostatic energy (step 2, $\Delta G_2 < 0$ kJ/mol) due to repulsive interactions among the many negative charges on apoflavodoxin's surface, i.e., $\Delta G_1 + \Delta G_2 < 0$.

The high efficiency of apoflavodoxin stabilization by Gdm^+ likely relates to its ability to bind specifically to protein surface residues [57,58]. Molecular dynamic simulations have indicated that Gdm^+ is prone to stacking and can interact with a number of planar protein side chains in a stacking manner [59]. Obviously, this interaction will be strengthened if the electrostatic surface potential is negative. To reveal possible cation binding sites on apoflavodoxin, the primary structures of flavodoxins from several species were compared (Fig. 1B). All sequences of flavodoxins from *Desulfovibrio* species show extra negative charges in the region from residues 50 to 77. Within this sequence stretch, there are several places where a few neighboring negative charges are clustered (and are surface exposed based on the model structure) that may create cation-binding sites (e.g., E63, D64, and E66; or D69 and D70; or E76 and E77). Interestingly, D69 and D70 as well as E76 and E77 are located on an unusually dynamic α-helix of *D. desulfuricans* flavodoxin as determined by NMR [60]. One may speculate that cation binding in this region may order the helix, which in turn would agree with our structural and thermodynamic observations. In kinetic experiments using urea as the denaturant, the presence of cations accelerates folding of apoflavodoxin (data not shown). This indicates that cation binding to apoflavodoxin takes place early in the folding process and also points to a binding site involving residues close in primary sequence.

These dramatic *in vitro* stability effects of salt raises the question of the role of ionic strength in apoflavodoxin structure and function *in vivo*. *D. desulfuricans* is a slight halophile and may accumulate high levels of salt inside the cells in order to osmoregulate in high-salt environments [28]. It appears that *D. desulfuricans* flavodoxin may have taken advantage of the presence of salt during the evolution of protein biophysical properties.

In vitro studies of flavodoxins from *D. desulfuricans* and other species have reported a range of T_{trs} values, chemical stabilities and intermediate species [23–27,61,62] that do not always agree. Inspection of experimental conditions used reveals different cation/anion concentrations and urea or GdmCl as denaturants. It appears possible to explain many observed variations by cation-specific effects not taken into account in these previous studies. We note that a so-called 'hidden' intermediate of *D. desulfuricans* flavodoxin found in GdmCl may in fact be a misinterpretation of Gdm^+-induced stabilization [24].

In conclusion, although cationic effects on protein properties are often smaller in comparison with anionic effects, we demonstrate that cationic effects on apoflavodoxin stability correlate with the position of the cations in the Hofmeister series. Our results indicate a direct role for cations (in biologically-relevant amounts)

in the stabilization of acidic proteins at neutral pH. Since the relative effect depends on the position of the cation in the Hofmeister series, water dehydration appears to be an important factor facilitating protein interactions. *In vivo*, variations in cation levels, analogously to anions [63], may be a way to modulate structure and stability of acidic proteins.

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