Anion binding to the ubiquitin molecule

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Abstract

Effects of different salts (NaCl, MgCl₂, CaCl₂, GdmCl, NaBr, NaClO₄, NaH₂PO₄, Na₂SO₄) on the stability of the ubiquitin molecule at pH 2.0 have been studied by differential scanning calorimetry, circular dichroism, and Tyr fluorescence spectroscopies. It is shown that all of the salts studied significantly increase the thermostability of the ubiquitin molecule, and that this stabilization can be interpreted in terms of anion binding. Estimated thermodynamic parameters of binding for Cl[−] show that this binding is relatively weak ($K_d = 0.15$ M) and is characterized by a negative enthalpy of −15 kJ/mol per site. Particularly surprising was the observed stabilizing effect of GdmCl through the entire concentration range studied (0.01–2 M), however, to a lesser extent than stabilization by NaCl. This stabilizing effect of GdmCl appears to arise from the binding of Cl[−] ions. Analysis of the observed changes in the stability of the ubiquitin molecule in the presence of GdmCl can be adequately described by combining the thermodynamic model of denaturant binding with Cl[−] binding effects.

Keywords: anion binding; circular dichroism; fluorescence; scanning calorimetry; ubiquitin

One of the components of milieu important for protein function and stability is ionic composition. This fact has been recognized for a long time, and comprehensive studies of the electrolyte effects on thermostability of a number of macromolecules have been exploited (von Hippel & Wong, 1964; von Hippel & Schleich, 1969a, 1969b). Development of methodologies both experimental and theoretical opened new perspectives for the study of thermodynamic principles underlying the effect of neutral salts on protein stability. Nevertheless, there is no common understanding of the stabilization/destabilization of proteins by neutral salts. A number of possible mechanisms such as specific and non-specific binding of ions to the protein molecule, electrostatic shielding of charges, etc., have been proposed (see for review Schellman, 1987, 1990; Timasheff, 1992, 1995; Yang & Honig, 1992; Garcia-Moreno, 1994; Gilson, 1995; Sharp, 1995; Sharp et al., 1995). One of these mechanisms, specific ion binding, can have very important biotechnological implications for the design of more thermostable proteins. There is “an obvious advantage of using ion binding for stabilization” via engineered ion binding sites on the surface of the protein because the activity of the enzyme can be preserved to a high

degree because surface substitutions usually do not significantly perturb the overall structure (Pace & Grimsley, 1988).

In this article we have studied the effects of increasing concentrations of electrolytes on the stability of the ubiquitin molecule. Ubiquitin is a suitable protein for such studies because of its physicochemical properties (Jenson et al., 1980; Breslow et al., 1986; Briggs & Roder, 1992; Khorasanizadeh et al., 1993). Ubiquitin is a small globular protein consisting of 76 amino acid residues, with a molecular weight of 8.4 kDa and without disulfide bonds or bound cofactors. It exists as a monomer in solution and is soluble up to high concentrations. The stability of human ubiquitin was recently studied by scanning calorimetry (Wintrobe et al., 1994). It was shown that the ubiquitin molecule unfolds as a cooperative two-state transition, and that this unfolding process is fully reversible. The results of this calorimetric study also indicated that ubiquitin is a highly thermostable protein with a transition temperature of 74 °C at pH 3.0. At neutral pH, human ubiquitin unfolds at temperatures above 100 °C (Makhatadze et al., unpub. obs.). The structures of human and yeast ubiquitins are virtually identical as determined by high-resolution X-ray crystallography and NMR (Vijay-Kumar et al., 1987a, 1987b; Weber et al., 1987; Harding et al., 1991). Using scanning calorimetry, circular dichroism, and fluorescence spectroscopies we have determined the effect of different salts, and in particular, details NaCl, Mg₂Cl, Ca₂Cl, and GdmCl on the thermodynamics of ubiquitin unfolding at pH 2.0. We report that the stability of ubiquitin increases with increasing concentrations of these salts up to 2 M. The results of these studies

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Abbreviations: PCR, polymerase chain reaction; CD, circular dichroism; DSC, differential scanning calorimetry; GdmCl, guanidinium chloride

are interpreted in terms of direct binding of anions to the ubiquitin molecule.

Results and discussions

Effect of neutral salts

Table 1 shows the results of the calorimetric experiments on ubiquitin at pH 2.0 with different concentrations of electrolytes. In all cases unfolding of ubiquitin is close to a two-state transition as judged by the comparison of experimental calorimetrically measured enthalpies of unfolding, ΔH_{cal} , and the enthalpies obtained by fitting the heat capacity profiles to a two-state transition, ΔH_{fit} , as described (Privalov & Potekhin, 1986). Ubiquitin in 10 mM glycine buffer at pH 2.0 has the transition temperature of 57 °C. Increasing the concentration of salt leads to a dramatic increase in

the transition temperature. Adding NaCl to 300 mM increases the transition temperature by 17°. Similar stabilizing effects are observed with other salts (MgCl₂, CaCl₂, NaBr, Na₂CIO₄, NaH₂PO₄, Na₂SO₄). One would predict such an effect of electrolytes from the general effects of neutral salts on electrostatic interactions in proteins. Indeed, at pH 2.0 the net charge on the ubiquitin molecule is +13. An increase in the ionic strength would decrease unfavorable electrostatic interactions between these charges thus stabilizing the native state. However, such a dramatic effect is not observed in the case of ribonuclease A or lysozyme (Fig. 1), indicating that the ubiquitin molecule has distinct properties. To further investigate this possibility we performed detailed analysis of the effects of monovalent (NaCl) and divalent (MgCl₂, CaCl₂) salts on the thermodynamic properties of ubiquitin. There are several advantages in using both monovalent and divalent salts. By using both types of salts we can vary the concentration of ions and the ionic strength in different ways because ionic strength, I , depends on the molar concentration as (Atkins, 1990):

$$I = \frac{1}{2} \cdot (m_+ \cdot z_+^2 + m_- \cdot z_-^2) \quad (1)$$

where z_+ and z_- are charge numbers for cation and anion, respectively, and m_+ and m_- are corresponding molar concentrations. So at the same molar concentration, the ionic strength of MgCl₂ or CaCl₂ will be three times higher than that of NaCl. Similarly, the concentration of anions (Cl⁻) at the same molar concentration of MgCl₂ or CaCl₂ will be twice higher than for NaCl, but the concentration of cations will be the same. Comparison of the transition temperatures for the ubiquitin molecule as a function of the total concentration of electrolyte (Fig. 2A), ionic strength (Fig. 2B), and concentration of Cl⁻ ions (Fig. 2C) clearly shows that the stabilizing effect of neutral salts, in the case of ubiquitin molecule, has

Table 1. Thermodynamic parameters of heat denaturation of human ubiquitin^a

Buffer ^b pH 2.0	T_t (°C)	ΔH_{cal} (kJ/mol)	ΔH_{fit} (kJ/mol)
0 mM NaCl	57.1	201	205
0 mM NaCl	55.6	200	204
0 mM NaCl	56.0	198	200
50 mM NaCl	63.4	236	238
100 mM NaCl	66.3	252	254
150 mM NaCl	68.7	260	255
200 mM NaCl	71.1	274	272
300 mM NaCl	74.4	277	264
500 mM NaCl	79.6	282	274
25 mM CaCl ₂	61.7	225	220
50 mM CaCl ₂	65.8	247	244
75 mM CaCl ₂	68.5	259	257
100 mM CaCl ₂	70.5	272	266
25 mM MgCl ₂	62.5	240	241
50 mM MgCl ₂	66.3	252	253
100 mM MgCl ₂	71.2	279	282
150 mM MgCl ₂	73.1	281	277
200 mM MgCl ₂	76.2	294	291
50 mM Na ₂ SO ₄	74.4	288	287
100 mM NaClO ₄	69.9	280	283
50 mM NaBr	63.9	253	254
0 mM NaH ₂ PO ₄ ^c	53.8	177	185
10 mM NaH ₂ PO ₄ ^c	59.4	212	214
50 mM NaH ₂ PO ₄ ^c	66.4	243	242
100 mM NaH ₂ PO ₄ ^c	70.4	259	263
25 mM GdmCl	59.5	214	200
50 mM GdmCl	62.8	236	215
100 mM GdmCl	65.3	249	238
150 mM GdmCl	67.2	254	248
250 mM GdmCl	68.7	257	250
500 mM GdmCl	70.8	256	259
1000 mM GdmCl	71.2	248	257
2000 mM GdmCl	63.7	218	228

^aEstimated error in the T_t values is ± 0.2 °C; ΔH_{cal} is calorimetric enthalpy and ΔH_{fit} is an enthalpy of the fit of a calorimetric profile to a two-state model. Estimated errors in ΔH_{cal} and ΔH_{fit} are on the order of 5–7%.

^bAll solutions contained 10 mM glycine, unless otherwise noted.

^cpH of the aqueous solutions was adjusted by phosphoric acid.

Fig. 1. Effect of increasing concentrations of NaCl on the transition temperature for human ubiquitin (filled circle), lysozyme (filled diamond), ribonuclease A (filled square), yeast ubiquitin (open down triangle), and K63R (open square), and E34S/K63R (open circle) variants of yeast ubiquitin at pH 2.0. Lines drawn through the points do not carry any meaning but to guide the eye.

Fig. 2. The dependence of the transition temperature of human ubiquitin at pH 2.0 on (A) molar salt concentrations, (B) ionic strength, or (C) Cl^- concentrations. Symbols are NaCl (filled circle), MgCl_2 (filled square), CaCl_2 (filled up triangle), GdmCl (filled down triangle), NaH_2PO_4 (open circle), NaBr (filled diamond), NaClO_4 (open square), and Na_2SO_4 (open down triangle).

a different nature than the consequence of ionic screening. The effect of mono- and divalent salts on the transition temperature of ubiquitin can not be represented by a single function if analyzed in terms of concentration of the electrolytes or their ionic strengths. Only when the transition temperature is plotted versus concentration of Cl^- ions does the stabilizing effect (increase in transition temperature) become independent of the salt type, and the observed effect converges to a single function (Fig. 2C). We interpret this effect in terms of anion (in this case Cl^-) binding to the ubiquitin molecule.

We estimated the difference in the number of binding sites Δn_{Cl^-} between unfolded, n_U , and native states, n_N , using the well-known equation (Privalov et al., 1969; Plaza del Pino & Sanchez-Ruiz, 1995):

$$\Delta n_{\text{Cl}^-} = n_U - n_N = -\frac{\Delta H_{\text{cal}}}{R \cdot T_t^2} \cdot \frac{dT_t}{d \ln[\text{Cl}^-]} \quad (2)$$

where R is the universal gas constant ($8.3145 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). Analysis of the calorimetric data according to this equation gives Δn_{Cl^-} equal to -1.9 ± 0.1 . This means that the native state binds about two molecules of Cl^- more than the unfolded state.

Increasing the concentrations of NaCl, MgCl_2 , or CaCl_2 leads to an increase not only in the transition temperature but also in the enthalpy of unfolding of ubiquitin compared to the enthalpy of unfolding in low salt concentrations (10 mM buffer only) at the same temperature (Fig. 3). This means that dissociation of Cl^- bound to the ubiquitin molecule is accompanied by a positive enthalpy change. Thus, one can use the enthalpy of ubiquitin unfolding in the presence and absence of Cl^- ions to get the thermodynamics of Cl^- binding to the ubiquitin molecule. To do so we fit the enthalpy of unfolding at different concentrations of Cl^- ions to the equation

$$\Delta H(T) = \Delta H^0(T) + \frac{K_{\text{Cl}^-} \cdot [\text{Cl}^-]}{1 + K_{\text{Cl}^-} \cdot [\text{Cl}^-]} \cdot \Delta n_{\text{Cl}^-} \cdot \Delta H_{\text{Cl}^-} \quad (3)$$

where $[\text{Cl}^-]$ is the concentration of Cl^- ions, K_{Cl^-} is the Cl^- binding constant, ΔH_{Cl^-} is the enthalpy of Cl^- binding per site,

and $\Delta H^0(T)$ is the enthalpy of ubiquitin unfolding at low concentration of ions (Wintrode et al., 1994) expressed as a second-order polynomial:

$$\begin{aligned} \Delta H^0(T) = & -170 + 8.77 \cdot (T - 273.15) \\ & - 0.0395 \cdot (T - 273.15)^2. \end{aligned} \quad (4)$$

Fitting to Equation 3 assumes that $\Delta n_{\text{Cl}^-} = -2$, and that the enthalpy of binding does not depend on temperature, i.e.,

$$\frac{\partial \Delta H_{\text{Cl}^-}}{\partial T} = 0.$$

There are a number of reasons required to incorporate these assumptions in the data analysis. First, $\Delta n_{\text{Cl}^-} \cdot \Delta H_{\text{Cl}^-}$ in Equation 3 are mutually linked parameters and cannot be resolved during fitting. This means that one needs independent observations to estimate one of these parameters. The use of $\Delta n_{\text{Cl}^-} = -2$ as determined according to Equation 2 is supported by the analysis of phosphate binding according to Equation 2, which gives a value of -2.2 ± 0.3 for Δn for phosphate, a value similar to that for Cl^- . This probably implies that there are two anion binding sites on the native state of the ubiquitin molecule (i.e., unfolded state does not bind Cl^- , $n_U = 0$). Second, it seems that we do not have a complete titration curve, which means that incorporation of the additional fitted parameters such as temperature dependence of enthalpy of binding makes the results of minimization unstable.

The thermodynamic parameters for Cl^- binding to the ubiquitin molecule are listed in Table 2. The binding constant is on the order of 7 M^{-1} (dissociation constant 0.15 M), which indicates that this binding is relatively weak. Binding is exothermic with the enthalpy of binding -15 kJ/mol per site.

Effect of guanidinium chloride

If there is a specific effect of Cl^- on the thermodynamic properties of ubiquitin, then how will this effect manifest itself in the case of GdmCl?

Fig. 3. The dependence of the enthalpy of human ubiquitin unfolding, ΔH_{cal} , on the transition temperature, T_t . Circles represent the experiments performed at in 10 mM buffer at different pH values of the solution (Wintrode et al., 1994). Dashed line is described by Equation 4. **A:** Symbols (filled square) represent the results obtained at pH 2.0 in the presence of different concentrations of NaCl, $MgCl_2$, or $CaCl_2$. The solid line represents fit to Equation 3 with the parameters listed in Table 2. **B:** Symbols (filled diamond) represent the results obtained at pH 2.0 in the presence of different concentrations of GdmCl. The solid line represents the fit to the Equation 5 with the parameters listed in Table 2.

Figure 2 shows the effect of GdmCl on the transition temperature of ubiquitin at pH 2.0. In the GdmCl concentration range studied (0.01–2 M) the transition temperature of ubiquitin is always higher than in the absence of GdmCl. Even in the presence of 2 M GdmCl the transition temperature increases by about 8°C (Fig. 2). At this concentration of GdmCl many proteins are already unfolded even at room temperature. What causes this stabilizing effect of GdmCl, a well-known protein denaturant? GdmCl is an electrolyte with a pK_a of ~ 11 . This means that at pH 2.0 it will be fully dissociated and in a first approximation one can assume that Cl^- and Gdm^+ ions will have additive effect on the thermodynamic parameters of ubiquitin unfolding. Thus, the stabilizing effect of GdmCl is caused by Cl^- ions. As can be seen from

Figure 2c, up to 0.2 M GdmCl affects the transition temperature in the same dependence as any other neutral salt and starts to deviate at GdmCl concentrations above 0.2 M. However, even at concentrations of GdmCl as high as 2 M, thermostability of the protein remains higher than in its absence. Interestingly, the enthalpy of unfolding in the presence of GdmCl also follows similar patterns as in the presence of NaCl or $MgCl_2$, i.e., the enthalpy of unfolding in the presence GdmCl concentration up to 0.2 M at any given temperature is higher than the enthalpy of unfolding in low salt solution. At around 0.2 M GdmCl the enthalpy function starts to decrease. This decrease is due to the negative enthalpy of protein interaction with Gdm^+ ions. It has been known for quite a while (see, e.g., Pfeil & Privalov, 1976) that protein interactions with GdmCl are very exothermic. This large heat effect has been used to calorimetrically measure protein GdmCl interactions using titration microcalorimetry (Makhatadze & Privalov, 1992). So what we observe in the case of ubiquitin is a combination effect Cl^- and Gdm^+ ions.

Interactions of denaturants with proteins have been analyzed using a number of different models: transfer model (Tanford, 1968; Alonso & Dill, 1991; Staniforth et al., 1993), preferential solvation (Lee & Timasheff, 1974; Arakawa et al., 1990; Timasheff, 1992, 1993, 1995), solvent exchange model (Schellman, 1987, 1990, 1994; Schellman & Gassner, 1996), and direct binding model (Pace, 1975, 1986; Makhatadze & Privalov, 1992). Model independent treatment of the solvent denaturation experiments has also been suggested (Ibarra-Molero & Sanchez-Ruiz, 1996). The direct binding model has been extensively studied using direct calorimetric methods, and enthalpies, binding constants and the number of binding sites have been estimated as a function of temperature for three different proteins (Makhatadze & Privalov, 1992). Thus, we have decided to use this model for the analysis of the effect of

Table 2. Thermodynamic parameters (enthalpy ΔH , binding constant K , and number of binding sites, Δn) of binding of Cl^- and Gdm^+ to ubiquitin at pH 2.0

	Binding of ^a	
	Cl^-	Gdm^+
ΔH (kJ/mol)	-15 ± 7	-8^b
K (M^{-1})	6.8 ± 2.1	0.3^b
Δn	-2^c	15 ± 3

^aErrors of fitted parameters are 95% confidence limits.

^bEstimated as described in the text.

^cCalculated from the effect of the Cl^- concentration of the transition according to Equation 2.

GdmCl on the stability of ubiquitin. The following equation has been used to fit the enthalpy of ubiquitin unfolding in the presence of different concentrations of GdmCl:

$$\Delta H(T) = \Delta H^0(T) + \frac{K_{Cl^-} \cdot [Cl^-]}{1 + K_{Cl^-} \cdot [Cl^-]} \cdot \Delta n_{Cl^-} \cdot \Delta H_{Cl^-} + \frac{K_{Gdm^+} \cdot [Gdm^+]}{1 + K_{Gdm^+} \cdot [Gdm^+]} \cdot \Delta n_{Gdm^+} \cdot \Delta H_{Gdm^+} \quad (5)$$

where $\Delta H(T)$ is the enthalpy of protein unfolding in the absence of electrolytes, and is defined by Equation 4, $[Gdm^+]$ is the concentration of Gdm⁺ ions, Δn_{Gdm^+} is the difference in the number of Gdm⁺ binding sites between unfolded and native states, ΔH_{Gdm^+} is the enthalpy of Gdm⁺ binding to the protein, and K_{Gdm^+} is the Gdm⁺ binding constant. In this equation we assume that Cl⁻ and Gdm⁺ ions bind independently. This assumption is supported by the observations made by Smith & Scholtz (1996), who studied the effect of NaCl and GdmCl on the helix-coil transition in peptide helices. In its present form, Equation 5 is still complex and simultaneous fit to estimate Δn_{Gdm^+} , ΔH_{Gdm^+} , and K_{Gdm^+} does not appear possible. So instead, we used estimates of ΔH_{Gdm^+} and K_{Gdm^+} using data reported by Makhatadze and Privalov (1992). Because the ubiquitin data (this study) were obtained at a higher temperature than experimentally measured by Makhatadze and Privalov, it is necessary to extrapolate ΔH_{Gdm^+} and K_{Gdm^+} values to 65 °, which is an average temperature for the data of ubiquitin unfolding in the presence of GdmCl. The enthalpy of binding of Gdm⁺ at 25 °C is -11 kJ/mol (Makhatadze & Privalov, 1992); however, ΔH_{Gdm^+} is temperature dependent, and its absolute value decreases with an increase in temperature. This decrease was estimated to be on the order of 3 kJ/mol, or -8 kJ/mol for Gdm⁺ binding to a site on a protein at 65 °C. The binding constant K_{Gdm^+} is also temperature dependent. At 10, 25, and 40 °C the average values for K_{Gdm^+} obtained from the calorimetric data on three proteins are 0.80, 0.60, and 0.52 M⁻¹, respectively in agreement with the previous estimates of 0.62 at 25 °C by Pace (1986). Thus, at 65 °C we estimated that K_{Gdm^+} is of the order 0.3 M⁻¹. Using these estimates and the thermodynamic parameters for Cl⁻ binding obtained from Equation 3 we can fit the experimental data of the effect of GdmCl on the thermodynamic parameters of ubiquitin unfolding by using Equation 5. This fit will have one adjustable parameter, Δn_{Gdm^+} , the difference in the number of Gdm⁺ binding sites in the unfolded and native states of ubiquitin. Keeping in mind the simplicity of Equation 5, the quality of the fit (Fig. 3B) is reasonably good, which indicates the validity, at least to a first approximation, of the applied formalism. Analysis gives an estimate for Δn_{Gdm^+} of 15 ± 3 . Considering the size of the ubiquitin molecule (76 amino acid residues) the estimate for Δn_{Gdm^+} seems to agree well with similar estimates for cytochrome *c* (104 amino acid residues, $\Delta n_{Gdm^+} = 19$), ribonuclease A (124 amino acid residues, $\Delta n_{Gdm^+} = 31$) and lysozyme (128 amino acid residues, $\Delta n_{Gdm^+} = 25$) (Fig. 4). Thus, to a first approximation the thermodynamic binding model for denaturants developed by Makhatadze and Privalov (1992) seems to adequately describe the effect of Gdm⁺ on the stability of the ubiquitin molecule. This, of course, does not imply that the only mode of action for denaturants is binding to protein groups via hydrogen bonding, although such interactions follow from the thermodynamic analysis (Makhatadze & Privalov, 1992; Sijpkens et al., 1993), hydrogen exchange kinetics (Lumb & Dobson, 1992; Kim & Woodward, 1993), and X-ray crystallogra-

Fig. 4. The dependence of the number of Gdm⁺ binding sites on the number of amino acid residues for ubiquitin (Ubq), cytochrome *c* (Cyt), ribonuclease A (RNase), and lysozyme (Lyz). Open symbols represent data taken from Makhatadze and Privalov (1992), solid symbol, this article.

phy (Snape et al., 1974; Thayer et al., 1993; Pike & Acharya, 1994; Dunbar et al., 1997). There are indications that denaturants can interact with aromatic side chains via non-conventional hydrogen bonding, i.e., hydrogen bond to aromatic π electrons (Duffy et al., 1993). One cannot exclude the earlier proposed effects of high concentrations of denaturants on the structure of water that will affect the thermodynamics of hydration of the protein groups (Tanford, 1968; Staniforth et al., 1993). Recent evidence for this obtained on model compounds is very convincing (Liu & Bolen, 1995).

GdmCl is believed to be much better denaturant than urea, implying that lower concentrations are required to unfold a protein by GdmCl than by urea. Ubiquitin is certainly an exception to this rule. Actually, in the case of ubiquitin, higher concentrations of GdmCl than of urea are required to unfold it at pH 2.0 and 25 °C. Figure 5 presents the results of denaturant induced unfolding of ubiquitin at pH 2.0 as monitored by the changes in the ellipticity at 222 nm and changes in Tyr-fluorescence at 310 nm. Clearly, the midpoint of the denaturant-induced transition is lower for the urea-induced transition than that for the GdmCl. Estimates based on the data presented in Figure 5 show that the midpoint of transition for urea is 3 M, whereas for GdmCl it is almost 1 M higher, at 3.8 M.

The first indication that GdmCl is an electrolyte and can have a dual effect on the stability of proteins has been demonstrated by Santoro and Bolen (1992). They showed that in order to get a correct value for the stability of thioredoxin using the linear extrapolation method (LEM) for denaturant-induced unfolding one has to use up to 1.5 M NaCl to compensate for the increase in the ionic strength of the protein solution due to the increase in the concentrations of GdmCl. Similar effects were observed on the third domain of turkey ovomucoid (Swint & Robertson, 1993).

Peculiar effects of GdmCl on the protein stability have also been reported by Mayr and Schmid (1993) and by Hagihara et al. (1993), and deserve comparison with the results presented in this article.

Fig. 5. Unfolding of human ubiquitin by urea (circles) and GdmCl⁻ (squares) as monitored by the changes in Tyr-fluorescence at 310 nm (open symbols) and CD at 222 nm (closed symbols). Experiments were performed at 25 °C as described in Materials and methods. Lines drawn through the points do not carry any meaning but to guide the eye.

Mayr and Schmid (1993) studied the effect of GdmCl and NaCl on the thermostabilities of ribonuclease A (RNase A) and ribonuclease T1 (RNase T1) at pH 5.0. They found that the addition of 1 M NaCl increases the transition temperature of RNase A by 4 °C. In the case of RNase T1, the addition of 1 M NaCl has a larger effect (10 °C) on the transition temperature. Addition of GdmCl to RNase A led to a decrease in the transition temperature in the entire concentration range studied (0–1 M). In contrast, addition of GdmCl did not lead to the immediate decrease in the transition temperature for RNase T1. Up to 0.1 M GdmCl actually led to an increase in the transition temperature. However, resultant thermostabilization was relatively small, only 0.4 °C. These results were interpreted in terms of stabilization by cation (Na⁺ and Gdm⁺) binding to the RNase T1 molecule. This conclusion was based on the fact that RNase T1 is a very acidic protein (12 Asp + Glu, 3 His, and one each Lys and Arg), and at pH 5.0 will have mostly negative charges. Calorimetric experiments also suggested Na⁺ binding to the RNase T1 molecule (Kiefhaber et al., 1990; Hu et al., 1992). In view of our results on the effect of neutral salts on the stability of ubiquitin, an alternative interpretation involving not only cation but anion binding as well can be suggested. Such an interpretation agrees with an earlier study by Pace and Grimsley (1988), which showed that both anion and cation binding sites are available on the RNase T1 molecule.

The stabilizing effect of anions on the acid denatured states of proteins have been studied extensively by Goto and Fink (Goto et al., 1990; Goto & Fink, 1990; Hagihara et al., 1993). It has been shown that anion binding promotes the transition of the acid unfolded state to the molten globule state (i.e., state with a large far UV-CD signal and a disordered tertiary structure). Recently, Hagihara et al. (1993) have shown that low concentrations of GdmCl also promote the formation of the molten globule state in cytochrome *c*. However, they interpreted the results in terms of non-

specific binding of anions to the charged groups of the protein, thus causing a decrease in the charge–charge repulsion, which then leads to the formation of the molten globule state. This molten globule state of cytochrome *c* is characterized by lower enthalpy and by lower stability than the native state. Compared with the results reported in this article, we note a number of important differences. (1) Ubiquitin at pH 2.0 in the absence of high concentrations of anions still maintains its native structure. (2) Binding of anions is specific and leads to an increase in the stability. (3) Binding of anions leads to an increase in the enthalpy of unfolding, as oppose to the decrease in enthalpy in cytochrome *c* (Potekhin & Pfeil, 1989). Although there were reports that one can populate a molten globule like state in the ubiquitin molecule, this can be achieved only in a 60% methanol solution (Harding et al., 1991; Woolfson et al., 1993). At pH 2.0 ubiquitin is in its native state, which unfolds cooperatively as judged by the simultaneous changes in the far UV-CD (probe for secondary structure) and Tyr-fluorescence (probe for tertiary interactions) as monitored by solvent denaturation experiments using both urea or GdmCl (Fig. 5).

Mapping of the anion binding sites

From the thermodynamic data presented above there is strong evidence that the stabilizing effect of anions in the case of ubiquitin occurs via specific binding implying that there are specific anion binding sites. Location of these sites remains unclear. Inspection of the crystal structure of ubiquitin (Vijay-Kumar et al., 1987a, 1987b) reveals that all 12 positively charged side chain are solvent exposed. We attempted to address the question of the location of the anion binding site using site-directed mutagenesis with yeast ubiquitin instead of human. The difference between human and yeast ubiquitin is only 3 out of 76 amino acid residues (Finley et al., 1987). The yeast ubiquitin molecule has Ser19, Asp24, and Ser28 in its sequence, whereas human ubiquitin has Pro, Asp, and Ala in these positions. It has been shown that the structures of ubiquitin molecules from yeast and human sources are virtually the same, in spite of the amino acid differences (Vijay-Kumar et al., 1987b). It appears that NaCl has a very similar stabilizing effect on the yeast ubiquitin molecule (Fig. 1). We also generated two mutations in the background of yeast ubiquitin, single substitution K63R, and double substitution K63R/E34S. The objective for design of the K63R mutant was to affect the nature of the charged group. The double-substituted ubiquitin molecule was motivated by the fact that glutamic acid at position 34 as judged by X-ray structure is in close proximity to the lysine at position 11 (distances from NZ-Lys11 to OE1-Glu34 and OE2-Glu34 are 3.9 and 3.3 Å, respectively), presumably forming a hydrogen bond/salt bridge. There are significant experimental (Waldburger et al., 1995) and theoretical (Yang & Honig, 1992; Hendsch & Tidor, 1994) evidences for buried or partially buried salt bridges to be destabilizing. The E34S substitution disrupts E34 to K11 interactions, and therefore, we anticipate that we will gain stability and eliminate or significantly affect the effect of salt on the stability of ubiquitin molecule. However, comparison of transition temperatures for E34S/K63R and K63R shows that thermostability of E34S/K63R is actually lower than for K63R, and there is no difference in the effect of NaCl on the thermostability of these two molecules. This implies that the E34-K11 interactions observed in the crystal structure are probably artifacts of crystal packing and are not present in the solution. Other possible candidates for the anion binding sites are three positive charged “clus-

ters" on the surface of the molecule. These clusters are formed by Lys6/His68, Lys27/Arg74, and Lys11/Lys33/Lys29. Site-directed mutagenesis will be an important tool in addressing the question of whether these positively charged residues are really involved in the formation of the anion binding sites, and such experiments are currently under way in our laboratory. One has to keep in mind that anion binding sites do not have to be formed by only charged residues. As has been shown by X-ray analysis of a number of proteins, the anion binding sites are formed predominantly by polar groups, and the role of charged groups in the formation of the anion binding site is not important (He & Quijcho, 1993; Ledvina et al., 1996; Oikonomakos et al., 1996; Quijcho, 1996; Yao et al., 1996). We note, however, that more complex anions (phosphate, sulphate, etc.) were studied in these cases.

Concluding remarks

There is an increasing amount of evidence that GdmCl can have dual actions on the stability of proteins: classical denaturing effect and its stabilizing effect presumably via ion binding and/or electrostatic screening of charges. This latter stabilizing contribution is tremendous in the case of ubiquitin: 2 M GdmCl stabilizes the protein by 8 °C. We believe that such effects of GdmCl on protein stability are common, and particular caution should be used in interpreting GdmCl-induced denaturation experiments.

Materials and methods

Bovine red blood cell ubiquitin, ribonuclease A, and hen egg white lysozyme were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

A plasmid for expression of ubiquitin in yeast has been provided by Dr. Daniel Finley (Harvard Medical School). The ubiquitin gene from this construct (YE52a) was amplified using PCR. Primers were designed so that *NdeI* and *EcoRI* sites were introduced at the 5' and 3' ends, respectively. These restriction sites were used to insert the ubiquitin gene into the pVEX plasmid (Presper et al., 1989), which is a modification of the T7 RNA polymerase expression system described by Studier et al. (1990). The resulting plasmid carrying the ubiquitin gene (pXYUB) was sequenced using a T7 promoter primer to confirm the sequence of the inserted gene. The BL21(DE3) strain of *Escherichia coli* carrying the *lacI* gene, the *lacUV5* promoter, the beginning of the *lacZ* gene, and the gene of T7 RNA polymerase, was transformed with the pXYUB plasmid. The *lacUV5* promoter is inducible by isopropyl- β -D-thiogalactopyranoside (IPTG). The protein purification protocol was modified and optimized from that of Burch and Haas (1994). The final yield of pure ubiquitin was on the order of 50 mg per liter of rich media.

Site-directed mutagenesis was carried out using sequential step PCR (Cormack, 1992). The sequence of the mutants was confirmed by sequencing the entire gene using the dideoxy chain termination method on ABI Prism 310 Genetic analyzer.

Concentration of ubiquitin in solution was measured spectrophotometrically at 280 nm using an extinction coefficient of 0.149 o.u.·mL·mg⁻¹·cm⁻¹ (Wintode et al., 1994).

Differential scanning calorimetry (DSC)

The calorimetric experiments were performed on a differential scanning calorimeter developed at The Johns Hopkins University

using principles described elsewhere (Privalov & Plotnikov, 1989). The protein sample at a concentration of 2–3 mg/mL was extensively dialyzed at 4 °C against the corresponding buffer using Spectrapor 3 dialysis membranes with a molecular weight cutoff of 3,500 Da. Insoluble material was removed by centrifugation for 15–20 min at 13,000 rpm. All experiments were performed at a heating rate of 1 °C/min. This heating rate is low enough to provide equilibrium conditions upon thermal unfolding. One of the indications of the equilibrium character of unfolding of any small globular human ubiquitin is equality of the calorimetric and fitted enthalpies as shown in Table 1 (see also Yu et al., 1994). Reversibility of the unfolding transition under several conditions was examined by rescanning the sample. As in the case of other proteins (see, e.g., Makhatadze et al., 1994), reversibility depends on the upper temperature of heating. For human ubiquitin in solution at pH 2.0 containing different concentrations of salts, rescanning after the sample was heated to the temperature just past the completion of the transition led to the recovery of at least 90% of the initial endotherm. Analysis of calorimetric profiles was performed as previously described (Wintode et al., 1994).

Solvent denaturation experiments

Solutions of ubiquitin at different concentrations of urea and GdmCl were prepared by mixing known amounts of buffer, buffered stock solutions of urea or GdmCl, and stock solution of ubiquitin in buffer. Concentrations of urea and GdmCl solutions were calculated from the refractive index as described by Pace (1986). Unfolding of the protein was monitored using CD or fluorescence spectroscopies. Far-UV CD spectra of ubiquitin at 25 °C were recorded on the Jasco J-700 spectropolarimeter. Rectangular quartz cells with a 1-mm path length were used in all experiments. The final protein concentration was on the order of 0.02 mg/mL. Changes in the maximum of fluorescence emission spectra ($\lambda_{\text{max}} = 310 \text{ nm}$) of ubiquitin were recorded on the SLM-4800C spectrofluorometer. Protein sample was excited at 278 nm. The final protein concentration was on the order of 0.05 mg/mL.

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