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Denaturant *m* values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding

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Abstract

Denaturant *m* values, the dependence of the free energy of unfolding on denaturant concentration, have been collected for a large set of proteins. The *m* value correlates very strongly with the amount of protein surface exposed to solvent upon unfolding, with linear correlation coefficients of $R = 0.84$ for urea and $R = 0.87$ for guanidine hydrochloride. These correlations improve to $R = 0.90$ when the effect of disulfide bonds on the accessible area of the unfolded protein is included. A similar dependence on accessible surface area has been found previously for the heat capacity change (ΔC_p), which is confirmed here for our set of proteins. Denaturant *m* values and heat capacity changes also correlate well with each other. For proteins that undergo a simple two-state unfolding mechanism, the amount of surface exposed to solvent upon unfolding is a main structural determinant for both *m* values and ΔC_p.

Keywords: denaturation; guanidine hydrochloride; heat capacity changes; *m* values; protein folding; protein stability; solvent-accessible surface area; urea

It has been known for many years that proteins can be unfolded in aqueous solution by high concentrations of certain reagents such as guanidine hydrochloride or urea. Denaturation with these chemicals is one of the primary ways of measuring the conformational stability of proteins and comparing the stabilities of mutant proteins. The use of these two denaturants is extremely widespread (Pace, 1986), even though the exact nature of the molecular interaction of denaturant molecules with protein surfaces is not well understood. It is known from solubility and transfer experiments with model compounds that the interaction of urea and Gdn HCl with the constituent groups of proteins is more favorable than the interaction of those groups with water (Tanford, 1970). These denaturants alter the equilibrium between the native (folded) and denatured (unfolded) states of the protein:

$$F \leftrightharpoons U.$$

The starting point for analysis of the free energy of the unfolding reaction is:

$$\Delta G = -RT\ln(U/F), \quad (1)$$

where F is the concentration of protein in the folded or native conformation, and U is the concentration of protein in the unfolded or denatured state at a particular denaturant concentration. The relative concentrations of F and U can be easily determined from studies where spectral probes are used to monitor the conformational state of the protein (see Pace, 1986). Naturally, Equation 1 only works if the protein molecules are in either one of two conformational states (a two-state unfolding reaction), which seems to be true for most small, globular proteins. If a three-state unfolding reaction is involved, or if the transition involves dimers or higher order species, an expression similar to Equation 1 can be used to determine ΔG for the transition in question (see the Materials and methods for the dimer

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Abbreviations: Gdn HCl, guanidine hydrochloride; LEM, linear extrapolation method; SPDS, solvent perturbation difference spectroscopy; ΔASA, change in solvent-accessible surface area; ΔASA_{np}, ΔASA_{pol}, the nonpolar and polar contributions to ΔASA; ΔC_p, denaturation heat capacity changes; DSC, differential scanning calorimetry; RNase, ribonuclease.

case). The free energy of unfolding so obtained is plotted against denaturant concentration in the transition region, and the free energy of unfolding in the absence of denaturant, ΔG^{H_2O}, is determined by extrapolating back to zero denaturant concentration using one of several extrapolation procedures.

The interaction of denaturants like urea or Gdn HCl with proteins shows a dependence of the free energy of unfolding on the molar concentration of denaturant that appears to be linear, at least at moderate to high denaturant concentrations where the transition typically occurs. This has led to the widespread use of the linear extrapolation method to estimate the conformational stability of the protein in the absence of denaturant. Application of this method gives two parameters, the free energy of unfolding at zero denaturant concentration (the intercept, ΔG^{H_2O}) and the dependence of free energy on denaturant concentration (the slope, which has been given the symbol m by Greene & Pace [1974]):

$$\Delta G = \Delta G^{H_2O} - m[\text{denaturant}]. \quad (2)$$

There are other methods that have been used to extract stability parameters from denaturation data, two of which derive from Tanford. Although this paper will concentrate on the parameter m from the LEM, these other methods deserve mention here as well, because all three of these alternative methods predict an upward curvature in the dependence of ΔG on denaturant resulting in a larger estimate of ΔG^{H_2O} than determined by the LEM.

The denaturant binding model (Aune & Tanford, 1968) assumes a discrete number of binding sites on the protein molecule for denaturant. The protein unfolds with increasing denaturant concentration because more binding sites are exposed in the unfolded form than in the folded form. If the sites are equivalent and noninteracting, then

$$\Delta G = \Delta G^{H_2O} - \Delta nRT \ln(1 + ka), \quad (3)$$

where Δn is the difference in the number of binding sites between U and F, k is the equilibrium binding constant (assumed equivalent for all sites), and a is the activity of denaturant. In this method, the parameters ΔG^{H_2O} and Δn are somewhat sensitive to the choice of k (Pace, 1986). Binding constants for the denaturation of proteins and peptide helices and from the study of denaturant interaction with model compounds are not in exact agreement, so the proper binding constants to use for urea and Gdn HCl are not clear (Pace, 1986; Makhatadze & Privalov, 1992; Scholtz et al., 1995, and references therein). Evidence for specific binding of denaturant molecules to protein is not very strong in any case. The very weak binding of denaturant leads to thermodynamic inconsistencies when a stoichiometric binding model is applied (Schellman, 1987).

Another method by Tanford (1970) makes use of model compound data on the solubility or transfer of amino acid analogs from water to aqueous urea or Gdn HCl solutions. By utilizing the following thermodynamic cycle:

$$\begin{array}{ccc} F_{H_2O} & \xleftrightarrow{\Delta G^{H_2O}} & U_{H_2O} \\ {\scriptstyle \Delta G_{tr,f}}\updownarrow & & \updownarrow{\scriptstyle \Delta G_{tr,u}} \\ F_{den} & \xleftrightarrow[\Delta G]{} & U_{den} \end{array}$$

one finds $\Delta G - \Delta G^{H_2O} = \Delta G_{tr,u} - \Delta G_{tr,f}$. The difference between $\Delta G_{tr,u}$ and $\Delta G_{tr,f}$ depends only on the groups exposed to solvent upon unfolding. If we let α_i represent the average fractional change in exposure of groups of type i, then

$$\Delta G_{tr,u} - \Delta G_{tr,f} = \sum_i \alpha_i n_i \delta g_{tr,i}, \quad (4)$$

where n_i is the total number of groups in the protein and $\delta g_{tr,i}$ is the free energy of transfer of one group from water to denaturant as determined from model compound data. For simplicity, the right side of Equation 4 is usually changed to $\Delta\alpha \sum_i n_i \delta g_{tr,i}$ where $\Delta\alpha$ represents the average change in exposure of all protein groups (Pace, 1986). Thus, the equation takes the final form:

$$\Delta G = \Delta G^{H_2O} + \Delta\alpha \sum_i n_i \delta g_{tr,i}. \quad (5)$$

This analysis generates ΔG^{H_2O} and $\Delta\alpha$, which represent the conformational stability of the protein in the absence of denaturant and the average change in accessibility of the protein groups upon denaturation, respectively.

A variation on this method was employed by Staniforth et al. (1993), in which the free energies of transfer from water to denaturant solution of model compounds were introduced into the analysis as follows:

$$\Delta G = \Delta G^{H_2O} + n\Delta G_{sm}[D]/(K_{den} + [D]), \quad (6)$$

where n is the number of internal side chains exposed during unfolding, and ΔG_{sm} and K_{den} are empirical constants representing the transfer behavior of internal side chains from water to denaturant, calculated from solubility data for model compounds and the composition of the protein being studied.

These three physical models require additional parameters representing the binding behavior of denaturant or the transfer behavior of protein groups into denaturant, as opposed to the empirical LEM, which has only two parameters. In any case, whether the binding model, Tanford's method, or the method of Staniforth et al. is used to analyze denaturation data, two key parameters are obtained: (1) the free energy of unfolding in the absence of denaturant, and (2) a parameter Δn, $\Delta\alpha$, or n, which is proportional to the amount of protein becoming exposed to solvent upon unfolding. In an analogous way, the LEM gives two parameters: the free energy of unfolding in the absence of denaturant and the m value. Consequently, we might expect m to be proportional to the amount of protein becoming exposed to solvent when the protein unfolds.

According to Schellman (1978), who first gave a theoretical, thermodynamic treatment of the interaction of denaturant molecules with proteins, the m value should be proportional to the surface area of protein exposed to solvent upon unfolding. A similar conclusion was reached by Alonso and Dill (1991) with their statistical thermodynamic model for the action of solvents on protein stability. To our knowledge, no one has attempted to correlate experimental m values with changes in accessible surface area upon unfolding, which are readily calculated from crystal structures using well-known methods (e.g., Lee & Rich-

Table 1. *Characteristics of 45 proteins that have m values and crystal structures available*[A]

Protein name	PDB	# Res.	# Crosslinks	ΔASA	ΔASA_{np}	ΔASA_{pol}	Gdn HCl m	Urea m	ΔC_p
Ovomucoid third domain (turkey)	1CHO	56	3	3,559	2,257	1,302	580[a]	250[a]	590[a]
IgG binding domain of protein G	1PGB	56	0	4,102	2,933	1,169	1,800[b]		620[b]
BPTI (A30, A51)	7PTI	58	2	4,288	2,949	1,339	1,200[c]		
BPTI (V30, A51)	1AAL	58	2	4,098	2,986	1,112	1,500[c]		
SH3 domain of α-spectrin	1SHG	62	0	4,560	3,439	1,121	1,880[d]	766[d]	813[d]
Chymotrypsin inhibitor 2	2CI2	64	0	4,868	3,552	1,316	1,890[e]		720[e]
Calbindin D9K	3ICH	75	0	6,162	4,361	1,801		1,140[f]	
Ubiquitin	1UBI	76	0	6,075	4,538	1,537	1,750[g]		1,360[h]
HPr (*B. subtilis*)	2HPR	88	0	7,090	5,212	1,878		1,050[i]	1,160[i]
Barstar	1BTA	89	0	7,402	5,913	1,489	2,400[j]	1,250[j]	1,460[j]
Lambda repressor (N-terminal)	1LMB	102	0	6,944	4,917	2,027	2,400[k]	1,090[l]	
Cytochrome *c* (tuna)	5CYT	103	1	9,756	6,732	3,024	2,800[m]		
Cytochrome *c* (horse heart)	2PCB	104	1	9,671	6,909	2,761	3,010[n]	1,200[n]	1,730[o]
Ribonuclease T1	9RNT	104	2	8,503	5,014	3,488	2,560[p]	1,210[q]	1,270[r]
Arc repressor[B]	1PAR	106	0	9,232	6,152	3,080	3,270[s]	1,910[s]	1,600[s]
FK binding protein (human)	1FKD	107	0	9,015	6,624	2,391		1,460[t]	
Iso-1-cytochrome *c* (yeast)	1YCC	108	1	9,170	6,235	2,935	3,400[u]	1,430[v]	1,370[w]
Thioredoxin (*E. coli*)	2TRX	108	1	9,123	6,828	2,295	3,310[x]	1,300[x]	1,660[y]
Barnase	1RNB	110	0	9,507	6,706	2,801	4,400[z]	1,940[aa]	1,650[bb]
Ribonuclease A	9RSA	124	4	10,117	6,819	3,638	3,100[p]	1,100[cc]	1,230[o]
ROP	1ROP	126	0	10,246	7,072	3,174	2,400[dd]		1,890[dd]
Che Y (*E. coli*)	3CHY	129	0	11,479	8,303	3,176	2,260[ee]	1,600[ee]	
Lysozyme (hen egg white)	6LYZ	129	4	11,406	7,474	3,932	2,330[ff]	1,290[gg]	1,540[o]
Lysozyme (human)	1LZ1	130	4	11,809	8,067	3,742	3,460[hh]		1,580[ii]
Fatty acid binding protein (rat)	1IFC	131	0	12,145	8,411	3,734	4,470[jj]	1,770[jj]	
Staphylococcal nuclease	2SNS	149	0	12,058	8,465	3,593	6,830[kk]	2,380[kk]	2,320[ll]
Interleukin 1-β	5I1B	153	0	13,971	10,180	3,791	5,580[mm]		1,890[nn]
Apomyoglobin (horse)	1YMB	153	0	13,557	9,909	3,648		1,800[oo]	
Apomyoglobin (sperm whale)[C]	5MBN	153	0	13,659	9,791	3,868		2,040[pp]	
Metmyoglobin (horse)	1YMB	153	0	14,735	10,862	3,873	3,710[qq]	2,140[qq]	1,870[rr]
Metmyoglobin (sperm whale)	5MBN	153	0	14,811	10,726	4,085	2,600[qq]	1,460[qq]	2,770[o]
Ribonuclease H	2RN2	155	0	13,726	9,615	4,111	4,500[ss]	1,930[ss]	
Dihydrofolate reductase (*E. coli*)	4DFR	159	0	13,639	10,286	3,353		1,900[tt]	
T4 lysozyme (T54, A97)	1L63	163	0	14,840	10,429	4,411	5,500[uu]	2,000[uu]	2,570[vv]
Gene V protein[B]	1BGH	174	0	13,512	9,644	3,868	3,600[ww]		
Adenylate kinase (porcine)	3ADK	194	0	16,759	11,924	4,835	4,800[xx]		
HIV-1 protease[B]	1HVR	198	0	18,834	12,564	6,270		2,050[yy]	
SIV protease[B]	1SIV	198	0	17,939	13,633	4,306		1,880[yy]	
Trp aporepressor[B]	3WRP	214	0	18,245	13,187	5,058		2,900[zz]	
α-Chymotrypsin	4CHA	241	5	21,699	15,714	5,985	4,100[cc]	2,070[cc]	3,020[o]
Chymotrypsinogen A	2CGA	245	5	21,933	15,586	6,347	4,440[aaa]	2,030[aaa]	
Tryptophan synthase, α-subunit[C]	1WSY	268	0	23,147	17,097	6,050		3,750[bbb]	4,600[ccc]
β-Lactamase[C]	3BLM	270	0	25,012	17,923	7,089	7,200[ddd]	3,210[ddd]	
Pepsinogen[C]	2PSG	370	3	35,002	25,607	9,395		7,800[ff]	6,090[o]
Phosphoglycerate kinase (yeast)	3PGK	415	0	36,125	26,212	9,913	9,700[eee]		7,500[fff]

[A] For each protein, the PDB file code, number of residues, and number of disulfides or covalent heme-protein crosslinks is shown. ΔASA and the nonpolar and polar contributions are calculated as described in the text. The last three columns give experimental m values for Gdn HCl or urea denaturation and the observed ΔC_p for each protein, when available. ΔASA values are in Å^2, m values in cal/(mol·M), and ΔC_p in cal/(mol·K)

[B] Dimer.

[C] Three-state mechanism.

[a] Swint and Robertson, 1993; [b] O'Neil et al., 1995; [c] Hurle et al., 1990; [d] Viguera et al., 1994; [e] Jackson et al., 1993; [f] Akke and Forsen, 1990; [g] Khorasanizadeh et al., 1993; [h] Wintrode et al., 1994; [i] Scholtz, 1995; [j] Agashe and Udgaonkar, 1995; [k] Lim et al., 1992; [l] Marqusee and Sauer, 1994; [m] McLendon and Smith, 1978; [n] Hagihara et al., 1994; [o] Privalov and Gill, 1988; [p] Pace et al., 1990; [q] Shirley et al., 1992; [r] Yu et al., 1994; [s] Bowie and Sauer, 1989; [t] Egan et al., 1993; [u] Ramdas et al., 1986; [v] Bryant et al., 1985; [w] Cohen and Pielak, 1994; [x] Kelley et al., 1987; [y] Santoro and Bolen, 1992; [z] Clarke and Fersht, 1993; [aa] Pace et al., 1992; [bb] Griko et al., 1994; [cc] Greene and Pace, 1974; [dd] Munson et al., 1994; [ee] Filimonov et al., 1993; [ff] Saito and Wada, 1983; [gg] Ahmad and Bigelow, 1982; [hh] Taniyama et al., 1992; [ii] Herning et al., 1992; [jj] Ropson et al., 1990; [kk] Shortle and Meeker, 1986; [ll] Carra et al., 1994; [mm] Craig et al., 1987; [nn] Makhatadze et al., 1994; [oo] De Young et al., 1993; [pp] Barrick and Baldwin, 1993; [qq] Pace, 1975; [rr] Kelly and Holladay, 1990; [ss] Dabora and Marqusee, 1994; [tt] Perry et al., 1987; [uu] Zhang et al., 1993; [vv] Hu et al., 1992a; [ww] Liang et al., 1993; [xx] Tian et al., 1988; [yy] Grant et al., 1992; [zz] Gittelman and Matthews, 1990; [aaa] Ahmad and Bigelow, 1986; [bbb] Stackhouse et al., 1988; [ccc] Yutani et al., 1991; [ddd] Mitchinson and Pain, 1985; [eee] Ritco-Vonsovici et al., 1995; [fff] Murphy and Freire, 1992.

ards, 1971; Miller et al., 1987; Lesser & Rose, 1990). Here we examine the relationship between *m* values and accessible surface areas and in the process hope to shed light on the interaction of denaturants with proteins, and on certain other aspects of protein denaturation.

Results

Change in surface area upon unfolding

Table 1 shows the 45 proteins, gathered from the literature, that have *m* values from denaturation experiments and also have a crystal structure available, along with the number of residues and disulfide bonds present in each protein.

The change in solvent-accessible surface area upon unfolding, as determined by the differences in solvent accessibility of the native form (calculated from the crystal structure) and the unfolded form (as modeled by an extended polypeptide chain) is given in Table 1, column 5. The nonpolar and polar contribution to the total ΔASA are given in columns 6 and 7. The amount of area buried in each protein correlates very strongly ($R = 0.99$) with the number of residues in each protein, as shown in Figure 1. Therefore, the ΔASA of a typical, globular protein can be estimated fairly accurately based simply on its size. On average, about 30% of the area buried in a folded protein is polar. Most of this is due to the burial of peptide groups.

Correlations of m values and heat capacity changes with ΔASA

Denaturant *m* values for Gdn HCl and urea are given in Table 1, columns 8 and 9. In Figure 2A and B, the dependence of denaturant *m* values on the change in accessible surface area upon unfolding is shown. There is a significant linear correlation in both cases, with an *R* value of 0.87 for Gdn HCl and 0.84 for urea. The slopes of the linear regression lines are 0.11 cal/(mol·M·Å^2) and 0.22 cal/(mol·M·Å^2) for urea and Gdn HCl, respectively. These represent the contribution to *m* per square

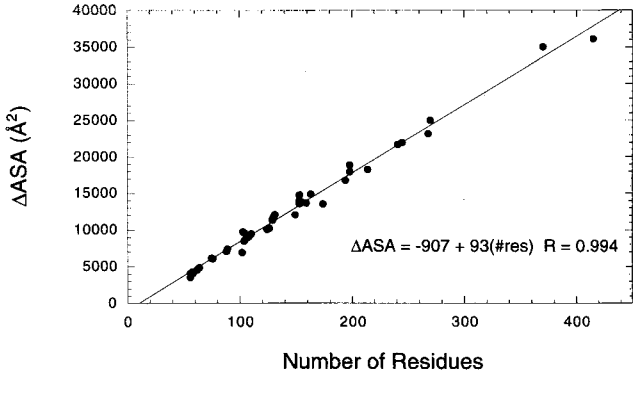

Fig. 1. Dependence of the calculated change in solvent-accessible surface area upon unfolding on the number of residues for the 45 proteins given in Table 1.

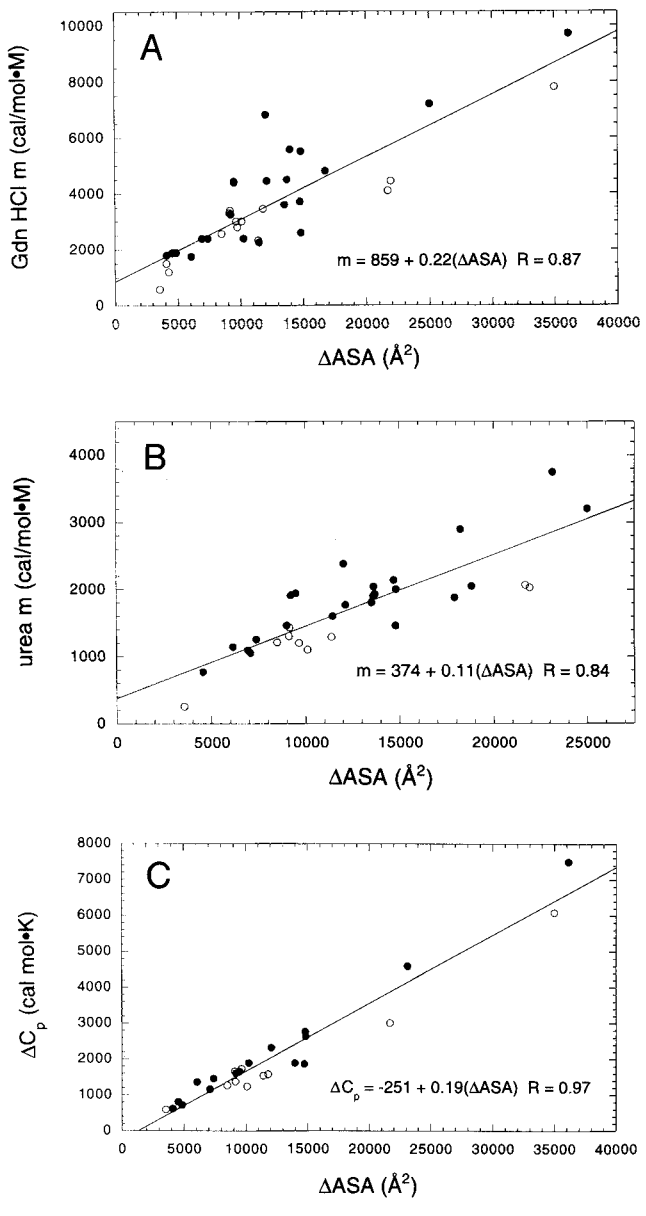

Fig. 2. Dependence of (**A**) *m* value for Gdn HCl denaturation, (**B**) *m* value for urea denaturation, and (**C**) heat capacity change upon unfolding on ΔASA for the 45 proteins shown in Table 1. Proteins with no crosslinks are shown as ● and those with crosslinks as ○.

Ångstrom of buried surface and indicate that Gdn HCl is twice as effective as a denaturant than is urea.

Although not the focus of this paper, denaturation heat capacity changes have been shown previously to be linearly dependent on ΔASA for several proteins (Livingstone et al., 1991; Spolar et al., 1992). For our set of proteins, ΔC_p values were also collected (given in Table 1, column 10, values in cal/[mol·K]) and correlated with the ΔASA values. The strong linear correlation ($R = 0.97$) is shown in Figure 2C. The average value of ΔC_p per residue is 14.2 ± 2.5 cal/(mol·K·residue). The values range from a low of 9.9 cal/(mol·K·residue) for RNase A to a high of 18.1 cal/(mol·K·residue) for sperm whale myoglobin.

Table 2. *Contribution of disulfide crosslinks to changes in accessible surface area (ΔASA) from solvent perturbation difference spectroscopy (SPDS)*[a]

	# -S-S-	% Trp + Tyr accessibility: Disulfides intact	Disulfides broken	Difference	*ASA*(unf)	*ΔASA*	*ΔASA*/ disulfide
Lysozyme	4	69	93	24	18,097	4,343	1,086
RNase A	4	68	88	20	17,001	3,400	850
RNase T1	2	86	95	9	13,863	1,248	624

[a] Data on Trp and Tyr accessibility are from Pace et al. (1992). Effects on *ΔASA* for folding are calculated as described in the text. ASA values are expressed in Å^2.

Correlations using ΔASA values corrected for crosslinks

It can be noticed in the plots of m against *ΔASA* that proteins with disulfide bonds or other crosslinks[4] tend to have lower m values than expected based on their *ΔASA* (they fall below the regression line). This is expected because the presence of crosslinks in the unfolded state will result in a more compact unfolded state, thus reducing the accessibility of the unfolded polypeptide chain to solvent. Consequently, the *ΔASA* computed using our method would be too high relative to a protein with no crosslinks. To compensate for the effect of crosslinks, we employ three different ways to estimate the magnitude of the reduction in *ΔASA* per disulfide bond. In the first method, the results provided by measurements of the accessibility of aromatic groups in proteins using solvent perturbation difference spectroscopy have been analyzed. The presence of certain reagents will change the extinction coefficients of aromatic residues. Because the amount of this change is proportional to the accessibility of the aromatic group to the perturbant, one can get an idea of the solvent accessibility of aromatic residues using this technique. Table 2 shows SPDS results for three proteins, taken from Pace et al. (1992), where the accessibility of the protein aromatic groups in three unfolded proteins is compared with and without disulfides. Clearly, the average accessibility of the aromatic groups increases when the disulfides are broken. Assuming that this change in accessibility applies to all protein groups, then the change in square Ångstroms is obtained by multiplying the total *ASA* of the extended chain by the percent difference. This total, divided by the number of disulfides, gives the average change in *ASA* of the unfolded state (and hence the change in *ΔASA*) per disulfide for the three proteins. The average of the three is about 900 Å^2.

Doig and Williams (1991) have estimated the change in ΔASA_{np} per disulfide bond from the dependence of hydration free energy and ΔC_p on crosslinks to be 590 Å^2 and 690 Å^2, respectively. Because the fraction of total area buried that is nonpolar is about 0.70, these values correspond to a change in total area of 850 Å^2 and 990 Å^2 per disulfide. These values are in good agreement with the value calculated from the SPDS results. The final method used to estimate the effect of crosslinks on *ΔASA* is discussed below.

Therefore, based on the above evidence, corrections of *ΔASA* for the effect of disulfide crosslinks on the accessibility of the unfolded state were made at 900 Å^2 per disulfide bond and the m values and ΔC_p values were correlated with the corrected *ΔASA* values. Linear correlation coefficients improve to 0.90 for both urea and Gdn HCl and improve to 0.98 for ΔC_p, as shown in Figure 3A, B, and C. The amount that a particular disulfide bond reduces the accessibility of a protein in the unfolded state depends on several factors: the size of the loop connected by the crosslink, the position relative to other crosslinks, and the overall size of the protein. The fact that using a single value for all crosslinks, obviously a major simplification, improves all three correlations suggests that our treatment is at least a reasonable approximation.

Other correlations of interest

If the mechanism of denaturation is similar for both urea and Gdn HCl, then the two m values should correlate with each other. For proteins with m values for both Gdn HCl and urea available, the two m values were correlated in Figure 4 (R = 0.90), indicating that the same factors are affecting both m values. Because m values and ΔC_p values both show a strong correlation with *ΔASA*, they should correlate with each other. Figure 5A and B shows the relation of Gdn HCl and urea m values to the ΔC_p of the same protein; a good correlation is found for each.

Nonlinear least-squares fitting of the data

As mentioned above, previous studies have already noted a correlation between *ΔASA* and heat capacity changes, ΔC_p. By fitting data for the transfer of model compounds from the liquid state to water, Spolar et al. (1992) determined an equation to estimate the ΔC_p from nonpolar and polar *ΔASA* values:

$$\Delta C_p = (0.32 \pm 0.04)(\Delta ASA_{np}) - (0.14 \pm 0.04)(\Delta ASA_{pol}). \quad (7)$$

[4] The three cytochromes contain a covalently attached heme group. For the purposes of the present analysis, we have assumed that this crosslinking can be treated in the same manner as a normal disulfide bond. This may be an oversimplification, but the paucity of data precludes a more detailed evaluation.

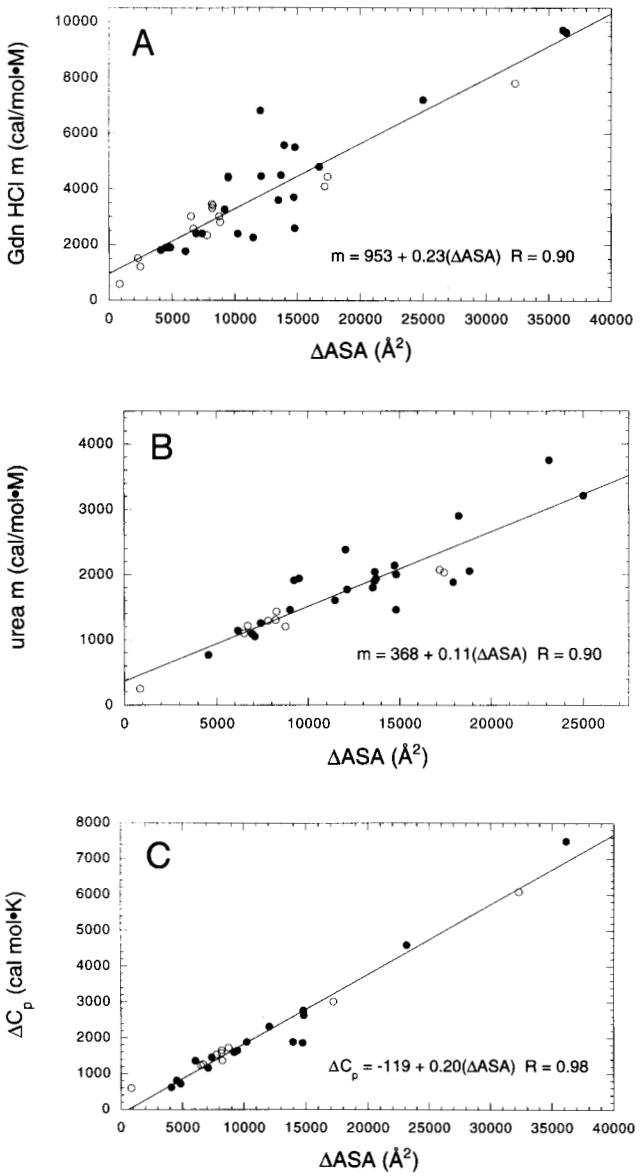

Fig. 3. Dependence of **(A)** m value for Gdn HCl denaturation, **(B)** m value for urea denaturation, and **(C)** heat capacity change upon unfolding on ΔASA for the 45 proteins in our data set, corrected for the effect of crosslinks by 900 Å^2 per crosslink (see text). Proteins with no crosslinks are shown as ● and those with crosslinks as ○.

By nonlinear fitting of data for the dissolution of solid model compounds, Murphy and Freire (1992) give as the best equation:

$$\Delta C_p = (0.45 \pm 0.02)(\Delta ASA_{np}) - (0.26 \pm 0.03)(\Delta ASA_{pol}). \quad (8)$$

The heat capacity changes for the proteins in our set were calculated using each of these equations and plotted versus the observed heat capacities in Figure 6. Both methods fit the data well ($R = 0.97$), but as can be seen from the slope of the regression line (slope = 0.72), Equation 8 overestimates the experimental

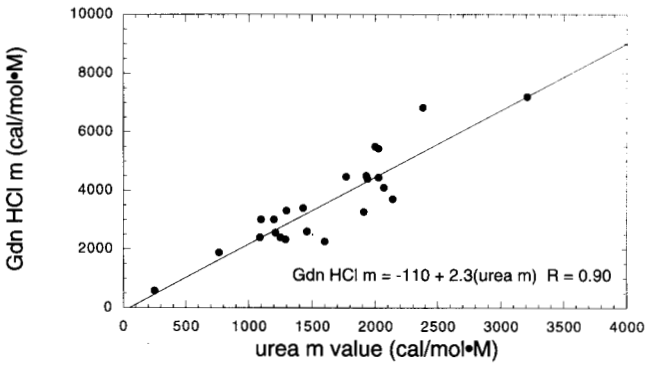

Fig. 4. Relationship between m values derived from denaturation with urea and Gdn HCl for proteins that had both m values available.

values of ΔC_p by about 25%. This is most likely due to the different methods of calculating ΔASA (see Murphy & Freire, 1992). However, the equation given by Spolar et al. (1992) is no better for estimating ΔC_p than simply using the equation from the fit in Figure 3C ($R = 0.97$), which uses the total ΔASA and thus does not require a separation into polar and nonpolar components. In addition, Equation 7 is only slightly better than simply multiplying the number of residues in a protein by 14 cal/mol·K·res). Still more accurate is using the regression equation given in Figure 4C, which features ΔASA corrected for cross-

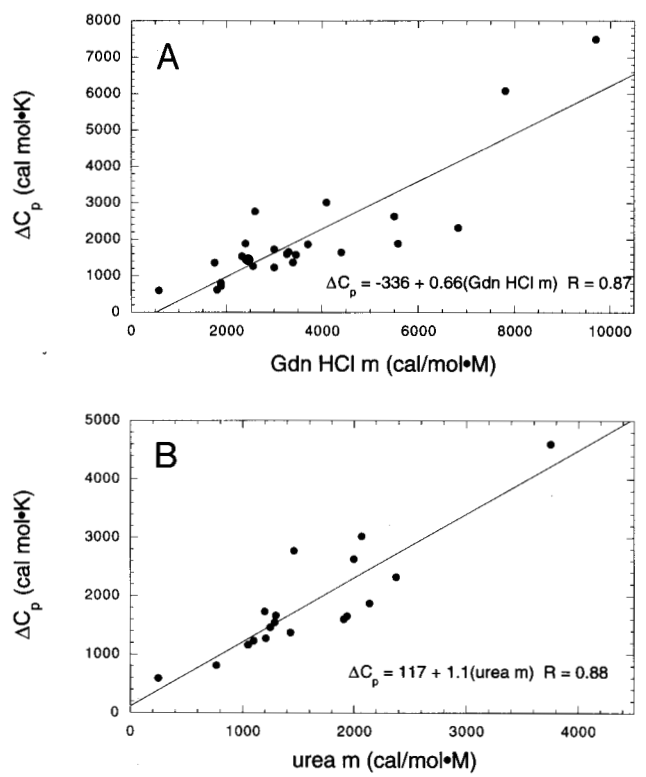

Fig. 5. Relationship between **(A)** Gdn HCl m values and **(B)** urea m values and heat capacity changes.

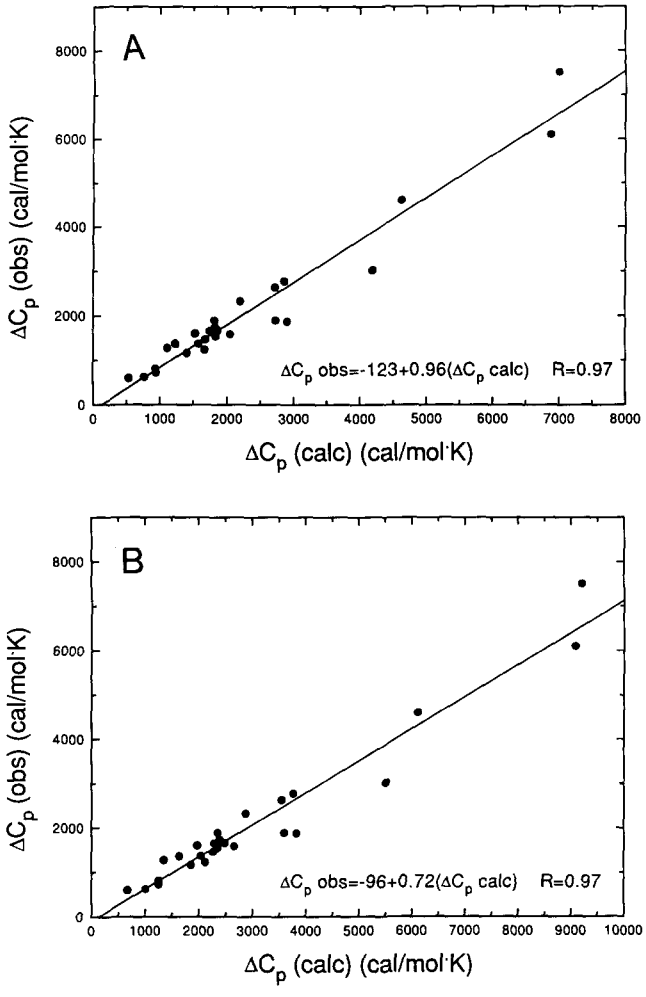

Fig. 6. Comparison of observed heat capacity changes given in Table 1 and heat capacity changes calculated using the equations given by (**A**) Spolar et al. (1992) and (**B**) Murphy and Freire (1992).

links ($R = 0.98$). Nonlinear least-squares fitting of our data to equations in the form given provides:

$$\Delta C_p = (0.28 \pm 0.12)(\Delta ASA_{np}) - (0.09 \pm 0.30)(\Delta ASA_{pol}), \quad (9)$$

which is within error of the values of Spolar et al. It gives a fit of the experimental data that is not significantly better, however ($R = 0.97$).

For denaturant m values, nonlinear least-squares fitting was used to try to separate the contribution to m of nonpolar and polar surface:

$$\text{Gdn HCl } m = (0.18 \pm 0.35)(\Delta ASA_{np}) + (0.50 \pm 0.87)(\Delta ASA_{pol}) \quad (10)$$

$$\text{Urea } m = (0.15 \pm 0.12)(\Delta ASA_{np}) + (0.08 \pm 0.29)(\Delta ASA_{pol}). \quad (11)$$

It appears from this that both denaturants interact favorably with both nonpolar and polar surfaces, but for Gdn HCl, the interaction with polar surfaces is more favorable than with nonpolar surfaces. This may be expected because Gdn HCl is ionic. The opposite appears to be true with urea, although the contributions are closer in value. Note that the separation into polar and nonpolar components of m or ΔC_p is difficult due to the high correlation of polar ΔASA with nonpolar ΔASA, resulting in large errors in the fitted parameters. The large errors in the respective fits hinder unequivocal interpretation.

Fits of the m values and ΔC_p's taking into account crosslinks gives the following:

$$\text{Gdn HCl } m = (0.28 \pm 0.03) \times [\Delta ASA - (792 \pm 780)(\#\text{ crosslinks})] \quad (12)$$

$$\text{Urea } m = (0.14 \pm 0.01) \times [\Delta ASA - (995 \pm 570)(\#\text{ crosslinks})] \quad (13)$$

$$\Delta C_p = (0.19 \pm 0.01) \times [\Delta ASA - (864 \pm 370)(\#\text{ crosslinks})]. \quad (14)$$

The disulfide bond corrections that maximize the fits are all close to the 900 Å^2 value calculated above.

Discussion

The correlations between m values and ΔASA are good, but the variations in m values for proteins close in ΔASA are quite large in some cases. We have addressed one possible cause, the effect of crosslinks on the ASA of the unfolded state, and the plots featuring the corrected ΔASA show improved correlation for Gdn HCl and urea m values and ΔC_p. But there are several other possible contributing factors.

One major consideration in any experimental measure of ΔG^{H_2O} is the possibility of deviation from a two-state unfolding mechanism. Deviation from a two-state mechanism should lower the m value (Pace, 1986). Some of the proteins in our set have been analyzed using a three-state model, but most have been analyzed using a two-state assumption. For only a few has the two-state model been confirmed experimentally. The most rigorous way of confirming the lack of intermediates present at equilibrium during thermal unfolding is to use differential scanning calorimetry. The calorimetric enthalpy can be compared to the van't Hoff enthalpy, which is calculated from the data with the two-state assumption. If these two enthalpies agree, it is good evidence for a lack of appreciable amounts of intermediate species. But this is only good for thermal unfolding. There is always the possibility that intermediates are present in unfolding by denaturants and not in thermal unfolding and there is no rigorous way of confirming a two-state denaturant unfolding mechanism. The best approach is to monitor denaturation by multiple spectral probes; CD, UV difference spectroscopy, fluorescence, and NMR have been used for this purpose. The coincidence of the unfolding curves is consistent with a two-state mechanism. For those proteins without such confirmation, a low m value may mean more than two equilibrium states. The variable amount of intermediates present at equilibrium is one explanation of the change in m values observed for staphylococcal

nuclease mutants (Carra & Privalov, 1995). An excellent review of mutational effects on *m* values in staphylococcal nuclease and other proteins has recently appeared (Shortle, 1995).

A possible factor affecting the *ASA* of the unfolded state of proteins is electrostatics. An unfolded protein with a high positive or negative charge would tend to be more extended than a neutral protein, due to the repulsion of like charges. The average accessibility of such a protein would be greater than for a neutral protein. This was an explanation suggested for the pH dependence of *m* found for RNase A, RNase T1 (Pace et al., 1990), and barnase (Pace et al., 1992). This effect could also be the cause of the curvature in the dependence of free energy on Gdn HCl observed for myoglobin (Pace & Vanderburg, 1979), because low pH was used to destabilize the protein to provide data at low denaturant concentrations (see below). Electrostatic effects are mediated by the ionic strength of the solution, and because Gdn HCl is a salt, this could add complications to the denaturation of proteins by Gdn HCl (for more about salt effects in Gdn HCl denaturation see Monera et al. [1994]). Various amounts of residual structure in the unfolded states of the proteins could also be a factor affecting the accessibility of the unfolded state (Shortle et al., 1988; Dill & Shortle, 1991).

Another factor affecting the *m* value besides ΔASA is the interaction of urea or Gdn HCl with different types of protein surface. For example, if denaturants interact more favorably with aromatic groups than with other residues (see e.g., Prakash et al., 1981) then a protein rich in aromatics should have a higher *m* value than a protein of average composition having the same ΔASA. Strong, specific binding of urea or Gdn HCl at one or more sites in the folded or unfolded protein would also affect the *m* value (Mayr & Schmid, 1993).

The validity of the linear extrapolation method for obtaining free energies of unfolding from chemical denaturation has come under question, mostly on the basis of its empirical nature. The physical models of protein denaturation mentioned in the introduction indicate that the dependence of free energy on denaturant concentration, $\delta\Delta G/\delta$[denaturant], should not be linear but should increase at low denaturant concentrations. Two studies have found such a curvature experimentally. Pace and Vanderburg (1979), in a study of the Gdn HCl denaturation of myoglobin, and Johnson and Fersht (1995), who studied urea denaturation of barnase, have both observed deviations from the LEM at low denaturant concentrations. It most cases, it is difficult to study denaturation at very low concentrations of denaturant because most proteins denature in moderate to high concentrations, and only data in the transition region can be analyzed. Pace and Vanderburg (1979) used acidic pH to lower the stability of myoglobin to obtain data at low denaturant concentrations and found an increase in $\delta\Delta G/\delta$[denaturant] at lower denaturant concentrations. It has been suggested by Yadav et al. (1992) that the curvature observed by Pace and Vanderburg (1979) at low denaturant concentrations is due to the presence of partially denatured intermediate states at low pH. Johnson and Fersht (1995) used DSC to measure the stability of barnase at different urea concentrations and found an upward curvature in the dependence of ΔG on urea concentration, as predicted by the physical models.

In contrast to these two cases, all other studies of protein denaturation at low denaturant concentrations have not observed any deviation from the LEM (Santoro & Bolen, 1992; Yadav et al., 1992; Ahmad et al., 1994; Agashe & Udgaonkar, 1995; Scholtz, 1995). Mutants of staphylococcal nuclease that denature at low denaturant concentrations show perfect linearity (Shortle & Meeker, 1986; Shortle et al., 1989; Shortle, 1995; according to Shortle [1995], the curvature in $\delta\Delta G/\delta$[denaturant] for urea apparent for some mutants was due to improper extrapolation of posttransition baselines and that correct extrapolation removes this curvature). In addition, studies of the chemical unfolding of helical peptides show that the effect of denaturants on the free energy of helix formation is described very well using the LEM, for both urea (Scholtz et al., 1995) and Gdn HCl (J.S. Smith & J.M. Scholtz, unpubl. results). In the case of helical peptides, denaturation data can be obtained to zero concentration of denaturant due to the low cooperativity of helix unfolding. Further evidence in support of the LEM is the fact that in nearly all cases, the ΔG^{H_2O} values derived from urea and Gdn HCl denaturation analyzed by linear extrapolation agree quite well with each other. This is not the case if the same data are analyzed with the binding model or Tanford's method (Pace, 1986). In addition, Bolen and Santoro (1988) and Santoro and Bolen (1988) have shown that the denaturation of phenylmethanesulfonyl α-chymotrypsin by urea, Gdn HCl, and 1,3-dimethyl urea analyzed by the LEM fit all the requirements of a thermodynamic cycle. The ΔG^{H_2O} values from linear extrapolation of urea denaturation for RNase T1 agree remarkably well with free energies obtained from thermal denaturation and DSC under a variety of conditions (Hu et al., 1992b; Yu et al., 1994). Similarly good agreement between urea and Gdn HCl data analyzed by the LEM and thermal denaturation by DSC was found by Santoro and Bolen (1992) for thioredoxin. Yao and Bolen (1995) have found good agreement between ΔG^{H_2O} found by the LEM on urea denaturation data at different values of pH and the dependence of free energy on pH using direct titrations on RNase A. Therefore, the preponderance of data suggests that the two reports of a nonlinear dependence of ΔG on denaturant concentration are exceptions, and at this point there is no compelling reason for using other, more complex analyses, despite many experiments addressing the issue. Certainly there is no reason for using the more complex methods unless deviation from the LEM is demonstrated experimentally for a particular protein.

A typical use for denaturation curves is to compare the stabilities of different proteins, for example, the differences in stability of several single amino acid mutants with the stability of the wild-type protein. If the stabilities of the proteins differ by small or moderate amounts, and the *m* values do not vary between proteins, the best way to obtain the differences in stability is by taking the difference in $[\text{denaturant}]_{1/2}$ values and multiplying by the wild-type *m* value or the average *m* value for all the proteins in the study. This gives $\Delta\Delta G$, or the change in free energy of unfolding due to the mutation. Because this is a stability difference at a denaturant concentration between the two $[\text{denaturant}]_{1/2}$ values, the necessity of a long extrapolation is eliminated and any curvature in $\delta\Delta G/\delta$[denaturant] at low denaturant concentrations has no effect on the analysis. Thus, for obtaining differences in stabilities between mutant proteins that are reasonably close in stability, the argument over different extrapolation procedures and possible curvature in $\delta\Delta G/\delta$[denaturant] is not an issue.

The good correlations found in the present study (Figs. 2, 3), even in the absence of any corrections and despite the caveats mentioned above, are good evidence for the strong relationship

between surface area (and more simply protein size) and m values. To illustrate the effect of ΔC_p and m on the unfolding of proteins, consider two hypothetical proteins A and B, which represent a small protein of about 100 residues and a medium-sized one of about 200 residues. Heat capacity change, the first unfolding parameter to be related to ΔASA, is the second derivative of the dependence of ΔG on temperature. The ΔC_p of a protein describes the amount of curvature in the plot of ΔG as a function of temperature, like the one shown in Figure 7A, called a stability curve by Becktel and Schellman (1987). The curves were generated using ΔH = 70 kcal/mol and ΔC_p = 1,000 cal/(mol·K) for protein A and ΔH = 120 kcal/mol and ΔC_p = 2,500 cal/(mol·K) for protein B. The higher the ΔC_p, the more sharply ΔG depends on temperature. So proteins with low ΔC_p's have a shallower curve as can be seen for protein A. This results in protein A having a higher denaturation midpoint (where $\Delta G = 0$; T_m = 90 °C) than protein B (T_m = 70 °C), but the conformational stability at ambient temperatures of protein B is higher: ($\Delta G_{25\,°C}$ is 8.0 kcal/mol for B and 6.3 kcal/mol for A). This explains why small proteins often have very high T_m's while still having average conformational stabilities, a phenomenon first noted by Alexander et al. (1992). Now let us consider chemical denaturation. Small proteins with low m values often have very high denaturation midpoint ([denaturant]$_{1/2}$) values while still having average stabilities. This is shown for proteins A and B in Figure 7B. The curves were generated using ΔG^{H_2O} = 6.3 kcal/mol for A and 8.0 kcal/mol for B, with m = 1,000 cal/(mol·M) for A and 2,000 cal/(mol·M) for B. From the LEM analysis in Figure 7B, one can see that the denaturation midpoint (where $\Delta G = 0$) is 6.3 M for protein A and 4.0 M for protein B, but again the conformational stability is higher for protein B. Therefore, it is improper to judge the conformational stability of a protein as high by a high T_m or a high [denaturant]$_{1/2}$, as some have done in the past. It would only be correct to say the stability against temperature, or the stability against denaturant, is high. The opposite holds for proteins with high m values and high ΔC_p's.

In summary, m values derived from analysis of urea and Gdn HCl denaturation curves are correlated strongly with the change in accessible surface area upon unfolding just as has been found with heat capacity changes. For proteins undergoing a simple two-state unfolding reaction, ΔASA is the main structural determinant for both the m value and ΔC_p.

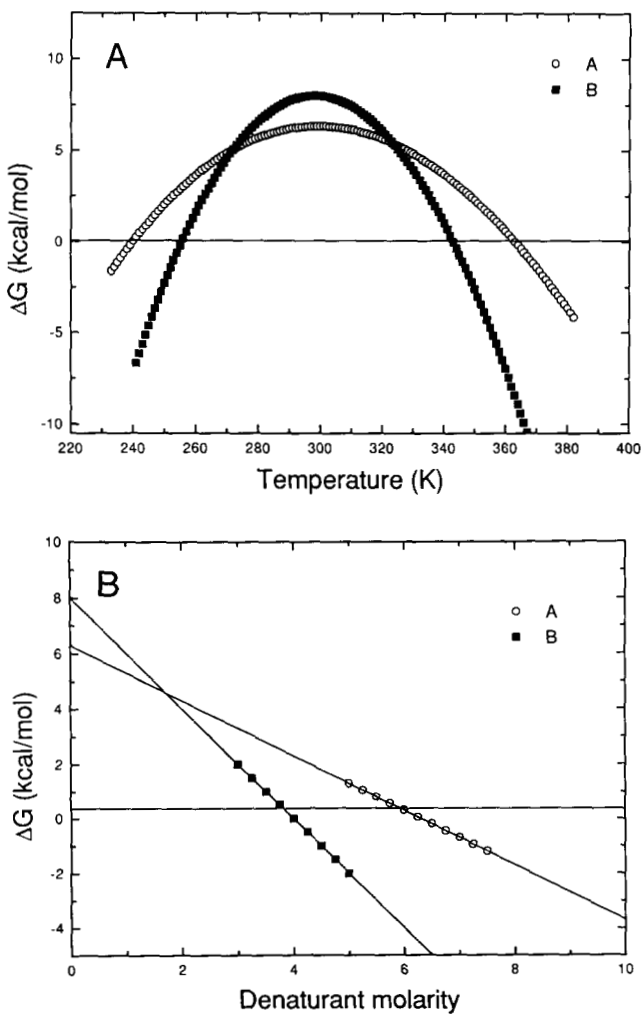

Fig. 7. Dependence of the free energy of unfolding on **(A)** temperature and **(B)** denaturant concentration for two hypothetical proteins, protein A (○) and protein B (■) (see text).

Materials and methods

A search was made of the literature to find denaturant m values of proteins that also have a good crystal structure available. Occasionally m is not given explicitly in the particular reference but can be calculated simply from the relation

$$m = \Delta G^{H_2O}/[\text{denaturant}]_{1/2}$$

or, for dimeric proteins, where ΔG^{H_2O} is concentration dependent,

$$m = (-RT \ln[\text{protein}] + \Delta G^{H_2O})/[\text{denaturant}]_{1/2},$$

where the [denaturant]$_{1/2}$ is the concentration at which the protein is half-denatured. In several proteins, m values have been measured only in one laboratory and under only one set of conditions. In some cases, like RNase A, m values from many different sources were available. Preference was given to m values measured at or near neutral pH and 25 °C. Usually m values measured under similar conditions in different laboratories are nearly identical. For proteins whose denaturation has been analyzed using a three-state model, the m value given is the sum of the m values for the two transitions.

The Brookhaven Protein Data Bank was used to obtain coordinate files for the structures of the proteins. Accessible surface areas of the native proteins were calculated using the program ACCESS (Lee & Richards, 1971), with a probe radius of 1.4 Å, a slice width of 0.25 Å, and atomic radii given by Richards (1977). Models of unfolded proteins were generated using a Silicon Graphics workstation and Insight II (Biosym). Polypeptide chains were given β-sheet extended dihedral angles after having any disulfide bonds broken. ACCESS was then used to measure the accessible surface areas of the extended chains. For those proteins in which heme groups were present the accessible surface area of the heme was calculated separately and added to the ASA of the extended chains. Surfaces were broken down into polar and nonpolar components by counting all

carbon atoms as nonpolar, and oxygen, nitrogen, and sulfur as polar. Nonlinear least-squares fitting of the data was performed using the program Nonlin for Macintosh (Johnson & Frasier, 1985; Brenstein, 1991).

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