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Thermodynamic prediction of structural determinants of the molten globule state of barnase

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Abstract

Recently, it has been demonstrated that the enthalpy and heat capacity changes for protein folding/unfolding can be predicted rather accurately from the crystallographic or NMR solution structure of a protein. (K.P. Murphy, V. Bhakuni, D. Xie and E. Freire, Mol. Biol. 227 (1992) 293–306.) Under some conditions proteins do not unfold completely, giving rise to states in which the molecule remains in a compact configuration after denaturation. These compact denatured or molten globule states retain a hydrophobic core, exhibit residual structure and a compactness close to that of the native state. This phenomenon is reflected in the thermodynamics of the process. By using the structural parametrization of the energetics, it is possible to develop an algorithm aimed at selecting partly folded states that conform to the experimental thermodynamic constraints of the molten globule. We have applied our molten globule search algorithm to the globular protein barnase. This approach has allowed a structure based selection of a unique family of structural states that satisfy the experimental criteria of the molten globule. The prediction of the molten globule search algorithm indicates that the first helix together with most of the β-sheet structure (β2, β3–5) and loop 5 constitute the main determinants of the molten globule intermediate, in agreement with the NMR data. These results open the prospect for an automated search of the structural determinants of the molten globule state of proteins and suggest that solvation parameters can be effectively used to probe structural states of proteins.

Key words: Molten globule state; Proteins; Structural determinants

1. Introduction

It is now clear that under certain solvent conditions, the heat denatured state of some proteins is not a completely unfolded and hydrated random coil, but a rather compact state exhibiting a significant hydrophobic core and different degrees of residual secondary structure. This state has been referred to as a "compact denatured" or "molten globule" state (see refs. [1–3] for reviews). In this paper, both terms will be used interchangeably and will denote a protein state characterized by: (1) Significant secondary structure; (2) Almost native compactness due to the presence of a sizable hydrophobic core; and (3) highly disrupted tertiary structure. These characteristics have been discussed elsewhere [4].

Recent experimental studies on the molten

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globule states of α-lactalbumin [5], cytochrome c [6,7], III^{Glc} [8] and the heat labile enterotoxin from E. Coli [9] have revealed that the molten globule state of these proteins has a lower heat capacity than the unfolded state, and that at low temperatures it has a higher enthalpy than the unfolded state. The lower heat capacity is consistent with the presence of a sizable hydrophobic core, while the higher enthalpy at low temperatures suggests that this state preferentially disrupts polar van der Waals and hydrogen bonding interactions [10,11].

The energetics of the molten globule state of globular proteins can be rationalized in terms of the known contributions of different interactions to the unfolding energetics [4,10,12]. Two terms are thought to account for most of the overall enthalpy change of unfolding: (1) a large positive term due to the disruption of hydrogen bonds and polar van der Waals interactions within the protein; and (2) a negative term resulting from the disruption of apolar van der Waals interactions and the subsequent hydration of apolar groups. The first term is only slightly temperature dependent while the second one has a strong positive dependence on temperature and is primarily responsible for the positive heat capacity observed upon unfolding. Of course, other partitionings of the energetics are also possible (see for example Refs. [13–16]) as researchers attempt to understand at different levels the origin of the forces responsible for protein stabilization. In this paper we are not concerned with a discussion of the origin of these forces but with the development of an automated computer search aimed at selecting protein structural states that will exhibit some predefined thermodynamic behavior. For this purpose, the important requirement is to have access to quantitatively reliable correlations between structural and thermodynamic parameters. Recently, an accurate structural parametrization of the enthalpy and heat capacity changes has been obtained [12]. This parametrization has been used to identify structural determinants of the molten globule state from an experimental knowledge of its energetics and to develop an automated search algorithm of the molten globule [17]. In this paper, the molten globule search algorithm has been applied to the globular protein barnase (MW 12.3 kDa) for which the folding pathway has been extensively studied by NMR and other techniques (see ref. [18] for a review).

2. Structural parametrization

The structural parametrization of the heat capacity and enthalpy is summarized below. The reader is referred to the original literature for details [11,12,19–21]. The relative heat capacity of an arbitrary conformational state of a protein can be expressed in terms of the difference in polar (ΔASA_{pol}) and apolar (ΔASA_{ap}) solvent accessible surface areas between that state and the native state, which is taken as the reference state,

$$\Delta C_p = 0.45\Delta ASA_{ap} - 0.26\Delta ASA_{pol}, \qquad (1)$$

where 0.45 and −0.26 are the elementary contributions per $Å^2$ of apolar and polar area exposed to water in cal $(K\ mol)^{-1}$. These two values have been obtained from a statistical analysis of the proteins for which high resolution thermodynamic and structural information is available, and the thermodynamics of aqueous dissolution of solid dipeptides [11]. Eq. (1) predicts the heat capacity change for unfolding of proteins with an accuracy close to the experimental error in the determination of ΔC_p (±9%). Unless otherwise noted, all thermodynamic quantities in this paper are referenced to the native state.

For the enthalpy change, however, different interactions contribute significantly to the observed difference between different conformations of a protein: (1) polar contributions arising from changes in the number of intramolecular hydrogen bonds; (2) van der Waals interactions between polar and apolar groups; (3) contributions arising from the hydration of protein groups that become exposed to the solvent; (4) the protonation or ionization of histidyl groups; (5) the binding or release of specific ligands or prosthetic groups, etc. The first three terms contribute most

of the enthalpy change, and can effectively be written as

$$\Delta H(T) = 34.5\Delta \mathrm{ASA}_{\mathrm{pol}} + \Delta C_{\mathrm{p}}(T - 100), \quad (2)$$

since the enthalpy change at 100°C scales linearly with the change in solvent accessible polar surface area that accompanies the transition [11,12]. This parametrization provides an accurate estimation of enthalpy values from structural parameters. At 100°C, the average error between the experimental and calculated values is 2.3%. Under standard conditions, Eq. (2) accounts for over 90% of the enthalpy change of unfolding at the experimental transition temperature (the median transition temperature for proteins in the database is 60°C [12]), since the enthalpies associated with protonation and other ionic effects are relatively small. Nevertheless, those additional contributions and those of specific ligands need to be taken into account explicitly, especially at low temperatures in which the contribution given by Eq. (2) is close to zero. The protonation of carboxylic groups has an enthalpy close to -1 kcal mol^{-1} and that of histidyl groups is close to -7 kcal mol^{-1}. The parametrization given by Eq. (1) and (2) is numerically accurate and has been used to predict protein unfolding parameters [12,20], binding of peptides to proteins [19], and the energetics of partly folded states [4,9,17].

Eqs. (1) and (2) establish a one to one corre-

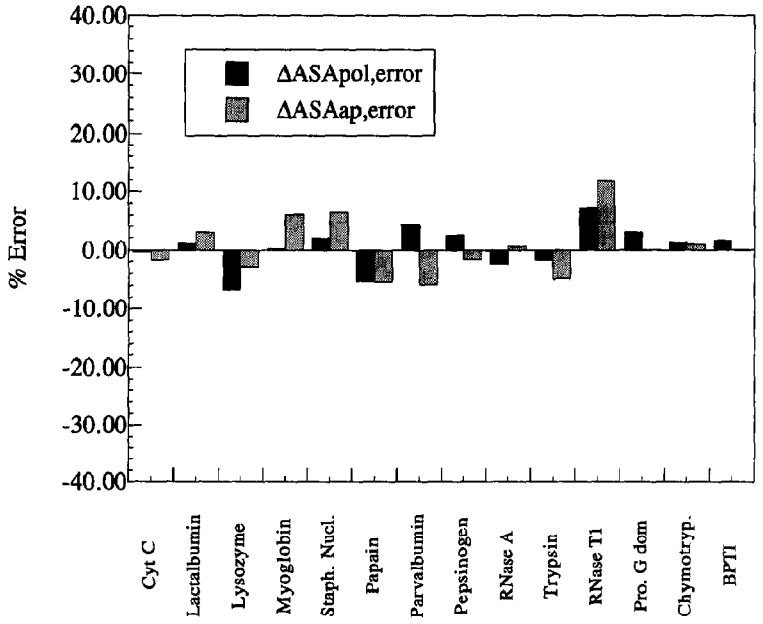

Fig. 1. Percent error in solvent accessible surface areas obtained from thermodynamic data and those calculated directly from the crystallographic structure of the proteins in the thermodynamic database, i.e. the set of proteins for which high resolution structure and thermodynamic information are available. The agreement between the calorimetric and crystallographic data is excellent, exhibiting a standard deviation of 2.8% for the polar and 3.6% for the apolar areas. At this writing the following proteins are included in the protein thermodynamic database: cytochrome c, carbonic anhydrase, chymotrypsin, α-lactalbumin, lysozyme, myoglobin, staph. nuclease, papain, parvalbumin, pepsinogen, ribonuclease A, ribonuclease T1, trypsin, protein G and and BPTI. In these and all calculations, accessible surface areas (ASA) of protein structures were analyzed as described before [12] using the program ACCESS (Scott R. Presnell, University of California, San Francisco), an implementation of the Lee and Richard's algorithm [23], with a probe radius of 1.4 Å and a slice width of 0.25 Å. Accessible surface areas in the unfolded state were modeled as the sum of the ASA values of the individual residues in the extended Ala-X-Ala tripeptide generated using Quanta (Polygen, Waltham, MA), taking into account the additional exposure at the carboxyl and amino terminus of the protein.

spondence between the enthalpy and heat capacity changes, and the polar and apolar solvent accessibilities. Rearrangement of Eqs. (1) and (2) yields

$$\Delta ASA_{pol} = [\Delta H(100)]/34.5, \quad (3)$$

$$\Delta ASA_{ap} = [\Delta C_p + (0.26/34.5)\Delta H(100)]/0.45, \quad (4)$$

and provide a way of estimating solvent accessibility changes directly from thermodynamic parameters. Eqs. (3) and (4) have been shown to predict ΔASA_{pol} and ΔASA_{ap} with high accuracy [22]. A comparison of the changes in solvent accessible surface areas obtained from calorimetric data and those calculated directly from the crystallographic structure of the proteins in the thermodynamic database yields a standard deviation of 2.8% for the polar and 3.6% for the apolar areas. These results are shown in Fig. 1.

3. Experimental thermodynamics of the molten globule state.

It has been shown that the molten globule states of cytochrome c [6] and α-lactalbumin [5] are enthalpically higher than the unfolded state at temperatures below 25°C. At high temperatures, however, the unfolded state is enthalpically higher because it has a larger heat capacity and, therefore, its enthalpy increases at a faster rate with temperature. For α-lactalbumin the enthalpy of the molten globule (ΔH_{MG}) and the enthalpy of the unfolded state (ΔH_U) are equal at approximately 45°C while for the cytochrome c the isoenthalpic temperature is close to 30°C. A qualitatively similar behavior has been observed for III^{Glc} [8] and the heat labile enterotoxin from E. Coli [9].

Analysis of the thermodynamic data for α-lactalbumin and cytochrome c in terms of Eqs. (3) and (4) indicates that a higher proportion of polar than apolar interactions are disrupted in the molten globule state. In terms of the experimental parametrization of the enthalpy, the ratio $\Delta H_{MG}/\Delta H_U$ at 100°C is equal to the fraction of the total polar area that becomes exposed to the solvent by the molten globule state. For α-lactalbumin this value is close to 0.6 indicating that 60% of the polar van der Waals and hydrogen bonding interactions become disrupted in the molten globule state. For cytochrome c the value is 45%. On the contrary, only 50% and 36% of the hydrophobic core is exposed by the molten globule of α-lactalbumin and cytochrome c respectively. These values are obtained by applying Eq. (4) to the measured thermodynamic parameters for the molten globule and unfolded states. From a structural point of view, this behavior occurs because the distribution of polar and apolar groups within the protein is not uniform, i.e. not all partly folded states expose to the solvent the same proportion of polar and apolar surface. For α-lactalbumin and cytochrome c, the apolar fractions of the total surface buried from the solvent in the native state are 0.59 and 0.61 respectively. The ratio of exposed polar to apolar groups is about 20–25% larger for the molten globule state than the unfolded state. These observations appear to be general and prompted us to develop a computer program aimed at performing a structural search of molten globule determinants based upon solvent accessibilities.

4. Automated search algorithm for molten globule state

The purpose of a molten globule search algorithm is to identify those native-like partly folded states that satisfy the thermodynamic constraints of the molten globule. This is achieved by generating a large ensemble of partly folded intermediates, using the structure of the native state as a template, and then calculate the expected enthalpy and heat capacity of each state. The algorithm used to generate partly folded states has been published before and will not be repeated here [17,24,25].

It has been experimentally observed that in most cases studied the folded regions in partly folded states preserve the overall folding of the native state and possess the same secondary structure elements of the native state ([26–28]; see also Ref. [4] for a recent review). If this is the

case the crystallographic or NMR solution structure of the native state can be used as a template to generate partly folded intermediates. The number of partly folded states that can be generated using the native structure as a template can be astronomically large and therefore computationally intractable since the total number of states is equal to 2^N, where N is the number of units. It is clear that the basic folding units cannot be defined at the residue level. However, one can take advantage of the known structural properties of partly folded intermediates discussed above and use secondary structure elements as the basic folding units. This partitioning is computationally tractable and has been successfully used before [24,25]. It must be noted that this partitioning strategy is independent of any folding mechanism and does not imply that secondary structure elements are the initial structures in the folding process and that they are intrinsically stable.

The relative enthalpies and heat capacities can be used as a criteria to select molten globule candidates from the entire ensemble of partly folded states. For computational purposes, the enthalpic properties of partly folded states are written in terms of the relative enthalpy of the partly folded state with respect the unfolded state, $\Delta\Delta H_i(T)$, defined as:

$$\Delta\Delta H_i(T) = \Delta H_i(T) - \Delta H_U(T). \quad (5)$$

For those states that are molten globule candidates, $\Delta\Delta H_i(T)$ must be the highest at low temperatures. The second criterium is the heat capacity. Because the molten globule preserves a hydrophobic core and a significant fraction of apolar groups do not become exposed to water, a low heat capacity is required. In this case, however, a normalized heat capacity value should be used since not all partly folded states have similar degrees of unfolding. The most appropriate quantity for an automated search is the heat capacity normalized with respect to the number of unfolded residues ($\Delta C'_{p,i}$):

$$\Delta C'_{p,i} = \Delta C_{p,i}/N_{U,i}, \quad (6)$$

where $N_{U,i}$ is the number of unfolded residues in state i. According to Eq. (6), the states characterized by a low $\Delta C'_{p,i}$ are those that expose to the solvent a small apolar surface per unfolded residue. Since, at any temperature, the solvent related entropy is directly related to ΔC_p, those states with the lowest ΔC_p also have the most favorable solvent related entropies, since ΔS_{solv} is given by $\Delta C_p \ln(T/T_s^*)$, where T_s^* is the temperature at which the solvent related entropy is zero. Eqs. (5) and (6) provide the test criteria to select molten globule candidates from an arbitrary ensemble of partly folded states.

In general, the algorithm for structure based selection of molten globule candidates can be summarized as follows:

– Generation of an ensemble of partly folded states using crystallographic or NMR structure as a template.

– For each state, calculation of structural parameters necessary for evaluation of enthalpy and heat capacity. ASA_{ap}, ASA_{pol}, number of protonizable groups (histidyl, carboxyl, etc.) that become exposed to water.

– Calculation of $\Delta\Delta H_i(T)$ and $\Delta C'_{p,i}$ for each state and identification of those states for which $\Delta C'_{p,i}$ is minimal and for which $\Delta\Delta H_i(T)$ is maximal at low temperatures.

5. Application to barnase

The three-dimensional structure of barnase has been determined crystallographically at 1.9 Å resolution [29,30] and by solution NMR [31]. For the purpose of generating partly folded states, the molecule was partitioned into eight folding units

Table 1

folding unit:	1	2	3	4	5	6	7	8
sequence:	{2–22}	{23–40}	{41–49}	{50–58}	{59–69}	{70–76}	{77–84}	{85–110}

corresponding to different elements of secondary structure (Table 1).

The first folding unit includes the amino terminal α-helix (6–18) and half of loop 1 (19–25). The second unit corresponds to the second α-helix (helix 2 (residues 26–34) according to the nomenclature of Fersht [18]), half of loop 1 and the entire loop 2 (residues 35–40). A one-turn α-helix (helix 3 (residues 41–46)) and turn 1 (residues 47–49) form the third cooperative unit. The fourth is simply the first β strand (β1) (residues 50–55). The fifth, sixth and seventh folding units correspond to loop 3 (residues 59–69), β2 (residues 70–76) and loop 4 (residues 77–84), respectively. The last unit includes a three-stranded anti-parallel β sheet (β3 (residues 85–91), β4 (residues 94–99) and β5 (residues 106–108)) and loop 5 (residues 100–105).

The above partitioning of barnase results in a total of 256 states, since the total number of states is 2^8. These states were generated with the computer using the crystallographic structure of the protein (PDB file 1RNB) as a template. Application of the molten globule search algorithm to these states yields the following results. Fig. 2 shows ΔASA_{pol} and ΔASA_{ap} for all the computer simulated states of barnase. As indicated in the figure, it is clear that some states expose to the solvent an anomalously low apolar area without exhibiting a similar anomaly in the polar area. In general, those states will be characterized by a low normalized heat capacity and a high enthalpy change. The states belonging to this subset preserve a sizable hydrophobic core and, in general, will satisfy the thermodynamic criteria of the molten globule state.

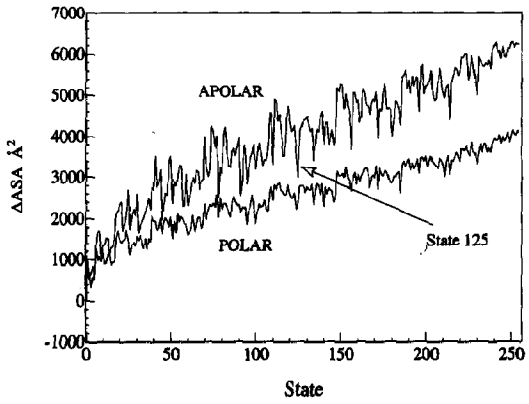

Fig. 2. The change in solvent accessible polar and apolar surface for the 256 computer simulated states of barnase. The states are numbered according to their degree of unfolding. 0 corresponds to the native state and 255 to the unfolded state. As discussed in the text, some states expose to the solvent an anomalously small apolar surface. Since the exposure of apolar surface to solvent water is characterized by a positive heat capacity change and a negative enthalpy, the protein states with anomalously small apolar exposure will tend to have low heat capacities and high enthalpies.

Table 2 summarizes the states identified by the search algorithm as better satisfying the enthalpy and heat capacity criteria. It is clear from these results that those states share common structural features. In particular, cooperative folding units 1 and 8 are folded, and cooperative folding units 2, 5 and 7 are unfolded in all six states. Cooperative

Table 2
Structural characteristics of molten globule state candidates for barnase

State	Binary code [a]	$\Delta H(25)$ (kcal/mol)	ΔC_p (cal/K mol)	$\Delta C'_p$ [b] (cal/K mol)
57	01001010	30.4	512	13.8
79	01001110	25.2	814	18.5
91	01101010	30.1	591	12.9
92	01011010	26.9	688	15.0
116	01101110	24.9	894	16.9
125	01111010	27.4	729	13.3
255	11111111	11.4	1745	16.0

[a] In this binary representation folding units in the folded state are represented by a 0 and folding units in the unfolded state are represented by a 1; e.g. in state 0 1 0 0 1 0 1 0, folding units 1, 3, 4, 6 and 8 are folded and folding units 2, 5 and 7 are unfolded.
[b] $\Delta C'_p$ is the heat capacity normalized with respect to the number of residues that are unfolded in each state as described by Eq. (6).

folding unit 6 is folded in four of the six states. These results suggest that the first helix together with most of the β-sheet structure (β2, β3–5) and loop 5 form the hydrophobic core of the protein, which is present in the molten globule state of the protein. Fig. 3 summarizes the structural features of this state. In this figure, the folded regions are represented by dark areas and the unfolded regions by light areas.

Fig. 3. The structural characteristics of state 125 (01111010). The dark areas represent the regions of the protein molecule that are folded in state 125. These areas correspond to residues 1–22, 70–76 and residues 85–110. The light areas correspond to regions that are unfolded. The folded regions in state 125 are folded in all six states selected by the molten globule search algorithm. This figure was generated with the program Molscript [32].

The folding pathway of barnase has been extensively studied by protein engineering and NMR experiments (see Ref. [18] for a recent review). It has been shown that helix 1, loop 5 and the β-sheet are formed early in the folding event. NMR studies indicate that the interactions between β-strands are present in the folding intermediate [33]. The most completely formed region is located in the center of the β-sheet; in particular, the interactions between β2–5 are especially well established [33]. The second turns of helix 2 and helix 3 are also formed early but the packing against each other and with the rest of the protein apparently does not occur until the protein is fully folded. For the case of β2, the protein engineering experiments show that it also folds early; however, the ϕ numbers, which were determined by mutations at residue 55, are the lowest among the β-strands. This suggests a relatively low stability for β2 in the β-sheet. Loop 1, loop 2 and loop 4 are shown not to be formed until after the rate-determining transition state. All of the above results are in excellent agreement with the prediction of the molten globule search algorithm. The only ambiguous region is loop 3, which has the guanosine binding site. The experimental studies show partial early formation of this region while the structural thermodynamic calculations predict that this region is unfolded in all molten globule candidates. It must be noted, however, that the results in this region might be affected by the fact that the crystal structure corresponds to the complex of the protein with the deoxy-dinucleotide inhibitor D(GpC).

6. Degree of unfolding of the molten globule of barnase

Table 3 summarizes different parameters that report the degree of unfolding of the molten globule candidates of barnase. The fraction of unfolded residues for the six states in the table range between 0.34 for state 57 to 0.5 for state 125. Similarly, the fraction of the hydrophobic core that becomes exposed varies between 0.37 and 0.56 and is always smaller than the fraction of polar residues that become exposed to water.

Table 3
Degree of unfolding of molten globule state candidates of barnase

State	F_U	$F_{\Delta ASA_{ap}}$	$F_{\Delta ASA_{pol}}$	$\Delta ASA_{ap}/\Delta ASA_{total}$
57	0.34	0.37	0.48	0.53
79	0.40	0.52	0.61	0.56
91	0.42	0.41	0.52	0.54
92	0.42	0.45	0.55	0.56
116	0.49	0.56	0.65	0.57
125	0.50	0.48	0.58	0.56
255	1.0	1.0	1.0	0.60

The ratio of polar to apolar surface area that becomes exposed to the solvent ranges between 0.77–0.87 for the molten globule candidates compared to 0.66 for the unfolded state. This result indicates that the molten globule state is predominantly stabilized by hydrophobic interactions, in agreement with the conclusion of Serrano et al. [34]. These authors concluded that the early folding events are characterized by the burial of hydrophobic surface without an extensive burial of hydrophilic residues and pointed out to the role of the β-sheet as the focal point in this process. This β-sheet is also the most persistent structure in the states selected by the molten globule search algorithm.

7. Implications for a folding pathway

If early folding intermediates of barnase are structurally analogous to the states listed in Table 2, as appear to be the case according to the results of Serrano et al. [34], it is tempting to speculate about the forces involved in the initial folding events. Clearly, a state characterized by an extensive burial of apolar residues without a parallel burial of polar ones will be characterized by a higher enthalpy than the unfolded state, as observed experimentally. From an enthalpic point of view, this state will be energetically unfavorable. Therefore, its stabilization will be necessarily of an entropic origin. Since the molten globule state is partially ordered and therefore has a lower configurational entropy than the unfolded state, the stabilization entropy must originate from the gain in solvent related entropy associated with the hydrophobic effect. The formation of secondary structure elements in the early stages of folding must be accompanied by a significant burial of hydrophobic surface. This is the case for the β-sheet and also the case for helix 1 which buries from the solvent a large hydrophobic surface independently of its packing with the rest of the protein [34,35].

8. Conclusions

The results presented here for barnase together with our results for α-lactalbumin [17], strongly suggest that a structure based search of folding intermediates that uses as the basis for selection criteria changes in solvent accessible surface areas the enthalpy and heat capacity changes provides an accurate identification of the structural determinants of the molten globule state. These results lend support to the idea that solvation parameters provide an important set of tools to understand protein folding. Previous attempts to use changes in accessible surface areas to interpret folding pathways have been recently reviewed in ref. [36]. As in the case of α-lactalbumin, the states selected by the search procedure for barnase belong to a single structural family suggesting that the structural determinants of the molten globule state are rather unique for a given protein. An ongoing area of research is the prediction of the physical and/or chemical conditions under which the molten globule state is thermodynamically stabilized and becomes significantly populated.

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Note added in proof

Recently we have found that the parametrization of the enthalpy can be improved by using 60°C as the reference temperature. This is close to the median denaturation temperature for proteins and the calculated values are less susceptible to extrapolation errors due to uncertainties in ΔC_p. The resulting equation is $\Delta H(60) = 31.4\ \Delta ASA_{pol} - 8.44\ \Delta ASA_{ap}$. If the new parameters are used then Eqs. (3) and (4) become

$$\Delta ASA_{ap} = \left[b(60)\Delta C_p - b'\Delta H(60)\right] / \left[a'b(60) - a(60)b'\right]$$

and

$\Delta ASA_{pol} = [a(60)\Delta C_p - a'\Delta H(60)]/[a(60)b' - a'b(60)]$, where $a(60) = -8.44$; $b(60) = 31.4$; $a' = 0.45$ and $b' = -0.26$ (see Ref. [17]). The conclusions of the analysis for barnase are the same in both cases.

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