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An alternative approach in the structure-based predictions of the thermodynamics of protein unfolding

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Abstract

A new approach for a first-order prediction of the thermodynamic properties of small globular proteins has been developed. The method put forward here has been shown to be successful in predicting, within acceptable margins of uncertainty, the denaturational heat capacity changes of a given protein if its amino acid composition is known. If compared with other models this method has the following advantages: (1) no details about the three-dimensional structure of the protein are required; (2) comparison with the thermodynamic properties of small model compounds is not necessary; (3) the temperature dependence of the denaturational heat capacity change is taken into account. Moreover, the equations developed have allowed us to point out the errors that can be made if the temperature-dependence of the denaturational heat capacity change is not taken into account in the calculation of the unfolding thermodynamic functions. © 1997 Elsevier Science B.V.

Keywords: Proteins; Thermodynamics; Structure-based predictions; Denaturational heat capacity; Hydrophobicity

1. Introduction

A major problem in protein engineering is that of selecting amino acid mutations that will alter the activity or stability of a protein in a predictable way. If the location and nature of the molecular replacements can be predicted with a high probability of success, biotechnology research laboratories will benefit greatly.

Studies of the correlations between thermodynamics and the structural features of proteins provide insight into the principles of protein energetics and can be therefore very useful in elucidating this problem [1]. The 'structural thermodynamic' approach appears to have potential for accurate stability predictions and for the development of strategies directed at selecting amino acid mutations that are likely to give rise to the desired effect.

Under equilibrium conditions the stability of a protein is dictated by its Gibbs free energy of stabilization (ΔG) [2]. In turn, the ΔG is determined by three thermodynamic parameters: the unfolding temperature (T_m), enthalpy (ΔH), and the heat capacity (ΔC_p) differences between the native and the unfolded state of the protein [3,4].

It is well known that protein denaturation is accompanied by a heat capacity increase, i.e., the heat capacity of the protein in its denatured state is significantly higher than in its native state. The importance of this general feature of proteins lies in the fact that the increase in heat capacity determines

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Table 1
Amino acid composition of lysozyme, myoglobin, ribonuclease A and cytochrome *c*

	Lysozyme		Myoglobin		Ribonuclease A		Cytochrome *c*	
	N_{res}	N_{CH}	N_{res}	N_{CH}	N_{res}	N_{CH}	N_{res}	N_{CH}
Lys	6	54	19	171	10	90	19	171
Arg	11	77	4	28	4	28	2	14
His	1	5	12	60	4	20	3	15
Asp	7	21	7	21	5	15	3	9
Glu	2	10	14	70	5	25	9	45
Gly	12	24	11	22	3	6	12	24
Asn	14	42	1	3	10	30	5	15
Gln	3	15	5	25	7	35	3	15
Cys	08	24	0	0	8	24	2	6
Ser	10	30	06	18	15	45	0	0
Thr	7	35	5	25	10	50	10	50
Tyr	3	21	3	21	6	42	4	28
Ala	12	48	17	68	12	48	6	24
Val	6	48	8	64	9	72	3	24
Leu	8	80	18	180	2	20	6	60
Ile	6	60	9	90	3	30	6	60
Pro	2	14	4	28	4	28	4	28
Phe	3	24	6	48	3	24	4	32
Met	2	16	2	16	4	32	2	16
Trp	6	48	2	16	0	0	1	8
Total	129	696	153	974	124	664	104	644

N_{res} is the number of residues; N_{CH} is the number of apolar hydrogens for each type of amino acid.

the temperature dependence of the thermodynamic parameters of protein denaturation and thus the stability of the protein native structure [2]. There are several explanations for the denaturation heat capacity effect. Heat capacity might increase because of: (1) the increase of the configurational freedom of the polypeptide chain upon unfolding, (2) the hydration of the groups that are exposed to water upon unfolding or (3) the gradual melting of the residual structure in the denatured protein with a continued increase in temperature [5].

How the above listed effects influence the denaturation heat capacity increase has been discussed at length by various researchers [6–8]. In particular, Privalov and Makhatadze [9] proposed a method for the calculation of the heat capacity increase by direct summation of the partial molar heat capacity contributions of the amino acid residues constituting the chain. Moreover, the heat capacity change caused by hydration of amino acid residues upon protein unfolding was also determined and it was shown that this is the main feature contributing to the observed heat capacity effect of unfolding.

The method put forward by Privalov and Makhatadze [9] gives excellent results in providing a correct revision of the thermodynamic parameters related to protein unfolding, but it requires details about the structural features of the protein. This structural information, (generally provided by X-ray analysis), is not always available. For this reason, Murphy and Gill [10] suggested an alternative method, based on solid model compounds, providing a sound rationale for the preliminary prediction of the thermodynamic behaviour of proteins. With this method, by combining the average structural features of globular proteins (i.e., number of residues, fraction of buried apolar groups and fraction of hydrogen bonds) with the heat capacity contributions of their specific heat capacity group, it should be possible to predict the thermodynamic properties of the proteins with reasonable success. In order to simplify their calculations, Murphy and Gill [10] assume that the temperature-dependence of the denaturation heat capacity change is constant in the (0–75°C) temperature range.

In order to overcome the limitations of the two approaches described above, in this paper we propose a third method for the calculations of ΔC_p

Table 2
Water-accessible surface area changes, $\Delta^U_N ASA_i$ (Å^2) upon transition from the native to the fully unfolded state for various groups of the four proteins considered (data from Ref. [9])

	Cytochrome *c*	Myoglobin	Ribonuclease A	Lysozyme
Aliphatic	4328	7386	4749	5385
Aromatic	1859	2689	1614	1894
–CHCONH–	3433	5300	4276	4106
Polar parts of:				
Trp	36	46	0	124
Met	86	85	171	86
Cys	70	0	279	278
Asn	230	35	276	320
Asp	25	183	143	187
Gln	187	259	386	97
Glu	140	536	200	111
Lys	198	239	117	122
Arg	195	272	239	347
Ser	38	171	304	283
Thr	188	86	195	129
Tyr	199	110	182	85
His	92	315	136	42

Table 3
Heat capacity contributions, per unit of surface-accessible area (ASA) for various constituent groups of proteins at various temperatures

	Temperature (°C)					
	5	25	50	75	100	125
Aliphatic	2.23	2.13	2.00	1.88	1.77	1.64
Aromatic	1.32	1.20	1.11	1.02	0.95	0.88
–CHCONH–	−1.16	−0.97	−0.80	−0.79	−0.78	−0.86
Polar parts of:						
Met	−4.03	−3.66	−3.19	−2.75	−2.33	−1.86
His	−0.96	−1.36	−1.42	−1.36	−1.23	−1.06
Ser	−1.66	−1.43	−1.20	−0.95	−0.71	−0.47
Asn	−1.29	−1.02	−0.67	−0.41	−0.15	0.09
Asp	−1.74	−1.42	−1.07	−0.71	−0.39	−0.11
Gln	−0.39	−0.24	−0.06	0.08	0.18	0.30
Glu	−0.73	−1.56	−0.35	−0.16	−0.03	0.09
Lys	−1.32	−1.53	−1.54	−1.30	−1.10	−0.90
Tyr	−0.06	0.0	−0.02	0.09	0.27	0.43
Arg	−0.39	−0.20	−0.11	−0.03	0.03	0.09
Thr	−1.13	−1.32	−1.19	−0.85	−0.25	0.59
Trp	3.87	3.94	3.72	3.53	3.27	3.05

based on studying average relationships between the thermodynamic properties of proteins and their amino acid composition. This method, which takes into account the temperature-dependence of denaturation heat capacity change, has several important advantages: (1) comparison with model compounds is not necessary; (2) There is no need for detailed information regarding the three-dimensional structure of the protein. Knowledge of the amino acid composition is sufficient for the calculus of all the denaturation thermodynamic variables of the proteins.

2. Theory and methods

The proteins studied in this paper are: lysozyme (Lys), cytochrome *c* (Cyt *c*), ribonuclease A (Rnase A) and myoglobin (Mb). The amino acid composition of these four proteins are given in Table 1. We have chosen these four proteins for the following reasons: (1) they are the most commonly studied proteins and there is a wealth of structural and thermodynamic information about them [11–15]; (2) they have already been analyzed using the methods proposed so far [9,10]; thus making it possible to compare previous results with the data obtained in the present work.

Unfortunately, no direct relationship exists between ΔC_p and the primary structure of a protein. Hence, ΔC_p has to be separated into its polar ($\Delta C_{p_{pol}}$) and apolar ($\Delta C_{p_{apo}}$) components. In order to do so, we will refer to the suggestions of Privalov [2,4] where the calculus of $\Delta C_{p_{apo}}$ and $\Delta C_{p_{pol}}$ is possible through detailed knowledge of the three-dimensional structure of the proteins.

The heat capacity change upon protein translation from the native state to the completely unfolded state, was calculated assuming that the heat capacity change of a group is proportional to its water-accessible surface area, and that the heat capacity contributions of the groups are additive:

$$\Delta C_p = \sum_i \Delta^{U}_{N} \mathrm{ASA}_i \cdot \Delta C_{p_i} \qquad (1)$$

where $\Delta^{U}_{N}\mathrm{ASA}_i$ is the water-accessible surface area change of all groups of a definite *i*th type in the protein upon its translation from the native to the completely unfolded state, and ΔC_{p_i} is the hydration heat capacity of the corresponding group calculated per ASA unit expressed in Å^2 (see Table 2).

The $\Delta\mathrm{ASA}_i$ for each residue were calculated in a previous paper [5] from the known atomic coordinates of the native proteins and their values are reported in Table 3.

Table 4
Polar ($\Delta C_{p_{apo}}$) and apolar ($\Delta C_{p_{pol}}$) contributions to the total denaturational heat capacity change of cytochrome *c*, ribonuclease A, lysozyme and myoglobin, at various temperatures. These values were obtained using the methods of Privalov and Makhatadze [9]

	5°C		25°C		50°C		75°C		100°C		125°C	
	$\Delta C_{p_{apo}}$	$\Delta C_{p_{pol}}$	$\Delta C_{p_{apo}}$	$\Delta C_{p_{pol}}$	$\Delta C_{p_{apo}}$	$\Delta C_{p_{pol}}$	$\Delta C_{p_{apo}}$	$\Delta C_{p_{pol}}$	$\Delta C_{p_{apo}}$	$\Delta C_{p_{pol}}$	$\Delta C_{p_{apo}}$	$\Delta C_{p_{pol}}$
Cyt *c*	12.1	−5.2	11.5	−4.8	10.17	−14	10	−4.3	9.4	−13.4	8.17	−3.7
Rnase A	13.7	−8.3	12	−7.2	11.2	−6	10.6	−5.6	9.9	−5.1	9.2	−5.3
Lys	14.5	−7.5	13.7	−6.4	12.8	−5.4	12	−5	11.3	−4.5	10.5	−4.6
Mb	20	−8.3	18.9	−7.3	17.7	−6	16.6	−5.6	15.6	−5.31	14.4	−5.68

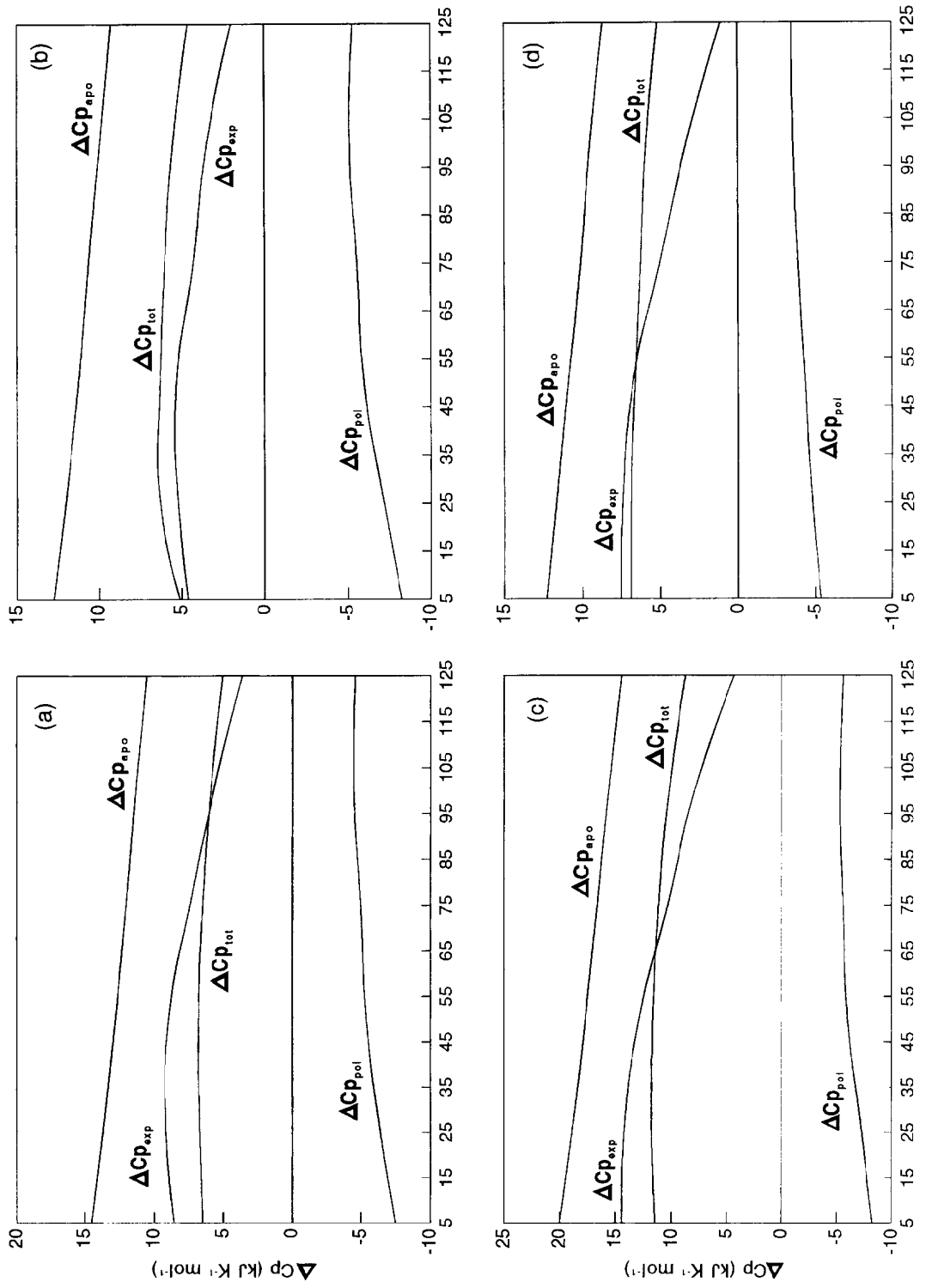

Fig. 1. Calculated denaturational heat capacity change ($\Delta C_{p_{tot}}$) and its polar ($\Delta C_{p_{pol}}$) and apolar ($\Delta C_{p_{apo}}$) components for: (a) lysozyme; (b) ribonuclease A; (c) myoglobin; (d) cytochrome *c*. The experimental heat capacity changes ($\Delta C_{p_{exp}}$) are derived from Ref. [9].

According to Eq. (1) and to the values reported in Tables 2 and 3, it is possible to calculate the polar and apolar components of the heat capacity changes for the four proteins by means of these data in the 5–125°C temperature range. The results of these calculations are reported in Table 4 and Fig. 1a–d.

In Section 3, we will examine the relationships (valid in the 5–125°C range) binding the amino acid composition and the polar and apolar contribution to ΔC_p.

3. Results and discussion

3.1. Apolar components of ΔC_p

It has long been known that the apolar components of ΔC_p for proteins is a linear function of temperature [11]:

$$\Delta C_{p_{apo}}(T) = a + bT$$

The change in the accessible surface area of the protein due to the unfolding is dependent on the number of apolar hydrogens (i.e., hydrogen atoms directly bound to a carbon) by a linear relationship [10]. It is therefore probable that the two coefficients a and b are, in turn, linearly bound to the number of apolar hydrogens in the proteins.

In order to discover these relationships, the apolar components of ΔC_p of the four investigated proteins were plotted vs. T and fitted by a linear function. The results for the four fits are reported in Table 5.

At this point, coefficients a and b calculated for the four proteins were plotted vs. the number of apolar hydrogens and then fitted by a linear function (see Fig. 2).

Table 5
Linear fits $\Delta C_{p_{apo}}$ components vs. T for the proteins lysozyme, myoglobin, cytochrome c, ribonuclease A

	N_{res}	N_{CH}	N_{CH}/N_{res}	a	b	R
Cyt c	104	644	6.19	12.18	−0.0282097	0.953
Rnase A	124	664	5.31	13.27	−0.0343065	0.962
Lys	129	696	5.39	14.54	−0.0328871	0.974
Mb	153	974	6.36	20.10	−0.0458226	0.985

Coefficient a is the intercept and coefficient b is the slope of the fitted lines. R is the correlation coefficient of each linear fit. N_{res} and N_{CH} are the number of residues and the number of apolar hydrogens, respectively.

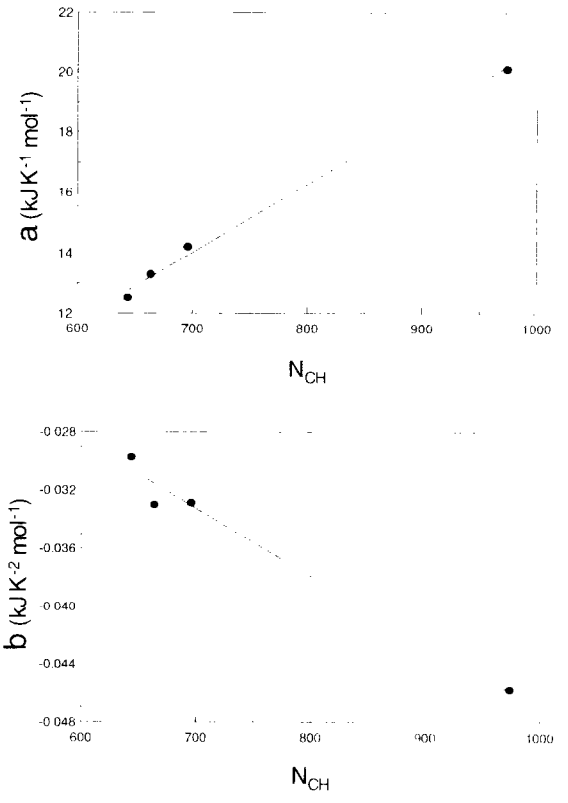

Fig. 2. Linear fits of coefficients a and b as a function of the number of apolar hydrogens. For the coefficient a the fitted equation is: $a = -2.43877 + 0.023205 \cdot N_{CH}$; the correlation coefficient is 0.973. For coefficient b the fitted equation is: $b = 0.00155 - 4.89819 \cdot 10^{-5} \cdot N_{CH}$; the correlation coefficient is 0.961.

If the amino acid composition of a protein is known, we can thus calculate the number of apolar hydrogens and therefore coefficients a and b which give the temperature dependence of $\Delta C_{p_{apo}}$ in the 5–125°C range.

The relationship between the number of apolar hydrogens of a protein and the apolar contribution to the overall denaturation change is as follows:

$$\Delta C_{p_{apo}} = a + bT = (-2.43877 + 0.023205 \cdot N_{CH}) + (0.00155241 - 4.89819 \cdot 10^{-5} \cdot N_{CH}) \quad (2)$$

3.2. Polar components of ΔC_p

From the data reported in Table 5 and Fig. 1a–d, it can be observed that the polar components of ΔC_p

do not vary linearly with temperature, but follow a parabolic trend:

$$\Delta C_{p_{pol}}(T) = \alpha + \beta T + \gamma T^2$$

The aim of this subsection is to find a relationship binding the three coefficients α, β and γ to the composition of the protein.

Previous works [9,10] have shown that $\Delta C_{p_{pol}}$ is, on average, related to the changes in the polar surface accessible to the solvent as a consequence of the unfolding. Since the number of peptide bonds plays a major role in determining the polar components of ΔC_p [9], it can be argued that some relationship should exist between the number of residues and the polar components of $\Delta C_{p_{pol}}$. In this respect, the coefficients α, β and γ for the four proteins studied were plotted vs. the number of amino acid residues and then fitted with a linear function. These fits are reported in Fig. 3.

Coefficients α, β and γ can therefore be calculated from the length of the polypeptide chain by the following formulae:

$$\alpha = 1.5563 - 0.00684997 \cdot N_{res} \quad (3)$$

$$\beta = -0.0599115 + 0.000867265 \cdot N_{res} \quad (4)$$

$$\gamma = 0.000429975 - 5.24359 \cdot 10^{-6} \cdot N_{res} \quad (5)$$

The values of coefficients α, β, γ and the error function δ are listed in Table 6.

The overall denaturational ΔC_p is the sum of the apolar and polar components of ΔC_p:

$$\Delta C_{p_{tot}}(T) = a + bT + \alpha + \beta T + \gamma T^2 \quad (6)$$

In Fig. 1a–d the calculated ΔC_p for the four proteins is reported ($\Delta C_{p_{tot}}$) and compared with the experimental data ($\Delta C_{p_{exp}}$).

In order to test how far the method proposed here can be applied to a wide variety of proteins, we compared the experimental values of denaturational heat capacity changes ($\Delta C_{p_{exp}}$) of Ref. [17] with the $\Delta C_{p_{tot}}$ values calculated by means of our method and with the denaturational heat capacity changes calculated by means of the method proposed by Privalov and Makhatadze [9] (Table 7).

As can be noted, the accuracy of the ΔC_p calculated by our method is quite satisfactory in the 5–50°C range. It should be pointed out, however, that this error, if compared with the uncertainties of previously developed methods [9,10] may be considered acceptable.

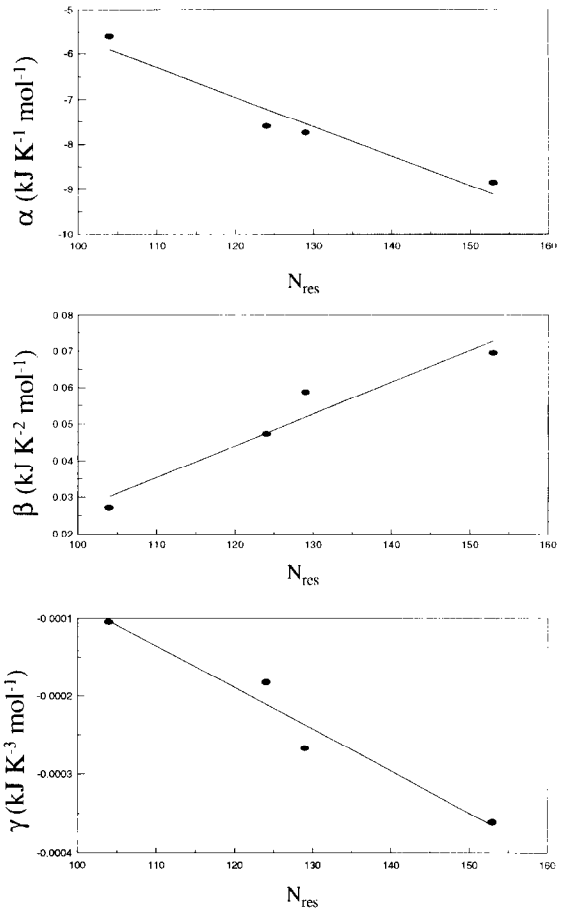

Fig. 3. Linear fits of coefficients α, β and γ as a function of the number of residues. The fitted equations are reported in Section 3.2. The correlation coefficients are 0.964, 0.971, 0.979 for coefficients α, β, γ, respectively.

3.3. The amino acid composition and the thermodynamics of protein unfolding

The enthalpy changes of the denaturation of globular proteins, when normalized to the number of amino acids, are seen to converge to a common value at a high temperature (near 100°C). Similarly, the normalized entropy changes of denaturation converge to a common value at high temperature close to the convergence temperature of the enthalpy changes.

Table 6
Parabolic fits ($\Delta C_{p_{pol}} = \alpha + \beta T + \gamma T^2$) of the polar components for the proteins lysozyme, cytochrome *c*, ribonuclease A, myoglobin

	N_{res}	α	β	γ	δ
Cyt *c*	104	−5.34	0.02702	−0.0001049	0.942
Rnase A	124	−6.28	0.04726	−0.0001828	0.964
Lys	129	−7.74	0.05863	−0.0002678	0.975
Mb	153	−8.68	0.06938	−0.0003614	0.958

The error function δ is a measure of the accuracy of the fitting operation. It is defined as: $\delta = |\Sigma_i (y^i_{fitted} - y^i_{exp})/n|$ [16], where, y^i_{exp} is the *i*th experimental value, y^i_{fitted} is the corresponding fitted value and *n* is the number of fitted points.

Similar behaviour is observed for the dissolution in water of liquid hydrocarbons. The convergence of ΔH or ΔS for the dissolution (or denaturation) of a series of model compounds (or proteins) can be represented by the following linear function of the form:

$$\Delta H^0(T) = \Delta H^* + \Delta C_p^0 (T - T_H^*) \quad (7)$$

and

$$\Delta S^0(T) = \Delta S^* + \Delta C_p^0 \ln\left(\frac{T}{T^*}\right) \quad (8)$$

where the asterisked temperature (T_H^* or T_S^*) is the temperature at which the enthalpy or entropy change for each compound converges to a common value of ΔH^* or ΔS^*.

Inspection of such plots for the denaturation of proteins reveals that all unfolding processes are characterized by the same value of T_S^* (namely 112°C) and T_H^* (namely 100.5°C).

Moreover, for protein denaturation ΔS^* is, on average 18.1 J K^{-1} (mol residue)$^{-1}$, and ΔH^* is 5.64 kJ (mol residue)$^{-1}$.

Table 7
Comparison between the experimental and the calculated denaturational heat capacity changes of Ref. [17]

T (°C)	$\Delta C_{p_{exp}}$ (kJ K^{-1} mol^{-1})	ΔC_p (kJ K^{-1} mol^{-1})[a]
5	5.8	5.0
25	5.7	4.7
50	5.1	4.3
75	3.8	4.1
100	2.5	3.8
125	0.8	3.6

[a]Denaturational heat capacity changes obtained by means of the equations set out in the present paper.

The overall free energy changes for protein unfolding (if the $\Delta C_p(T)$ function is known) is thus given in terms of the various enthalpic and entropic contributions:

$$\Delta G_{(T)} = 5.64 \text{ kJ (mol residue)}^{-1} \cdot N_{res} + \Delta C_p(T - 373.6\text{ K}) - T \cdot 18.1 \text{ J K}^{-1} \text{ (mol residue)}^{-1} - T \cdot \Delta C_p \cdot \ln\left(\frac{T}{385\text{ K}}\right) \quad (9)$$

3.4. Effect of composition on the thermodynamics of proteins

The analysis reported in this paper, together with previously published data [10] indicate that, for glob-

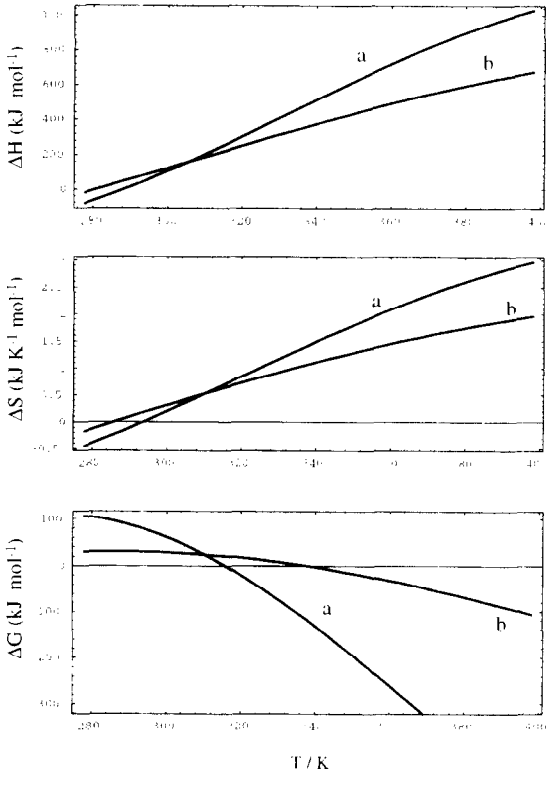

Fig. 4. Effect of composition (hydrophobicity) on the thermodynamic properties (ΔH, ΔS and ΔG) of proteins. The thermodynamic functions were calculated, according to the equations reported in Section 3, for two hypothetical proteins of the same size (100 residues). The curves are for a protein of seven apolar hydrogen atoms per residue (curve a) and for a six apolar hydrogen atoms per residue (curve b).

ular proteins, all thermodynamic denaturation parameters are functions of the number of hydrogen bonds and the number of buried apolar hydrogens which, on average, are simple function of the size and the composition of the protein.

The effect of protein composition (hydrophobicity) is illustrated in Fig. 4. In this figure the enthalpy (upper panel), entropy (middle panel) and free energy changes as a function of temperature are plotted for a hypothetical protein of 100 residues. Lines 'a' are for an average composition of seven apolar hydrogen atoms per residue and lines 'b' are for an average composition of six apolar hydrogen atoms per residue. It can be seen that an increase in the number of apolar hydrogen atoms per residue, which can be thought of as an increase in the average hydrophobicity of the protein, results in an increase in the enthalpy and entropy changes. Moreover, this results in an increase in the unfolding temperature T_m and a decrease in the hypothetical cold denaturation temperature (see Fig. 4, panel c). Maximum stability does not appear to be changed significantly. These results are in direct contrast with previous literature data supplied by Murphy and Gill [10], who, in very similar situations, predicted an increase in cold denaturation temperature on increasing hydrophobicity. This fact suggests that when temperature ranges outside the denaturation range (typically 40–70°C) are explored, approximation of a constant ΔC_p can lead to gross errors.

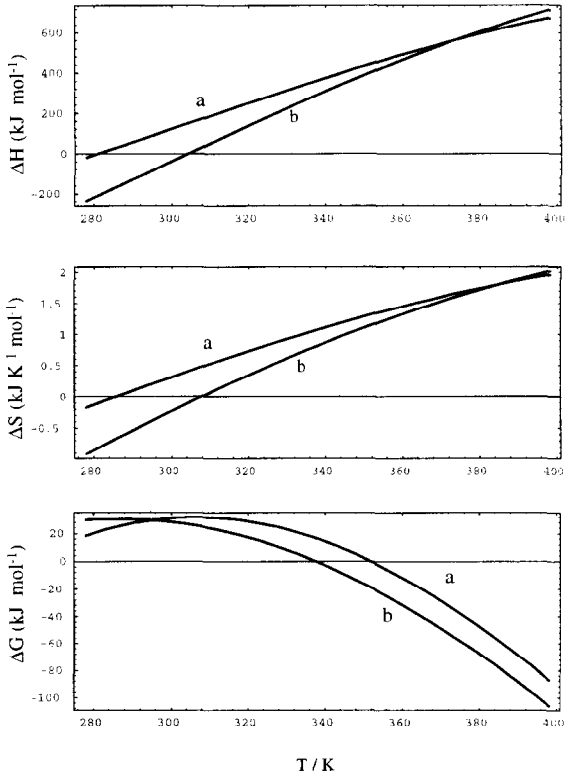

Fig. 5. Effect of size on protein thermodynamic functions. ΔH, ΔS and ΔG were calculated for two hypothetical proteins of the same composition ($N_{CH}/N_{res} = 6$). The curves are for a protein of 150 (curve a) and 100 (curve b) residues.

3.5. *Effect of size on the thermodynamics of protein unfolding*

The effect of size is illustrated in Fig. 5 where we show the enthalpy (upper panel), entropy (middle panel) and free energy changes as a function of temperature for a hypothetical protein with an average of six apolar hydrogen atoms per residue. In this figure we compare a protein of 150 residues (line a) with a protein of 100 residues (line b). The difference in size obviously results in a difference both in the number of hydrogen bonds and in the number of buried apolar hydrogen atoms. An increase in size provides a significant increase in the melting temperature, but the main effect is that of increasing maximum stability. These effects are compatible with the data provided by Murphy and Gill [10].

4. Conclusions

The principal aim of this paper was to build an easy-to-use but effective tool for the calculus of the ΔC_p of proteins if their amino acid composition is known. The analysis developed also takes into account the temperature dependence of ΔC_p and shows that in some cases (at low temperatures for instance) the temperature dependence of ΔC_p cannot be neglected.

Our model must be compared with the detailed analysis of heat capacity effects in protein unfolding given by Privalov and Makhatadze [9]. In their analysis, Privalov and Makhatadze determined the partial molar heat capacities in water of all the constituent

groups of a protein as a function of temperature. The ΔC_p value for the transfer of these groups from the gas phase to water was then calculated and the value of the hydration component of the heat capacity change was determined using detailed information about the three dimensional structure of the same four proteins studied here.

A weakness in the work of Privalov and Makhatadze [9] is that it needs the knowledge of the detailed X-ray structure of the proteins is necessary and this is not always available. In addition, the calculation of the solvent accessible surface area of the various groups is significantly dependent on the choice of the correct Van der Waals ratio of water molecules. The analysis of Privalov and Makhatadze is also based on the assumption that the unfolded state of the protein does not have any residual secondary structure, but several experimental and theoretical works disfavour this assumption.

The analysis of ΔC_p proposed by Murphy and Gill [10], on the other hand, does not depend on the detailed knowledge of the three-dimensional structure of the protein, but does not take into account the temperature-dependence of ΔC_p.

The analysis put forward in the present paper summarizes the advantages of the two previous methods, providing a sound rationale for the correct prediction of denaturational thermodynamic changes, also taking into account their temperature dependence. The errors in the calculus of thermodynamic quantities are in general acceptable, but if more precise values for specific proteins are required they can be found if actual structural information is used in the analysis.

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