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DSC studies of the conformational stability of barstar wild-type

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Abstract

The temperature induced unfolding of barstar wild-type of bacillus amyloliquefaciens (90 residues) has been characterized by differential scanning microcalorimetry. The process has been found to be reversible in the pH range from 6.4 to 8.3 in the absence of oxygen. It has been clearly shown by a ratio of $\Delta H_{vH}/\Delta H_{cal}$ near 1 that denaturation follows a two-state mechanism. For comparison, the C82A mutant was also studied. This mutant exhibits similar reversibility, but has a slightly lower transition temperature. The transition enthalpy of barstar wt (303 kJ mol^{-1}) exceeds that of the C82A mutant (276 kJ mol^{-1}) by approximately 10%. The heat capacity changes show a similar difference, ΔC_p being 5.3 ± 1 kJ mol^{-1} K^{-1} for the wild-type and 3.6 ± 1 kJ mol^{-1} K^{-1} for the C82A mutant. The extrapolated stability parameters at 25 °C are ΔG^0 = 23.5 ± 2 kJ mol^{-1} for barstar wt and ΔG^0 = 25.5 ± 2 kJ mol^{-1} for the C82A mutant.

Keywords: aggregation prevention; barstar wild-type; DSC; oxidization; thermodynamics

Barstar is the inhibitor of barnase (Hartley, 1988). The structure of barstar has been solved by X-ray crystallography (C40,82A mutant) (Guillet et al., 1993) and by NMR (wild-type (wt)) (Lubienski et al., 1994). The structure comprises of four α-helices and a three-stranded parallel β-sheet as schematically shown in Figure 1. Buckle et al. (1994) demonstrated in studies on complex formation between barnase and barstar that there is a high degree of complementarity both in the shape and in the charge of the interacting protein surfaces. The dissociation constant, $K_d = 6 \times 10^{-14}$ M, of the complex between barstar wt and barnase wt is three times smaller than that observed for the dissociation of barstar C40,82A double mutant from barnase wt, $K_d = 2 \times 10^{-13}$ M (Hartley, 1993). The conformational stability of several mutants of barstar has been studied (Martinez et al., 1995; Wintrode et al., 1995), but up to now barstar itself escaped each attempt of direct microcalorimetric measurement. There are, however, a few optical studies that deal quantitatively with the conformational stability of barstar wt (Schreiber & Fersht 1993; Khuruana & Udgaonkar, 1994; Agashe & Udgaonkar, 1995; Frisch et al., 1995; Khuruana et al., 1995; Ramachandran & Udgaonkar 1996). The inability to carry out DSC studies on barstar wt was generally ascribed to the fact that thermal unfolding of the protein involves irreversible processes that prevented quantitative evaluation of the transition curves. The main cause of the irreversibility of thermal unfolding was assumed to stem from the oxidization of the two cysteine residues at positions 40 and 82, a process that leads to inter- and intra-molecular disulfide bond formation (Frisch et al., 1995; Martinez et al., 1995). These side reactions usually disturb the directly determined calorimetric transition enthalpies much more strongly than the indirectly obtained van't Hoff enthalpies. To avoid such complications, preferentially Cys to Ala mutated barstar proteins have been employed in the calorimetric measurements. As interesting as these studies are, they suffer, however, from one severe interpretative disadvantage—they lack the wild-type thermodynamic parameters as reference values. We managed to measure directly by DSC the stability parameters of barstar wild type in two ways: (a) by keeping the protein in the reduced state in the presence of DTT, and (b) by working under strictly oxygen-free conditions without DTT. Through these provisions we have been able to obtain reversible unfolding conditions that permitted quantitative thermodynamic analysis of the heat capacity transition curves. The present DSC results are in good agreement with the stability parameters derived for barstar wt from the denaturant unfolding studies of Agashe and Udgaonkar (1995).

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Results and discussion

Reversibility of barstar wt and C82A mutant unfolding

Analysis of the heat capacity measurements in terms of thermodynamic equilibrium parameters is most straightforward for re-

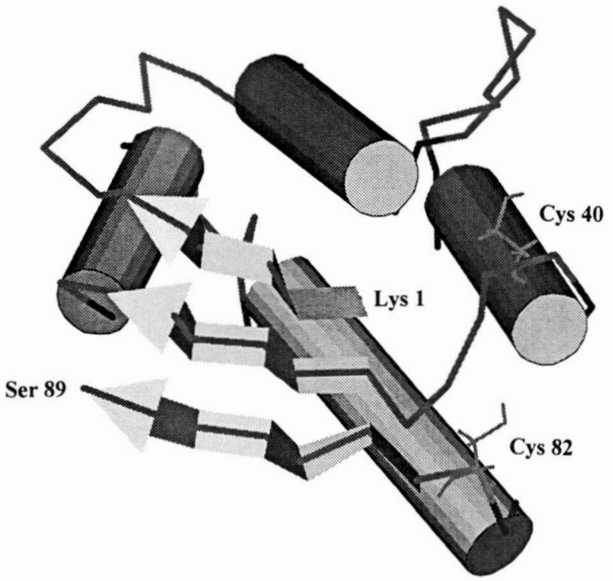

Fig. 1. Structure of barstar wt. The structure of barstar wt comprises four α-helices and a three stranded parallel β-sheet. The protein studied by us has methionine at position 1 and, therefore, 90 amino acids. The molecule shown here lacks Met 1. It is the 1 BTB structure deposited into the Brookhaven Data Bank by M.J. Lubienski (9 May 1994).

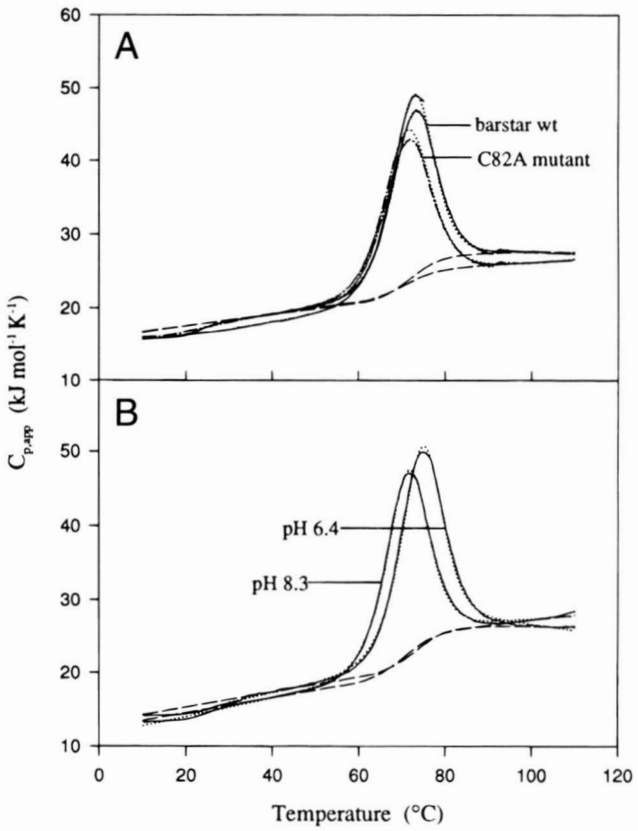

Fig. 2. DSC transition curves of barstar wt and C82A mutant. **A:** Reversibility tests of barstar wt and the C82A mutant. Two successive DSC scans with barstar wt (—) and the C82A mutant (–·–), respectively, are shown. Each first scan was interrupted 2–3 °C above the corresponding transition temperature. Barstar wt: concentration: 1.08 mg/mL; C82A mutant: concentration: 1.12 mg/mL. The dashed lines (- - - -) represent sigmoidal baselines. The dotted lines (· · · ·) show the results of fitting the experimental C_p values to a two-state model using Equations 1–5. The numerical results are listed in Table 1. Buffer: 50 mM $NaPO_4$, 1 mM EDTA, pH 7.4; heating rate: 2 K/min. **B:** pH dependence and two-state analysis of barstar wt C_p transition curves. pH 6.4 concentration: 0.858 mg/mL; pH 8.3 concentration: 1.22 mg/mL. Results of the DSC curve analysis assuming a two-state model (solid line: experimental curves, dotted lines: fitted curves, dashed lines: sigmoidal baselines). The dotted lines were obtained by least-squares fitting of the experimental data to Equations 1–5. The thermodynamic parameters are listed in Table 2. Buffer: 50 mM $NaPO_4$, 10 mM DTT, 1 mM EDTA; heating rate 2 K/min.

versible processes and the superimposability of transition curves in two sequential DSC runs using the same sample is a good criterion for reversibility. Figure 2A shows two sequential DSC curves for both proteins—wild-type barstar and the C82A mutant. The close similarity of the transition curves of the first and second scan is generally accepted as a clear indication of the reversibility of the unfolding transitions of the proteins. Strictly speaking, this test provides of course only a measure of repeatability, because thermodynamic reversibility implies that at every point throughout the transition the observed C_p signal is an equilibrium measurement. Because DSC instruments usually do not permit a direct control by going up and down in temperature during a scan or by interrupting the scan in the middle of the transition, another indirect control is provided by proving the lack of scan rate dependence. We have used different heating rates and observed no changes in the transition curves. Therefore, we conclude that the unfolding transition of barstar is a reversible process. The postdenaturational C_p functions show no sign of aggregation, and the solutions were clear even after three measurements. The heat capacity curves were numerically integrated and also fitted according to the two-state model described by Equations 1–5. The dotted curves show the results of the fit. It is obvious that the data of the DSC measurements can be very well approximated by a two-state model with a sigmoidal baseline. The thermodynamic parameters obtained from these studies at pH 7.4 are summarized in Table 1.

The transition temperature, $t_{1/2}$, of barstar wild type is 72.8 °C in 50 mM sodiumphosphate, 1 mM EDTA buffer, pH 7.4. It is slightly higher than that of the C82A mutant ($t_{1/2}$ = 71.4 °C). This is paralleled by the transition enthalpy, which has an average value of 303 kJ mol^{-1} for barstar wt and 276 kJ mol^{-1} for the C82A mutant. Thus, the transition enthalpies of barstar wt exceed those of the C82A mutant by approximately 10% at pH 7.4. The average ΔC_p value is 5.3 kJ mol^{-1} K^{-1} for the wild-type protein and 3.6 kJ mol^{-1} K^{-1} for the C82A mutant, respectively. The decrease in ΔH_{cal} and ΔC_p caused by exchange of one cysteine by an alanine is consistent with the loss of secondary and tertiary structure that has been demonstrated by circular dichroism for C82A relative to the wild-type protein (Ramachandran & Udgaonkar, 1996).

We observed that for both proteins, independent of the lower transition enthalpy and lower ΔC_p value of the mutant, the ratios of van't Hoff enthalpy to directly measured calorimetric enthalpy are unity within the limits of error. This is a clear indication of the cooperative nature of the transition of both wt barstar and the C82A mutant, which can be described by a two-state process.

pH dependence of the transition parameters

Denaturation curves were obtained in the pH range of 6.4 to 8.3 using 50 mM $NaPO_4$ buffer, containing 1 mM EDTA and 10 mM DTT. The pH was adjusted to the respective value by adding

Table 1. *Thermodynamic parameters of barstar wt and the C82A mutant at pH 7.4 in the presence of 10 mM DTT and in the absence of DTT in oxygen free buffer*[a]

	$t_{1/2}$ (°C)	ΔH^0_{cal} (kJ/mol)	ΔH^0_{vH} (kJ/mol)	$\frac{\Delta H^0_{vH}}{\Delta H^0_{cal}}$	$\overline{\Delta H}$ (kJ/mol)	ΔC_p (kJ/mol K)	$\Delta G^0_{25\,°C}$ (kJ/mol)
Barstar wt							
Without DTT							
1st heating (fit)	72.6 ± 0.3		313 ± 15				
2nd heating (int)	73.0 ± 0.3	296 ± 15					
2nd heating (fit)	72.9 ± 0.3		299 ± 15				
	72.8 ± 0.3*			1.03 ± 0.1	303 ± 15*	5.3 ± 1*	23.5 ± 2
With DTT							
1st heating (fit)	72.6 ± 0.3		307 ± 15				
2nd heating (int)	72.6 ± 0.3	314 ± 15					
2nd heating (fit)	72.8 ± 0.3		289 ± 15				
	72.7 ± 0.3			0.95 ± 0.1	303 ± 15		
Barstar C82A							
Without DTT							
1st heating (fit)	71.5 ± 0.3		282 ± 15				
2nd heating (int)	71.2 ± 0.3	270 ± 15					
2nd heating (fit)	71.5 ± 0.3		277 ± 15				
	71.4 ± 0.3*			1.04 ± 0.1	276 ± 15*	3.6 ± 1*	25.5 ± 2
With DTT							
1st heating (fit)	70.8 ± 0.3		283 ± 15				
2nd heating (int)	71.3 ± 0.3	279 ± 15					
2nd heating (fit)	71.2 ± 0.3		276 ± 15				
	71.1 ± 0.3			1.00 ± 0.1	279 ± 15		

[a] 50 mM sodium phosphate, 1 mM EDTA (fit) refers to a least-squares fit assuming a two state model (Vogl, 1995) using Equations 1–5; (int) refers to numerical integration using a sigmoidal baseline. ΔG^0 values have been calculated with Equation 10 using the average of ΔH_{cal} and ΔH_{vH} values termed $\overline{\Delta H}$ in the table. The cooperativity parameter $\Delta H^0_{vH}/\Delta H^0_{cal}$ has been calculated from the averaged ΔH^0_{vH} values of the first and second heating and the average ΔH^0_{cal} value.

* The values carrying the asterisk have been used for the calculation of the standard Gibbs energy at 25 °C.

Table 2. *Variation with pH of the thermodynamic parameters of barstar wt*[a]

	$t_{1/2}$ (°C)	ΔH^0_{cal} (kJ/mol)	ΔH^0_{vH} (kJ/mol)	$\frac{\Delta H^0_{vH}}{\Delta H^0_{cal}}$	$\overline{\Delta H}$ (kJ/mol)	ΔC_p (kJ/mol K)	$\Delta G^0_{25\,°C}$ (kJ/mol)
pH 6.4							
2nd heating (int)	74.1 ± 0.3	320 ± 15					
2nd heating (fit)	74.4 ± 0.3		321 ± 15				
	74.3 ± 0.3			1.00 ± 0.1	321 ± 15	4.9 ± 1	27.4 ± 2
pH 7.4							
1st heating (fit)	72.6 ± 0.3		307 ± 15				
2nd heating (int)	72.6 ± 0.3	314 ± 15					
2nd heating (fit)	72.8 ± 0.3		289 ± 15				
	72.7 ± 0.3			0.95 ± 0.1	303 ± 15	5.3 ± 1	23.5 ± 2
pH 8.0							
2nd heating (int)	71.9 ± 0.3	308 ± 15					
2nd heating (fit)	72.1 ± 0.3		299 ± 15				
	72.0 ± 0.3			0.97 ± 0.1	304 ± 15	4.7 ± 1	25.7 ± 2
pH 8.3							
2nd heating (int)	71.4 ± 0.3	309 ± 15					
2nd heating (fit)	71.5 ± 0.3		309 ± 15				
	71.5 ± 0.3			1.00 ± 0.1	309 ± 15	5.0 ± 1	25.3 ± 2

[a] (fit) refers to a least-squares fit assuming a two state model (Vogl, 1995) using Equations 1–5; (int) refers to numerical integration using a sigmoidal baseline. Buffer: 50 mM sodium phosphate, 1 mM EDTA, 10 mM DTT.

sodium hydroxide solution. Decreasing the pH from 8.3 to 6.4 results in an increase in the transition temperature (Fig. 2B) from 71.5 to 74.3 °C paralleled by an increase in the average transition enthalpy from 309 kJ mol^{-1} to 321 kJ mol^{-1}.

The change in pH does not affect the cooperativity of the unfolding transition, as indicated by the finding that the ratio of $\Delta H_{vH}/\Delta H_{cal}$ remains near unity. This result corroborates the observations of Khuruana et al. (1995), who reported that the assumption of a two-state transition was appropriate for the description of their optical measurements in the same pH range.

Determination of the heat capacity change ΔC_p from a ΔH vs. $t_{1/2}$ plot is not very precise for the barstar system. This results from the fact that the pH range, in which unfolding proceeds reversibly, is restricted and the concomitant changes in transition temperature are limited to about 3 °C. Therefore, the ΔC_p value has a large error attached to it. We obtained for barstar $\Delta C_p = 4.3 \pm 2$ kJ mol^{-1} K^{-1} from the slope of such a plot, which nevertheless agrees relatively well with the average ΔC_p value of 5.3 kJ mol^{-1} K^{-1} obtained from the individual transition curves.

Stability of barstar wt and the C82A mutant

At 25 °C the extrapolated $\Delta G^0_{25\,°C}$ value for barstar wt is 23.5 ± 2 kJ mol^{-1} and that of C82A 25.5 ± 2 kJ mol^{-1}. Thus, at 25 °C the mutated protein apparently exhibits higher stability than the wild-type protein. However, because the extrapolation involved in the calculation of $\Delta G^0_{25\,°C}$ extends over more than 45 °C in both cases, even small errors in ΔC_p could have led to the apparent inversion of stabilities. Therefore, we are inclined to consider this difference at 25 °C between wild-type and the C82A-mutated protein as insignificant. The results for barstar wt are in fair though not perfect agreement with those determined previously with spectroscopic methods (CD, UV, Fluorescence). Khuruana et al. (1995) found at pH7 the following parameters: $t_{1/2}$ = 71.5 °C, ΔH_{vH} = 251 kJ mol^{-1}, ΔC_p = 5 kJ mol^{-1} K^{-1}, and $\Delta G^0_{25\,°C}$ = 17.4 kJ mol^{-1}, while Agashe and Udgaonkar (1995) reported for pH8 the values: $t_{1/2}$ = 69.7 °C, ΔH_{vH} = 291 kJ mol^{-1}, ΔC_p = 6.2 kJ mol^{-1} K^{-1}, $\Delta G^0_{25\,°C}$ = 18.8 kJ mol^{-1}. For the C82A mutant Ramachandran and Udgaonkar (1996) determined $\Delta G^0_{25\,°C}$ = 16.7 kJ mol^{-1} and for the wild type $\Delta G^0_{25\,°C}$ = 19.2 kJ mol^{-1} at pH 7 from denaturant induced unfolding curves. For the C40A,82A double mutant three stability values have been reported so far. On the basis of guanidine hydrochloride induced unfolding at pH 7, a value of 18.4 kJ mol^{-1} was derived by Khuruana et al. (1995), while Schreiber and Fersht (1993) observed $\Delta G^0_{25\,°C}$ = 20.1 kJ mol^{-1}. Thermal unfolding studies at pH 8 resulted in the following parameters for the di-ala mutant: $t_{1/2}$ = 69.6 °C, ΔH_{cal} = 178.2 kJ mol^{-1}, ΔC_p = 5.9 kJ mol^{-1} K^{-1} (Wintrode et al., 1995). These thermodynamic quantities yield a surprisingly low stability value of $\Delta G^0_{25\,°C}$ = 5.3 kJ mol^{-1} for the di-ala mutant.

The temperature dependence of the standard Gibbs energy of barstar wt and the C82A mutant as derived from the present DSC studies is shown in Figure 3 by the solid and dash-dotted curves, respectively. The experimental stability curves of barstar wt and the C82A mutant show maxima at 20.4 °C and 3 °C, respectively. There is no indication in the stability curve that between pH 6.4 and 8.3 in the absence of denaturants either the wild-type protein or the mutant protein could exhibit cold denaturation. This observation is consistent with the results reported by Agashe and Udgaonkar (1995) for barstar wt that cold denaturation could only be achieved above 0 °C by the addition of at least 1.2 M GdnHCl at

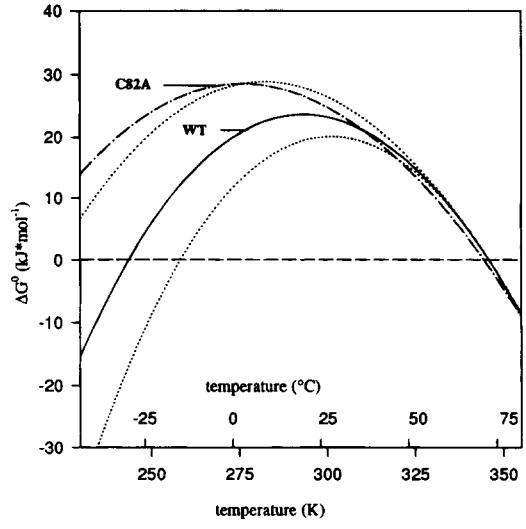

Fig. 3. Stability of barstar wt and the C82A mutant protein at pH 7.4. The stability of barstar wt (—) and the C82A mutant (–·–) was calculated from the average values of the measurements of barstar without DTT marked by an asterisk (*) in Table 1 according to Equation 10: wild type: ΔH^0 = 303 kJ mol^{-1}, ΔC_p = 5.3 kJ mol^{-1} K^{-1}, $t_{1/2}$ = 72.8 °C; C82A: ΔH^0 = 276 kJ mol^{-1}, ΔC_p = 3.6 kJ mol^{-1} K^{-1}, $t_{1/2}$ = 71.4 °C. The dotted stability curves were calculated using ΔH^0 = 303 kJ mol^{-1} and $t_{1/2}$ = 72.8 °C of the wild-type protein but ΔC_p = 4.3 or 6.3 kJ mol^{-1} K^{-1} for the heat capacity changes to demonstrate the strong dependence of the shape of the stability curve on the magnitude of the ΔC_p value.

pH 8.0. We can support these observations by the present DSC measurements on barstar wt under similar buffer conditions at pH 7.4. The result is shown in Figure 4. We clearly see a heat denaturation peak centred at $t_{1/2}$ = 48.8 °C and approximately 50% of the cold denaturation peak having a maximum at about 3.2 °C. The apparent enthalpy involved in high temperature unfolding of barstar wt in the presence of 1.87 M GdnHCl at pH 7.4 is about +103 kJ mol^{-1} at $t_{1/2}$ = 48.8 °C. This apparent enthalpy value

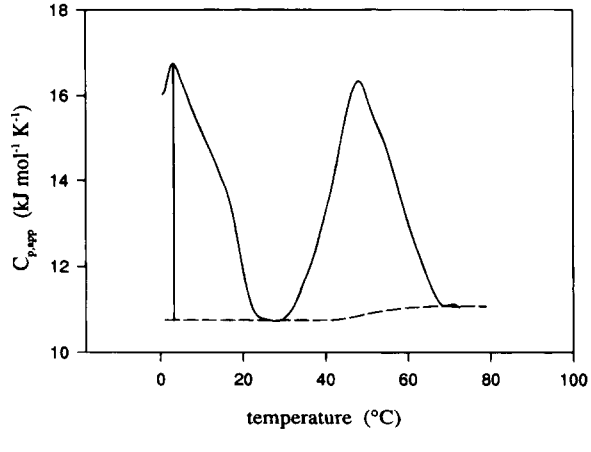

Fig. 4. Barstar wt heat and cold denaturation in the presence of 1.87 M GdnHCl at pH 7.4. Solid line: DSC measurement of barstar wt; dashed line: baseline used for evaluation. Start temperature: 0.5 °C; concentration: 1.02 mg/mL; heating rate: 1 K/min; buffer: 50 mM $NaPO_4$, 1 mM EDTA, 1.87 M GdnHCl, pH 7.4. Due to technical limitations the calorimetric measurements could not be extended to temperatures below 0 °C.

refers to unfolding of approximately 64% of the total barstar population, as an inspection of the ellipticity values in the presence of 1.8 M GdnHCl reveals (Fig. 3A; Agashe & Udgaonkar, 1995). Therefore the molar transition enthalpy of barstar wt in the presence of 1.87 M GdnHCl would amount to about 160 kJ mol^{-1}. The existence of a considerable percentage of unfolded protein at about 30 °C in the presence of 1.87 M GdnHCl could also be the reason why we observe an apparent ΔC_p only 0.5 kJ mol^{-1} K^{-1}. Agashe and Udgaonkar (1995) had calculated a ΔC_p of about 6 kJ mol^{-1} K^{-1} for heat denaturation. This value is close to the value they observed for cold denaturation and also similar to the apparent ΔC_p value in the presence of GdnHCl. Because in their treatment of the CD transition curves ΔC_p is a fit parameter that describes the curvature of the ΔG^0 vs. T curve over a large temperature range, their value of the apparent ΔC_p in the presence of GdnHCl is less prone to errors than ours. However, the choice of the ΔC_p is rather immaterial to the numerical integration of the C_p transition peak. Therefore, the enthalpy value calculated from the area of the DSC peak is probably correct. This ΔH value is, however, considerably larger than the ΔH value reported by Agashe and Udgaonkar on the basis of their CD measurements. A possible explanation is that the experiments of Agashe and Udgaonkar (1995) were done in very low ionic strength buffers.

To illustrate the extent to which the calculation of ΔG^0 could be influenced by small errors in the determination of ΔC_p we show in Figure 3 two alternative ΔG^0 curves for the wild-type protein as dotted lines. These stability curves have been calculated using ΔC_p values that differ by ±1 kJ mol^{-1} K^{-1} from the average ΔC_p value of 5.3 kJ mol^{-1} K^{-1}. All other parameters remained unchanged. It is quite informative to realize the significant errors that could be involved in stability estimates that are based on inaccurate ΔC_p values. Wintrode et al. (1995) reported a stability curve of the C40,82A double mutant at pH 8 with vanishing ΔG values at about +15 °C and 64.9 °C. This would mean that cold denaturation should be visible in the C_p curves at about 15 °C. However, there is no indication of a cold denaturation peak in the curves given in that study. Obviously, the small ΔH value observed by Wintrode et al. with the di-ala mutant of barstar is responsible for the misleading stability curve, because both the ΔC_p value and the transition temperature are similar to the values reported by Martinez et al. (1995) for the same mutant. The small variations in the $T_{1/2}$ and ΔC_p values published by these two groups are likely to result from the slightly different buffer conditions used and are insignificant for the divergent ΔG^0 estimates. It is only the enthalpy that is significantly different 265 kJ mol^{-1} at pH 7 (Martinez et al., 1995) versus 178 kJ mol^{-1}, pH 8 (Wintrode et al., 1995). Because we did not have the di-ala mutant for direct comparison we cannot provide new quantitative evidence for the discussion. Indirectly, it can, however, be argued that it is only the surprisingly low enthalpy value that leads to the apparent instability of the di-ala mutant of 5.3 kJ mol^{-1} at 25 °C. Both the transition temperature and the heat capacity change are very close to what has been observed for the wild-type protein and the mono-ala mutant in the present study and for the di-ala mutant by Martinez et al. (1995). In view of the relatively small changes in the thermodynamic properties that are caused by the C82A mutation ($\Delta t_{1/2}$ = 1.4 °C; $\Delta\Delta H^0$ = 27 kJ mol^{-1}; $\Delta\Delta C_p$ = 1.7 kJ mol^{-1} K^{-1}) in barstar a much more drastic ΔH^0 change is not very likely to result from the C40A mutation. However, the final answer must be given by an experiment.

Calculation of the heat capacity of unfolded barstar

The molar heat capacity values of barstar wt obtained from the DSC measurements are 17.4 ± 1.3 kJ mol^{-1} K^{-1} (1.68 ± 0.13 J g^{-1} K^{-1}) at 25 °C in the native state and 27.4 ± 1.3 kJ mol^{-1} K^{-1} (2.65 ± 0.13 J g^{-1} K^{-1}) at 95 °C in the denatured state. The heat capacity values do not depend on pH in the pH range used here. The variation with temperature of the molar predenaturational heat capacity is $C_p^N(t) = 8.48 \times 10^{-2} t + 15.28$ (kJ mol^{-1} K^{-1}). We can rule out that an exothermic or irreversible process occurs after the transition with barstar wt in the pH range of 6.4 to 8.3 or with the C82A mutant at pH 7.4. The native state C_p^N value at 25 °C of the C82A mutant is 17 ± 1.3 kJ mol^{-1} K^{-1} (1.65 ± 0.13 J g^{-1} K^{-1}) that of the denatured state at 95 °C, C_p(D), is 26.2 ± 1.3 kJ mol^{-1} K^{-1} (2.54 ± 0.13 J g^{-1} K^{-1}). A very similar native state C_p^N value of 1.55 ± 0.13 J g^{-1} K^{-1} (16.7 kJ mol^{-1} K^{-1}) at 20 °C was reported by Wintrode et al. (1995) for the C40,82A double mutant. The values of the absolute heat capacities of barstar wt with or without DTT were identical within the limits of error.

It was tempting to calculate the absolute heat capacity of the denatured protein on the basis of C_p increments derived from tripeptide model compounds measured in collaboration with G.R. Hedwig in our laboratory (Vogl et al., 1995; Häckel et al., 1997). The polynomial equation describing the variation with temperature of the unfolded state is $C_p^D(t) = 24.6 + 0.0669\,t - 3.499 \times 10^{-4}\,t^2$; t refers to °C. The calculated heat capacity (Fig. 5) agrees very well with the measured value. The C_p^D value at 95 °C is 27.8 kJ mol^{-1} K^{-1}, and thus only by 0.4 kJ mol^{-1} K^{-1} (+0.04 J g^{-1} K^{-1}) higher than the experimental value. If we assume that the heat capacity increments determined from tripeptides reflect C_p contributions for optimally hydrated single amino acid residues lacking any interference from neighboring moieties, it should be expected that they provide the maximal C_p values attainable by a random polypeptide chain. The fact that our experimental C_p values for unfolded barstar are somewhat below this limiting value appears reasonable and is in-

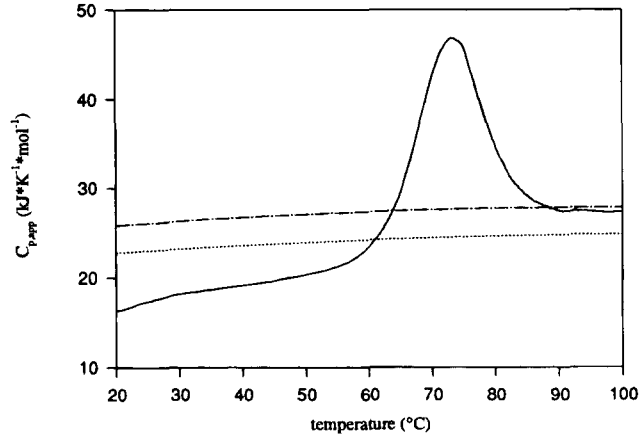

Fig. 5. Comparison of experimental and calculated partial molar heat capacity of denatured barstar wt. Measurement (solid line): barstar wt, concentration: 1.08 mg/mL. The dashed line is the heat capacity of the denatured protein calculated with an C_p increment system based on C_p measurements on tripeptides performed in collaboration with G.R. Hedwig in our laboratory ($C_p^D(t) = 24.6 + 0.0669\,t - 3.499 \times 10^{-4}\,t^2$) (Downes & Hedwig, 1995; Vogl et al., 1995; Häckel et al., 1997). The dotted line is calculated using the increment system based on model compounds published by Makhatadze and Privalov (1990). $C_p^D(t) = 21.76 + 0.055\,t - 2.466 \times 10^{-4}\,t^2$ Buffer: 50 mM $NaPO_4$, 1 mM EDTA, pH 7.4; heating rate: 2 K/min.

dicative of the presence of some minor residual structure in the unfolded chain. This conclusion is consistent with the observation by Khurana et al. (1995), who found at 90 °C and 220 nm a residual mean residue ellipticity of approximately −5,500 deg cm^2 mol^{-1}. This signal is significantly larger than that of −2,600 deg cm^2 mol^{-1} expected for a random chain configuration of (Chen et al., 1972).

For comparison we also computed the heat capacity of denatured barstar wt with the increment system given by Makhatadze and Privalov (1990) on the basis of non-peptide model compounds. The resulting $C_p^D(t) = 21.76 + 0.055\,t - 2.466 \times 10^{-4}\,t^2$ function is considerably lower than the experimental curve. This discrepancy suggests that at least for barstar the C_p increments derived from tripeptides reflect the heat capacity of the unfolded chain better than those derived predominantly from model compounds other than amino acid residues.

Dependence of the transition parameters on the presence of DTT

To prevent intermolecular disulfide bond formation by oxidation we performed measurements under reducing conditions provided by the presence of DTT and under strictly oxygen-free conditions. Inspection of the results shown in Table 1 suggests that transition temperatures are not altered by the presence of DTT, while the corresponding calorimetric transition enthalpies are slightly higher in the presence of DTT. This DSC result indicating a negligible influence of DTT on the transition temperatures is consistent with the CD evidence provided by Ramachandran and Udgaonkar (1996). The slightly increased ΔH_{cal}^0 values could be a buffer effect resulting from the presence of DTT and/or reflect the finding of our mass spectrometry studies that about 30% of the barstar population appeared to have reacted covalently with one DTT after the first heating to 100 °C. Thus, although on the average calorimetric measurements in the presence of DTT showed similar results as under oxygen free conditions, it is preferable to avoid DTT, if possible, because DSC studies in the presence of this reducing agent are difficult to perform. Therefore, the complete exclusion of oxygen from the solution appears to be the preferable procedure for DSC studies on oxidation-sensitive proteins.

Materials and methods

Chemicals

All chemicals used in the studies were of reagent grade: $NaH_2PO_4 \cdot H_2O$ p.a. (Merck), $Na_2HPO_4 \cdot 2H_2O$ p.a. (Merck), EDTA p.a. (Merck), NaCl p.a. (Riedel de Haën), 1,4-Dithio-DL-threitol microselect (Fluka).

Expression and purification of barstar wt

Expression and purification of barstar wt, the mutants C82A and C40A, followed published procedures (Khurana & Udgaonkar, 1994; Ramachandran & Udgaonkar, 1996).

Concentration determination

Protein concentrations were determined by UV absorption at 280 nm. The following absorption coefficients were used ϵ (Barstar WT) = 2.22 mg^{-1} mL cm^{-1} (determined in the lab of Udgaonkar), ϵ = 2.14 mg^{-1} mL cm^{-1} for C82A, and ϵ = 2.24 mg^{-1} mL cm^{-1} for C40A (these two values have been calculated following Martinez et al., 1995). The molar quantities in this paper have been calculated using the following molecular masses (WT) M = 10.346(±4) D, and (C82A and C40A) M = 10.315(±2) D. The partial specific volume used for the C_p calculations was 0.73 mL g^{-1} (Martinez et al., 1995).

The calculated molar masses of Barstar WT, the C82A, and the C40A mutants have been experimentally verified by by high-resolution mass spectrometry (MALDI). The average value of five measurements on barstar wt was M = 10.346 ± 4 Da, that of three measurements on C82A was 10.315 ± 2 Da. All mass spectrometry measurements were carried out at pH 7.4 in 10 mM $NaPO_4$ buffer. The results indicate that in all cases the methionine at position 1 is still present. This result is consistent with the observation of Frisch et al. (1995). It is interesting to note that barstar expressed in bacillus amyloliquefaciens was found to lack the methionine (Hartley, 1988).

In some of our DSC and mass spectrometry measurements 10 mM DTT was used in thermal unfolding experiments to avoid oxidation of the cysteines. When wild-type barstar was analyzed after heating to 100 °C in the presence of DTT the mass spectrometrical analysis revealed an increase in molar mass by 117 ± 4 D for approximately 30% of the molecules. This can be explained by partial condensation of DTT with one of the cysteines.

DSC studies

The measurements were carried out in an N-DSC instrument from Calorimetric Science Corporation having a cell volume of 0.897 mL. The heating rate was 2 K/min, and sample concentrations were between 0.6–1.8 mg mL^{-1}. Data were recorded every 0.1 K. All studies were carried out in 50 mM $NaPO_4$, 1 mM EDTA buffer. The pH was adjusted to the respective value by adding small amounts of sodium hydroxide solutions. In one set of measurements 10 mM DTT was present in the DSC runs. To quantify the effect of DTT on the stability of barstar wt and the C82A mutant control experiments with and without DTT containing buffer were performed.

Addition of DTT can be avoided, if the buffer and the sample are properly saturated with nitrogen at 4 to 8 °C prior to the experiments. We performed the dialysis in a closed air free retort under nitrogen. The reported oligomerization of barstar in the presence of oxygen (Frisch et al., 1995; Martinez et al. 1995) could be avoided completely by this procedure. Under these conditions as well as under reducing conditions in the presence of DTT barstar wt exists as monomer without disulfide bonds.

Reversibility of the unfolding process was checked by heating the protein in the first scan to a temperature 2 to 3 K above $t_{1/2}$ followed by cooling and reheating the same sample fully through the transition.

The calorimetric transition enthalpies ΔH_{cal}^0 were obtained by numerical integration of the area under the excess heat capacity peaks employing a sigmoidal baseline to take the heat capacity changes between the native and denatured state into account.

Curve analysis

Barstar wt is thought to undergo a temperature-induced transition of the simple [N]↔[D] type in the pH range from pH 6 to 9 (Khuruana et al., 1995). To verify the two-state model the C_p data were fitted to an [N]↔[D] transition employing a sigmoidal base-

line (C_p(base)). In such a model the temperature dependence of the equilibrium constant $K(T)$ is given by the van't Hoff equation.

$$K(T) = \left(\frac{T}{T_{1/2}}\right)^{\Delta C_p/R} \cdot \exp\left[-\frac{\Delta H^0}{R}\left(\frac{1}{T} - \frac{1}{T_{1/2}}\right)\right] \cdot \exp\left[\Delta C_p \cdot \frac{T_{1/2}}{R}\left(\frac{1}{T} - \frac{1}{T_{1/2}}\right)\right] \quad (1)$$

and the degree of unfolding α_D is given by the equation

$$\alpha_D = \frac{K}{K+1}. \quad (2)$$

To calculate the sigmoidal baselines according to Equation 3 the pre- and post-denaturational C_p functions were linearly extrapolated. b and d are the slopes of the pre- and post-denaturational baselines, a is the Y-axis intercept at 0 K (Vogl, 1995).

$$C_p(\text{base}) = (1 - \alpha_D)(a + b \cdot T) + \alpha_D(c + d \cdot (T - T_{1/2})). \quad (3)$$

The constant c is the C_p value of the denatured state of the protein at $T_{1/2}$ and can be calculated by

$$c = a + b \cdot T_{1/2} + \Delta C_{p,T_{1/2}}. \quad (4)$$

The transition curve has been simulated by the following equation for the heat capacity function of a two-state transition.

$$C_p(\text{calc}) = C_p(\text{base}) + \frac{K \cdot \Delta H^2}{(1+K)^2 \cdot R \cdot T^2}. \quad (5)$$

To allow for a direct comparison of the stability parameters obtained from our DSC and previous denaturation studies the thermodynamic parameters were extrapolated to 298.15 K. The relevant equations for the extrapolation are

$$\Delta S^0_{T_{1/2}} = \frac{\Delta H^0_{T_{1/2}}}{T_{1/2}} \quad (6)$$

$$\Delta H^0_{25\,^\circ\text{C}} = \Delta H^0_{T_{1/2}} + \Delta C_p(298.15 - T_{1/2}) \quad (7)$$

$$\Delta S^0_{25\,^\circ\text{C}} = \Delta S^0_{T_{1/2}} + \Delta C_p \ln \frac{298.15}{T_{1/2}} \quad (8)$$

$$\Delta G^0_{25\,^\circ\text{C}} = \Delta H^0_{25\,^\circ\text{C}} - 298.15 \Delta S^0_{25\,^\circ\text{C}}. \quad (9)$$

The temperature dependence of the standard Gibbs energy is therefore given by

$$\Delta G^0(T) = \Delta H^0_{T_{1/2}}\left(1 - \frac{T}{T_{1/2}}\right) - \Delta C_p\left[T_{1/2} - T + \left(T \ln \frac{T}{T_{1/2}}\right)\right]. \quad (10)$$

DSC in the presence of GdnHCl

A DSC measurement was carried out with guanidine hydrochloride in the buffer using a heating rate of 1 K/min. The concentration of GdnHCl was determined with a refractometer according to Nozaki (1972). Because due to technical limitations of the calorimetric measurements could not be performed to low enough temperatures that would allow registration of the full cold denaturation peak, the transition enthalpy of the cold denaturation was estimated as follows. A linear baseline was used for both heat and cold denaturation. The area corresponding to approximately half of the cold denaturation peak was determined by planimetry, as indicated by the line dividing the cold denaturation peak. The area of the heat denaturation peak was used as reference value for the calculation of the cold denaturation enthalpy. The apparent ΔH value estimated in this manner for cold denaturation was corrected for the percentage of residual native structure seen by CD absorption under these experimental conditions.

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