Thermodynamic and Kinetic Analysis of the SH3 Domain of Spectrin Shows a Two-State Folding Transition†

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ABSTRACT: The folding and unfolding reactions of the SH3 domain of spectrin can be described by a two-state model. This domain is a β-sheet barrel containing 62 amino acids. Equilibrium unfolding by urea, guanidine hydrochloride, and heat is completely reversible at pH values below 4.0. At higher pH values the unfolding is reversible as long as the protein concentration is below 1 mg/mL. The Gibbs energy of unfolding in the absence of denaturant, ΔG_{H_2O}, at pH 3.5 and 298 K is calculated to be 12 kJ mol^{-1} for urea, chemical, and temperature denaturation. The stability of the protein does not change noticeably between pH 5.0 and 7.0 and is around 15.5 kJ mol^{-1}. Since heat effects of unfolding are relatively small and, as a result, heat-induced melting occurs in a wide temperature range, the analysis of scanning calorimetry data was performed taking into account the temperature dependence of unfolding ΔC_p. The free energy of unfolding obtained for this domain (ΔG_{H_2O} = 14 ± 2 kJ mol^{-1}) was, within experimental error, similar to those obtained in this work by other techniques and with those reported in the literature for small globular proteins. Kinetics of unfolding and refolding at pH 3.5, followed both by fluorescence and by circular dichroism, provide evidence of the simplest folding mechanism consistent with the two-state approximation. A value for ΔG_{H_2O} = 13 ± 0.7 kJ mol^{-1} can be extrapolated from the kinetic data. No intermediate can be seen to accumulate by equilibrium denaturation followed by fluorescence and circular dichroism, refolding kinetics and calorimetry, and a concomitant recovery of secondary and teritary structure is observed during refolding. This suggests that the two-state model can properly describe the folding of this domain from both the equilibrium and kinetic points of view and raises the question of whether the accumulation of kinetic intermediates is merely a result of the size of the protein being studied.

It is clear that a polypeptide chain cannot explore all the conformational space in order to reach the native state, and it thus follows that one or more folding pathways should exist. The analysis of small monomeric proteins has shown that in many cases they follow a single unfolding transition in equilibrium and can consequently be studied as a two-state system. However, there is now increasing evidence for the existence of one or more intermediates in proteins with more than 70 residues. These intermediates have been detected when the folding reactions of these proteins have been studied kinetically or under certain conditions of pH, solvent, etc. (Ikeguchi *et al.*, 1986; Kuwajima *et al.*, 1988; Griko *et al.*, 1988; Udgaonkar & Baldwin, 1990; Briggs & Roder, 1992; Lu & Dahlquist, 1992; Radford *et al.*, 1992; Serrano *et al.*, 1992; Varley *et al.*, 1993; Mann & Matthews, 1993). On the other hand, there are two cases, the chymotrypsin inhibitor CI2 (Jackson & Fersht, 1991) and the G-domain (Alexander *et al.*, 1992b), for which a kinetic or equilibrium intermediate could not be detected, using a wide variety of techniques. The protein inhibitor CI2 has 80 amino acids, but only 60 of them form a globular compact domain (McPhalen & James, 1987). The G-domain forms part of the IgG binding protein and contains around 57 residues (Fahnestock *et al.*, 1986). In both cases there is a central α-helix packing against an antiparallel β-strand (Lyan *et al.*, 1991; Gronenborn *et al.*, 1991; Orban *et al.*, 1992; McPhalen & James, 1987). This raises the following question: are the intermediates accumulating during refolding in other proteins the result of size and consequently of complexity? If this is the case, then we should expect that other proteins or domains with around 60 residues, and no disulfide bridges, should also lack any populated intermediate and behave as true two-state systems.

The SH3 domain is a single small domain of around 60 residues, consisting almost exclusively of β-structure. It is found in a number of proteins and the homology pattern between all of them is well established (Musacchio *et al.*, 1992a). The crystal structures of the SH3 domains of α-spectrin (Musacchio *et al.*, 1992b) and human Fyn (a *src* family tyrosine kinase) (Noble *et al.*, 1993) have recently been determined by X-ray diffraction. Solution structures of SH3 domains from other sources have also been obtained by NMR: human phosphatidylinositol 3′-kinase (PI3K) (Koyama *et al.*, 1993; Booker *et al.*, 1993), *src* (Yu *et al.*, 1992, 1993), and phospholipase Cγ (PLC-γ) (Kohda et al., 1993). All the resolved SH3 domains are highly conserved structures consisting mainly of two antiparallel β-sheets perpendicularly disposed to form a compact β-barrel, with loops of different extensions between the β-strands. This domain is quite interesting, not only because of its small size but also because few all-β-structure proteins (Varley *et al.*, 1993) have been studied from a thermodynamic and kinetic point of view.

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[1] *Abbreviations*: Gdn·HCl, guanidine hydrochloride; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; OD, optical density; CD, circular dichroism; SH3, *src* homology region 3; DSC, differential scanning calorimetry.

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For this paper, we have analyzed the thermodynamic and kinetic behavior of the SH3 domain from α-spectrin to see if it behaves in a similar way as the CI2 protein or the G domain of the IgG binding protein. We have used urea or guanidine hydrochloride to denature the protein, and the changes in fluorescence and/or CD were measured in order to follow the unfolding process. Differential scanning calorimetry experiments were also performed in the pH 2.0–4.0 range. The kinetics of unfolding and refolding, using urea concentration jumps, was used to detect transient intermediates not detected under equilibrium conditions.

EXPERIMENTAL PROCEDURES

Chemicals. Guanidine hydrochloride (Gdn·HCl) and urea were purchased from BRL (Gaithersburg, MD). Buffers for equilibrium denaturation were citric acid, glycine, and sodium phosphate from Merck (Darmstadt, Germany) and PIPES from Sigma (St. Louis, MO). All other reagents were of the highest purity available. Double-distilled deionized water was used throughout.

Expression and Purification of the Recombinant SH3 Domain. pET3c plasmid coding for wild-type SH3 domain was a generous gift from Dr. Saraste. The domain was expressed in *Escherichia coli* BL21 strain. The protein was purified essentially by the method of Musacchio et al. (1992b).

Protein Concentration Determination. The extinction coefficient was calculated by the method of Gill and von Hippel (1989), consisting of the measurement of absorbance at 280 nm of a protein preparation in 6 M Gdn·HCl. Only two tryptophans and the three tyrosines contribute to this signal. The extinction coefficient at 280 nm for the domain in 6 M Gdn·HCl is calculated to be 15 220 and is 16 147 for the native protein.

Chemical Denaturation Experiments. Urea and Gdn·HCl solutions were prepared gravimetrically in volumetric flasks. For each data point 100 or 50 μL of SH3 in the appropriate buffer was mixed with 750 μL of a given denaturant solution. At pH values lower than 3.5, high concentrations of urea changed the pH. Since the stability of this domain is very sensitive to pH values lower than 5.0, we adjusted the pH of the different urea solutions plus buffer, with concentrated hydrochloric acid. The mixtures of the protein plus the buffer in the appropriate urea solutions were left to equilibrate for at least 1 h.

(*a*) *Fluorescence Spectroscopy Analysis.* Fluorescence emission spectra of tryptophan residues in SH3 were used to monitor any changes in the environment of Trp 41 and 42 upon unfolding of the protein. Fluorescence was measured in an Aminco Bowman Series 2 luminescence spectrometer. Excitation was at 290 nm with a 2-nm slit. Fluorescence was detected through an 8-nm slit. In these experiments protein concentration was kept at 3 μM, which renders an OD at 280 nm of 0.05. All experiments were performed at 298 K. The fluorescence spectrum corrected for the instrument response of the SH3 domain is reduced in intensity and red-shifted from 336 to 347 nm upon denaturation with urea or guanidine hydrochloride (data not shown). We found the maximum difference between the native and denatured states to be at 329 nm. However, we have chosen 315 nm to monitor protein denaturation in order to minimize the contribution of Raman scattering from the buffer to the signal. The equlibrium constant for denaturation was calculated, for each denaturant concentration, using

$$K_U = (F_N - F)/(F - F_U) \quad (1)$$

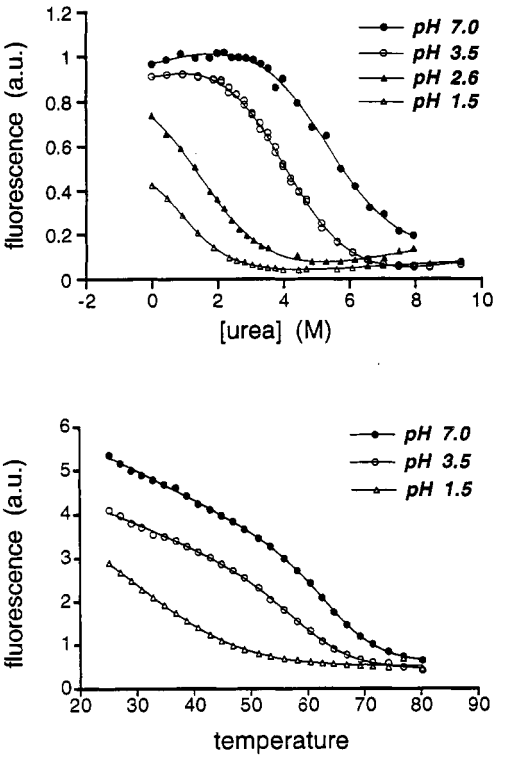

FIGURE 1: Chemical and temperature denaturation profiles of spectrin SH3 domain, followed by fluorescence. Fluorescence emission registered at 315 nm (excitation at 290 nm) after at least 1 h of treatment with denaturant at 298 K. The following buffers were used: 50 mM citric acid, pH 3.5 (○); 50 mM PIPES, pH 7.0 (●); 50 mM glycine, pH 1.5 (△) and 2.5 (▲). Traces were fit to eq 3. (Top) Urea denaturation. (Bottom) Temperature denaturation. In all cases at low pH, a destabilization of the domain can be observed although it cannot be quantified.

where F is the fluorescence value at a certain concentration of denaturant and F_N and F_U are the corresponding fluorescence values for the fully folded and unfolded states in the absence of denaturant. It has been found experimentally that the free energy of unfolding of proteins in the presence of urea is linearly related to the concentration of the denaturant (Pace, 1986):

$$\Delta G_U = \Delta G_{H_2O} - m[\text{denaturant}] \quad (2)$$

The value of m and ΔG_{H_2O}, the apparent free energy of unfolding in the absence of denaturant, may be calculated from eq 1, because $\Delta G_U = -RT \ln K_U$. The proportionality constant m reflects the cooperativity of the transition and is believed to be related to the difference in hydrophobic surface exposed to the solvent between the native and denatured states.

The quantum yield of the fluorescence of the native and denatured protein increases with urea (Figure 1, top). In the case of Gdn·HCl the fluorescence of the native protein decreases with the denaturant, while that of the unfolded state increases. Consequently the fluorescence yield of the unfolded (F_U) protein in water needs to be linearly extrapolated from higher denaturant concentrations, at which the protein is denatured.

Taking all these dependencies into account, the fluorescence data can be fitted to the following equation

$$F = \{(F_N + a[\text{denat}]) + (F_U + b[\text{denat}]) \exp(m[\text{denat}] - \Delta G_{H_2O})/RT\}/\{1 + \exp(m[\text{denat}] - \Delta G_{H_2O})/RT\} \quad (3)$$

in which the dependence of the intrinsic fluorescence upon

denaturant concentration, in both the native and denatured state, is taken into account by the terms a[urea] and b[urea], respectively (linear approximation). With this kind of analysis we are assuming a two-state model for denaturation, with no species accumulating significantly apart from the native and denatured forms of the domain.

The values obtained for ΔG_{H_2O} and m are only reliable when both a and b can be calculated accurately, in other words, when the total transition can be observed, and we have several values of the fluorescence of the native and denatured states over a large range of denaturant concentrations.

(*b*) *Circular Dichroism.* CD equilibrium measurements were performed in a Jobin-Yvon C-VI machine. Far UV circular dichroism spectra were recorded in a 0.2-cm path length cuvette at 298 K. Secondary structure was followed at 235 nm as a function of urea concentration. We did not find any dependence of the ellipticity of the native or denatured states with the urea concentration and, as a consequence, the data could be fitted to the following simpler equation:

$$F = F_N - \{(F_N - F_U)\exp(m[\text{urea}] - \Delta G_{H_2O})/RT\}/\{1 + \exp(m[\text{urea}] - \Delta G_{H_2O})/RT\} \quad (4)$$

Thermal Denaturation Experiments. Thermally induced unfolding was monitored by fluorescence spectroscopy at different pHs. Fluorescence measurements were made through the temperature range 298–363 K. The temperature was monitored by a thermocouple immersed in the cuvette under observation. Reversibility of the transition can be tested by almost complete recovery of the fluorescence signal after cooling back to 298 K. There is a linear dependence of the fluorescence of the native (F_N) and denatured states (F_U) (Figure 1, bottom) with temperature, and consequently the values of F_N and F_U are estimated at each temperature by linear extrapolation (Pace, 1986).

The enthalpy, $\Delta H_U(T)$, and the enthropy, $\Delta S_U(T)$, of unfolding at a given temperature T can be calculated from the following equations:

$$\Delta H_U(T) = -R[\mathrm{d}(\ln K_U)/\mathrm{d}(1/T)] \quad (5)$$

$$\Delta S_U(T_m) = \Delta H_U(T_m)/(T_m) \quad (6)$$

where T_m is the midpoint of thermal denaturation. van't Hoff plots ($\ln K_U$ vs $1/T$) are approximately linear through the T_m region, thus allowing the estimation of the enthalpy and enthropy of unfolding at T_m. Using the average ΔC_p value obtained from differential scanning calorimetry and applying the following equations

$$\Delta H_U(T) = \Delta H_U(T_m) + \Delta C_p(T - T_m) \quad (7)$$

$$\Delta S_U(T) = \Delta S_U(T_m) + \Delta C_p \ln(T/T_m) \quad (8)$$

$$\Delta G_U(T) = \Delta H_U(T) - T\,\Delta S_U(T) \quad (9)$$

it is possible to calculate the free energy of unfolding, ΔG_U, at a certain temperature T.

Differential Scanning Calorimetry. Calorimetric experiments were performed with the computerized version of the DASM-4 microcalorimeter (Privalov & Potekhin, 1986) at heating rates of 0.5, 1, and 2 K/min. The samples were routinely heated up to 383 K and then cooled slowly inside the calorimeter and reheated once again to check the reversibility of the unfolding. Before filling into the cell, the samples were extensively dialyzed against buffers with the appropriate pH. Since the experiments were conducted between pH 2 and 4, buffers were either 10 mM sodium glycine or 10 mM sodium acetate. The concentration of the protein in calorimetric experiments was between 2 and 5 mg/mL.

The molar partial heat capacity was calculated as previously described (Privalov & Potekhin, 1986) assuming that the partial specific volume of SH3 is 0.73 mL/g (the average value for globular proteins) and its molecular mass equal to 7150 Da. The molar partial heat capacity was further analyzed using SCAL software (Filimonov *et al.*, 1982) and the curve-fitting option of Sigmaplot (Jandel Co.).

The necessity of using a nonlinear $\Delta C_{p,u}$ function to analyze calorimetric experiments was demonstrated by Privalov *et al.* (1989), who showed that $C_{p,u}$ is a nonlinear function of temperature and who suggested a simple procedure for estimating $C_{p,u}$ from the amino acid content of the protein (Makhatadze & Privalov, 1990; Privalov & Makhatadze, 1990). For many years the heat effect of the unfolding was calculated by a direct integration of the C_p^{exc} function in accordance with the following definitions (Filimonov *et al.*, 1982; Privalov & Potekhin, 1986);

$$\langle \Delta C_{p,0}(T) \rangle = C_{p,p}(T) - C_{p,0}(T) = \mathrm{d}\langle \Delta H \rangle/\mathrm{d}T = \delta C_p^{int} + \delta C_p^{exc} \quad (10)$$

$$\delta C_p^{int} = \sum_i \Delta C_{p,i} F_i \qquad \delta C_p^{exc} = \sum_i \Delta H_i \,\mathrm{d}F_i/\mathrm{d}T \quad (11)$$

Here $\langle \Delta C_{p.0} \rangle$ is the excess of C_p over $C_{p,0}$, the heat capacity of the initial state (which from now on will be considered as the reference state), and $\langle \Delta H(T) \rangle$ is the overall excess of the enthalpy, while ΔH_i and $\Delta C_{p,i}$ refer to the increments corresponding to the macroscopic states of protein with relative population F_i. Then $\delta C_p^{int}(T)$ is related to the "internal" heat capacity increase, while $\delta C_p^{exc}(T)$ is the excess heat absorbance of the reaction.

To find δC_p^{exc} from C_p one has to approximate both $C_{p,0}$ and δC_p^{int} by some reasonable functions. Usually the first one is approximated by a linear function of temperature (Privalov, 1979; Privalov & Potekhin, 1986; Privalov *et al.*, 1989) and the second one by a sigmoid proportional to the integral of δC_p^{exc} (Takahashi & Sturtevant, 1981; Filimonov *et al.*, 1982). Such an approximation seems to be very reasonable and will result in a relatively correct estimation of the δC_p^{exc} whenever the following restrictions are fulfilled:

$$\delta C_{p,max}^{int} \ll \delta C_{p,max}^{exc} \quad (12)$$

$$\Delta C_{p,i} \approx k\,\Delta H_i \quad (13)$$

where k is the same constant for all the possible macroscopic states of the protein. Since the first condition is usually valid for proteins with relatively high enthalpy of unfolding (>300 kJ/mol), the error in evaluation of δC_p^{exc} introduced by such approximate estimation of δC_p^{int} is negligible, and δC_p^{exc} is useful for the calculation of T_m and of the overall heat effect. However, the applicability of this simple algorithm to the more detailed thermodynamic analysis, e.g., for simply checking the two-state model validity, is very questionable when conditions 12 and 13 are not valid or ΔC_p of unfolding depends on temperature.

It seems that in general, and in particular in those cases when the heat effects of unfolding are small, the most correct approach to the calorimetric data analysis is to perform the global fitting of the data to some reasonable model, which will impose the restrictions on the variable parameters and reduce

the fitting uncertainty (Filimonov *et al.*, 1982). For example, for SH3 one can assume that

$$C_{p,0}(T) = a_0 + b_0(T - T_0) \quad (14)$$

where a_0 is the partial molar heat capacity of the native protein at some temperature $T = T_0$ and b_0 is the slope. Then, by analyzing the initial parts of all the experimentally accumulated molar heat capacity functions, rather strict constraints on the values of a_0 and b_0 can be deduced. A similar procedure might be used to impose constraints on the $C_{p,u}$ and, as a result, on the δC_p^{int} function. In addition, the heat capacity of the unfolded state can be estimated with the method suggested by Makhatadze and Privalov (1990) and Privalov and Makhatadze (1990).

In our analysis we have performed a direct global fitting of the C_p curves to a two-state model under the assumption of the linearity of $C_{p,0}$ and the variability of $\Delta C_{p,u}$. The latter was presented as $f(T) = a + bT + cT^2$ since, as it will be shown later, a first-order polynomial cannot adequately describe the heat capacity of the unfolded state, $C_{p,u}$.

For a two-state monomolecular process we can write

$$N \overset{K_u}{\Longleftrightarrow} U \quad (15)$$

$$K_u = [U]/[N] = f_u/f_n \quad (16)$$

$$f_n + f_u = 1 \quad (17)$$

where N and U stand for the native and unfolded states, f_n and f_u for their fractions, and K_u for the monomolecular equilibrium constant.

Then for excess heat capacity we have

$$\langle \Delta C_{p,0} \rangle = \delta C_p^{int} + \delta C_p^{exc} = \Delta C_{p,u} f_u + \Delta H_u \, df_u/dT \quad (18)$$

Here $\Delta H_u(T)$ and $\Delta C_{p,u}(T)$ are the enthalpy and the heat capacity increments of unfolding. Introducing T_m as the temperature where at given solvent conditions $K_u(T_m) = 1$ and taking into account that

$$d\Delta H_u/dT = \Delta C_{p,u} \quad (19)$$

and that $\Delta C_{p,u}$ is a parabolic function

$$\Delta C_{p,u}(T) = a + bT + cT^2 \quad (20)$$

we get for the enthalpy

$$\langle \Delta H_u(T) \rangle = \Delta H_u(T_m) + a(T - T_m) + [b(T^2 - T_m^2)/2] + [c(T^3 - T_m^3)/3] \quad (21)$$

In a similar way, since

$$d\Delta S_u/dT = \Delta C_{p,u}/T \quad (22)$$

one can find ΔS_u, ΔG_u, and K_u as temperature functions. Finally

$$\delta C_p^{int} = \Delta C_{p,u} K_u/(1 + K_u) \quad (23)$$

$$\delta C_p^{exc} = \frac{\Delta H_u^2}{RT^2} \frac{K_u}{(1 + K_u)^2} \quad (24)$$

Kinetic Analysis. Kinetics were followed in a Biologic stopped-flow machine both by fluorescence and by circular dichroism. For fluorescence experiments the unfolding reaction was performed by dilution of the native SH3 domain in 50 mM citric acid, pH 3.5, with the appropriate ratio of denaturating buffer containing different concentrations of urea in 50 mM citric acid, pH 3.5. For the refolding reaction, the unfolded domain in 50 mM citric acid, pH 3.5, containing 7.5 and 9 M urea, was mixed with an excess of the same buffer without urea to give various final urea concentrations. Fluorescence was measured through a 320-nm cutoff filter (excitation at 290 nm). In the CD refolding experiments the buffer used was 5 mM citric acid, pH 3.5. The CD signal was followed at 235 nm. In all the experiments the pH of the buffer containing the urea solution was adjusted to pH 3.5 with hydrochloric acid. The cell chamber and the syringes were kept at 298 K.

RESULTS

Chemical Denaturation Followed by Circular Dichroism and Fluorescence Spectroscopy. (a) Fluorescence Spectroscopy. In Figure 1 we show the chemical denaturation by urea (top) of the SH3 domain followed by fluorescence. At pH 3.5 the values for ΔG_{H_2O} and m are 12 ± 0.2 kJ mol^{-1} and 3.1 ± 0.03 kJ mol^{-1} M^{-1}, respectively, depending on the slope of the effect of urea on the denatured state. The stability of the protein decreases at lower pH values and increases at higher pH values. However, it is not possible to quantify the data with reasonable accuracy, due to the lack of points for the native (low pH) or denatured state (high pH) (Figure 1, top). In the case of Gdn·HCl denaturation, there is a sharp dependence of the native fluorescence on the Gdn·HCl concentration (data not shown). This dependency precludes any quantitative analysis at pHs below or equal to 3.5. Only between pH 5.0 and 7.0 is it possible to estimate ΔG_{H_2O} and m with reasonable accuracy. Between these two pHs the values for ΔG_{H_2O} and m are 15.5 ± 1.3 kJ mol^{-1} and 7.85 ± 0.5 kJ mol^{-1} M^{-1}, respectively.

(b) Circular Dichroism. Tryptophan fluorescence seems to be dependent on very local changes in its environment. In order to test if the transition monitored by fluorescence reflected a total disruption of the overall structure of the domain, or just a local unfolding, we analyzed the chemical denaturation of SH3 by urea at pH 3.5. The SH3 far UV CD spectrum is a complex one (Figure 2, top). It presents two minima at 228 and 203 nm and two maxima at 220 and 235 nm. Upon urea denaturation the overall spectrum flattens, although the spectrum could only be registered down to 220 nm because of the high urea absorbance below this value. For this reason we have chosen 235 nm to follow urea denaturation (Figure 2, bottom). An increase in the negative ellipticity is observed upon urea addition. The fitting of ellipticity data at 235 nm to eq 3 gives ΔG_{H_2O} = 12 ± 0.1 kJ mol^{-1} and m = 3.2 ± 0.06 kJ mol^{-1} M^{-1}.

(c) Thermal Denaturation Followed by Fluorescence. It is not possible to perform an accurate analysis of the thermal denaturation of the SH3 demain at pH 7.0, due to the high midpoint of the thermal denaturation (Figure 1, bottom). At pH 3.5 the midpoint of the thermal denaturation is good enough to obtain an accurate extrapolation of the fluorescence of the native and denatured states. The T_m of the thermal denaturation was found to be 333 ± 6 K. The $\Delta H_u(T_m)$ of the thermal denaturation at pH 3.5 was calculated from a van't Hoff plot (ln K_u vs $1/T$) (data not shown) and was found to be 196 ± 3 kJ mol^{-1}. Applying eqs 6–9, and using the ΔC_p

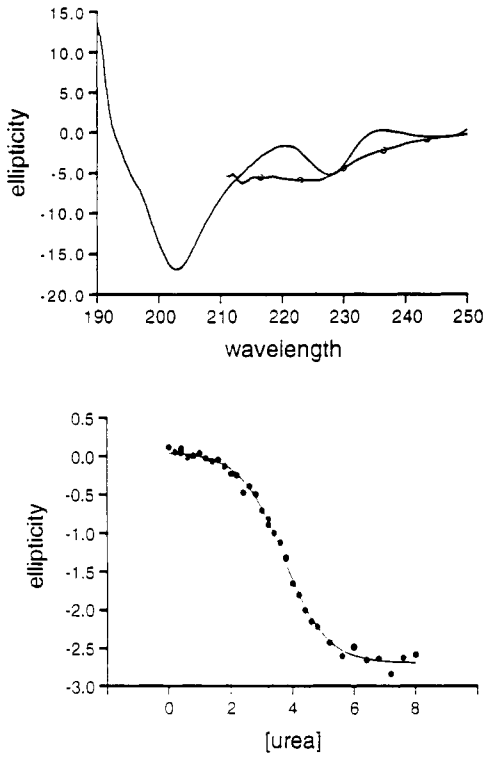

FIGURE 2: Circular dichroism spectra of native and denatured SH3 domain and chemical denaturation profiles of spectrin SH3 domain. (Top) CD spectra at 298 K of different conformations of SH3. Native SH3 in 5 mM citric acid, pH 3.5 (—); unfolded SH3 in 5 mM citric acid, pH 3.5, and 8 M urea (○). (Bottom) Urea denaturation of the SH3 domain in 5 mM citric acid, pH 3.5, at 298 K, followed by changes in the circular dichroism signal at 235 nm.

value at 298 K (3.9 kJ deg^{-1} mol^{-1}) obtained from the calorimetric analysis (see below), we find a value for ΔG_{H_2O} of 13 ± 3 kJ mol^{-1}, which is within experimental error similar to that determined by chemical denaturation.

Differential Scanning Calorimetry. In acidic solutions with pH between 2 and 3.5 and at low ionic strength, the thermal unfolding of SH3 fragment is almost 100% reversible. In other words, the first and the second records of the heat capacity of the protein solutions were indistinguishable within the limits of experimental error. At pH 3.5 and 4.0 the reversibility of unfolding started to decrease, but the recovery was still reasonably high (about 70%) even after heating the samples up to 383 K. Such a decrease in reversibility was expected since the solubility of the native protein also decreases above pH 4 (Musacchio *et al.*, 1992b).

Due to the small size of SH3 its overall molar heat effect of unfolding is not large, i.e., the C_p peaks are small and broad (see Figure 3), which complicates an accurate evaluation of the thermodynamic parameters. To get reliable records of C_p we had to work at relatively high protein concentration (up to 5 mg/mL); nevertheless, neither concentration nor heating rate dependence of the heat capacity was observed. It was concluded, therefore, that under our experimental conditions of low pH and low ionic strength the SH3 unfolding in the calorimeter cell occurs at equilibrium. Careful consideration of the curves presented in Figure 3 reveals two important factors for the data analysis and evaluation of thermodynamic parameters. First, it is seen that the heat capacity of the native SH3 increases with temperature in full agreement with the known behavior of globular proteins (Privalov, 1979; Filimonov *et al.*, 1982; Privalov & Potekhin, 1986). At the same time, the heat capacity of the unfolded state under our

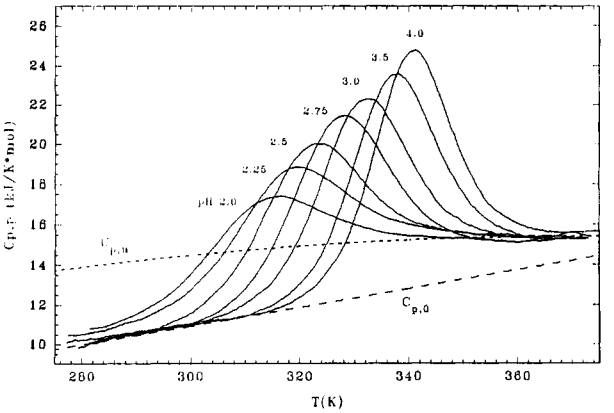

FIGURE 3: Temperature dependence of the molar partial heat capacity of the SH3 at acidic pH (—). The dashed lines show average temperature dependencies of $C_{p,0}$ (– – –) and $C_{p,u}$ (- - -), as found by the global curve-fitting procedure (for the parameters see text). The average value of $C_{p,0}$ at 293 K is equal to 10.5 ± 0.8 kJ K^{-1} mol^{-1}. pH values are shown on top of the curves.

experimental conditions is much less dependent on temperature, at least above 340 K. If the initial state corresponds to the monomer of the native protein and the final state corresponds to the monomer of the unfolded molecule, such a difference in C_p slopes should mean that the ΔC_p of unfolding is not constant and changes with temperature. A similar effect was first pointed out by Takahashi and Sturtevant (1981) and later analyzed and explained by Privalov and co-workers (Privalov *et al.*, 1989; Makhatadze & Privalov, 1990; Privalov & Makhatadze, 1990), who have shown that, in general, the $\Delta C_{p,u}$ (the heat capacity increment of unfolding) of globular proteins depends on temperature. It seems, however, that most of the enthalpy values in the literature are correct within the limits of experimental error, despite the fact that, most frequently, the calorimetric data for globular proteins used to be analyzed under the simplified assumption of a constant ΔC_p (Privalov, 1979) (see Experimental Procedures).

The calculated function $C_{p,u}$ for SH3 fragment is shown in Figure 5. Since the data on partial heat capacities of the elements of protein chemical structure are available only for a few temperatures (Makhatadze & Privalov, 1990), an analytical expression for $C_{p,u}(T)$ was found as a second-order least-square regression through the six calculated data points:

$$C_{p,u}(T) = -9.42 + 0.1366T - 0.0001795T^2 \quad (\text{kJ K}^{-1}\text{ mol}^{-1}) \tag{25}$$

or after introducing T_0 = 293 K

$$C_{p,u}(T) = 15.2 + 0.137(T - T_0) - 0.0001795(T^2 - T_0^2) \tag{26}$$

For the same T_0, the average values of a_0 and b_0 in function (10) turned out to be equal to 10.5 kJ K^{-1} mol^{-1} and 0.047 kJ K^{-2} mol^{-1}, respectively. Thus for the estimated $\Delta C_{p,u}(T)$ function (Figure 4) we have

$$\Delta C_{p,u}(T) = (a - a_0) + (b - b_0)(T - T_0) + c\,(T^2 - T_0^2)$$

$$= \Delta a + \Delta b(T - T_0) + c(T^2 - T_0^2) \tag{27}$$

$$= 4.7 + 0.090(T - T_0) - (1.795 \times 10^{-4})(T^2 - T_0^2)$$

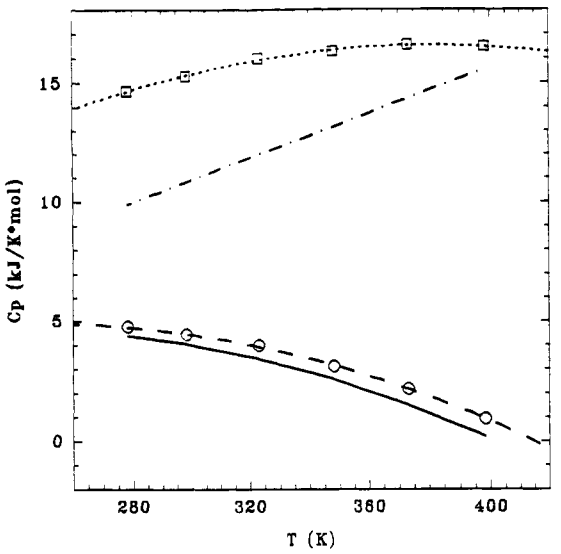

FIGURE 4: Temperature dependencies of the molar heat capacities of SH3. The temperature dependencies of the molar heat capacities of the SH3 in the native state, $C_{p,0}$ (---), were found by global fitting of experimental data, and in the unfolded state, $C_{p,u}$ (□) was calculated according to Privalov et al. (1989), Makhatadze & Privalov (1990). Their difference, $\Delta C_{p,u}^{calc}$, is plotted with (○), while the solid line shows $\Delta C_{p,u}(T)$ found by global fitting of the experimental data.

In some of our direct global fittings only Δa was allowed to vary. In others b_0 and Δb were also allowed to vary, while c was always kept constant. There are two reasons for unfreezing b_0. First, since only small regions of $C_{p,0}$ could be registered in the case of SH3 (Figure 3), the scatter of b_0 around an average value of 0.047 kJ K^{-2} mol^{-1} was rather high, namely, about 12%. Second, at pH below 2.25 (see Figure 3) even at 280 K about 10% of the protein is already in the unfolded state. Of course, in such a case $C_{p,0}$ is unknown to us and both a_0 and b_0 had to be adjusted.

The typical example of the curve fitting is shown in Figure 5 where the best-fit parameters are listed in the figure legend. In addition to Δa, Δb, and b_0, the fitting gave us the values of T_m and $\Delta H_u(T_m)$, which are shown in Table 1 and plotted in Figure 6 together with the polynomial regression through these points.

It is seen that the dependence of $\Delta H_u(T_m)$ on T_m in the experimentally accessible temperature range fits very well to the linear regression line with the slope of 3.4 ± 0.1 kJ K^{-1} mol^{-1}, despite the fact that in reality $\Delta C_{p,u}$ varies between 3.7 kJ K^{-1} mol^{-1} at pH 2.0 (T_m = 307 K) and 2.8 kJ K^{-1} mol^{-1} at pH 4.0 (T_m = 339 K) (Figure 7). For this reason, $\Delta H_m(T_m)$ points fit better to function 21, although the quality of fitting is not drastically increased. The difference between the two regressions becomes remarkable only below and, in particular, above the experimentally attainable range of T_m. A similar paradox was observed by Privalov and co-workers for some other globular proteins (Privalov *et al.*, 1989).

The global fitting with variable Δa and b_0 introduced some corrections into the $C_{p,u}(T)$ function in comparison with the calculated one (see Figure 4). The experimentally observed heat capacity of the unfolded state is on average about 0.7 kJ K^{-1} mol^{-1} lower than the estimated one. This is a very good agreement, taking into consideration the fact that in the case of SH3 the absolute value of the partial heat capacity of the protein was determined with a precision of about 0.8 kJ K^{-1} mol^{-1}.

The thermodynamic parameters derived from the global calorimetric analysis are shown in Table 1.

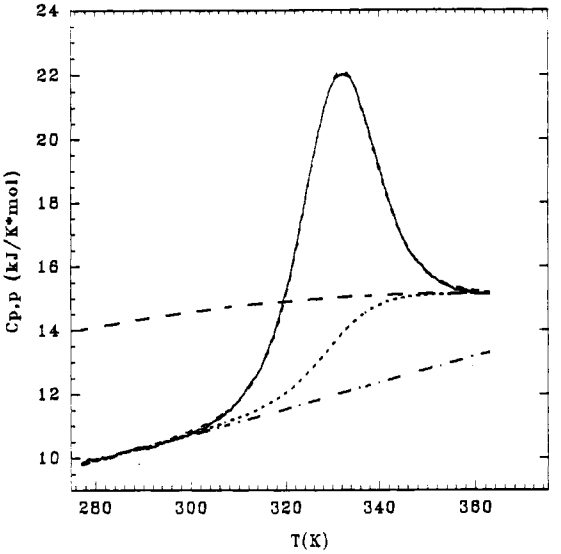

FIGURE 5: Best fit of the experimental temperature dependence of the partial molar heat capacity of SH3 at pH 3.0. The best fit (- - -) of the experimental temperature dependence of the partial molar heat capacity (—) recorded at heating rate 1 K min^{-1} and total protein concentration of 2 mg/mL. The best-fit parameters for this experiment were T_m = 329.7 K; $\Delta H_u(T_m)$ = 176 kJ/mol; $C_{p,0}(T)$ = 10.4 + 0.044(T − 293) (---); $\Delta C_{p,u}(T)$ = −5.95 + 0.087T − (1.795 × 10^{-4})T^2. $C_{p,u} = C_{p,0} + \Delta C_{p,u}$ is shown with (— — —) and δC_p^{int} with (- - -).

Table 1: Thermodynamic[a] Parameters of SH3 Fragment Unfolding at Acidic pH and Low Ionic Strength

pH	T_m (K)	$\Delta H_u(T_m)$ (kJ mol^{-1})	$\Delta G_u(298)$ (kJ mol^{-1})	$\Delta C_{p,u}(T_m)$ (kJ K^{-1} mol^{-1})
2.0	307	93	2.3	3.7
2.25	313	114	4.2	3.6
2.5	320	139	6.9	3.4
2.75	327	162	9.8	3.2
3.0	331	174	11.6	3.0
3.5	336	188	13.9	2.9
4.0	339	197	15.6	2.8

[a] The thermodynamic parameters derived from the calorimeter model are shown. The error is around 8% for $\Delta H_u(T_m)$ and $\Delta C_{p,u}(T_m)$, 20% for ΔG_u, and around 0.7 K for T_m.

Kinetic Analysis of the Folding and Unfolding Reactions of the SH3 Domain. It has been found that in many proteins in which an intermediate in equilibrium conditions is not found, the kinetic analysis of the refolding reaction shows unequivocally the presence of an intermediate (Matouschek *et al.*, 1992). In order to see if we could detect a kinetic intermediate in the refolding of the SH3 domain, we performed a kinetic analysis at pH 3.5. The results of this analysis are shown in Figure 7. As expected for an unfolding reaction in which the strong denaturation conditions preclude the accumulation of an intermediate (Matouschek et al., 1990), there is a linear dependence of the logarithm of the rate constant of unfolding (k_U) with urea. In the refolding reaction two main transitions are observed: a fast transition, completed in less than 5 s, and a slow one with a kinetic constant of 0.0176 ± 0.0023 s^{-1}, which is independent of the urea concentration (data not shown). Contrary to what has been found in many other proteins (Ikeguchi *et al.*, 1986; Kuwajima *et al.*, 1988; Matouschek *et al.*, 1990), the logarithm of the rate constant of refolding (k_F), versus urea, can be fitted to a linear equation. Folding kinetics have also been followed by circular dichroism at 235 nm. Points are totally superimposable with those obtained by fluorescence (Figure 7). This indicates a concomitant recovery of both secondary and tertiary structure

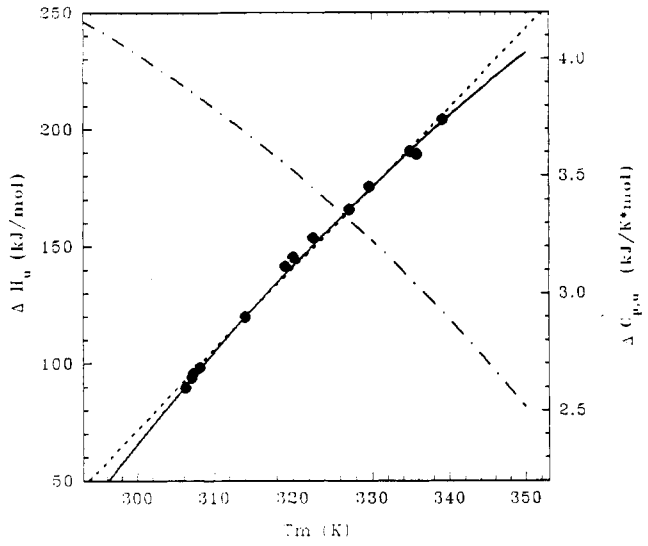

FIGURE 6: Plot of $\Delta H_u(T_m)$ versus T_m. The plot of $\Delta H_u(T_m)$ *versus* T_m as found from the global fitting of the DSC curves for SH3 at acidic pH (●, left axis) is shown. The solid line shows the parabolic function $\Delta H_u(T)$ corresponding to $\Delta C_{p,u} = -5.95 + 0.087T - (1.795 \times 10^{-4})T^2$ (---, right axis), while (- - -) corresponds to the linear regression through the points with the slope of 3.4 kJ K^{-1} mol^{-1}.

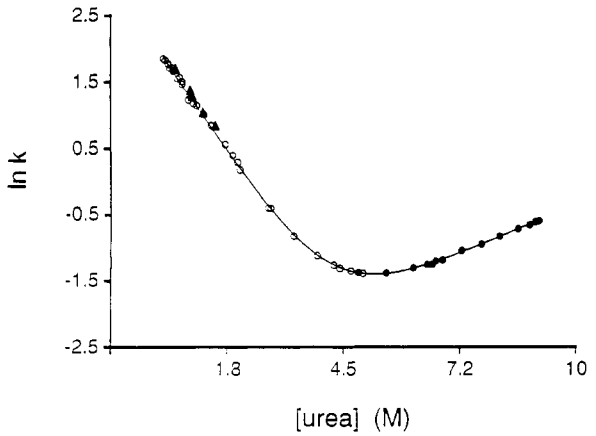

FIGURE 7: Kinetics of unfolding and refolding. Urea concentration dependence of the natural logarithm of the rate constants for refolding and unfolding is shown. The unfolding or refolding reactions were initiated by an urea concentration jump in a stopped-flow machine equilibrated at 298 K. The buffer used for fluorescence measurements was 50 mM citric acid, or 5 mM in the case of CD measurements, at pH 3.5. (○) Refolding experiments followed by fluorescence. (▲) Refolding experiments followed by CD. In this case it was not possible to do the experiments at high urea concentrations due to signal to noise ratio problems. (●) Unfolding experiments followed by fluorescence. The solid curve is the best fit of the whole set of data to eq 28.

upon diluting into refolding buffer. Values within the transition region obtained by either unfolding or refolding experiments were indistinguishable. The whole reaction can be fitted to the following equation (Jackson & Fersht, 1991):

$$\ln k = \ln [k_{f,H_2O} \exp(-m_{kf}[\text{urea}]) + k_{u,H_2O} \exp(-m_{ku}[\text{urea}])] \quad (28)$$

where k is the rate constant at a given concentration of denaturant, k_{f,H_2O} is the rate constant of refolding in water and k_{u,H_2O} is the rate constant of unfolding in water, and m_{kf} and m_{ku} are the slopes of the refolding and unfolding reactions, respectively. The kinetic parameters calculated from this equation are as follows: $k_{f,H_2O} = 8.14 \pm 0.08$ s^{-1}, $k_{u,H_2O} = 0.045 \pm 0.002$ s^{-1}, $m_{kf} = 4 \pm 0.03$ kJ mol^{-1} M^{-1}, and $m_{ku} = -1.14 \pm 0.03$ kJ mol^{-1} M^{-1}.

The spectrin SH3 domain has two proline residues in a *trans* conformation, in the native state. The relative amount of *cis* and *trans* conformation in the unfolded state could, in principle, be calculated by the relative amplitudes of the fast and slow kinetic phases of refolding, as long as the isomerization takes place in the unfolded state. For spectrin SH3 the slow phase of refolding accounts for 4% ± 1% of the total amplitude of fluorescence recovery. This number is small compared with other proteins of similar size with the same number of *trans* prolines (Jackson & Fersht, 1991) and could indicate that the isomerization of at least one of the prolines takes place in a folded conformation.

The values of the free energy of unfolding ΔG_{H_2O} and the constant m, which reflects the cooperativity of the transition, can be estimated from the above parameters, considering the following equilibrium:

$$\text{Uc} \overset{K_{iso}}{\Leftrightarrow} \text{Ut} \underset{k_{u,H_2O}}{\overset{k_{f,H_2O}}{\Leftrightarrow}} \text{N} \quad (29)$$

K_{iso} is defined as the ratio between the amount of protein in the *cis* and *trans* conformation in the unfolded state. When the native conformation of the proline residues is a *trans* conformation, then the contribution of the *cis–trans* isomerization to the free energy of unfolding is negligible (Jackson & Fersht, 1991) and we can obtain a reasonable estimation of ΔG_{H_2O} from the second equilibrium using

$$\Delta G_{H_2O} = -RT \ln (k_{f,H_2O}/k_{u,H_2O}) \quad (30)$$

$$m = m_{kf} + m_{ku} \quad (31)$$

then $\Delta G_{H_2O} = 12.9$ kJ mol^{-1} and $m = 2.9 \pm 0.06$ kJ mol^{-1} M^{-1}.

DISCUSSION

Criteria for a Two-State Transition. For the equilibrium denaturation of a protein to be described as a two-state process, several criteria must be fulfilled by the protein: (i) the unfolding of the protein by a denaturant agent must fit to a single transition curve and must be independent of the probe used to monitor it, (ii) the DSC unfolding endotherm must fit the $C_p(T)$ function corresponding to the two-state model, and (iii) the fitting of the logarithm of the rate constant of unfolding and refolding, with urea must be linear and the values for ΔG_{H_2O} and m obtained from equilibrium and kinetics must be identical within experimental error. This third criterion is in many cases the most sensitive of the three criteria for the detection of transient intermediates (Jackson & Fersht, 1991).

The equilibrium denaturation of SH3, by urea at pH 3.5, fits a single transition curve and within experimental error seems to be independent of the probe used to monitor it (fluorescence of CD). Differential scanning calorimetry analysis of the protein at different pHs, although somehow complicated because of the dependency of $\Delta C_{p,u}$ on temperature, indicates that the denaturation of this domain follows a two-state transition. The ratio between the van't Hoff's to calorimetric enthalpy is 1.03 ± 0.08, which is within the range of 1.00 ± 0.05 observed for the two-state denaturation of several globular proteins (Privalov, 1979). Kinetic analysis of the folding and unfolding reactions, followed by CD or fluorescence, clearly indicates that there is no intermediate accumulating at low urea concentrations. Also, the values for ΔG_{H_2O} and m obtained from equilibrium chemical denaturation, thermal denaturation, calorimetry, and kinetics

Table 2: Comparison of the Thermodynamic Unfolding Values of SH3 Obtained from Chemical and Temperature Denaturation, Differential Scanning Calorimetry, and Kinetics at pH 3.5 and 298 K

method	ΔG_{H_2O} (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	T_m (K)	$\Delta H_u(T_m)$ (kJ mol^{-1})
DSC[a]	14 ± 3.0		336 ± 0.8	188 ± 15
temp[b]	13 ± 3.0		333 ± 6	196 ± 3
fluorescence[c]	12 ± 0.2	3.1 ± 0.03		
CD[d]	12 ± 0.1	3.2 ± 0.06		
kinetics[e]	13 ± 0.7	2.9 ± 0.06		

[a] Differential scanning calorimetry. [b] Temperature denaturation followed by fluorescence. [c] Urea chemical denaturation followed by fluorescence. [d] Urea chemical denaturation followed by circular dichroism. [e] Kinetics of urea-induced unfolding and refolding.

are identical within experimental error (Table 2). Therefore, from the equilibrium and kinetic points of view the two-state model applies for the SH3 folding–unfolding reaction. Intermediates of folding in this case should be transient ones, whose energy is indistinguishable from the transition state.

SH3 Stability. The thermodynamic parameters obtained from the equilibrium, kinetic, and calorimetric analysis indicate that the free energy of unfolding of SH3 at pH 3.5 and 298 K must be around 13 kJ mol^{-1} with a value of m around 3.1 kJ mol^{-1}. The stability of the protein at this pH is rather low compared with the majority of the globular proteins. However, we must take into account that this is not the optimal pH for maximum stability of this domain. This optimal pH, which lies between pH 5.0 and 8.0, cannot be used for calorimetry analysis due to the low solubility that this protein has above pH 4.0 at concentrations higher than 1 mg/mL (Musacchio *et al.*, 1992b). Analysis of the stability of this domain between pH 5.0 and 7.0, by Gdn·HCl denaturation, indicates that the stability of the protein is around 15.5 ± 1.3 kJ mol^{-1} with a m value of 7.82 ± 0.5 kJ mol^{-1} M^{-1}. This value for ΔG at pH 7.0, which could be lower than the real value by 10–15% due to the different ways in which urea and Gdn·HCl interact with the protein (Makhatadze & Privalov, 1992), is relatively low for a monomeric globular protein. It has been found for other small monomeric proteins of similar size that the free energy of unfolding at 298 K is around 25–29 kJ mol^{-1} (Jackson & Fersht, 1991; Alexander *et al.*, 1992a). On the other hand this small value is not surprising, taking into account the fact that SH3 is a protein domain and not a monomeric protein. Consequently, it is reasonable to think that interactions with other domains of the protein will stabilize it. The m value is proportional to the average exposure of residues on denaturation. This value is around 10.9–18.8 kJ mol^{-1} M^{-1} for monomeric proteins with 100–150 amino acids, denatured by Gdn·HCl (Pace *et al.*, 1974; Greene & Pace, 1974; Kellis *et al.*, 1989). On the other hand, our m value is quite similar to the value found for the chymotrypsin inhibitor CI2 (7.8 kJ mol^{-1} M^{-1}), which has structured core of 60 amino acids (Jackson & Fersht, 1991).

From the calorimetry point of view, the SH3 domain seems to be an ordinary representative of the small, single-domain globular proteins despite its small molecular weight. Thus the specific heat capacity of the native state at 293 K, 1.47 ± 0.13 J K^{-1} g^{-1}, its temperature dependence, (6.7 ± 1.7) × 10^{-3} J K^{-2} g^{-1}, the specific denaturation enthalpy at the corresponding T_m (e.g., 26.3 ± 2 J g^{-1} at 336 K), and the average specific heat capacity change on denaturation (0.47 ± 0.04 J K^{-1} g^{-1}) all compare very well with what has been described for compact globular proteins (Privalov, 1979; Privalov & Khechinashvili, 1974). In addition, the ΔH_u (mol of amino acid residue)$^{-1}$ reaches a value of 5.2 ± 0.4 kJ (mol of amino acid)$^{-1}$ when $\Delta C_{p,u}$ equals zero, which is close to the values recently found for the ΔH of unfolding of globular proteins at high temperature (Privalov *et al.*, 1989; Privalov & Makhatadze, 1990). For the members of this protein family there is a correlation between $\Delta H_u(298)$ and $\Delta C_{p,u}(298)$, as was pointed out by Murphy *et al.* (1992) (it should be mentioned, however, that these authors used the average values of $\Delta C_{p,u}$ for all proteins). The values found by us for SH3 [$\Delta H_u(298)$ = 917 J (mol of residue)$^{-1}$ and $\Delta C_{p,u}(298)$ = 62 J K^{-1} (mol of amino acid)$^{-1}$] fit rather well to that correlation if the actual value of $\Delta C_{p,u}(298)$ = 3.9 kJ K^{-1} mol^{-1} is taken instead of the average value of 3.4 kJ K^{-1} mol^{-1} (data not shown).

SH3 Folding. As discussed above, the kinetic analysis of protein folding seems to be very sensitive to the presence of kinetic intermediates. In the majority of monomeric domain proteins in which this analysis has been carried out, a significant deviation of linearity has been found which is attributed to the accumulation of a folding intermediate (Ikeguchi *et al.*, 1986; Kuwajima *et al.*, 1988; Matouschek et al., 1990; Schreiber & Fersht, 1993). At least in the last two cases, this intermediate is not due to any *cis–trans* prolyl isomerization. However, in two small monomeric proteins of similar size to the SH3 domain, the CI2 chymotrypsin inhibitor (Jackson & Fersht, 1991) and the G-domain (Alexander *et al.*, 1992b), no intermediate was found to accumulate in the refolding reaction. In the SH3 domain we find the same phenomena for those molecules which in the unfolded state contained the two prolines in the native conformation. It is possible that the slow phase we detected could be due to the isomerization of any of the two prolines in a folded nonnative structure. This will count, in a strict sense, as a folding intermediate. Although the *cis–trans* intermediates are well-known in the literature, the intermediates we are seeking are those accumulating in the folding reactions of unfolded proteins with all the prolines in the native conformation (Matouschek *et al.*, 1989; Schreiber & Fersht, 1993). Our data clearly demonstrate that these intermediates do not accumulate significantly in the refolding reaction of SH3.

Kinetic analysis using quench-flow of a large β-sheet barrel protein, interleukin 1β, has revealed the presence of a kinetic intermediate. It has been suggested, on the basis of this study, that the formation of the β-sheet in β-sheet proteins follows a different folding pathway compared to the formation of the β-sheet in α/β mixed proteins (Varley *et al.*, 1993). In this study it was found that folding to the native structure involves the rapid formation of a β-structure around a hydrophobic core, followed by a slow stabilization of the secondary structure. However, the kinetic analysis by CD and fluorescence of the SH3 domain indicates that this is not the case for all β-sheet proteins.

It is now generally believed that there must be kinetic intermediates in the folding pathway of proteins [for a review see Kim and Baldwin (1990)]. In the case of the CI2 protein, the G-domain, and the SH3 domain, no intermediate can be seen to accumulate. These proteins have in common that they have a very small core within the limits of what has been postulated to be a stable folding domain without disulfide bridges (Privalov & Gill, 1988). On the other hand, their fold is very different. In the case of CI2 (McPhalen & James, 1987) and the G-domain (Lyan *et al.*, 1991; Gornenborn *et al.*, 1991; Orban *et al.*, 1992), there is an α-helix packing against a β-sheet, while in the SH3 domain there is only a β-sheet barrel (Musacchio *et al.*, 1992b). It is puzzling that the only three proteins for which a kinetic intermediate has

not been found to accumulate correspond to proteins which have a globular unit of around 60 residues. Although more proteins of this size should be studied, it is tempting to speculate that only in those proteins for which the size imposes the existence of one or more large hydrophobic cores, or two or more subdomains, will there be kinetic intermediates that will accumulate in the folding pathway, whereas in one-domain proteins with a single small hydrophobic core, the acquisition of secondary structure will be simultaneous with that of the tertiary structure.

CONCLUSIONS

In summary, SH3 represents a very good model for protein folding due to the simplicity of its mechanism, following a pure two-state transition. In addition, the coincidence of Gibbs energy of unfolding from all the methods used, urea and calorimetry, suggest that the final denatured state reached by both methods is energetically the same, and so it is expected to be a fully denatured state. The differential scanning calorimetry of the spectra SH3 domain requires $\Delta C_{p,u}$ to be temperature-dependent in order to carry out an appropriate calorimetric data analysis. This agrees with earlier findings and shows that only under certain conditions would the use of a constant $\Delta C_{p,u}$ lead to the same results within experimental uncertainty.

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