Differential Scanning Calorimetric Study of the Thermal Unfolding of β-Lactamase I from *Bacillus cereus*†

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ABSTRACT: The irreversible thermal unfolding of the class A β-lactamase I from *Bacillus cereus* has been investigated at pH 7.0, using differential scanning calorimetry (DSC) and inactivation kinetic techniques. DSC transitions showed a single peak with a denaturation enthalpy of 646 kJ·mol^{-1} and were moderately scan rate dependent, suggesting that the process was partially kinetically controlled. The inactivation kinetics at constant temperature showed that the irreversible denaturation of the enzyme occurs as the sum of two exponential terms whose amplitudes are strongly temperature dependent within the transition range so that, at the lowest temperatures within this interval, irreversible inactivation would proceed mainly through the slow phase. The fraction of irreversibly denatured enzyme (D) as a function of temperature for a given scanning rate was calculated by numerical integration of the kinetic equation with temperature, using previously determined kinetic parameters. This D form was the most populated of the unfolded states only at temperatures well above the maximum in the calorimetric transition. Combination of the results of kinetic and DSC experiments has allowed us to separate the contribution of the final D state to the excess enthalpy change from the contribution arising from the reversibly denatured forms of the enzyme (I_i, $i = 1, ..., n$), with the resulting conclusion that the scan rate dependence of the calorimetric traces was the result of two different dynamic effects, viz., the irreversible step and a slow relaxation process during formation of the reversibly denatured intermediate states. Finally, the problems of using results obtained at a single scan rate to validate the two-state kinetic model are commented on.

Penicillin resistance in bacteria is mainly due to the action of β-lactamases (EC 3.5.2.6), which protect these microorganisms against the lethal effects of β-lactam antibiotics on cell wall synthesis. These enzymes hydrolyze the β-lactam ring of the penicillin or cephalosporin molecule, and as a result, the compounds lose their antibiotic activity. Despite their common function, β-lactamases form a diverse family depending on their substrate preference. They have been divided into three classes, viz., A, B, and C, on the basis of their amino acid sequence (Ambler, 1980). Class B β-lactamases are metalloenzymes (Davies & Abraham, 1974) and are clearly distinct from the members of classes A and C, which contain an active-site serine residue involved in the formation of an acylenzyme with β-lactam substrates during catalysis (Knott-Hunziker et al., 1979; Joris et al., 1984). There is poor sequence similarity between the A and C classes of β-lactamases; however, class A enzymes share common structural features with both class C and the DD-carboxypeptidases, suggesting that classes A and C of β-lactamases and β-lactam target enzymes may be evolutionarily related (Pollock, 1971; Kelly et al., 1986; Samraoui et al., 1986).

β-Lactamase I from *Bacillus cereus*, a member of class A β-lactamases, consists of a single polypeptide chain of 270 amino acid residues and has a molecular mass of 28 000 daltons (Thatcher, 1975; Lloyds & Peacocke, 1970). The spatial arrangement of the secondary structural elements may be described as a five-stranded β-pleated sheet with a group of three α-helices on one side and five helices on the other. At the present stage of resolution, the molecule does not appear to consist of two separate domains as is the case for other class A or class C β-lactamases (Dideberg et al., 1987; Herzberg & Moult, 1987; Oefner et al., 1990), but regions of different structural types, both α and α/β, are present (Samraoui et al., 1986) at whose interface is located the active site.

Differential scanning calorimetry can be a useful tool in the study of protein structure since it allows the recognition of individual domains as they undergo thermal denaturation and permits the direct calculation of all the thermodynamic functions of unfolding (Privalov, 1979). Although thermodynamic equilibrium of the sample is a prerequisite for calculation of the thermodynamic functions of the process, it has been shown that the DSC thermograms for the thermal denaturation of various proteins can still be interpreted in terms of the equilibrium relationships, despite the fact of calorimetric irreversibility (Edge et al., 1985; Sturtevant, 1987). However, an increasing number of proteins whose denaturation thermograms are kinetically controlled and, therefore, scan rate dependent have appeared in recent years (Sánchez-Ruiz et al., 1988a,b; Gûzman-Casado et al., 1990; Galisteo et al., 1991), and models including an irreversible step have been proposed to describe their thermal denaturation as well as the influence of ligand binding on stability (Sanchez-Ruiz et al., 1988a; Freire et al., 1990; Conejero-Lara et al., 1991).

In the present paper, we have studied the irreversible unfolding of β-lactamase I by differential scanning calorimetry and inactivation kinetics. The scanning rate effect on the DSC transitions was examined in order to evaluate to what extent the heat absorption profile is determined by the rate of formation of the final irreversibly denatured state. The analysis of the experimental results showed that the scanning rate dependence of the excess heat capacity curves resulted from the combination of two dynamic effects involving the irre-

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versible step and a slow equilibrium between native and intermediate reversibly denatured states.

Materials and Methods

β-Lactamase I from *Bacillus cereus* was obtained from the Centre for Applied Microbiology and Research, Porton Down, England, and was used as supplied. Enzyme solutions were prepared by exhaustive dialysis with 60 mM phosphate buffer, pH 7. Enzyme concentrations were measured spectrophotometrically using the reported absorption coefficient of $\epsilon_{280}^{0.1\%} = 1.07$ (Cartwright & Waley, 1987). All the absorbance measurements were carried out in a Cary 210 spectrophotometer at 25 °C. The purity of β-lactamase I was checked by SDS–polyacrylamide electrophoresis (Laemmli, 1970). Nitrocefin was obtained from Glaxo (Greenford, U.K.), and all other chemicals were of reagent grade.

The enzyme activity was assayed spectrophotometrically by following the hydrolysis of nitrocefin (O'Callaghan et al., 1972) and had a specific activity of 42.9 μmol·mg^{-1}·min^{-1} at 25 °C. The kinetics of irreversible thermal inactivation of β-lactamase I were followed using the following procedure: 5 μL of a enzyme solution was introduced into previously thermostated tubes immersed in a water bath at the desired temperature. At given times, the tubes were removed from the bath and immediately chilled in a water–ice mixture to stop the inactivation process. The remaining activities were assayed at 25 °C, after appropriate dilution of the sample. Control experiments were run in parallel at 4 °C.

Calorimetric measurements were carried out in a Microcal MC-2 ultrasensitive DSC (MicroCal Inc., Northampton, MA) at scan rates varying from 10 to 60 °C/h and under an extra constant pressure of 2 atm. The standard DA-2, Cpcalc, and Dynacp software packages were used for data acquisition and analysis. The experimental calorimetric traces were corrected for the effect of the instrument response time using the procedure described elsewhere (López-Mayorga & Freire, 1987). The reversibility of the thermally induced transition was checked by reheating the solution in the calorimeter cell immediately after cooling from the first run. The kinetic analysis of the DSC curves was carried out according to the method outlined by Galisteo et al. (1991). The relaxation time of the process involving equilibrium states was determined as a function of temperature from the scanning rate dependence of the excess enthalpy function of the reversibly unfolded states as described by Freire et al. (1990). All protein and buffer solutions were degassed for about 1 min under vacuum (with gentle stirring) before being loaded into the calorimeter. The enzyme concentrations in the solution for the calorimetric experiments were in the range 1.4–3.0 mg·mL^{-1}.

Results

Figure 1 shows an original DSC recording for the thermal denaturation of β-lactamase I at a scan rate of 20 °C/h. The thermogram shows a single peak with a transition temperature, T_m, corresponding to the maximum heat capacity of 51.03 °C and a denaturation enthalpy, ΔH, of 646 kJ mol^{-1}, somewhat higher than that found by Arnold and Viswanatha (1983). As previously reported by these authors, the thermal denaturation of β-lactamase I was irreversible, and no discernible transition was observed in a second run of the samples. As can be seen in Figure 2, T_m values are scan rate dependent, even after correction for the effect of the instrumental response. Denaturation of β-lactamase I cannot be described solely in terms of the two-state kinetic model. However, deviations from the behavior predicted by this model were not immediately evident using only data at a single scan rate. The dependence of the

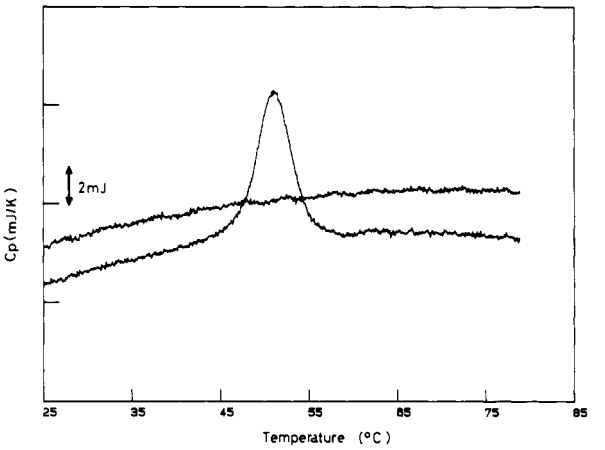

FIGURE 1: Excess heat capacity versus temperature profile of β-lactamase I in phosphate buffer, pH 7.0 at 20 °C/h. The protein concentration was 1.4 mg/mL. The buffer–buffer base line is also shown.

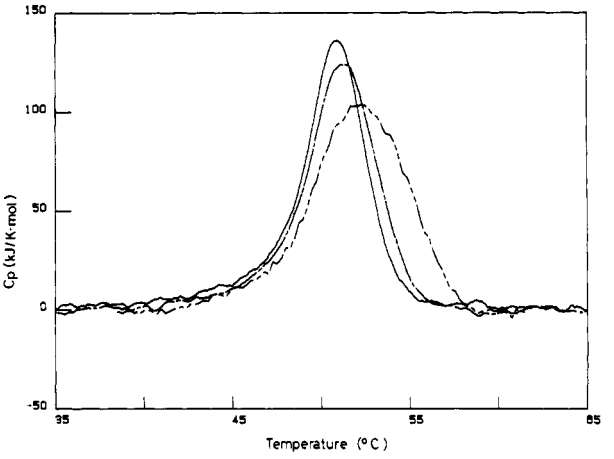

FIGURE 2: DSC traces of β-lactamase I at different scanning rates: 12 °C/h (solid line); 20 °C/h (dotted line); and 60 °C/h (double-dotted line).

excess heat capacity curves on temperature was analyzed using eq 1–3 where k is the rate constant, v the scan rate, Q_t the

$$k = vC_p/(Q_t - Q) \quad (1)$$

$$\ln\left(\ln\frac{Q_t}{Q_t - Q}\right) = \frac{E}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right) \quad (2)$$

$$v/T_m^2 = \frac{AE}{R}\exp(-E/RT_m) \quad (3)$$

total heat of the calorimetric transition, Q the heat evolved at a given temperature, C_p the excess heat capacity at this temperature, E the activation energy, and A the frequency factor of the Arrhenius equation.

Figure 3A,B shows the plots of ln k and ln {ln $[Q_t/(Q_t - Q)]$} versus $1/T$. As could be expected if thermal unfolding followed the two-state kinetic model, linear relationships can be established for both the Arrhenius plot and eq 2, and the linear regression lines yielded correlation coefficients of 0.99–0.97. However, the values of the rate constants obtained from data at different scan rates were clearly different, particularly at high temperatures, and the values of T_m, calculated from the y-axis intercept in eq 2, deviated from those obtained from the calorimetric traces by as much as one Celsius degree.

The shifts found in T_m when passing from the faster scan rates to scan rates of 20 and 12 °C/h are well below those expected from eq 3 (see Figure 3C). This deviation in the

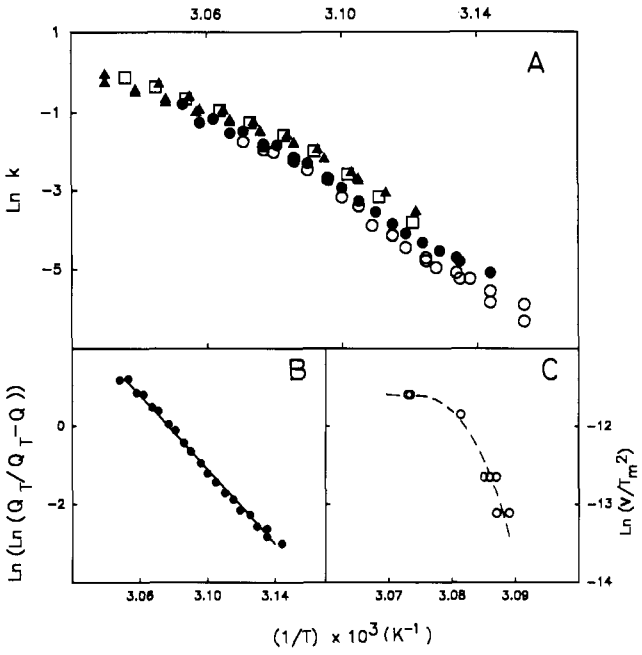

FIGURE 3: (A) Arrhenius plot of k data (min^{-1}) assuming the two-state kinetic model. Scanning rates: (▲) 60 °C/h; (□) 45 °C/h; (●) 20 °C/h; (○) 12 °C/h. (B) Plot of the heat evolved according to eq 2 at a scanning rate of 20 °C. The solid line is the least-squares fit of the data. (C) Plot of ln (v/T_m^2) versus $1/T_m$ (v in degrees centigrade per minute).

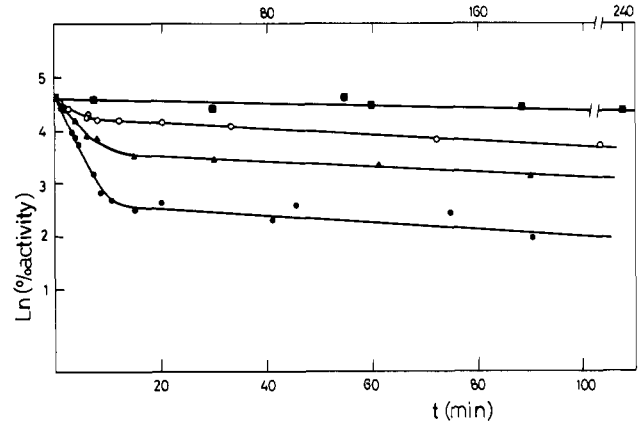

FIGURE 4: Kinetics of irreversible inactivation of β-lactamase I at 1.6 mg/mL in 60 mM phosphate buffer, pH 7.0. Semilogarithmic plot of the percentage of the recovered activity ($1 - P_D$) as a function of the incubation time at 44.8 (■) (upper scale), 51 (○), 53 (▲), and 55.6 °C (●). Solid lines are the curves calculated from eq 5 using α_i and λ_i values shown in Figure 5.

scanning rate dependence of T_m, from the behavior predicted by the two-state kinetic model, indicated the presence of other than native and irreversible denatured states during thermal denaturation of β-lactamase I.

The kinetics of thermal inactivation of β-lactamase I were investigated following the time course of irreversible activity loss, at several temperatures within the range 43–56 °C and at the same protein concentrations used in the calorimetric experiments (see Materials and Methods). A semilogarithmic plot of activity as a function of time at four temperatures is shown in Figure 4. The inactivation profiles can be described as a sum of two exponential terms, for which the observed rate constants were designated λ_1, for the fast phase, and λ_2, for the slow phase. The slow phase constitutes the main component of the inactivation curves at temperatures below T_m values while above 54 °C the fast phase accounts for most of the irreversible inactivation. The Arrhenius plots of λ_1 and

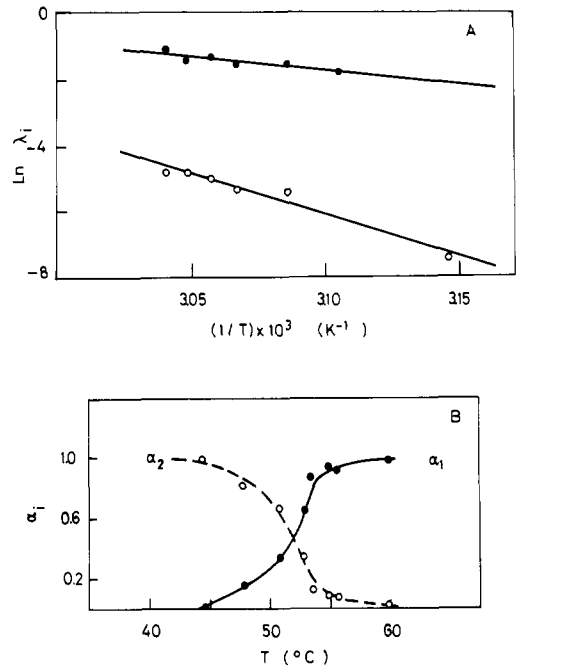

FIGURE 5: (A) Arrhenius plot of the rate constants for the fast (●) and the slow (○) phases of irreversible inactivation of β-lactamase I. (B) Temperature dependence of the amplitudes of the fast, α_1, and slow, α_2, phases.

λ_2 and the dependence of the amplitudes of the phases, α_1 and α_2, with temperature are shown in Figure 5.

The enzyme concentration, [E], can be expressed as

$$[E] = [D] + \sum_{i=0}^{n}[I_i] \quad (4)$$

where [D] is the concentration of the irreversibly denatured enzyme and $[I_i]$ is the protein concentration in state I_i, I_0 being the native state. The time course of the fraction of active enzyme measured experimentally (see Materials and Methods), $\sum_{i=0}^{n}P_i$, can be described by

$$\sum_{i=0}^{n}P_i = \alpha_1 \exp(-\lambda_1 t) + \alpha_2 \exp(-\lambda_2 t) \quad (5)$$

Now, as the scanning rate, v, is the time derivative of temperature (dT/dt), the temperature derivative of the left-hand side of eq 5 can be expressed as

$$d(\sum_{i=0}^{n}P_i)/dT = -(1/v)[\alpha_1\lambda_1 \exp(-\lambda_1 t) + \alpha_2\lambda_2 \exp(-\lambda_2 t)] \quad (6)$$

Thus, integration of eq 6 from a temperature at which the inactivation rate is negligible ($P_D = 0$) to a temperature T will allow the determination of the fraction P_D at any temperature and scan rate. Curves of P_D obtained by numerical integration of eq 6 using the Kutta—Simpson algorithm are shown in Figure 6 for two different scanning rates.

The excess enthalpy is given by

$$\langle \Delta H \rangle = P_D \Delta H + \sum_{i=0}^{n}P_i \Delta H_i \quad (7)$$

where ΔH_i is the enthalpy difference between the state I_i and the reference state I_0 (Freire et al., 1990). The enthalpy for state D was assumed to be equal to the measured transition enthalpy, ΔH, since at a scanning rate of 12 °C/h the enzyme was fully irreversibly denatured at temperatures at which the transition was completed (above 58.5 °C). The irreversible

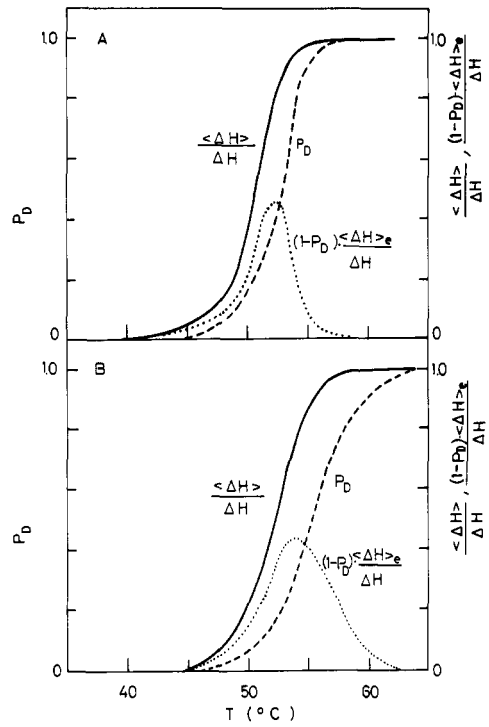

FIGURE 6: Fraction of irreversibly denatured enzyme as a function of temperature for thermal denaturation of β-lactamase I (---) at two different scanning rates: (A) 12 and (B) 60 °C/h. The fractions of the experimental excess enthalpy (solid line) and that contributed by the reversibly denatured protein (dotted line) versus temperature profiles, at the same scanning rates, are also shown.

step itself would probably not be associated with a significant heat effect since the value of the denaturation enthalpy was scan rate independent, despite the fact that the P_D values reached immediately after completion of the transition slightly depend on the scanning rate (see Figure 6). The fraction of heat absorbed, $\langle\Delta H\rangle/\Delta H$, has been also represented in Figure 6 as a function of temperature. The values of $\langle\Delta H\rangle/\Delta H$ are, in general, well above those of P_D, so it can be concluded that reversible and irreversible denatured forms of β-lactamase I coexist within the temperature range of the transition and that only at those temperatures where the transition is almost completed does the final irreversible state account for most of the excess enthalpy.

Equation 7 can be transformed into eq 8 where the excess enthalpy $\langle\Delta H\rangle_e$ is defined as $\sum_{i=0}^{n} y_i\Delta H_i$ and y_i is second molar

$$\langle\Delta H\rangle = P_D\Delta H + (1 - P_D)\langle\Delta H\rangle_e \qquad (8)$$

fraction for only those states which are reversibly interconverted ($y_i = [I_i]/\sum_{i=0}^{n}[I_i]$). $\langle\Delta H\rangle_e$ is an average over the states I_i only and contains the thermodynamic information associated with the reversible unfolding steps of the transition (Freire et al., 1990). The fraction of the excess enthalpy from reversibly denatured forms at scanning rates of 12 and 60 °C has also been represented in Figure 6 as a function of temperature. Equation 8 was used to calculate the $\langle\Delta H\rangle_e$ function using the P_D values determined above, and the computed curves are represented in Figure 7. As can be seen in this figure, the $\langle\Delta H\rangle_e$ functions were also scan rate dependent, and there was a good relationship between the experimental T_m value found at a given scan rate and the temperature at which the corresponding $C_{p,e}$ function shows a maximum (data not shown). These results suggest that a slow relaxation process between I_i forms could also be responsible, in combination with the irreversible step, for the observed dependence of the calorimetric traces of β-lactamase I on the scanning rate. To

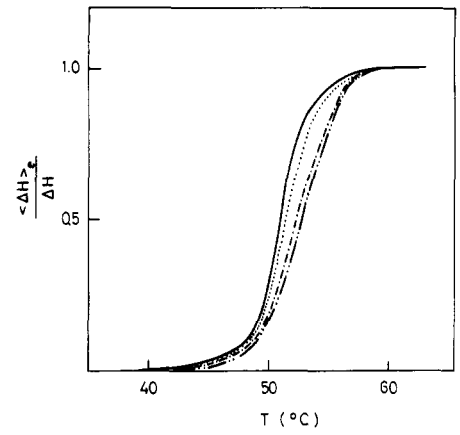

FIGURE 7: Excess enthalpy functions for the reversibly unfolded states of β-lactamase I calculated from data at different scanning rates: 60 °C/h (-·-); 45 °C/h (—); 20 °C/h (···); and 12 °C/h (—). The values have been normalized with respect to ΔH.

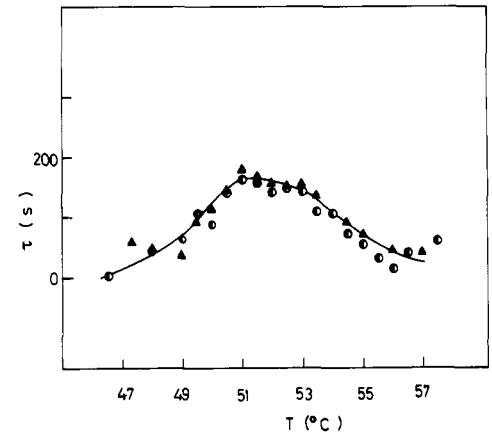

FIGURE 8: Transition relaxation time vs temperature for reversible thermal unfolding of β-lactamase I obtained using $\langle\Delta H\rangle_e$ data at 20 (◐) and 12 °C/h (▲). In both cases, the reference scanning rate was 60 °C/h.

check this hypothesis, $\langle\Delta H\rangle_e$ functions obtained from different scanning rate measurements were analyzed using eq 9, which

$$\tau = -[\mathrm{d}\ln(\langle\Delta H\rangle_{e,v_2} - \langle\Delta H\rangle_{e,v_1})/\mathrm{d}t]^{-1} \qquad (9)$$

described the relationship between the excess enthalpy values obtained at two scanning rates for slow equilibrium transitions (Freire et al., 1990), where τ is the relaxation time of the $\langle\Delta H\rangle_e$ function and $\langle\Delta H\rangle_{e,v_i}$ is the excess enthalpy value obtained at a scanning rate v_i. Figure 8 shows the results obtained by dynamic analysis of $\langle\Delta H\rangle_e$ using data at 12 and 20 °C/h; in both cases, the reference scanning rate, v_1, was 60 °C/h. As seen in Figure 8, the observed differences of $\langle\Delta H\rangle_e$ may be explained in terms of a slow relaxation time, strongly dependent on temperature, with a maximum value of about 170 s around the transition temperature.

Discussion

The results obtained in this work show that thermal inactivation of β-lactamase I from *Bacillus cereus* is a complex process and that both reversibly and irreversibly denatured states of the protein may be present within the temperature range of the transition. This situation can be depicted using the scheme:

$$I_0 \leftrightarrow I_1 \longleftarrow\cdots\longrightarrow I_n \rightarrow D$$

Formation of the final irreversibly denatured D form of the enzyme is particularly significant at high temperatures and low scanning rates. Combination of calorimetric and kinetic

methods lead to the conclusion that the influence of the scanning rate on the DSC transition reflects two different dynamic effects, viz., the irreversible step leading to the final D state and a slow relaxation process during the formation of the reversibly denatured intermediate states ($I_0 \longleftarrow\cdots\longrightarrow I_n$). The slowness of this process could result in the appearance of the two phases observed for irreversible activity loss. Studies by Goto and Fink (1989) have shown the capability of β-lactamase I to adopt different conformational states depending on the denaturation conditions. Thus, at low ionic strength and extreme pHs, it is close to the fully unfolded state, but the presence of increasing salt concentrations stabilizes a compact state with the properties of a molten globule. Transitions between the different states were reversible, although complicated by the tendency of the molten globule found at acidic pH to undergo irreversible denaturation.

The difference in the $\epsilon_{280}^{0.1\%}$ value employed in this work to determine β-lactamase I concentration and that used by Arnold and Viswanatha (1983) would account for the difference found between the present value of the enthalpy of denaturation and that previously reported. On the basis of DSC data collected at a single scan rate of 150 °C/h, analyzed using the model derived by Fujita et al. (1979), Arnold and Viswanatha (1983) reported that thermal denaturation of this enzyme could be described as an irreversible two-state process. These authors took as further proof of the validity of the model the fact that thermal inactivation of the enzyme at 54.9 °C was a first-order kinetic process, although deviations were noted after 80% inactivation was achieved. At this temperature, the present results show that about 90% activity is lost through the fast phase, for which the kinetic rate constant was of the same order as that previously reported by these authors. Furthermore, as has been shown in the present work, linearity of DSC data represented according to eq 2 (the equivalent to that employed by Arnold and Viswanatha) is not sufficient evidence that the transition follows the two-state kinetic model, and in this particular case, it could well result from the combination of the two kinetic effects characterizing the thermal unfolding of β-lactamase I. On this point of general interest, comparison of data obtained under several scan rate conditions seems to be essential in order to validate a given kinetic model to describe the influence of an irreversible process during the unfolding of a protein. In particular, the accordance of the observed scanning rate dependence of T_m with the behavior predicted by eq 3 seems to be the most unequivocal test for the two-state kinetic model.

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