Effect of irreversibility on the thermodynamic characterization of the thermal denaturation of *Aspergillus saitoi* acid proteinase

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The thermal denaturation of the acid proteinase from *Aspergillus saitoi* was studied by CD and differential scanning calorimetry (DSC). This process seemed to be completely irreversible, as protein samples that were heated to temperatures at which the transition had been completed and then cooled at 25 °C did not show any reversal of the change in the CD signal. Similar results were obtained with DSC. Nevertheless, we were able to detect the presence of reversibly unfolded species in experiments in which the enzyme solution was heated to a temperature within the transition region, followed by rapid cooling at 25 °C. Accordingly, the denaturation behaviour of the acid proteinase seems to be consistent with the existence of one (or more) reversible unfolding transition followed by an irreversible step. The van’t Hoff enthalpy, \( \Delta H_{\text{NH}} \), which corresponds to the reversible transition was calculated from extrapolation to infinite heating rate as 310 kJ·mol\(^{-1}\). This parameter was also determined from direct estimation of the equilibrium constant at several temperatures (\( \Delta H_{\text{NH}} = 176 \text{ kJ·mol}^{-1} \)). Comparison of the average \( \Delta H_{\text{NH}} \) with the calorimetric enthalpy (\( \Delta H_{\text{cal}} = 770 \text{ kJ·mol}^{-1} \)) gave a value of 3.2 for the \( \Delta H_{\text{cal}}/\Delta H_{\text{NH}} \) ratio, indicating that the molecular structure of the enzyme is probably formed by three or four co-operative regions, a number similar to that of the acid proteinase, pepsin. It should be noted that a completely different conclusion would be obtained from a straightforward analysis of the calorimetric curves, disregarding the effect of irreversibility on the denaturation process.

INTRODUCTION

Differential scanning calorimetry (DSC) has been widely employed during the last two decades to study thermal transitions in proteins. It can provide detailed information on the energetics and mechanism of the folding/unfolding processes of these macromolecules [1]. For example, it can be used to test the validity of the two-state assumption for the analysis of protein unfolding. This test is based on a comparison of the calorimetric enthalpy change, \( \Delta H_{\text{cal}} \), with the van’t Hoff enthalpy, \( \Delta H_{\text{NH}} \), determined from the temperature-dependence of the equilibrium constant [2,3]. Thus it has been found that many small globular proteins unfold as single co-operative units, showing a ratio of \( \Delta H_{\text{cal}}/\Delta H_{\text{NH}} \) that is close to 1.0 [1,3]. However, multistate proteins often display more complex processes where two or more individual two-state transitions are present [4,5]. Although in some instances the transitions largely overlap, deviation from the two-state mechanism is indicated by a value of \( \Delta H_{\text{cal}}/\Delta H_{\text{NH}} \) considerably greater than 1.0. Furthermore, it is generally considered that this ratio gives an indication of the number of co-operative regions (thermodynamic domains) that are present in the macromolecule [1,4]. It should be recalled that, in the determination of \( \Delta H_{\text{NH}} \), concepts derived from equilibrium thermodynamics are employed. Thus the co-operativity analysis described above can only be applied to reversible transitions that are not under kinetic control [6].

It is well known that several proteins undergo unfolding transitions that are irreversible as judged by the calorimetric criterion, i.e. they show no endotherm when rescanned after being subjected to a previous heating and cooling cycle [7]. Nevertheless, in several of these cases the formalism of equilibrium thermodynamics has been used to analyse the DSC curves [8,9]. For instance, thermal denaturation of pepsin has been studied at several pH values [5,10]. In spite of the irreversible character of this transition, \( \Delta H_{\text{NH}} \) values derived from single calorimetric curves are used to sustain the proposal that the number of co-operative units in this enzyme varies with pH [10].

In the present work we studied the thermal denaturation of the acid proteinase from *Aspergillus saitoi*, an enzyme that belongs to the pepsin family. Although the global process appeared to be irreversible, an unfolded species in equilibrium with the native protein was detected. \( \Delta H_{\text{NH}} \) pertaining to the equilibrium transition was estimated by two methods and compared with \( \Delta H_{\text{cal}} \). The \( \Delta H_{\text{cal}}/\Delta H_{\text{NH}} \) quotient so obtained suggests that the proteinase molecule consists of three or four co-operative regions.

EXPERIMENTAL

Materials

*A. saitoi* acid proteinase (crude extract) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade. Distilled deionized water was used throughout.

Enzyme purification

Approx. 1 g of crude proteinase extract was dissolved in 25 ml of 0.05 M phosphate buffer, pH 4.0, and filtered through a 0.42 μm Millipore membrane. It was then submitted to gel filtration in a column (2.5 cm x 54 cm) packed with TSK HW-40 (Merck, Darmstadt, Germany). The sample was eluted with the phosphate buffer at a flow rate of 0.67 ml/min. Fractions were collected and assayed for proteolytic activity against heat-denatured haemoglobin as described elsewhere [11]. Fractions showing significant proteolytic activity were combined and the resulting solution was concentrated to 1 mg of protein/ml; 1 ml of this preparation was then injected on to a TSK DEAE-5PW column (0.75 cm x 7.5 cm) installed in a Varian 9000 chromatograph. Protein components were eluted at a flow rate of 0.7 ml/min, by a linear gradient of

Abbreviation used: DSC, differential scanning calorimetry.

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The isolated acid proteinase was submitted to SDS/PAGE [12], only one band was observed (results not shown), corresponding to a molecular mass of 34 kDa. This value is similar to that of pepsin [13], but differs considerably from those of other fungal acid proteinases [11]. The optimum pH for proteolytic activity against haemoglobin was found to be 2.3. Activity was reduced to 50% of maximum at pH 1.6 and 4.0, and fell to zero below pH 1.0 and above pH 5.0.

CD spectroscopy

CD spectra were recorded in a JASCO J-500A spectropolarimeter calibrated with (+)-10-camphorsulphonic acid [14]. Measurements were made on protein solutions of 0.282-0.425 mg/ml employing 0.10 and 0.05 cm-pathlength cells. Protein concentration was determined spectrophotometrically using the absorption coefficient reported for the acid proteinase (\(A_{1cm,280nm} = 13.15\)) [15]. A water-jacketed cell holder and a Haake NK-22 circulating bath were used for temperature control. The actual temperature inside the cell was measured with a thermistor probe. Mean residue ellipticity, \(\theta_{m,r.w.}\), was calculated using a mean residue molecular mass of 117 Da [15].

Thermal denaturation of the enzyme was followed by continuously monitoring ellipticity changes at a fixed wavelength while the sample temperature was increased at constant rate. Careful adjustment of the heating-control knob of the circulating bath permitted us to select heating rates in the range 0.1-1.5 K/min with a precision of \(\pm 0.03\) K/min. Observed ellipticities were transformed to apparent fractions of denatured protein, \(f_{D,app}\), by using the well-known relation:

\[
f_{D,app} = (\theta_\tau - \theta_n) / (\theta_\tau - \theta_w)
\]

where \(\theta_\tau\) is the sample ellipticity at a particular temperature and \(\theta_n\) and \(\theta_w\) are the corresponding values for the denatured and native states, extrapolated to the same temperature.

DSC

Calorimetric endotherms were obtained with a MicroCal MC-2 differential scanning calorimeter (Microcal, Northampton, MA, U.S.A.). Enzyme solutions of 1-1.3 mg/ml were dialysed against 0.05 M phosphate, pH 5.0. All solutions were degassed under vacuum for 20 min; after degassing, pH and protein concentration were checked and samples were then loaded into the calorimeter cells. Calorimetric scanning was carried out at 1.25, 0.9 or 0.6 K/min, under a total pressure of 2.0 kg/cm\(^2\). Buffer-buffer tracings were recorded under the same conditions and subtracted from sample endotherms. Excess-heat-capacity curves were then obtained by subtraction of sigmoidal baselines. DA-2 and Origin software packages (MicroCal) were used for automatic data collection and analysis including baseline subtraction and determination of denaturation enthalpies.

RESULTS AND DISCUSSION

The CD spectrum of the enzyme from A. saitoi at pH 5.0 and 25 °C is shown in Figure 2. The positive peaks at 197 nm and 230 nm as well as the negative band around 212 nm seem to be characteristic of fungal acid proteinases, as they have been found in other acid proteinases [11].
in CD curves of three other enzymes of this family [11,16,17]. In the pH range 2.5–5.5, the spectral features of the proteinase remained approximately constant, with only minor variations in magnitude (results not shown). However, outside this range, spectral shapes changed dramatically, indicating that gross conformational modifications were taking place under these conditions. Considering this limited pH region of stability, we decided to perform all thermal denaturation studies at pH 5.0 as activity of the proteinase was close to zero at this pH, thus largely avoiding the risk of autolysis as a source of irreversibility.

**Effect of heating rate on the thermal denaturation**

At 80 °C (pH 5.0) the proteinase CD spectrum (Figure 2) resembled those typical of heat-denatured proteins [18]. Undoubtedly, the protein has lost its native conformation at this temperature. Especially noticeable is the large variation in CD signal at 230 nm on heating of the sample, making ellipticity measurements at this wavelength very appropriate for following the denaturation process. Figure 3 shows thermal transition curves obtained at different heating rates by continuous recording of ellipticity at 230 nm. In some experiments the transition was also followed at 197 and 212 nm; the resulting curves were almost completely superimposable on those of Figure 3, indicating that at these three wavelengths the CD signal was monitoring the same process.

As can be seen in Figure 3, the denaturation transition was strongly dependent on heating rate, as expected for an unfolding process that is under kinetic control due to the presence of an irreversible reaction [6,19,20]. Indeed, samples that were heated up to 75 °C (at which temperature the transition had been completed) failed to show any reversal of the CD signal when cooled back to 25 °C; after standing at this temperature for a few minutes, sample solutions became appreciably turbid. However, at high temperature no turbidity was observed, and the absorption spectrum of the acid proteinase showed no significant increase in light scattering on denaturation.

In view of the irreversible character of the acid proteinase denaturation, we first analysed its transition curves in terms of a simple two-state model,

\[ N \rightarrow D \]  

where N and D represent the native and irreversibly denatured forms of the enzyme respectively and \( k_{\text{app}} \) is the apparent first-order rate constant for the reaction. Sánchez-Ruiz et al. [7] have derived a set of mathematical relations that can be employed to estimate the apparent activation energy, \( E_{\text{app}} \), of the process. One relates the temperature at the maximum of the heat-capacity curve \( (T_m) \) to the heating rate, \( v \):

\[
\ln \frac{v}{T_m^2} = \ln \frac{AE}{RT_m} - \frac{E}{RT_m}
\]  

where \( A \) is the frequency or pre-exponential factor in the Arrhenius equation. Since for an irreversible two-state process \( f_T = 1 - (1/e) = 0.63 \) at \( T = T_m \) [7], eqn. (3) can be used to analyse the transition curves for acid proteinase denaturation. A plot of \( \ln (v/T_m^2) \) versus \( 1/T_m \) (constructed from data in Figure 3) showed a noticeable curvature (Figure 4), suggesting that \( k_{\text{app}} \) is not an elementary rate constant. To explain this behaviour we then considered a Lummey–Eyring mechanism [21] which takes into account the presence of a reversibly unfolded state, U, in addition to states N and D:

\[ N \rightarrow U \rightarrow D \]

where \( K \) is the equilibrium constant for the N to U transition. It has been shown [6,22] that DSC curves for proteins that follow a Lummey–Eyring model are strongly affected by the heating rate.
Figure 5 $\Delta H_{vH,app}$ as a function of the reciprocal of the heating rate ($v$)

- Values obtained from CD experiments (Figure 3);
- Values from DSC; ---, linear extrapolation of $\Delta H_{vH,app}$ to infinite heating rate, obtained by considering the three data points corresponding to the highest heating rates.

Of course, this effect should also be observed in curves of $f_D$ against $T$. Nevertheless, if it is assumed that equilibrium is always established, certain thermodynamic information pertaining to the N to U process can be gained from data extrapolation to infinite heating rate [6,23]. In particular, it can be demonstrated (see the Appendix) that the $\Delta H_{vH,app}$, as defined by eqns. (5) and (6):

$$\frac{d(\ln K_{app})}{d(1/T)} = -\frac{\Delta H_{vH,app}}{R}$$

(5)

$$K_{app} = \frac{f_{D,app}}{1-f_{D,app}}$$

(6)

can be expressed as a linear function of $1/v$ at high values of $v$:

$$\Delta H_{vH,app} \approx RT^2k \left(\frac{1}{v}\right) + \Delta H_{vH}$$

(7)

Therefore extrapolation of $\Delta H_{vH,app}$ to $1/v = 0$ would give the equilibrium $\Delta H_{vH}$. Figure 5 shows that for acid proteinase denaturation, $\Delta H_{vH,app}$ seems to follow a linear dependence on $1/v$ when $v$ is higher than 0.7 K/min. $\Delta H_{vH,app}$ values were calculated from the transition curves illustrated in Figure 3. Although eqn. (7) states that $\Delta H_{vH,app}$ values must be evaluated at the same temperature, in practice their evaluation from individual transition curves does not introduce serious errors as $\Delta H_{vH}$ can be taken as a constant within the relatively narrow temperature range studied here (plots of $\ln K_{app}$ versus $1/T$ were linear; results not shown). In Figure 5, different sets of data points corresponding to heating rates between 0.9 and 1.5 K/min, were linearly extrapolated to zero $1/v$. With three, four or five data points we obtained correlation coefficients of 0.95–0.99, while $\Delta H_{vH}$ varied from 234 to 334 kJ·mol$^{-1}$. The dashed line in Figure 5 shows the extrapolation obtained using three data points. When a sixth data point ($v = 0.7$ K/min) was included in the set, the correlation coefficient decreased to 0.80. These results suggest that the average of extrapolated values obtained with five or fewer data points ($310 \pm 30$ kJ·mol$^{-1}$) can be considered as a reasonable estimate for $\Delta H_{vH}$.

Direct estimation of $\Delta H_{vH}$

The data analysis just described is based on the assumption that the thermal denaturation behaviour of the acid proteinase is consistent with a Lumry–Eyring model [eqn. (4)]. Further support for this assumption would be obtained by direct detection of the reversibly unfolded state (U) which should exist in equilibrium with the native protein. Indeed, the presence of the U species was demonstrated in an experiment in which the protein solution was heated ($v = 1.5$ K/min) to a temperature ($T'$) at which the transition was approximately half-completed; when the solution was rapidly cooled (10 K/min) to 25 °C, the change in CD signal observed on heating was reversed to a considerable extent (Figure 6). Furthermore, if changes in ellipticity are expressed as $f_{D,app}$ (as in Figure 6) and it is assumed that U and D states display the same ellipticity, it is evident that $f_{D,app}$ should be equal to the sum of fractions of reversibly unfolded ($f_U$) and irreversibly denatured ($f_D$) protein. Therefore, when the sample is at a temperature $T'$ and then rapidly cooled from $T'$ to 25 °C, the extent of reversal in $f_{D,app}$ would give a quantitative estimation of the value of $f_U$ at $T'$. In addition, since $f_{D,app} = 1 - f_{U,app}$, the equilibrium constant for the reversibly unfolding step can be evaluated as $K = f_U/f_D = f_D/(1 - f_{D,app})$.

By performing a series of experiments in which sample heating was stopped at different temperatures, we were able to determine several values of $f_U$ within the transition-temperature range. The equilibrium constant corresponding to each of those $f_U$ values was calculated as outlined above, and the van’t Hoff plot (ln $K$ versus $1/T$) shown in Figure 7 was constructed. From the slope of the straight line, a $\Delta H_{vH}$ of 176 kJ·mol$^{-1}$ was obtained. It must be recognized that the $\Delta H_{vH}$ determined by this method is a lower limit to the true value. This is due to the fact that sample cooling took a finite time during which some of the protein molecules in the U state were transformed to the D state, thus causing an underestimation of $f_D$ and $K$. Indeed, the value of 176 kJ·mol$^{-1}$ is considerably smaller than the previous estimation

**Figure 5**

**Figure 6** Partial reversibility in the thermal denaturation of the acid proteinase

A sample of the enzyme was heated to 65 °C and then cooled rapidly to 25 °C. The process was monitored by ellipticity changes at 230 nm and the apparent fraction of the denatured protein, $f_{D,app}$, calculated as indicated in the text. Also shown are the estimates for the fractions of reversibly unfolded ($f_U$) and irreversibly denatured ($f_D$) states at 65 °C.
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Equilibrium at temperatures monitored using enzyme nearly at 4 mg/ml respectively.

From Table 1.3

75 - 1.25 K/min respectively. As shown in Figure 5, ΔH_vH.app. values calculated from calorimetric measurements followed the same trend as ΔH_vH.app. values obtained from CD experiments. This observation suggests that the two data sets would give a common ΔH_vH when extrapolated to infinite heating rate (actually, it was not possible to attain scanning rates higher than 1.25 K/min in our calorimeter).

A direct analysis of calorimetric data would result in a ratio of ΔH_vH/ΔH_vH of 1.4-1.7, suggesting that the acid proteinase molecule is formed by two thermodynamic co-operative units. It should be stressed, however, that because of the irreversible character of the denaturation transition, ΔH_vH values derived from calorimetric curves are only apparent. Therefore a more realistic calculation of ΔH_vH/ΔH_vH would be obtained by using the ‘true’ ΔH_vH, e.g. 243 kJ·mol⁻¹ (average of the ΔH_vH.app. extrapolated to infinite heating rate and the value determined directly by CD). ΔH_vH/ΔH_vH would then be 3.2, indicating that there are three or four co-operative regions in the enzyme. This value is the same as that found for pepsin at pH 6.5 [5,10]. For this latter protein it is observed that ΔH_vH/ΔH_vH ratios (derived from calorimetric experiments performed at a single scanning rate) decrease from 3.7 to 2.5 when the pH is lowered from 6.5 to 5.0, eventually reaching a value of 1.7 at pH 2.0 [10]. Accordingly, Makarov et al. [10] have proposed that the number of thermodynamic domains in the pepsin molecule changes with pH. Nevertheless, given the completely irreversible behaviour of pepsin denaturation in acidic media, and in the light of the results found in our study, it is clear that the above proposal should be closely re-examined.

Concluding remarks

We have shown in this work that the thermal denaturation of the acid proteinase from A. saitoi follows a Lurny-Eyring model [eqn. (6)]. This conclusion was substantiated by the detection of the reversibly unfolded species, U. In spite of the global irreversibility of the process, we were able to estimate the ΔH_vH for the equilibrium between N and U. Comparison of ΔH_vH with ΔH_vH, led to the proposal that the number of co-operative regions in the acid proteinase is similar to that of pepsin. A completely different conclusion would be reached if ΔH_vH values obtained directly from the calorimetric curves were used.

The procedures employed to estimate ΔH_vH were based on the measurement of ellipticity; the choice of this method was appropriate in our case as loss of the enzyme’s native structure seemed to be equally well monitored by CD and DSC. In addition, the spectroscopic method required less sample than the calorimetric method. This can be an invaluable advantage for work with proteins that are available in very limited quantities.

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REFERENCES

APPENDIX

For a Lumry–Eyring model of the type depicted in eqn. (4) of the main paper, Sánchez-Ruiz [1] has shown that, assuming that equilibrium is always established, the fractions of the three states considered in the model are given by:

\[ f_u = \frac{1}{K+1} \exp \left( -\frac{1}{v} F(T) \right) \]  
\[ f_d = \frac{K}{K+1} \exp \left( -\frac{1}{v} F(T) \right) \]  
\[ f_D = 1 - \exp \left( -\frac{1}{v} F(T) \right) \]  

where

\[ F(T) = \int_T^\infty \frac{kK}{K+1} dT \]  

(A4)

In these equations \( K = f_u/f_d \), \( v \) is the scanning rate and \( k \) is the first-order rate constant for the process \( U \rightarrow D \). At this point it should be recalled that \( f_{D,app} \), as defined by eqn. (1) in the main paper, is related to \( f_u \) and \( f_D \) by the expression [2]:

\[ f_{D,app} = f_u + f_D (\Theta_U - \Theta_D)/(\Theta_D - \Theta_U) \]  

(A5)

where \( \Theta \) is a spectroscopic value observable at a particular wavelength. If \( U \) and \( D \) states have different spectral properties, according to eqn. (A5), one would expect to observe a dependence of \( f_{D,app} \) on the wavelength used to monitor the transition. As this was not the case for the thermal denaturation of the acid proteinase, it can be assumed that states \( U \) and \( D \) possess very similar dichroic characteristics. Furthermore, considering that ellipticities in the far-UV region are mainly a reflection of the folding pattern of a polypeptide chain, the above assumption seems to be a reasonable one. Thus eqn. (A5) can be rewritten as

\[ f_{D,app} = f_u + f_D. \]  

As \( f_u + f_D = 1 \), the apparent equilibrium constant can be expressed as:

\[ K_{app} = f_{D,app} / (1 - f_{D,app}) = (1 - f_u)/f_u \]  

(A6)

Introducing eqn. (A1) leads to:

\[ K_{app} = (K+1) \exp \left( \frac{1}{v} F(T) \right) - 1 \]  

(A7)

or

\[ \ln K_{app} = \ln \left( (K+1) \exp \left( \frac{1}{v} F(T) \right) - 1 \right) \]  

(A8)

Differentiation of eqn. (A8), followed by rearrangement gives:

\[ \frac{d(\ln K_{app})}{dT} = \exp \left( \frac{1}{v} F(T) \right) \left( \frac{kK + dK}{v + dT} \right) \]  

(A9)

where we have used eqn. (A4) in the form \( dF(T) = kK/(K+1) \).

From eqn. (A9) and the general relationship \( d(\ln K)/dT = \Delta H/RT^4 \),

\[ \Delta H_{VH,app} = \frac{kK}{K} \left( R T^4 k + \Delta H_{VH} \right) \]  

(A10)

When \( v \gg F(T) \), this equation would be simplified to:

\[ \Delta H_{VH,app} \approx RT^4 k(1/v) + \Delta H_{VH} \]  

(A11)

which is eqn. (7) in the main paper.

REFERENCES