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Determination of enzyme global thermostability from equilibrium and kinetic analysis of heat inactivation

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An equilibrium-kinetic description for the two-stage irreversible thermoinactivation of enzymes (N $\Leftrightarrow$ D $\Rightarrow$ I) is examined by using chymotrypsin as a model. The parameter ΔG for enzyme unfolding (N $\Leftrightarrow$ D) and activation free energy for the D $\Rightarrow$ I reaction ($\Delta G_i^{\#}$) were summed to provide an index of enzyme global thermostability ($\Delta G^{\#}$; $\Delta G^{\#} = \Delta G + \Delta G_i^{\#}$). There was good agreement between calculated and experimental global thermostability (i.e. enzyme stability with respect to reversible and irreversible thermoinactivation reaction steps) at 0–60°C. The results indicate that enzyme global thermostability may be markedly dependent on the rate of enzyme folding.

NOTATION

ΔC_p	Heat-capacity for unfolding
D	Denatured or unfolded enzyme
ε_o	Total enzyme concentration
f_u	Fraction of unfolded enzyme
ΔG	Free energy for enzyme reversible unfolding
$\Delta G^{\#}$	Activation free energy for irreversible inactivation or global-thermostability index
$\Delta G_f^{\#}$	Activation free energy for folding
$\Delta G_i^{\#}$	Activation free energy for D $\Rightarrow$ I reaction
$\Delta G_u^{\#}$	Activation free energy for unfolding
h	Planck's constant
ΔH	Enthalpy change for unfolding
I	Irreversibly inactivated enzyme
k	Observed rate constant for thermoinactivation
k_f	Folding-rate constant
k_i	Rate constant for D $\Rightarrow$ I step
k_u	Unfolding-rate constant
K	Equilibrium constant for unfolding (D/N)
K^*	Boltzmann constant
N	Native enzyme
R	Gas constant
ΔS	Entropy change for unfolding
T	Temperature
v	Rate of irreversible thermoinactivation

INTRODUCTION

Enzyme irreversible thermoinactivation involves unfolding of the native (N—) enzyme to form a denatured (D—) state. This is then converted to an irreversibly inactivated (I—) state. This two-stage irreversible thermoinactivation can be summarised by the scheme: N $\Leftrightarrow$ D $\Rightarrow$ I (Lumry & Eyring, 1954; Tombs, 1985; Ahern & Klibanov 1988, Krisjansson & Kinsella, 1991). Possible D $\Rightarrow$ I reactions and I-forms for enzyme heat inactivation have received attention (Ahem & Klibanov, 1988). However, the relationship between enzyme unfolding, enzyme folding, and the rate of irreversible thermoinactivation deserves further study (Brown & Yada, 1991, Iwata *et al.*, 1991). The applicability of equilibrium and irreversible protein inactivation models to differential scanning calorimetry (DSC) has been discussed recently (Sanchez-Ruiz, 1992 and references cited therein).

In this paper, the link between enzyme reversible and irreversible thermoinactivation is examined using chymotrypsin as a model system. The free energy for chymotrypsin unfolding (ΔG) and activation free energy for the D $\Rightarrow$ I thermoinactivation reaction ($\Delta G_i^{\#}$) were estimated at 0–60°C and 40–130°C, respectively. The sum of ΔG and $\Delta G_i^{\#}$ terms produced

a calculated global thermostability ($\Delta G^{\#}$) index, which was then compared with experimentally observed activation-free-energy values for irreversible thermoinactivation. Previous results showed that the thermoinactivation of chymotrypsin, dissolved in 1–2M guanidine hydrochloride (GnHCl), led to a biphasic Arrhenius plot in accord with the two-stage model (Owusu & Berthalon, 1993). The topic addressed now is whether the global rate of enzyme thermoinactivation can be estimated on the basis of precepts from the two-stage scheme. The approach illustrated may be useful for relating enzyme conformational stability changes, such as those produced by chemical modification or protein engineering, directly to changes in enzyme half-life.

MATERIALS AND METHODS

Chymotrypsin (Type I; free of autolysis products and low molecular weight contaminants) was purchased from Sigma (UK). GnHCl was Micro select grade (> 99·5% purity with an absorbance of 0·03 at 260 nm and pH 4·5–6·0 for a 6M solution at 25°C) from Fluka Chemicals Ltd (UK). All other materials were of AnalaR grade from the British Drug Houses Ltd. Ultra-violet (UV) difference spectra were recorded using a Pye Unicam SP1800 spectrophotometer fitted with a heated cuvette block.

Study of chymotrypsin irreversible thermoinactivation

The techniques used were essentially as reported previously (Owusu *et al.*, 1992; Owusu & Berthalon, 1993). Inactivation studies were performed using 0·1M sodium chloride (pH 5·0), with or without 4M GnHCl as solvent and at 40–130°C. Residual enzyme activity was determined using the azocasein assay.

Study of the reversible heat unfolding of chymotrypsin

Solutions of chymotrypsin (0·5–10 mg/ml in 0·1M sodium chloride, pH 5·0) in a pair of matched quartz cuvettes were heated at 25–65°C (±0·1°C) by means of a water bath. The reference sample was maintained at room temperature. The heat-unfolding of chymotrypsin was monitored by UV difference spectrophotometry.

Theory

A two-stage scheme and the rate of irreversible thermoinactivation of enzymes (v) can be expressed by eqns (1) and (2) respectively:

$$N \underset{k_f}{\overset{k_u}{\Leftrightarrow}} D \overset{k_i}{\Rightarrow} I \qquad (1)$$

$$v = k_i[D] \qquad (2)$$

where, k_u, k_f, and k_i, are the rate constants for unfolding, folding, and irreversible steps as shown in eqn (1).

The concentration of reversibly unfolded or denatured enzyme ([D]) can be expressed in terms of k_u, k_f, k_i, total enzyme concentration (ε_o; where ε_o = [N] + [D] under initial conditions), and the fraction of unfolded enzyme (f_u; where f_u = [D]/ε_o). As the [D] state reaches a steady state during thermoinactivation, then, from eqn (1), we have:

$$k_u[N] = [D]\{k_f + k_i\} \qquad (3)$$

Combining eqns (2) and (3) and since [N] = ε_o (1 − f_u), then eqn (4) describes the rate of irreversible thermoinactivation in terms of the rates for enzyme unfolding, folding, enzyme folding, and the irreversible (D ⇒ I) steps.

$$v = \frac{k_i k_u \varepsilon_o (1 - f_u)}{(k_i + k_f)} \qquad (4)$$

Equation (4) can be contrasted with the expression

$$v = k_i K \varepsilon_o / (1 + K)$$

obtained by adopting an equilibrium approximation; K (the equilibrium constant for enzyme reversible unfolding) = [D]/[N] (Owusu *et al.*, 1992). In general, the steady state and equilibrium approximations are complementary. The latter is more appropriate where irreversible inactivation is preceded by a fast and reversible unfolding step. The steady state approximation (cf. eqn (3)) is a more general description (Pyun, 1971). Paradoxically eqn (4) is probably only valid over a limited range of f_u values. Note that the condition $f_u \rightarrow 1$ leads to the unrealistic result that $v \rightarrow 0$; that is, eqn (4) is likely to be valid only under low/moderate temperature conditions. The analogous expression to eqn (4) based on the equilibrium approximation does not exhibit such limitations.

In eqn (4), the magnitudes of k_f and k_i affect the overall kinetics of irreversible thermoinactivation. The extent of activity recovery or regeneration, after heating, may depend on whether the D-state is converted into the I- or N-state. Equations (5) and (6) result, where $k_i \gg k_f$ or $k_i \ll k_f$, respectively;

$$v = k_u \varepsilon_o (1 - f_u) \qquad (5)$$

$$v = k_u k_i \varepsilon_o (1 - f_u)/k_f = k_i K \varepsilon_o (1 - f_u) \qquad (6)$$

According to eqn (5), where the folding rate is slower than the rate of irreversible change, then thermoinactivation will reflect the rate of enzyme unfolding with an apparent rate constant (k) equal to k_u (cf. eqn (5); $k \cong k_u$). However, for an enzyme exhibiting fast folding, then according to eqn (6), thermoinactivation will reflect the rate unfolding, folding and irreversible steps, i.e.

$$k \cong k_i k_u / k_f$$

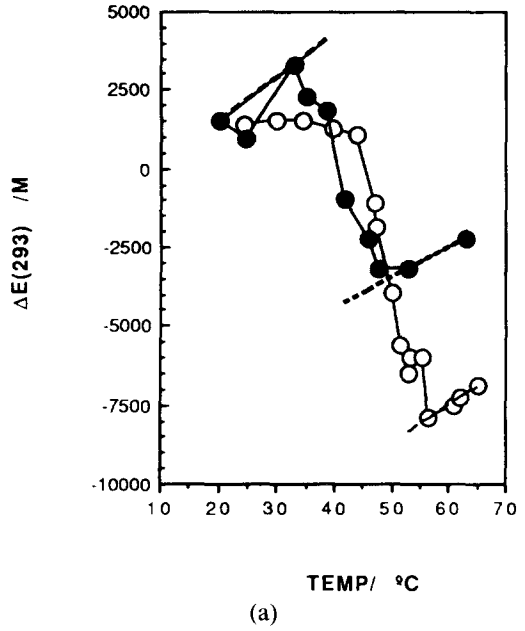
(Sanchez-Ruiz, 1992). The temperature dependence of k can be described eqn (7), where K^*, h, and R are the Boltzman, Planck and gas constants respectively:

$$k = (K^* T/h) \exp(\Delta G^{\#}/RT) \qquad (7)$$

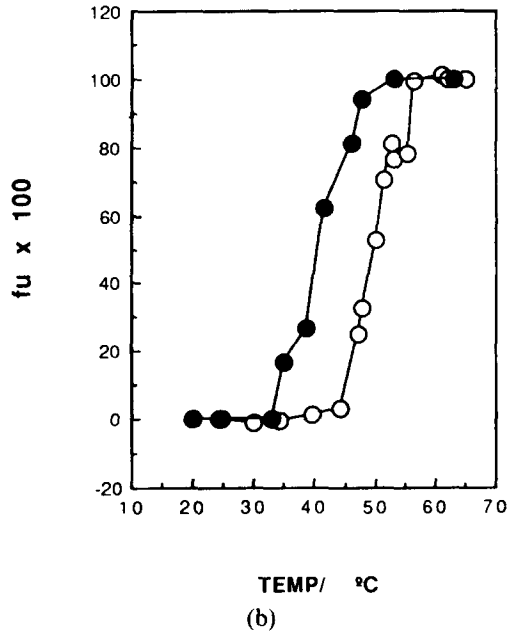
The parameter $\Delta G^{\#}$ is the experimental activation free energy for enzyme irreversible thermoinactivation; $\Delta G^{\#}$ and k values are indices of enzyme global thermostability or a measure of the resistance to both reversible and irreversible thermoinactivation. Equations (5)–(7) reveal that $\Delta G^{\#}$ is a composite of the activation free energy values for enzyme-unfolding ($\Delta Gu^{\#}$), folding ($\Delta Gf^{\#}$), and irreversible change ($\Delta Gi^{\#}$) (Owusu *et al.*, 1992):

$$\Delta G^{\#} = \Delta G_u^{\#} - \Delta G_f^{\#} + \Delta G_i^{\#} = \Delta G + \Delta G_i^{\#} \qquad (8)$$

In this paper, observed $\Delta G^{\#}$ values for chymotrypsin irreversible thermoinactivation are compared with global thermostability indices computed from eqn (8).



(a)



(b)

Fig. 1. Reversible thermal unfolding of chymotrypsin. (a) Plot of UV difference changes at 293 nm (ΔE293) versus temperature, T (°C). Conditions, 0·1M aqueous sodium chloride, pH 5·0 without (○) or with 1·5M GnHCl (●); (– – – –) values for N and D states. (b) Plot of fraction of unfolded enzyme (f_u) versus temperature.

RESULTS AND DISCUSSION

The heat unfolding of chymotrypsin produced UV difference spectral changes consistent with the exposure of buried tryptophan residues from the non-polar interior of an enzyme to the relatively polar environment of the bulk solvent. The net UV difference change (ΔE, M^{-1}) of -10085 M^{-1} for the heat-unfolding of chymotrypsin (Fig. 1(a)) is comparable with that given in previous reports (Martin & Bhatnagar, 1966). The value of ΔE was —8830 M^{-1} in the presence of 1·5M GnHCl owing to the solvent polarity decrease resulting from the presence of denaturant.

Values for K and f_u were determined from UV difference changes at 293 nm (ΔE293) using

$$f_u = (\Delta E293_N - \Delta E293_{OB})/(\Delta E293_D - \Delta E293_N)$$

and

$$K = (\Delta E293_N - \Delta E2930_{OB})/(\Delta E293_{OB} - \Delta E293_D),$$

where the subscripts refer to values for native enzyme (N), denatured enzyme (D; at 55—65°C) and observed (OB) $\Delta E293$ values at intermediate temperatures. The heat-unfolding of chymotrypsin at pH 5·0 (Fig. 1) was found to be reversible for a heating time of about 60 min. Cooling a sample of heated enzyme from 60 to 25°C restored it to the UV difference spectra for the native enzyme. Figure 1(b) shows a plot of f_u versus temperature for experiments performed with and without 1·5M GnHCl.

Thermodynamic parameters for the thermal unfolding of chymotrypsin were determined from the temperature dependence of K and the heat-capacity difference between the N- and D-states (ΔC_p). The parameter ΔC_p was determined directly from the temperature dependence of ΔG (i.e. RT ln K) expressed as a second-order polynomial

$$\Delta G = A + BT + CT^2.$$

The constants A, B and C are a function of thermodynamic parameters, i.e.:

$$\Delta S = -(B - 2CT),$$

$$\Delta H = A - CT^2$$

and

$$\Delta C_p = 2CT$$

(Brandt, 1964). The parameters A, B and C were determined by non-linear regression of ΔG versus temperature data. The complete stability curve or ΔG versus temperature graph is shown in Fig. 2. Thermodynamic parameters for chymotrypsin-reversible heat-unfolding are reported in Table 1.

As a check on the reliability of parameters obtained by non-linear regression, ΔC_p was also determined by direct calculation using the relation

$$\Delta C_p = \Delta H/\Delta T.$$

The heat unfolding of chymotrypsin was determined in three solvents (a) 0·1M aqueous NaCl (pH 5·0), (b) 0·1M aqueous NaCl (pH 3·8) and (c) 0·1M aqueous

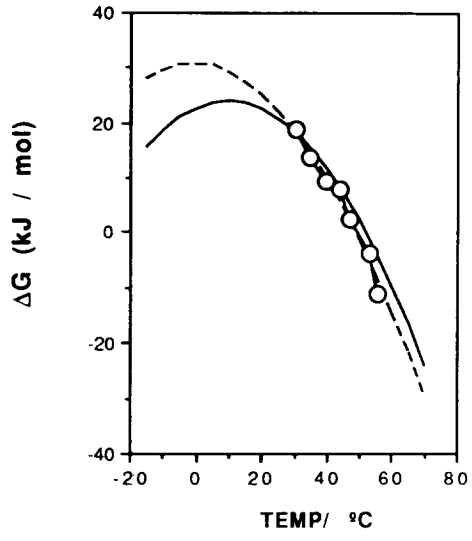


Fig. 2. Stability curve for chymotrypsin. A graph of unfolding free energy (ΔG) versus temperature, T (°C) determined by non-linear regression (——) of experimental points (○) or by direct calculation (– – – –) (see text).

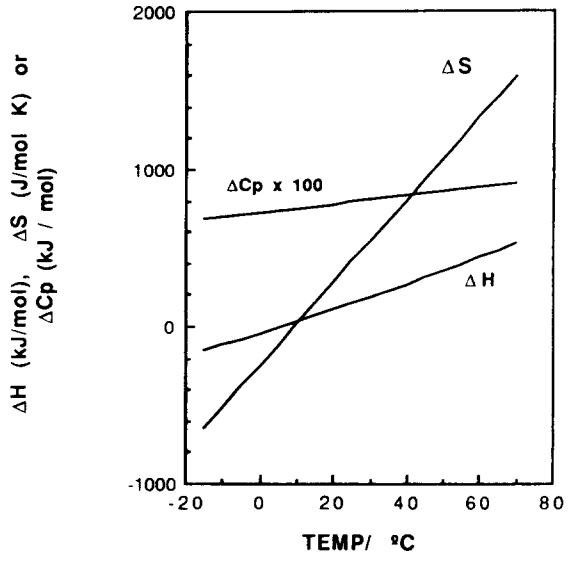


Fig. 3. The temperature dependence of enthalpy (ΔH), entropy (ΔS), and heat capacity (ΔC_p) changes for chymotrypsin reversible unfolding (solvent was 0·1M aqueous sodium chloride, pH 5·0).

NaCl (pH 5·0) with 1·5M GnHCl. A van't Hoff plot of log K versus $1/T$ (results not shown) led to values for T_m and ΔH_m (i.e. ΔH values at the T_m)). With solvents (a)–(c), ΔH_m and T_m estimates were (a) 384 kJ mol^{-1} and 49°C, (b) 368 kJ mol^{-1} and 49·3°C and (c) 326 kJ mol^{-1} and 40·7°C, respectively. By taking the combination of results for solvents (a) and (c) or (b) and (c), ΔC_p was estimated as (7·1 ± 0·1) kJ mol^{-1} K^{-1} (Table 1). To obtain reliable ΔC_p estimates by means of this method required the use of conditions under which ΔT was reasonably large.

Profiles of ΔG versus temperature profiles (Fig. 2) were also calculated from ΔH_m, T_m, and ΔC_p values using

$$\Delta H = \Delta H_m - \Delta C_p (T_m - T)$$

$$\Delta S = \Delta S_m - \Delta C_p \ln (T_m/T)$$

and

$$\Delta G = \Delta H - T\Delta S$$

(Shiao *et al.*, 1971). The stability curve for chymotrypsin (Fig. 2) shows the resistance of the native enzyme to reversible thermal unfolding at temperatures from −15 to 70°C. The profiles determined by two separate methods are shown. There was agreement between the stability curves obtained by non-linear regression and by direct calculation (Fig. 2). Figure 3 shows the temperature dependence of ΔH and ΔS values. These are similar to profiles reported for other small globular proteins. The curvature in ΔG versus temperature graphs is usually attributed to a large ΔC_p change for protein-unfolding associated with the exposure of non-polar amino acid side chains in the D-state to solvent. The motional restriction imposed on solvent molecules produces a decrease in the specific heat capacity of the system (Brandt, 1964; Shiao *et al.*, 1971; Becktell & Schellman, 1987).

Table 1. Thermodynamic parameters for the reversible heat-unfolding of chymotrypsin[a]

Parameter	*a*	*b*
A	−1034	—
B	7·78	—
C	$-1{\cdot}32 \times 10^{-2}$	—
ΔH (kJ/mol)	213 (±5)	217 (±0·9)
ΔS (J/mol K)	636 (±10)	664 (±20)
ΔC_p (kJ/mol K)	7·1 (±0·06)	7 (±0·1)
ΔG (kJ/mol)	24 (±2·5)	22 (±0·3)

[a] Values for 25°C. Data are means of three replicates with SD in parenthesis. Parameters were determined by (*a*) non-linear regression or (*b*) direct calculation (see text for details). Solvent was 0·1M NaCl, pH 5·0.

Estimation of $\Delta G_i^{\#}$ values

Chymotrypsin was pre-unfolded in 4M GnHCl and heated for varying time periods in order to determine $\Delta G_i^{\#}$ values. The thermoinactivation reaction for unfolded chymotrypsin is D ⇒ I. These experiments and the analysis of data to obtain $\Delta G_i^{\#}$ values have been described previously (Owusu & Berthalon, 1993). The parameter was linearly dependent on temperature. Over the range 40–130°C, the relation

$$\Delta G_i^{\#} = 0{\cdot}17\ T + 82{\cdot}1$$

applies (cf curve B in Fig. 4). The parameter $\Delta G_i^{\#}$ was also sensitive to the concentration of GnHCl. At 40°C, $\Delta G_i^{\#}$ was 79·4 kJ mol^{-1}, 82·0 kJ mol^{-1}, and 87·1 kJ mol^{-1} at GnHCl concentrations of 1·0M, 1·5M and 4·0M, respectively. Assuming a linear dependence on GnHCl concentration, $\Delta G_i^{\#} \approx 76$ kJ mol^{-1} in the absence of GnHCl. Pre-unfolding of chymotrypsin using 4M GnHCl could therefore result in the over estimation of $\Delta G_i^{\#}$ values by about 8 kJ mol^{-1} (see below).

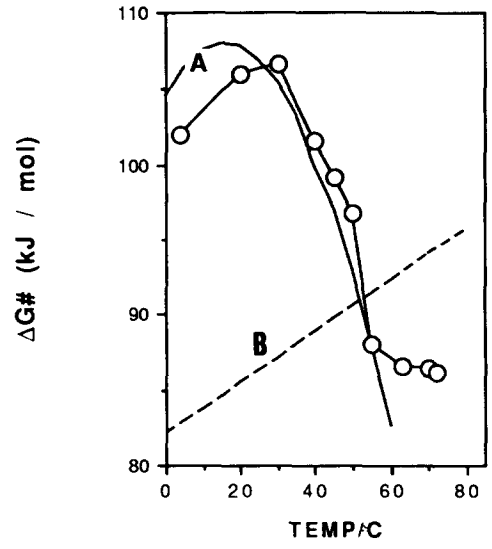


Fig. 4. Temperature-global thermostability ($\Delta G^{\#}$) profile for chymotrypsin. (A) Experimental (○) and calculated (——) $\Delta G^{\#}$ values versus temperature. (B) $\Delta G_i^{\#}$ values for the D ⇒ I step in 4M GnHCl.

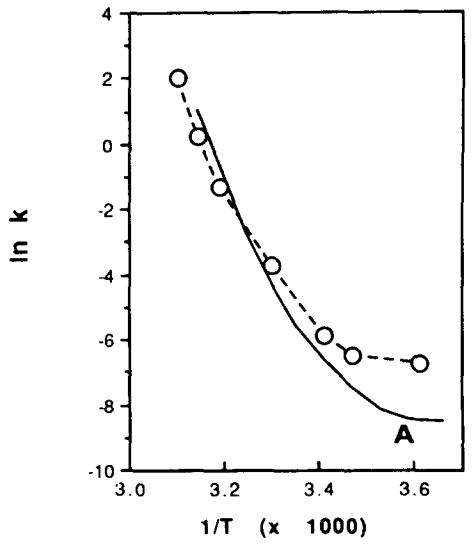


Fig. 5. Arrhenius plot for chymotrypsin irreversible thermoinactivation; (A) calculated from data in Fig. 4 using eqn (7)). Symbols are as in Fig. 4.

Global thermostability estimates

Variations in the global thermostability index ($\Delta G^{\#}$) with temperature are shown in Fig. 4 (Curve A). At 25–60°C, there was reasonable agreement between calculated and experimental global-thermostability values. The intersection of curves A and B (Fig. 4) occurred at about 50°C, corresponding to the value of T_m for chymotrypsin in 0·1M sodium chloride (pH 5·0). The graph of $\Delta G^{\#}$ versus temperature deviated from curve A (Fig. 4) at temperatures exceeding the enzyme-unfolding temperature, This deviation of observed from calculated $\Delta G^{\#}$ values is expected at high temperatures, where the rate-limiting step for irreversible thermoinactivation involves the D ⇒ I step.

A graph of $\Delta G^{\#}$ versus temperature (Fig. 4) or log k versus $1/T$ (Fig. 5) showed curvature at low temperatures. This is attributable to a large activation heat capacity change ($\Delta C_p^{\#}$) for the irreversible thermoinactivation of chymotrypsin at moderate temperatures. It is known that the transition of native chymotrypsin to form a transition state ($N^{\#}$) results in a negligible change in enzyme conformation or degree of solvent accessibility to hydrophobic amino acid residues. Hence $\Delta C_p^{\#}$ for chymotrypsin unfolding is approximately zero. By contrast, there is a large $\Delta C_p^{\#}$ value for the folding (D ⇒ $N^{\#}$) transition (Lumry & Eying, 1969). It may be inferred, from the preceding discussion, that the log k versus $1/T$ curvature (Fig. 5) and associated notional $\Delta C_p^{\#}$ term are indications of the importance of k_f (rate constant for enzyme folding) in determining the apparent rate of irreversible thermoinactivation (cf eqn (6) and accompanying discussion). The non-linear character of the graph shown in Fig. 5 will be important where thermostability results are to be extrapolated from high to low temperatures.

The accuracy of global thermostability estimates may depend on the accuracy with which ΔG and in particular $\Delta G_i^{\#}$ values can be determined. Difficulties can be expected where, for example two or more D ⇒ I reactions occur in parallel. The pre-unfolding strategy (here exposure to GnHCl) could also change the type of D ⇒ I thermoinactivation reaction. For instance, 4 M GnHCl will prevent non-covalent aggregation of heat-unfolded proteins (Ahern & Klibanov, 1988). As discussed above, the presence of 4M GnHCl probably led to an over-estimation of $\Delta G_i^{\#}$ values by about 8 kJ mol^{-1}. Such constant errors in $\Delta G_i^{\#}$ values lower or raise calculated global thermostability values by a constant degree along the Y-axis in Fig. 4. The curvature of this graph, which shows the optimum temperature for enzyme stability, should remain unaffected. In general, a comparison of calculated and actual global-thermostability indices should readily establish the degree of error in $\Delta G^{\#}$ estimates.

In conclusion, the two-stage thermoinactivation scheme has been examined quantitatively on the basis of a combination of equilibrium and kinetic studies. Calculated and observed global-thermostability indices were in good agreement. However, the present method appears limited by the accuracy with which $\Delta G_i^{\#}$ estimates can be determined. Further studies are in progress with the chymotrypsin system used at low pH to enable ΔG and $\Delta G_i^{\#}$ values to be determined in the absence of a denaturant. The generality of the equilibrium-kinetic analyses should be established by using, as model systems, enzymes known to undergo fully reversible unfolding under certain conditions, e.g., lysozyme, ribonuclease and other serine proteinases. The results reported above suggest that the estimation of enzyme global thermostability from precepts based on the two-stage thermoinactivation model is feasible.

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