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The conformational stability of a lipase from a psychrotrophic *Pseudomonas fluorescens*

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The reversible unfolding by heat or guanidine hydrochloride of lipase from a psychrotrophic *Pseudomonas fluorescens* was studied by fluorescence spectrophotometry. Lipase conformational stability was assessed based on thermodynamic parameters, e.g. free energy (ΔG), enthalpy (ΔH), entropy (ΔS) and heat capacity (ΔC_p) changes for unfolding. The degree of reversibility was 70% and 100% for heat or guanidine hydrochloride unfolding. ΔG(25°C), $\Delta G(H_2O)$, ΔH_m, ΔS_m and ΔC_p values for lipase unfolding were 9·5, 6·5 and 151 kJ/mol, 475 J/mol/K and 4·06 kJ/mol/K respectively. The ΔG values determined by direct monitoring of unfolding were comparable to the value of 15 kJ/mol estimated from the enzyme temperature–activity profile. The conformational stability of lipase from the psychrotrophic *Pseudomonas fluorescens* is shown to be significantly lower than the stability usually observed for mesophilic enzymes.

NOTATION

ΔC_p	Heat capacity change for the unfolding reaction
$[D]_{1/2}$	GnHCl concentration at 50% unfolding
F_n	Fraction of native enzyme
F_u	Fraction of unfolded enzyme
ΔG	Free energy change
ΔG(25°C)	Standard free energy change from thermal-unfolding
$\Delta G(H_2O)$	Standard free energy change from GnHCl-unfolding
ΔH_m	Enthalpy change at T_m
ΔS_m	Entropy change at T_m
T_m	Melting temperature, temperature at 50% unfolding
T_s	Temperature of maximum stability
V_c	Calculated enzyme activity
V_{obs}	Observed enzyme activity
y_n	Fluorescence intensity for the native conformation
y_{obs}	Observed fluorescence intensity
y_u	Fluorescence intensity for the unfolded conformation

INTRODUCTION

Psychrotrophic bacteria, mainly of the genus *Pseudomonas*, produce heat-resistant lipases which can withstand pasteurization and ultra high-temperature sterilization. The enzymes can cause spoilage of milk and dairy products during storage (Dring & Fox, 1983; Stead, 1986; Mottar, 1989). In spite of the high heat resistance, high-temperature treatment is an important means of controlling psychrotroph lipases and other quality-related enzymes in foods (Adams, 1991).

The thermal stability of enzymes refers to the temperature at which an enzyme protein molecule spontaneously unfolds. This is related to the thermodynamic stability, or Gibbs free energy difference between the unfolded and native enzyme conformations (Myers, 1990).

Approaches used for the measurement or comparison of enzyme and protein stability include determination of the melting temperature (T_m), measurement of the free energy of stabilization (ΔG), estimation of the temperature of maximum stability (T_s) and measurement of the degree of persistence of functional activity at or after exposure to a particular temperature (O'Fagain & O'Kennedy, 1991). This last criterion was largely used in previous investigations of the thermal stability of psychrotroph enzymes (Kroll, 1989).

Irreversible heat-inactivation of enzymes occurs in two stages. There is a reversible unfolding followed by an irreversible covalent or noncovalent step (Pain, 1977; Zale & Klibanov, 1983; Ahern & Klibanov, 1988). The unfolding and irreversible steps are in series. Therefore, greater resistance to conformational change will result in enhanced stability against irreversible inactivation.

The quantitative relationship between lipase reversible unfolding and the overall rate of irreversible inactivation

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was recently discussed (Owusu *et al.*, 1992). It was shown that enzyme unfolding was rate-limiting for lipase inactivation at low temperatures. However, at UHT temperatures, lipase inactivation rate was probably determined by enzyme chemical composition. Psychrotroph lipases have been described as 'flexible' (Mitchell *et al.*, 1986; Kroll, 1989), and it seems likely that in spite of the high heat resistance of psychrotroph lipases, these enzymes possess a low intrinsic heat stability. That is, psychrotroph lipase unfolding occurs at lower than ultra-high temperatures. We recently described an indirect method for estimating the conformational or thermodynamic stability of a range of psychrotroph lipases and proteases based on their temperature–activity profiles (Owusu *et al.*, 1991). This approach, however, reflects enzyme active-site stability in the presence of substrate.

In this paper we describe the direct measurement of conformational stability of a lipase purified from the psychrotrophic *Pseudomonas fluorescens* strain 2D.

Changes in enzyme conformation in response to heating or guanidine hydrochloride (GnHCl) concentration were directly measured using fluorescence spectrophotometry. The heat and GnHCl unfolding profiles obtained, as well as studies of the temperature–activity profile, confirm the view that psychrotroph lipases are not exceptionally resistant to heat unfolding. This view is based on measurement of ΔG, ΔH, ΔS and ΔC_p values for lipase reversible unfolding.

MATERIALS AND METHODS

Cultural conditions and lipase purification

Pseudomonas fluorescens strain 2D was isolated from raw milk on tributyrin agar. The microorganism was cultured in half-strength peptone water at 10°C for up to 4 days. Lipase was purified using hydrophobic interaction chromatography on Phenyl-Sepharose CL 4B, followed by gel filtration on Sephadex G-100 and G-75, respectively. Homogeneity was confirmed using SDS-PAGE analysis with silver staining (Makhzoum, unpublished).

Lipase assay

For the determination of the temperature–activity profile, lipase activity was assayed using the pH-stat method (Parry *et al.*, 1966). The pH-stat equipment (Radiometer, Copenhagen, Denmark) consisted of an autoburette (type ABU 80), pH-meter (type PHM 82), titrator (type TTT 80) and a titration assembly. The reaction mixture consisted of 5 ml of a 2% tributyrin emulsion and 2 ml of the lipase solution. The activity was expressed in milliequivalents of fatty acids released per minute per ml of lipase solution.

Unfolding studies

The direct monitoring of lipase structural changes involved the use of fluorescence spectrophotometry (Perkin-Elmer 204 Beaconsfield, Bucks, UK, fluorimeter fitted with a digital output and a thermoregulated cuvette holder). Temperature control was by way of a connected water bath. The temperatures inside cuvettes were monitored using a thermocouple. Fluorescence measurements were made at excitation and emission wavelengths of 283 and 340 nm, respectively.

The fraction of unfolded lipase (F_u) and unfolding equilibrium constant (K_u) were determined from the changes in lipase fluorescence emission as a function of temperature using eqns (1) and (2):

$$F_u = (y_f - y_{obs})/(y_f - y_u) \quad (1)$$

$$K_u = (1 - F_u)/F_u \quad (2)$$

where y_{obs} is the observed fluorescence intensity and y_f and y_u represent the values of y_{obs} characteristic of the folded and unfolded states, respectively (Baldwin & Eisenberg, 1987; Pace, 1990). The molar enthalpy of denaturation, ΔH_m, was determined from an Arrhenius plot of ln K_u *versus* $1/T$. The gradient of such a plot is $\Delta H_m/R$, where R is the gas constant. Subsequently, the molar entropy change at the melting temperature (T_m) is given by

$$\Delta S_m = \Delta H_m/T_m \quad (3)$$

because $\Delta G = \Delta H_m - T\Delta S_m$, and at the T_m the change in ΔG is zero. In order to determine standard enthalpy and entropy parameters, the heat capacity change (ΔC_p) for lipase unfolding is required. ΔC_p was determined by following the thermal unfolding of lipase in the presence of 1·33 M GnHCl. The change in heat capacity was then calculated from

$$\Delta C_p = (\Delta H_m - \Delta H^*_m)/(T_m - T^*_m) \quad (4)$$

where ΔH^*_m and T^*_m are the enthalpy change and melting temperature in the presence of GnHCl (Pace & Laurents, 1989).

Enthalpy, entropy and free energy changes as a function of temperature, i.e. $\Delta H(T)$, $\Delta S(T)$ and $\Delta G(T)$ were then determined from

$$\Delta H(T) = \Delta H_m - \Delta C_p(T_m - T) \quad (5)$$

$$\Delta S(T) = \Delta S_m - \Delta C_p \ln (T_m/T) \quad (6)$$

and since $\Delta G(T) = \Delta H(T) - T\Delta S(T)$, then

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (7)$$

(Privalov & Khechinashvili, 1974; Becktel & Schellman, 1987; Pace & Laurents, 1989).

Lipase conformational stability was also estimated from the GnHCl unfolding profile in the same way as previously described (Owusu, 1992).

The effect of calcium was studied by addition of calcium chloride to a final concentration of 10 mM to the enzyme solution prior to heat and GnHCl unfolding.

The analysis of the lipase temperature–activity profile to yield thermodynamic stability indices in the presence of substrate has been described previously (Owusu *et al.*, 1991).

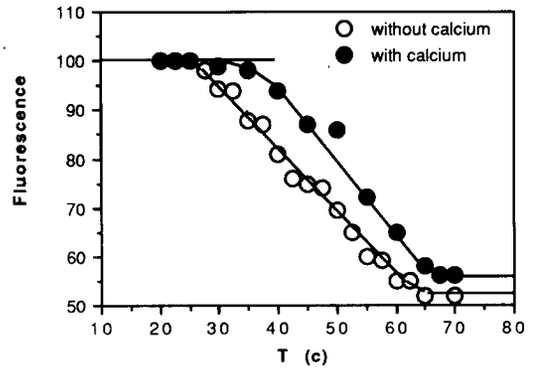

Fig. 1. Thermal-unfolding of 2D lipase (enzyme ~0·3 mg/ml in 0·05 M tris-HCl buffer, pH 8·0) in the presence and absence of calcium.

RESULTS AND DISCUSSION

Lipase thermal- and GnHCl-unfolding, as followed by fluorescence spectrophotometry (Figs 1 and 2, respectively), showed well-defined transition regions. For the purpose of the analysis below, the denaturation reaction is assumed to be two-state. The applicability of such a mechanism is supported by the linearity of the Van't Hoff plot (Fig. 3).

Heat-unfolding was shown to be 70% reversible. Thus a heated lipase sample, when cooled, gave a refolding profile similar to the unfolding one. GnHCl-unfolding was 100% reversible. Unfolded samples, when diluted, recovered their initial fluorescence properties.

Thermodynamic parameters for the heat- and GnHCl-unfolding of 2D lipase at 25°C are summarized in Table 1. ΔG values were 9·5 and 6·5 kJ/mol for thermal-unfolding and GnHCl-unfolding, respectively. Changes in $\Delta G(T)$, $\Delta H(T)$ and $\Delta S(T)$ for heat-unfolding of 2D lipase, as a function of temperature, are shown in Figures 4 and 5. From this it may be seen that the optimal stability (maximum $\Delta G(T)$) value occurs at around 11°C. Both $\Delta H(T)$ and $\Delta S(T)$ were linear functions of temperature over the range 0–60°C (Fig. 5). The linear increase of $\Delta H(T)$ and $\Delta S(T)$ with increasing temperature may indicate an increase in the number of bonds being broken and the extent of structural disorder as the enzyme is heated. On the other hand, as the temperature is increased, hydrophobic

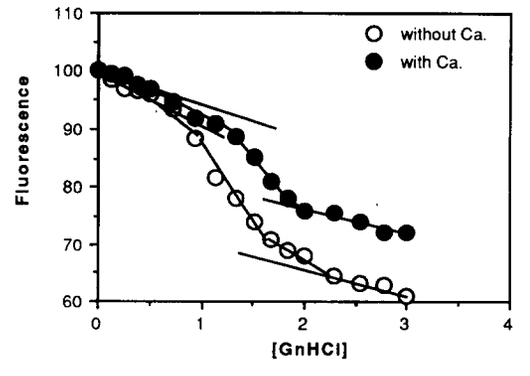

Fig. 2. GnHCl-unfolding of 2D lipase in the presence and absence of calcium.

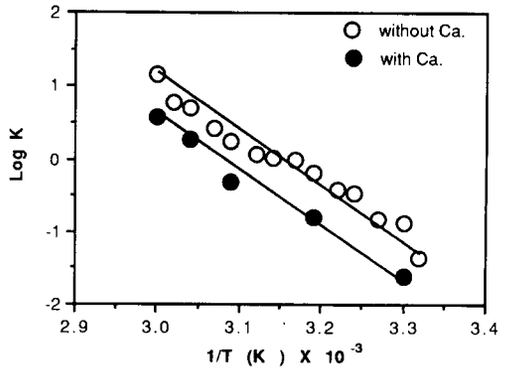

Fig. 3. Van't Hoff plot for the thermal unfolding.

interactions may strengthen. This process, which is endothermic, may also contribute to an increase in the enthalpy of unfolding at high temperatures (Myers, 1990). ΔH and ΔS values reflect the number of bonds broken and the change in structural disorder upon unfolding. The absolute values (Table 1) of ΔH and ΔS are each only slightly lower than those reported for lysozyme (Privalov & Khechinashvili, 1974) and bovine pancreatic ribonuclease A (Greene & Pace, 1974) However, the combined effects of ΔH and ΔS differences lead to relatively lower ΔG values for psychrotroph lipase (see below).

The thermodynamic stability of 2D lipase as measured by ΔG is significantly lower than values of about 40–60 kJ/mol reported for other globular proteins and enzymes, like bovine pancreatic ribonuclease A (Salahudin & Tanford, 1970; Greene & Pace, 1974; Privalov & Khechinashvili, 1974), hen egg white lysozyme (Aune & Tanford, 1969; Privalov & Khechinashvili, 1974), α-chymotrypsin (Greene & Pace, 1974; Knapp & Pace, 1974; Privalov & Khechinashvili, 1974) and α-lactalbumin (Kuwajima *et al.*, 1976). The reason for such differences in stability between psychrotroph lipase and other globular proteins is uncertain. Presumably differences in ΔG reflect the lower strength and/or numbers of stabilizing forces, e.g. hydrophobic interactions, hydrogen bonds, disulfide bridges and electrostatic interactions (Mozhaev & Martinek, 1984).

T_m and the midpoint of the GnHCl unfolding concentration ($[D]_{1/2}$) are also useful predictive quantities for enzyme stability. The T_m value for 2D lipase was 44·8°C, while the ($[D]_{1/2}$) was 1·26 M. The T_m value for

Table 1. Thermodynamic parameters for 2D lipase unfolding

Method	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol)	T_m (°C)	$[D]_{1/2}$
Thermal-unfolding (fluorescence)	9·5 11·8[a]	151·03 141·62[a]	475 435[a]	44·8 52·2[a]	—
Thermal-unfolding (temperature–activity data)	15·3	199·6	618	49·7	—
GnHCl-unfolding	6·5 7·4[a]	—	—	—	1·26 1·35[a]

[a] Values in the presence of added calcium (10 mM).

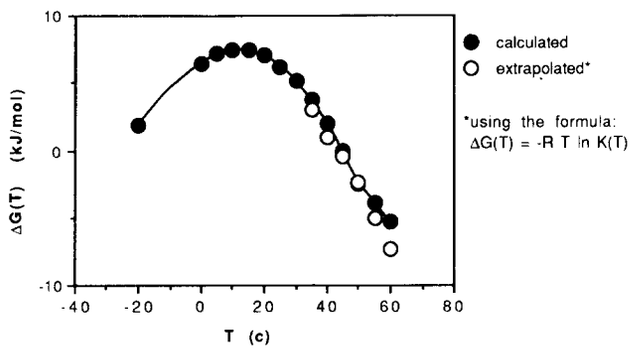

Fig. 4. Stability curve for 2D lipase.

2D lipase is close to the temperature optimum for lipase activity. The T_m corresponds to the temperature at which 50% of the enzyme molecules are unfolded. This unfolding to expose buried nonpolar groups occurs at temperatures where there is a strengthening of the hydrophobic interactions (at $T < 70°C$) and may lead to aggregation and loss of activity. However, conformational changes at moderate temperatures need not result in activity loss. In thermophilic enzymes such changes may lead to a rearrangement of structure (Mozhaev & Martinek, 1984). A thermal transition was seen to take place at 40–50°C for the enzyme prolamine transferase from the thermophilic *Sulfobus solfataricus* which resulted in an adjustment of its conformation to allow it to function at higher temperatures (Ragone *et al.*, 1992).

That the maximum structural ability of 2D lipase is at 11°C may be of practical importance. This temperature is close to the refrigeration temperatures used for milk storage. Hence there is a real potential for the spoilage of milks and dairy products stored for long periods at such refrigeration temperatures, where psychrotroph lipases are most stable.

ΔC_p is commonly accepted as a measure of the extent of exposure of nonpolar groups to water (Privalov, 1979). Such changes in heat capacity can be taken as constant for a given protein (Privalov & Khechinashvili, 1974). In the present study, the ΔC_p value for the thermal unfolding of 2D lipase was found to be 4 kJ/mol, which is comparatively lower than values of 6–8 kJ/mol/K reported for ribonuclease T1 (Pace & Laurents, 1989; Thomson *et al.*, 1989) and ribonuclease A (Pace & Laurents, 1989). The smaller ΔC_p for psychrotroph lipases compared to other globular proteins is consistent with two views. First, the native conformation in psychrotroph lipases may be less compactly folded and, secondly, the nonpolar groups in the denatured state could be less extensively exposed. The first postulate is highly probable, as supported by the low conformational stability of psychrotroph lipases (Owusu *et al.*, 1991).

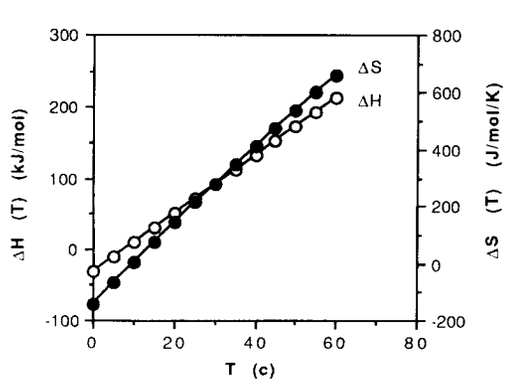

Fig. 5. Variation of enthalpy and entropy changes with temperature.

Addition of calcium (10 mM) to lipase prior to unfolding seems to have a stabilizing effect. In fact the presence of calcium increased the stabilization free energy by more than 2 kJ/mol and the T_m by more than 5°C. In the case of GnHCl-denaturation, the increase was around 1 kJ/mol and 0·1 M for $\Delta G(H_2O)$ and $[D]_{1/2}$, respectively.

The stabilizing effect of calcium has also been shown with other proteins. Calcium may stabilize thermolysin against denaturation by protecting the region near its binding sites from a cooperative conformational change (Dahlquist *et al.*, 1976). Likewise, bovine α-lactalbumin was found to assume a more rigid and compact native structure in the presence of calcium (Owusu, 1992).

Analysis of the 2D lipase temperature–activity profile resulted in an Arrhenius plot as described previously (Owusu *et al.*, 1991) there was a coincidence of calculated (V_c) and observed (V_{obs}) enzyme activity at low temperatures, indicating that no significant lipase denaturation had occurred at this range of temperatures. At higher temperatures, however, a deviation was observed, presumably resulting from lipase heat-unfolding. Similar observations were made for lipases from *Acinetobacter* and *H. lanuginosa* (Owusu *et al.*, 1991), *Ps. fluorescens* MC 50 (Adams & Brawley, 1981) and *Pseudomonas* strains P38, P46 and IOL3 (Makhzoum, unpublished).

The ΔG and T_m values obtained from temperature–activity profile data (Table 1) were higher than the values from direct studies. The former results may be higher due to the stabilizing effect of the substrate. The value of ΔG can also depend upon the type of detector used to monitor unfolding.

Ps. fluorescens 2D lipase exhibits the same significant UHT resistance and kinetic stability characteristics as *Ps. fluorescens* P38 lipase (Owusu *et al.*, 1992; Makhzoum, unpublished). Therefore the thermodynamic stability of 2D lipase discussed here may be at least partially representative of other psychrotroph lipases. Such a projection must be treated with some caution, but based on present results, it seems likely that psychrotroph lipases will in general exhibit low thermodynamic stability. Further study on lipases and other psychrotroph enzymes is needed to shed light on the mechanisms of heat resistance in these proteins.

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