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Urea and Heat Unfolding of Cold-Adapted Atlantic Cod (*Gadus morhua*) Trypsin and Bovine Trypsin

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Abstract: The reversible unfolding reactions for phenylmethylsulphonyl fluoride (PMSF)-modified trypins from Atlantic cod (cod PMS-trypsin) and cattle (bovine PMS-trypsin) were monitored by fluorescence spectrophotometry as a function of urea concentration and temperature. For urea unfolding at 25°C, the free energy change at zero concentration of urea ($\Delta G(H_2O)$) for cod PMS-trypsin was 11($\pm$4·4) kJ mol^{-1} compared with 18($\pm$1·14) kJ mol^{-1} for bovine PMS-trypsin, while the mid-point concentration for urea unfolding curve ($[urea]_{1/2}$) was 3·0($\pm$0·57) M and 4·1($\pm$0·16) M, respectively. From studies of enzyme heat unfolding, the mid point temperature of the thermal unfolding curve (T_m) was 46($\pm$1·4)°C for cod PMS-trypsin compared with 57($\pm$2)°C for bovine PMS-trypsin. The standard free energy change ($\Delta G°$) for reversible thermal unfolding of cod PMS-trypsin was 9($\pm$1) kJ mol^{-1} compared with 19($\pm$1) kJ mol^{-1} for bovine PMS-trypsin. Values for the enthalpy (ΔH_m), entropy (ΔS_m) and heat capacity (ΔC_p) for heat unfolding are compared. Results from urea and thermal unfolding studies show that cod PMS-trypsin has a significantly lower conformational stability than bovine PMS-trypsin.

Key words: fish trypsin, bovine trypsin, urea unfolding, thermal unfolding, heat stability.

INTRODUCTION

Cold-adapted proteinases from fish have some unique properties such as a lower inactivation temperature, high molecular activity at low temperature and an increased ability to catalyse the hydrolysis of native protein substrates when compared with proteinases from mammals, thermophilic organisms and plants (Simpson and Haard 1984, 1987; Simpson *et al* 1990). Highly active and thermolabile fish proteinases have potential applications in the commercial manufacture of cheese and protein hydrolysates (Simpson and Haard 1987; Haard 1992; Bjarnason and Asgeirsson 1993). The combination of high catalytic activity and low thermal stability is thought to be a consequence of the greater molecular flexibility of cold-adapted enzymes (Hultin 1978; Hochachka and Somero 1984). A general relationship between enzyme conformational stability, dynamics and catalytic efficiency appears to have been confirmed for enzymes isolated from mesophilic and thermophilic microorganisms (Huber 1979; Vihinen 1987; Varley and Pain 1991).

However, the conformational flexibility of fish proteinases has yet to be extensively studied. The crystal structures of bovine trypsin and trypsin from salmon were recently compared with the view of elucidating some possible mechanisms of cold adaptation (Smalas *et al* 1994). The isolation, characterisation and possible applications of cod digestive proteinases have been extensively studied (Asgeirsson *et al* 1989; Raae and Walther 1989; Simpson *et al* 1989, 1990; Haard 1992; Bjarnason and Asgeirsson 1993). For the time being, only a few reports concerning the irreversible inactivation of fish proteinases have been published (Bjarnason and Asgeirsson 1993; Amiza and Owusu Apenten 1994). At the time of writing, there have been no previous reports concerning urea and/or heat unfolding of trypsin from marine sources.

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The aim of this study is to compare the conformational stability of cold-adapted trypsin from Atlantic cod (*Gadus morhua*) with that of bovine trypsin. The conformational stability of a globular protein may be defined as the free energy change for the following reaction:

$$\text{native (folded)} \Leftrightarrow \text{denatured (unfolded)} \quad (1)$$

The thermodynamic or conformational stability of a protein can be measured as a function of temperature or denaturant concentration. Reversible unfolding studies allow measurement of conformational or thermodynamic stability. The methods of protein unfolding reaction thermodynamics involve a number of assumptions (Brandt 1964, 1965; Hermans 1965; Aune and Tanford 1969; Pfeil and Privalov 1979; Privalov 1979; Schellman and Hawkes 1980; Creighton 1990) such as, that the unfolding proceeds via a two-state mechanism and that the free energy may be obtained from the apparent values in the presence of denaturants by extrapolation to zero denaturant concentration (Aune and Tanford 1969; Schellman and Hawkes 1980; Pace 1986, 1990; Becktel and Schellman 1987; Santoro and Bolen 1988). For proteinases, it is important to perform unfolding studies under conditions where autolysis does not occur. Thus, it is essential to use covalently modified (eg inhibited) enzymes or extremes of pH. Cod trypsin is acid labile, and therefore studies cannot be performed at acidic pH. In this study, cod and bovine trypsins were modified with PMSF in order to avoid autolysis.

MATERIALS AND METHODS

Atlantic cod trypsin (Type XX-S, purified by affinity chromatography), bovine trypsin (both EC 3.4.21.4), Trizma base, benzoyl arginine nitrophenyl anilide (BAPA), phenylmethylsulphonyl fluoride (PMSF) and acrylamide were supplied by Sigma Chemical Co (Poole, UK). Urea was puris grade (Fluka Chemical Ltd, Glossop, UK). All other chemicals were of AnalaR grade from the BDH/Merck Ltd, (Lutterworth, UK).

Preparation of cod PMS-trypsin and bovine PMS-trypsin

PMSF-inhibited trypsin was prepared as follows. Trypsin powder was dissolved in Tris-HCl buffer (50 mM, pH 7·0) (1 mg ml^{-1}). Then PMSF solution (0·2 M in acetone; 100 μl for 10 mg trypsin) was added to the enzyme solution. The mixture was allowed to stand for 3 h at room temperature and then dialysed exhaustively against several changes of Tris-HCl (50 mM, pH 8·0) at 4°C overnight. The resulting bovine or cod PMS-trypsin was shown to retain less than 1% of the activity of native trypsin when assayed using benzoyl arginine nitrophenyl anilide (BAPA) as substrate.

Urea unfolding of cod PMS-trypsin and bovine PMS-trypsin

To determine the unfolding profile as a function of urea concentration, the same amounts of trypsin (0·2 ml; 1 mg ml^{-1} in 0·05 M Tris-HCl buffer, pH 8·0) were added to various concentrations of urea (prepared from a 10 M urea stock solution). The mixtures were allowed to stand for 3 h at 25°C to reach equilibrium. Fluorescence measurements were then made using an excitation and emission wavelengths of 295 nm and 340 nm for cod PMS-trypsin and 295 nm and 320 nm for bovine PMS-trypsin. These emission wavelengths were chosen to reflect the largest difference between the fluorescence of native and urea-unfolded enzyme (Fig 1). The instrument used was a fluorescence spectrophotometer (Perkin Elmer 203, Beaconsfield, Bucks, UK) fitted with a digital output and a thermoregulated twin cuvette

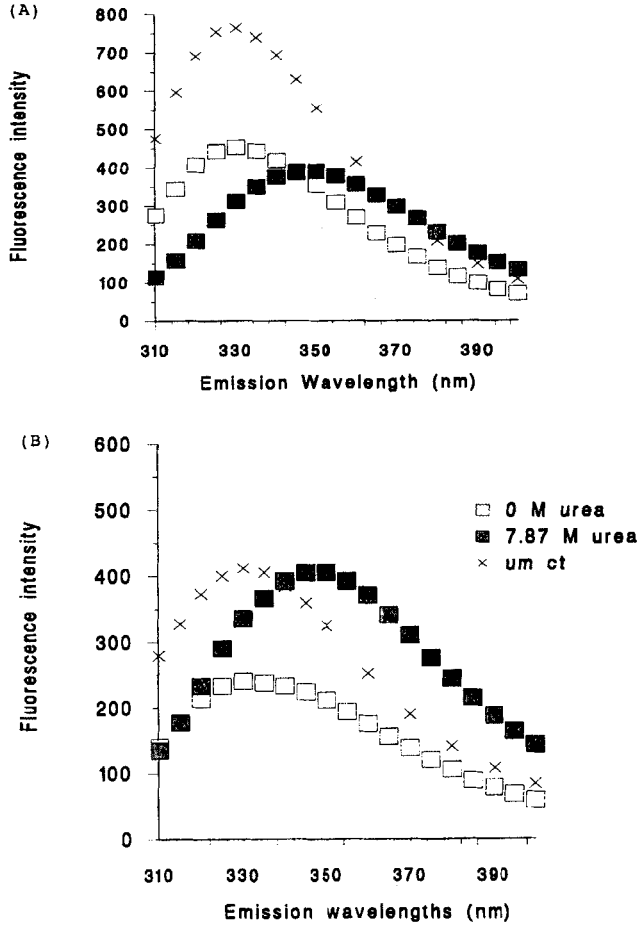

Fig 1. Fluorescence emission spectrum for X, unmodified trypsin; □, PMS-trypsin; and ■, urea unfolded PMS-trypsin. (A) Data for bovine trypsin, and (B) data for cod trypsin.

holder, essentially as described previously (Owusu Apenten 1992). Temperature control was by means of circulation from a water bath.

Thermal unfolding of cod PMS-trypsin and bovine PMS-trypsin

Cod PMS-trypsin or bovine PMS-trypsin samples (0·03–0·1 mg ml^{-1} in 50 mM Tris-HCl buffer, pH 8·0) were heated in a Teflon-stoppered quartz cuvette at 2–90°C. The fluorescence excitation and emission wavelength used in all thermal unfolding experiments was 295 nm and 315 nm, respectively. Sample temperatures were monitored using a thermocouple immersed in a 'reference' cuvette situated adjacent to the sample cuvette. After each experiment heated enzyme samples were cooled to room temperature in order to discover whether thermal transitions were reversible. Urea and thermal unfolding profiles were calculated from fluorescence measurements (see below) with the aid of spread sheets (Microsoft Excel, Microsoft Corp, USA). The algorithm used in conjunction with the spreadsheet is described below (eqns **2–4**).

Fluorescence quenching studies with acrylamide

Acrylamide monomer was dissolved in Tris-HCl buffer (0·5 M, pH 8·0) to form a 0·6 M stock solution. To examine the degree of solvent accessibility of tryptophan residues, a measurement of acrylamide quenching was made by adding 0–1·5 ml of acrylamide stock solution to a sample of PMS-trypsin. Fluorescence readings were then taken with an excitation wavelength of 295 nm and emission wavelength of 315 nm, as described before.

Stability estimates from urea and thermal unfolding profiles

Analysis of unfolding profiles

For both urea- and thermal-unfolding profiles, the fraction of unfolded enzyme (F_u) and unfolding equilibrium constant (K_u) were determined from the changes in fluorescence emission using eqns (**2**) and (**3**):

$$F_u = (y_N - y_{obs})/(y_N - y_D) \quad (2)$$

$$K_u = (1 - F_u)/F_u = (y_N - y_{obs})/(y_{obs} - y_D) \quad (3)$$

where y_{obs} is the observed fluorescence intensity while y_N and y_D represent the fluorescence intensity of the folded and unfolded states, respectively. y_N and y_D are assumed to show a linear dependence on urea concentration and temperature (Permyakov and Burhstein 1984; Santoro and Bolen 1988; Pace 1990).

Conformational stability towards urea unfolding

From the graph of free energy change (ie $\Delta G = -RT \ln K_u$, where R is the gas constant and T is the absolute temperature) versus urea concentration, a straight line relation exits according to eqn (**4**)

$$\Delta G = \Delta G(H_2O) - m[\text{urea}] \quad (4)$$

where $\Delta G(H_2O)$ is the free energy change at zero concentration of urea and m (kJ mol^{-1} M^{-1}) is the slope of the plot (Pace 1986, 1990; Santoro and Bolen 1988). The mid-point concentration for urea unfolding curve, $[\text{urea}]_{1/2}$ can be obtained from the plot of fraction unfolded versus urea concentration or from eqn (**4**), ie when ΔG is zero the $[\text{urea}]_{1/2}$ value is given by $\Delta G(H_2O)/m$.

Conformational stability towards thermal unfolding

The temperature dependence of ΔG can be expressed as a second order polynomial (Brandt 1964, 1965; Shiao *et al* 1971; Cantor and Schimmel 1980; Ragone *et al* 1992; Owusu Apenten and Berthalon 1994).

$$\Delta G = A + BT + CT^2 \quad (5)$$

The constants A, B and C were determined by non-linear regression of ΔG versus T (K) data. A, B and C are a function of the enthalpy (ΔH), entropy (ΔS) and heat capacity change (ΔC_p) for enzyme unfolding according to eqn (**6**).

$$\Delta H = A - CT^2 \quad (6a)$$

$$\Delta S = -(B - 2CT) \quad (6b)$$

$$\Delta C_p = 2CT \quad (6c)$$

The use of eqn (**5**) and eqn (**6**) has been described previously in connection with the heat unfolding of chymotrypsinogen, ribonuclease, chymotrypsin and propylamine transferase (Brandt 1964, 1965; Shiao *et al* 1971; Ragone *et al* 1992; Owusu Apenten and Berthalon 1994).

RESULTS AND DISCUSSION

Urea unfolding of cod and bovine PMS-trypsins

Figure 1(A) and (B) show the fluorescence emission spectrum for unmodified trypsins, PMS-trypsins and urea-unfolded PMS-trypsins. In all cases the fluorescence excitation wavelength was 295 nm at which wavelength only tryptophan residues were excited. It is generally accepted that there is some relationship between the wavelength of the fluorescence emission spectrum peak maximum (λ_{max}) and the exposure of tryptophan in a protein (Brand and Witholt 1967; Teale 1960; Burhstein *et al* 1973). It is notable that λ_{max} was the same (330 nm) for cod PMS-trypsin, bovine PMS-trypsin and unmodified cod or bovine trypsins.

These data suggest that the tryptophan residues in unmodified trypsins and PMS-trypsins were equally accessible to solvent. It would seem that PMSF modification of the active site serine within trypsin does not result in a global conformational change affecting the rest of the enzyme (see below).

A suitable emission wavelength for monitoring urea unfolding was determined from the fluorescence spectra (Fig 1) by choosing the wavelength where the greatest difference in fluorescence emission was noted for PMS-trypsin and urea-unfolded PMS-trypsin. The fluorescence intensity increased for urea-unfolded cod PMS-trypsin over the whole range of wavelengths (300–400 nm). For urea-unfolded bovine PMS trypsin the fluorescence intensity decreased before 345 nm and increased after 345 nm. Figure 2 shows the urea-unfolding profiles for cod PMS-trypsin and bovine PMS-trypsin at 25°C. The straight lines fitted for y_N and y_D (obtained by using least square method) represent the pre- and post-unfolding correction (Pace 1986, 1990; Santoro and Bolen 1988). Using the analysis described above, the fraction unfolded (F_u) can be calculated. Figure 3 shows the fraction of unfolded PMS-trypsins at the various urea concentrations. The profiles show clearly that the concentration of urea needed to denature bovine PMS-trypsin was higher compared with the concentration of urea required to denature cod PMS-trypsin. This indicates that the conformation of cod trypsin is less stable than bovine trypsin.

Figure 4 shows the plot of free energy change (ΔG) versus urea concentration. The extrapolation to zero urea concentration was done by using a linear extrapo-

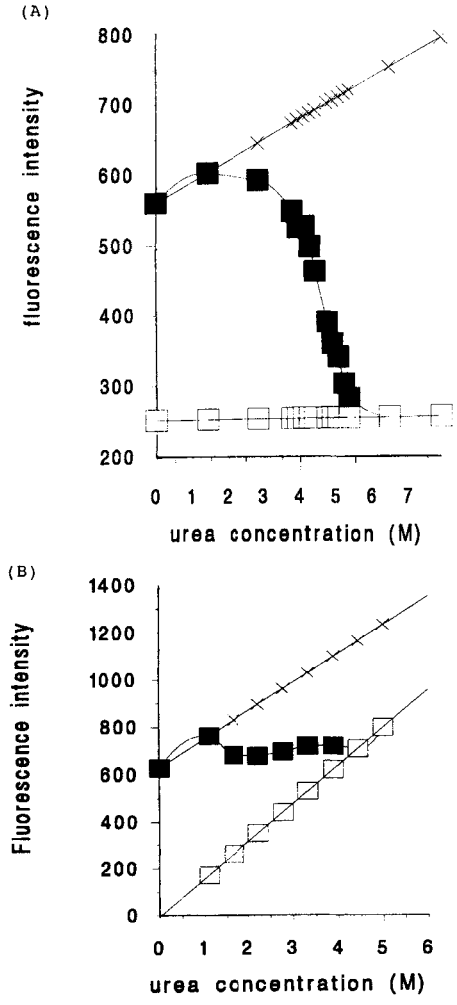

Fig 2. ■, The effect of urea on intrinsic fluorescence emission intensity for (A) bovine PMS-trypsin, and (B) cod PMS-trypsin. The linear lines represent the fluorescence intensity of X, native; and □, unfolded PMS-trypsin. Conditions (1 mg ml^{-1} PMS-CT in 50 mM Tris-HCl buffer solution, pH 8·0; temperature = 25°C).

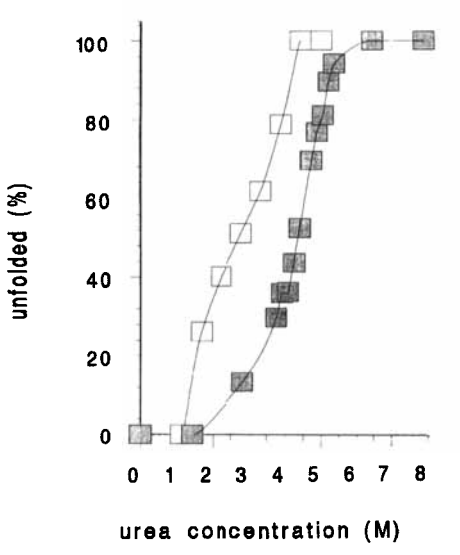

Fig 3. Urea unfolding profiles for □, cod PMS-trypsin, and ■, bovine PMS-trypsin.

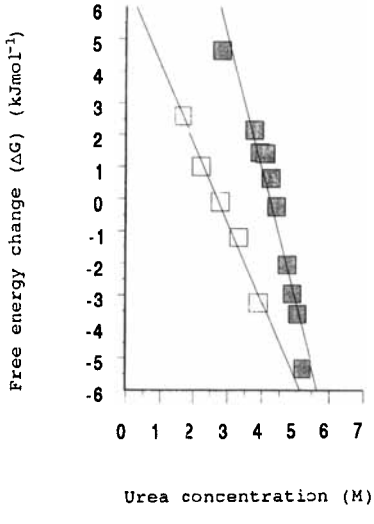

Fig 4. Determination of conformational stability ($\Delta G(H_2O)$) of □, cod PMS-trypsin; and 8 ■, bovine PMS-trypsin by linear extrapolation of urea unfolding data (see text for details).

TABLE 1
Thermodynamic parameters for the reversible urea unfolding of Atlantic cod PMS-trypsin and bovine PMS-trypsin[a]

Parameter	*Atlantic cod PMS-trypsin*	*Bovine PMS-trypsin*
Slope	3.5 (±0·96) kJ mol^{-1} M^{-1}	4·4 (±0·44) kJ mol^{-1} M^{-1}
$\Delta G(H_2O)$	11 (±4·4) kJ mol^{-1}	18 (±1·14) kJ mol^{-1}
$[Urea]_{1/2}$	3·0 (±0·57) M	4·1 (±0·16) M

[a] Data is the average of at least three experiments. Standard deviation in parentheses.

lation method. The results are summarised in Table 1. Values of $\Delta G(H_2O)$, $[urea]_{1/2}$, and m (kJ mol^{-1} K^{-1}) for cod PMS-trypsin urea unfolding were all lower than the corresponding values for bovine PMS-trypsin. These results are all consistent with the view that the native conformation of cod trypsin is less stable to urea unfolding compared to bovine trypsin. The slope (m) in Fig 4 is a measure of the suddenness or co-operativity of the protein unfolding transition in urea. The slope parameter (m) increases for proteins possessing a highly compact globular structure (Pace 1990). Therefore, under the present conditions, cod trypsin would appear to present a less compact and co-operative structural system compared with bovine trypsin.

Thermal unfolding of cod and bovine PMS-trypsins

The intrinsic fluorescence of cod PMS-trypsin and bovine PMS-trypsin changes with temperature as shown in Fig 5. Figure 6 shows the thermal unfolding profiles for cod PMS-trypsin and bovine PMS-trypsin. At temperatures of 20–90°C both enzymes produced a classical sigmoidal unfolding profile consistent with a two-state process, $N \Leftrightarrow D$. The thermal unfolding transitions were fully reversible under the conditions of this study. Thus cooling a sample of enzyme, which had previously been heated to 90°C, restored the initial fluorescence characteristics (data not shown). Cooling both PMS-trypsin below about 10°C appeared to lead to a small but significant increase in the folded structure. In the following discussion only the major thermal unfolding transition occurring between 25 and 90°C will be discussed.

It is clear from Fig 6 that the native conformation of cod PMS-trypsin is heat labile compared with bovine PMS-trypsin. Thermal unfolding of cod PMS-trypsin began at 40°C compared with 50°C for bovine PMS-trypsin. Such results are in agreement with the finding that heating to about 40°C leads to irreversible thermal unfolding of Atlantic cod trypsin (Simpson *et al* 1990; Amiza and Owusu Apenten 1994). The T_m value for cod PMS-trypsin was 46(±1·4)°C (Table 2). This value for T_m is consistent with the observed activity–temperature optimum (T_{opt}) of about 40–50°C for Atlantic cod trypsin (Asgeirsson *et al* 1989; Simpson *et al* 1990). The T_m for bovine PMS-trypsin was 57(±2)°C showing that this enzyme is significantly more heat stable than Atlantic cod trypsin.

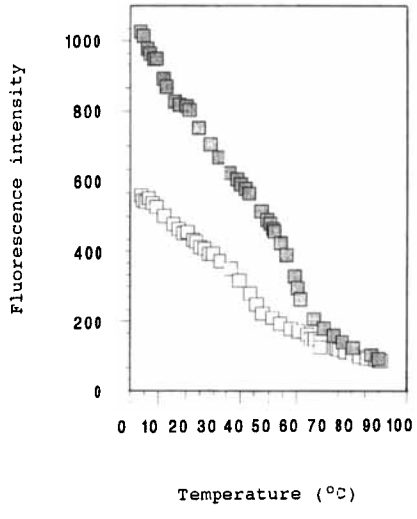

Fig 5. Effect of temperature on the fluorescence intensity of □, cod PMS-trypsin; and ■, bovine PMS-trypsin.

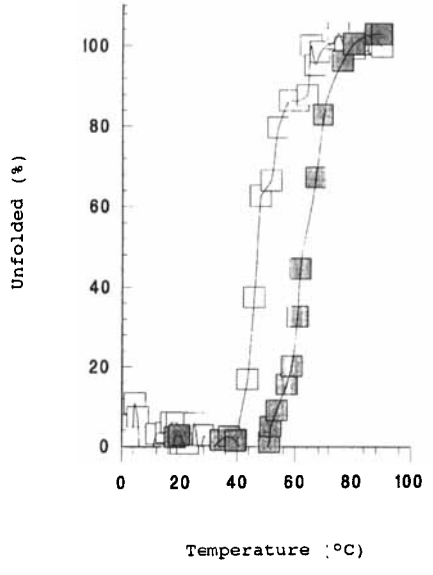

Fig 6. Thermal unfolding profiles for □, cod PMS-trypsin; and ■, bovine PMS-trypsin.

TABLE 2
Thermodynamic parameters for the reversible thermal unfolding of Atlantic cod PMS-trypsin and bovine PMS-trypsin[a]

Parameter	*Atlantic cod PMS-trypsin*	*Bovine PMS-trypsin*
A	-1.59×10^6	-2.16×10^6
B	1.08×10^4	1.45×10^4
C	−18·20	−24·00
ΔH_m (kJ mol^{-1})[b]	286 [8·7]	446 [6.9]
ΔS_m (kJ mol^{-1} K)[b]	812 [−0·1]	1350 [−40·4]
ΔC_p (kJ mol^{-1} K)[c]	11·6	16·0 [14·4]
$\Delta G°$ (kJ mol^{-1})[c]	9 (±1)	19 (±1)
T_m (°C)	46 (±1·4)	57 (±2)
T_{max} (°C)	23	29

[a] PMS-trypsin (0·03–0·1 mg ml^{-1}) in Tris-HCl buffer (50 mM, pH 8·0). Precision of thermodynamic parameters ((x/SD) × 100) was 13·5%.
[b] Thermodynamic values in square brackets are estimates when $T = T_{max}$ (see Fig 7).
[c] Mean of three experiments, standard deviation in parentheses.

The stability curves for cod and bovine PMS-trypsins are given (Fig 7) as a graph of experimental and theoretical ΔG values (calculated according to eqn (5), continuous line) plotted against temperature. Values for the constants, A, B, C together with thermodynamic parameters (see eqn (6)) are listed in Table 2. The average precision (ie (mean/SD) × 100) for the data shown was 13·5%.

There are essentially two approaches for determining ΔC_p for protein unfolding. The direct approach uses differential scanning calorimetric (DSC) measurements. The ΔC_p value can be directly recorded from the shift in the baseline of a thermogram before and after the main DSC peak (Privalov 1979). Indirect methods for estimating ΔC_p are ultimately based on the use Kirchoff's and related functions (eqn (7))

$$d\Delta H/dT = \Delta C_p \quad (7a)$$

$$\Delta H = \Delta H_m + \Delta C_p(T - T_m) \quad (7b)$$

$$\Delta S = \Delta S_m + \Delta C_p \ln (T/T_m) \quad (7c)$$

In eqn (7), T_m is defined as the mid-point temperature for heat-unfolding whilst ΔS_m and ΔH_m are, respectively, the entropy and enthalpy change for heat-unfolding at a temperature equal to T_m. As ΔH for protein heat-unfolding is generally independent of pH (Privalov 1979), thermal unfolding experiments carried out at different pH values affords a set of ΔH and T_m values from which ΔC_p can be estimated using $\Delta C_p = \Delta H_m/\Delta T_m$. In relation to eqn (7a), changes in urea, guanidine hydrochloride as well as pH can be employed in order to reduce the T_m. Difficulties may arise where a change in the solvent conditions lead to a change in the pathway for thermal unfolding (Blumenfeld 1981; Pace and Laurent 1989; Pace 1990).

A further method for estimating ΔC_p indirectly, employs a combination of eqns (7b) and (7c) leading to the Gibbs–Helmholtz relation (eqn (8))

$$\Delta G = \Delta H_m(1 - T/T_m) + \Delta C_p(T - T_m + \ln (T/T_m)) \quad (8)$$

which shows the temperature dependence of ΔG or the stability curve for a given enzyme (Fig 7). The stability curve, which is produced by a form of extrapolation, apparently shows the complete enzyme thermal unfolding profile even at temperatures where ΔG is not measurable experimentally. The limitations and possible uses of enzyme stability curves have been extensively discussed by Becktel and Schellman (1987).

Provided that ΔG values can be determined experimentally over a sufficiently wide temperature range, non-linear regression of ΔG versus T data using eqn (8) will yield precise estimates ΔC_p, ΔH_m and T_m (Becktel and Schellman 1987; Pace 1990). To increase the reliability of ΔC_p values determined using the Gibbs–Helmholtz equation (eqn (8)) it has been proposed that ΔG values from thermal unfolding experiments might be combined with $\Delta G(H_2O)$ values determined from thermal unfolding studies carried out in the presence of urea. This innovation has the effect of providing ΔG data at relatively low temperatures where uncertainty associated with curve-fitting tends to be greatest (Pace and Laurent 1989).

In the current work we have also employed non-linear regression curve fitting although using Brandt's empirical expression (eqn (5)) to describe the relation

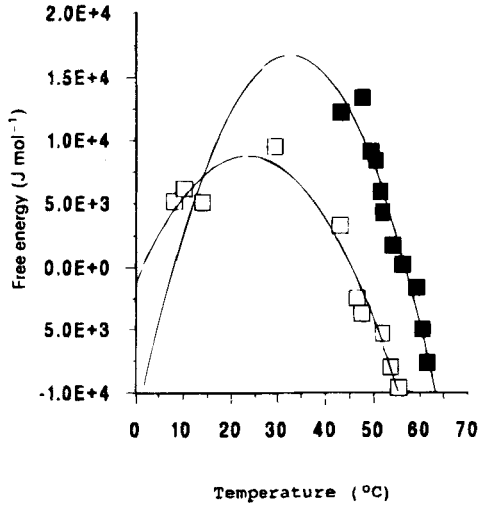

Fig 7. Stability curves for □, cod PMS-trypsin; and ■, bovine PMS-trypsin. Continuous lines show non-linear regression line fit to the eqn (5) (see text). Parameters A, B and C are in Table 2.

between ΔG and T. The advantage of eqn (**5**) over eqn (**8**) is that the former allows readier access to ΔS and ΔH values and their temperature dependence according to eqn (**6**). The use of Brandt's function has been shown to lead to reliable results in heat-unfolding studies for chymotrypsinogen, irreversibly inactivated chymotrypsins (Brandt 1964; Shiao *et al* 1971), ribonuclease (Brandt 1965; Shiao *et al* 1971) propylamine transferase (Ragone *et al* 1992) and non-modified α-chymotrypsin (Owusu Apenten and Berthalon 1994). Indeed, Shiao *et al* (1971) have shown that, for the case of ribonuclease, using Brandt's expression (eqn (**5**)) or the Gibbs–Helmholtz relation (eqn (**8**)) leads to similar ΔC_p estimates.

ΔG estimates from urea and thermal unfolding (Tables 1 and 2) agree, within experimental error. This suggest that urea-unfolding and thermal unfolding transitions occur via a similar two-state reaction.

The stability curves (graph of ΔG versus T, Fig 7) for cod PMS-trypsin and bovine PMS-trypsin show that the former enzyme is apparently more thermolabile. From the turning point in Fig 7 ($\delta\ \Delta G/\delta T \approx 0$ or where ΔG reaches a maximum) the temperature for maximum conformational stability (T_{max}) for cod PMS-trypsin was 23°C compared with a T_{max} value of about 28°C for bovine PMS-trypsin. At the T_{max}, the free energy change for enzyme heat-unfolding (ΔG_{max}) was 8·7 (kJ mol^{-1}) for cod PMS-trypsin and 19·0 (kJ mol^{-1}) for bovine PMS-trypsin (Table 2). Because ΔG shows only a slight temperature dependence near room temperature the standard free energy value ($\Delta G°$) given in Table 2 is not markedly different from the ΔG_{max} values discussed above.

The difference in the conformational stability of Atlantic cod trypsin and bovine trypsin can be assessed from the values of K_u (ie [native]/[unfolded] *cf* eqn (**1**)) at 25°C. For each enzyme $K_u = \exp(-\Delta G°/RT)$. Using data from in Table 1, the ratio of K_u values for Atlantic cod trypsin and bovine trypsin is 57 : 1. That is, at a temperature of 25°C cod PMS-trypsin was approximately 60-fold less heat stable compared with bovine PMS-trypsin. The differences in heat stability diminishes at lower temperatures; at about 0–5°C cod trypsin may be more stable than bovine trypsin (Fig 7).

Cod trypsin has a greater number of charged amino acids as well as a higher ratio of acidic to basic amino acids (Asgeirsson *et al* 1989; Simpson *et al* 1990). The average hydrophobicity of cod trypsin is also significantly lower than the value for bovine trypsin (Asgeirsson *et al* 1989; Simpson *et al* 1990). It has been suggested that such structural differences would render cod trypsin more susceptible to charge–charge (de)stabilisation under low ionic strength conditions. By comparison with bovine trypsin, hydrophobic interactions would also be less important for the stabilisation of cod trypsin (Asgeirsson *et al* 1989; Simpson *et al* 1990; Asgeirsson and Bjarnason 1980). However, the literature is replete with reports of homogeneous enzymes from mesophilic and thermophilic enzymes with greatly differing heat stability (Amelunxen and Murdock 1978; Jaenicke 1981; Daniel et al 1982; Owusu and Cowan 1989; Varley and Pain 1991; Cowan 1992). So far, natural mechanisms for enzyme thermal stabilisation have been difficult to identify.

The comparative heat stability of cod and bovine trypsins is perhaps only approximately reflected by results of this study. Specifically it is uncertain whether modification with PMSF increases the heat stability of trypsins. Shreier *et al* (1984) have shown that binding of low molecular weight inhibitors can stabilise proteinases against thermal unfolding. In experiments involving a thermostable proteinase (thermitase) it was shown that the degree of stabilisation was positively correlated with the enzyme–inhibitor binding constant. Modification of the active site serine of thermitase with dansyl chloride or diisopropyl fluorophosphate produced a 10–50% increase in the heat stability of thermitase. The stability of PMS-chymotrypsin toward urea unfolding is apparently greater than the stability of the unmodified enzyme (Santoro and Bolen 1988). The degree of stabilisation arising from inhibitor binding may depend on the nature of the enzyme. Based on the fluorescence results referred to above, it is unlikely that modification with PMSF leads to any significant changes in the heat stability of cod trypsin relative to that of bovine trypsin.

Comparing ΔH_m values (Table 2) it is clear that the thermal unfolding reaction for cod PMS-trypsin appears to be less endothermic compared with the unfolding reaction for bovine PMS-trypsin. The entropy change for unfolding (ΔS_m) was also lower for cod trypsin. Assuming that the heat-unfolded state is the same for both enzymes the lower ΔS_m implies that the native conformation for cod PMS-trypsin has a higher conformational entropy. That is, the native conformation of cod PMS-trypsin appears to be more disordered or flexible compared with native bovine PMS-trypsin.

A comparison of ΔH_m and ΔS_m values is complicated by temperature dependence of both parameters (eqn (**7**)). There was a significant heat capacity (ΔC_p) change for the thermal unfolding of cod PMS-trypsin and bovine PMS-trypsin (Table 2). Allowing for the observed 10°C difference in T_m values there is apparently no numerical difference in ΔH and ΔS values for the two enzymes. For example, using results from Table 2, the ΔH value for bovine PMS-trypsin unfolding (*were unfolding to occur at 46°C*) can be calculated (using eqn (**7b**) or $\Delta H = \Delta H_m + \Delta C_p$ [46–57]) as 286 kJ mol^{-1} which is identical to the enthalpy change for Atlantic cod PMS-trypsin unfolding. In view of these observations, it would appear that the important differences lie in the value of ΔC_p.

The results of fluorescence quenching studies (Fig 8) suggest that there are at least two classes of tryptophan residues in bovine or cod trypsin distinguishable by the

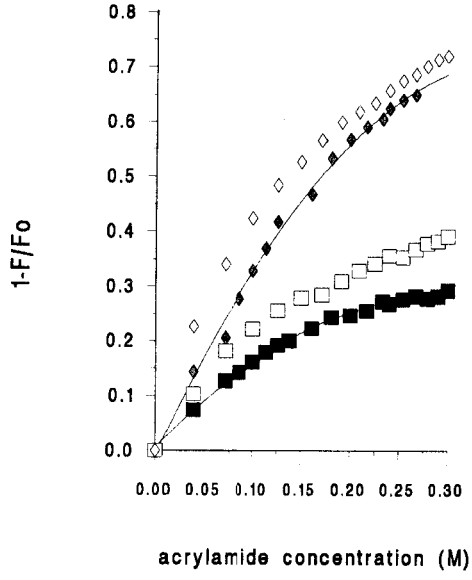

Fig 8. Quenching studies with acrylamide for □, cod PMS-trypsin; and ■, bovine PMS-trypsin at pH 8 (50 mM Tris-HCl) compared to that of cod ◇, cod PMS-trypsin; and ◆, bovine PMS-trypsin at pH 4·4 (50 mM Gly–Gly buffer).

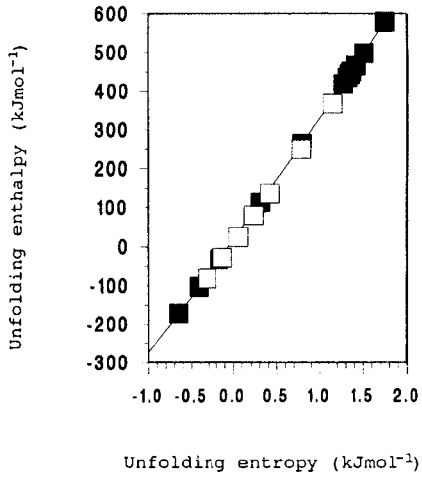

Fig 9. Enthalpy/entropy interdependence for thermal denaturation reactions for □, cod PMS-trypsin; and ■, bovine PMS-trypsin.

degree of accessibility to acrylamide. The slope in Fig 8 is proportional to the bimolecular rate constant for collision between quencher and chromophore. The greater slopes observed for cod trypsin indicate that this enzyme is more flexible compared with the bovine trypsin. Interestingly, the crystal structures of bovine trypsin and a cold adapted trypsin from salmon show no differences in their overall molecular flexibility. However, there were noticeable differences in the inter-domain mobility and in the autolysis loop of salmon and bovine trypsins (Smalas *et al* 1994).

In view of the temperature dependence of thermodynamic parameters, ΔH and ΔS terms were also compared at standard temperature (25°C). The standard thermodynamic parameters are related according to the relation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, and it is significantly that for the thermal unfolding of bovine PMS-trypsin $-T\Delta S^\circ > \Delta H^\circ$. By contrast, for the thermal unfolding of Atlantic cod PMS-trypsin $\Delta H^\circ > -T\Delta S^\circ$. A larger $-T\Delta S^\circ$ term compared with ΔH° can be taken as indicating that hydrophobic or solvophobic interactions play a major role (compared with charge–charge interactions or hydrogen bonding) in stabilising the native conformation of bovine PMS-trypsin. In the case of Atlantic cod PMS-trypsin, a predominant enthalpy term suggests that stabilisation is mainly by electrostatic or H-bonding interactions. Such differences in thermodynamic parameters are apparently consistent with the lower degree of hydrophobicity and higher net charge present in the Atlantic cod trypsin molecules (Nojima *et al* 1977).

Finally, Fig 9 shows a graph of ΔH values plotted against values of ΔS. The thermodynamic data for both enzymes fit a single line as might be expected for two essentially similar proteins. The slope of this line was 312 K. The linear correlation between ΔH and ΔS values may be evidence that there is an enthalpy–entropy compensation phenomenon during trypsin thermal unfolding. An entropy–enthalpy phenomenon is characteristic of physicochemical processes (such as protein unfolding, (eqn (**1**)) where formation of a reaction product (unfolded enzyme) is associated with changes in solvation (Labuza 1980, and references therein). The unfolding of a globular protein is allied with an increase in the solvent accessibility of hydrophobic amino acid residues initially buried in the interior of the native protein. The resulting immobilisation of solvent molecules via clathrate formation results in an increase in the specific heat capacity (C_p) of a solution containing unfolded protein compared with native protein (Brandt 1964, 1965; Shiao *et al* 1971; Privalov 1979; Pfeil and Privalov 1979; Becktel and Schellman 1987). From Table 2 it can be seen that ΔC_p (the specific heat capacity difference between the native and thermally unfolded enzyme) is significantly greater for the thermal unfolding of bovine PMS-trypsin compared with the value for Atlantic cod PMS-trypsin. Therefore nonpolar residues within the *native* conformation of bovine PMS-trypsin appear to be much more completely removed from solvent than the corresponding groups in Atlantic cod PMS-trypsin. It has been demonstrated that the magnitude of ΔC_p is positively correlated with the number of non-polar residue contacts within a globular protein (Privalov 1979).

In conclusion, the results of this study suggest that the native conformation of cod trypsin is less heat stable compared with the native conformation of bovine trypsin at room temperature. The differences in the conformational stability of the two enzymes may arise from a change in the ΔC_p value for the heat unfolding. It is tempting to infer that the major strategy for cold-

adaptation in cod trypsin appears to be the reduction of hydrophobic contacts within the globular structure. Further studies, involving a wider range of solvent conditions, are necessary in order to produce a better appreciation of the molecular strategies for cold-temperature adaptation in Atlantic cod trypsin.

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REFERENCES

Amelunxen R E, Murdock A L 1978 In *Microbial Life in Extreme Environments*, ed Kushner D J Academic Press, New York, USA, pp 217–278.

Amiza M A, Owusu Apenten R K 1994 Thermal inactivation for alkaline proteinases from North Sea cod (*Gadus morhua*) and bovine α-chymotrypsin. *J Sci Food Agric* **66** 389–391.

Asgeirsson B, Bjarnason J B 1989 Structural and kinetic properties of chymotrypsin from Atlantic cod (*Gadus morhua*). Comparsion with bovine chymotrypsin. *Comp Biochem Physiol* **99B** 327–335.

Asgeirsson B, Fox J W, Bjarnason J B 1989 Purification and characterisation of trypsin from the poikilotherm *Gadus morhua*. *Eur J Biochem* **180** 85–94.

Aune K C, Tanford C 1969 Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. II. Dependence on denaturant concentration at 25°C. *Biochemistry* **8** 4586–4590.

Becktel W J, Schellman J A 1987 Protein stability curves. *Biopolymers* **26** 1859–1877.

Bjarnason J B, Asgeirsson B 1993 Psychrophilic proteolytic enzymes from Atlantic cod: their characteristics and applications. *Genetic Engr Biotechnol* **13** 31–39.

Blumenfeld L A 1981 *Problems in Biological Physics*. Springer-Verlag, Berlin, Germany, pp 49–91.

Brand L, Witholt B 1967 Fluorescence measurements. *Meth Enzymol* **11** 776–856.

Brandt J F 1964 The thermodynamics of protein denaturation 1. The denaturation of chymotrypsinogen. *J Am Chem Soc* **86** 4291–4301.

Brandt J F 1965 The nature of the complexities in the ribonuclease conformational transition and implications regarding clathirating. *J Am Chem Soc* **87** 2759–2760.

Burhstein E A, Vedenskina N S, Ivkona M N 1973 Fluorescence study of actin. *Photochem Photobiol* **18** 263–279.

Cantor C R, Schimmel P R 1980 *Biophysical Chemistry III: The behaviour of Biological Macromolecules*. W H Freeman & Co., San Francisco, CA, USA, pp 1075–1107, A-17.

Cowan D A 1992 Enzymes from thermophilic *Archaebacteria*—current and future applications in biotechnology. *Biochem Soc Symp* **58** 149–169.

Creighton T E 1990 Protein folding. *Biochem J* **270** 1–16.

Daniel R M, Cowan D A, Morgan H W, Curan M P 1982 Correlation between protein heat stability and resistance to proteolysis. *Biochem J* **207** 641–644.

Gildberg A 1992 Recovery of proteinases and protein hydrolysates from fish viscera. *Bioresource Technol* **39** 271–276.

Haard N F 1992 A review of proteolytic enzymes from marine organisms and their applications in the food industry. *J Aquatic Food Product Technol* **1** 17–35.

Hermans J 1965 Methods for the study of reversible denaturation of proteins and interpretation of data. *Meth Biochem Anal* **13** 81–111.

Hochachka P W, Somero G N 1984 *Biochemical Adaptation*. Princetown University Press, Surrey, UK.

Huber R 1979 Conformational flexibility and its functional significance in some proteins. *Trends Biochem Sci* **4** 271–274.

Hultin H O 1978 Enzymes from organisms acclimated to low temperatures. In *Enzymes: The Interface Between Technology and Economics*, eds Danehy J D & Wolnak B. Marcel Dekker, New York, USA, pp 161–178.

Labuza, T P 1980 Enthalpy/entropy compensation in food reactions. *Food Technol* **Feb** 67–77.

Nojima H, Ikai A, Oshima T, Noda H 1977 Reversible thermal unfolding of thermostable phosphoglycerate kinase. Heat stability associated with mean zero enthalpy change. *J Molec Biol* **116** 429–442.

Owusu R K 1992 The effect of calcium on bovine α-lactalbumin conformational transitions by ultraviolet difference and fluorescence spectrophotometry. *Food Chem* **43** 41–45.

Owusu Apenten R K 1995 A three-state heat denaturation of bovine α-lactalbumin. *Food Chem* **52** 131–133.

Owusu R K, Cowan D A 1989 Correlation between microbial protein heat stability and resistance to denaturation in aqueous: organic two-phase systems. *Enzyme Microb Technol* **11** 468–474.

Owusu Apenten R K, Berthalon N 1994 Determination of enzyme global heat stability from equilibrium and kinetic analysis of heat inactivation. *Food Chem* **51** 15–20.

Pace C N 1986 Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Meth Enzymol* **131** 266–280.

Pace C N 1990 Measuring and increasing protein stability. *Trends Biotechnol* **8** 93–97.

Pace C N, Laurent D V 1989 A new method for determining the heat capacity for protein folding. *Biochemistry* **28** 2520–2525.

Permyakov E A, Burhstein E A 1984 Some aspects of studies of thermal transitions in proteins by means of their intrinsic fluorescence. *Biophys Chem* **19** 265–271.

Pfeil W, Privalov P L 1979 Conformation changes in proteins. In *Biochemical Thermodynamics*, ed Jones N M. Elsevier, Oxford, UK, pp 75–115.

Privalov P L 1979 Stability of proteins. Small globular proteins. *Adv Protein Chem* **33** 161–241.

Raae A J, Walther B T 1989 Purification and characterisation of chymotrypsin, trypsin and elastase like proteinases from cod (*Gadus morhua*). *Comp Biochem Physiol* **93B** 317–324.

Ragone R, Facchiano F, Cacciapouti G, Porcelli M, Collonna G 1992 Effect of temperature on the propylamine transferase from *Sulfolobus solfataricus* an extreme thermophilic archaebacterium. 2. Denaturation and structural stability. *Eur J Biochem* **204** 483–490.

Santoro M M, Bolen D W 1988 Unfolding free energy changes determined by the linear extrapolation method. I. Unfolding of phenylmethylsulfonyl alpha-chymotrypsin using different denaturants. *Biochemistry* **27** 8063–8068.

Schellman J A, Hawkes R B 1980 *Protein Folding: Proc. 28th German Biochemical Society, Regensburg*, ed Jaenicke R. Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands, pp 331–341.

Shiao D F, Lumry R, Fahey J 1971 Studies of the chymotrypsinogen family of proteins. XI. Heat capacity changes

accompanying reversible thermal unfolding of proteins. *J Am Chem Soc* **93** 2024–2035.

Shreier E, Fittkau S, Horne W E 1984 Influence of synthetic peptide inhibitors on the thermal stability of thermitase, a serine proteinase from *Thermoactinomyces vulgaris*. *Int J Peptide Protein Res* **23** 134–141.

Simpson B K, Haard N F 1984 Trypsin from Greenland cod, *Gadus ogac*. Isolation and comparative properties. *Comp Biochem Physiol* **79B** 613–622.

Simpson B K, Haard N F 1987 Cold adapted enzymes from fish. In *Food Biotechnology*, ed Knorr D. Marcel Dekker Inc, New York, USA, pp 495–527.

Simpson B K, Simth J P, Yaylayan V 1989 Kinetic and thermodynamic characteristics of a digestive protease from Atlantic cod, *Gadus morhua*. *J Food Biochem* **13** 201–213.

Simpson B K, Simpson M V, Haard N F 1990 Properties of trypsin from pyloric ceca of Atlantic cod (*Gadus morhua*). *J Food Sci* **55** 959–961.

Smalas A O, Heimstad E S, Hordvik A, Willassen N P, Male R 1994 Cold adaptation of enzymes: Structural comparison between salmon and bovine trypsins. *Proteins Struct Funct Genet* **20** 149–166.

Teale F W J 1960 Ultraviolet and fluorescence of protein in neutral solutions. *Biochem J* **76** 381–388.

Varley P G, Pain R H 1991 Relationship between stability, dynamics and enzyme activity in 3-phosphoglycerate kinases from yeast and *Thermus thermophilus*. *J Molec Biol* **220** 531–538.

Vihinen M 1987 Relationship of protein flexibility to heat stability. *Protein Engr* **1** 447–480.