Structural features responsible for kinetic thermal stability of a carboxypeptidase from the archaeobacterium *Sulfolobus solfataricus*

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Investigations were performed on the structural features responsible for kinetic thermal stability of a thermostable carboxypeptidase from the thermoacidophilic archaeobacterium *Sulfolobus solfataricus* which had been purified previously and identified as a zinc metalloprotease [Colombo, D'Auria, Fusi, Zecca, Raia and Tortora (1992) *Eur. J. Biochem.* **206**, 349–357]. Removal of Zn^{2+} by dialysis led to reversible activity loss, which was promptly restored by addition of $80\ \mu M$ $ZnCl_2$ to the assay mixture. For the first-order irreversible thermal inactivation the metal-depleted enzyme showed an activation energy value of $205.6\ kJ\cdot mol^{-1}$, which is considerably lower than that of the holoenzyme ($494.4\ kJ\cdot mol^{-1}$). The values of activation free energies, enthalpies and entropies also dropped with metal removal. Thermal inactivation of the apoenzyme was very quick

at $80\ ^\circ C$, whereas the holoenzyme was stable at the same temperature. These findings suggest a major stabilizing role for the bivalent cation. Chaotropic salts strongly destabilized the holoenzyme, showing that hydrophobic interactions are involved in maintaining the native conformation of the enzyme. However, the inactivation rate was also increased by sodium sulphate, acetate and chloride, which are not chaotropes, indicating that one or more salt bridges concur in stabilizing the active enzyme. Furthermore, at the extremes of the pH–stability curve, NaCl did not affect the inactivation rate, confirming the stabilizing role of intramolecular ionic bonds, as a pH-dependent decrease in stability is likely to occur from breaking of salt bridges involved in maintaining the native conformation of the protein.

INTRODUCTION

A number of enzymes from thermophilic micro-organisms have been identified in recent years [1–11]. They interest researchers mainly because they can be used in many areas of biotechnology [12–14]. Investigations on these molecules are also expected to contribute to a better understanding of their thermal stability mechanisms. This prompted us to research the intracellular proteolytic activities in the thermoacidophilic archaeobacterium *Sulfolobus solfataricus*. We identified five different proteases, including one carboxypeptidase [15] which was subsequently purified to homogeneity and characterized [16]. It was a metalloprotease with a tetrameric structure; the molecular mass of the subunit was 42 kDa. It also displayed interesting properties, such as an unusually broad substrate specificity and the ability to withstand some denaturing agents and organic solvents up to $40\ ^\circ C$, in particular 8 M urea, 0.1 % SDS, 50 % methanol and 99 % ethanol. In aqueous medium, it showed good thermal stability up to $85\ ^\circ C$ [16].

Our investigations tried to elucidate the molecular forces that contribute to the kinetic thermal stability of the carboxypeptidase. Our study assigned primary stabilizing roles to the bonded zinc ion and to the presence of salt bridges.

EXPERIMENTAL

Materials

Benzoylglycylated arginine (Bz-Gly-Arg), Mes and triethanolamine were purchased from Sigma (St. Louis, MO, U.S.A.). All other buffers and salts were purchased from Merck (Darmstadt, Germany).

Enzyme activity assay

Carboxypeptidase activity was determined as previously reported

in [16]. The procedure took advantage of the enzyme's ability to hydrolyse Bz-Gly-Arg, which results in a decrease in u.v. absorbance. The assay mixture (total volume 0.8 ml) consisted of 0.1 M potassium Mes buffer, pH 6.5, containing 0.1 mM Bz-Gly-Arg. After incubation of this mixture for 5 min at $60\ ^\circ C$, the enzyme was added and the mixture incubated for another 30 min at the same temperature. After chilling on ice, the absorbance was measured immediately at 240 nm against a blank sample. The blank was incubated for 35 min at $60\ ^\circ C$; the enzyme was added and the absorbance determined after chilling. When assaying zinc-depleted carboxypeptidase (see the Results section), $80\ \mu M$ $ZnCl_2$ was also present in the mixture, allowing rapid reactivation of the apoenzyme. Each activity value was calculated from the mean of at least three independent determinations. Standard deviations never exceeded $\pm 10\%$ of the respective mean values. The substrate was not spontaneously hydrolysed to any significant degree during incubation.

Enzyme purification

Pure carboxypeptidase was obtained according to the procedure described in [16], starting from *S. solfataricus* cells, strain MT-4 (ATCC 49155), kindly supplied by the Servizio di Fermentazione dell'Istituto per la Chimica di Molecole di Interesse Biologico del C.N.R., Arco Felice, Italy. Cells were grown aerobically in a mineral medium as reported in [17], using yeast extract (2 g/l) as the carbon source. They were collected during the stationary phase.

Activity decay measurements

The enzyme ($20\ \mu g\cdot ml^{-1}$) was incubated in sealed vials at the indicated temperatures in, unless otherwise stated, 50 mM triethanolamine/HCl buffer, pH 7.5 (measured at $25\ ^\circ C$), brought to 1.0 M with NaCl. At predetermined times, $20\ \mu l$

Abbreviation used: Bz-Gly-Arg, benzoylglycylated arginine.

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samples were removed, cooled immediately on ice and assayed for residual carboxypeptidase activity as described above. pH values other than 7.5 were measured at the reported temperatures.

Calculation of activation parameters

On the basis of activity measurements, we detected first-order inactivation kinetics under all conditions, irrespective of pH and I . Activation parameters were then calculated as described in [18]. To determine activation energy values (E_a) for thermal inactivation of holo- and apo-enzyme, inactivation rate constants were plotted in an Arrhenius plot. Activation enthalpies ($\Delta H^\ddagger$) for each temperature were calculated according to:

$$\Delta H^\ddagger = E_a - RT$$

where R ($8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$) is the universal gas constant and T the absolute temperature. Activation free energies ($\Delta G^\ddagger$) of the inactivation process were calculated according to:

$$\Delta G^\ddagger = -RT \ln \frac{kh}{kT}$$

where k (s^{-1}) is the inactivation rate constant, h ($6.6256 \times 10^{-34} \text{ J} \cdot \text{s}$) the Planck constant and k ($1.3805 \times 10^{23} \text{ J} \cdot \text{K}^{-1}$) the Boltzmann constant. Activation entropies ($\Delta S^\ddagger$) were then calculated according to:

$$\Delta S^\ddagger = \frac{\Delta H^\ddagger - \Delta G^\ddagger}{T}$$

RESULTS AND DISCUSSION

Contribution of bonded zinc ion to the kinetic thermal stability of carboxypeptidase

This investigation was aimed at identifying the structural features responsible for thermal stability of proteins from *S. solfataricus*. A previously isolated carboxypeptidase [16] was used as a model molecule. Since the enzyme was irreversibly denatured, we were prevented from studying the thermodynamics of the process; so we investigated its kinetic thermal stability. In the aforementioned paper [16], we found that dialysis of the carboxypeptidase, first at pH 5.5 in the presence of 10 mM EDTA, then at pH 7.5 in its absence, completely eliminated enzyme activity, and that it was promptly restored by addition of Zn^{2+} in micromolar concentrations. This clearly supported identification of the molecule as a metalloprotease; at the same time, dialysis allowed us to obtain the zinc-depleted enzyme easily, starting from the holoenzyme form. This makes it possible to determine activation parameters of the inactivation process on both holo- and apo-enzyme. In fact, although the apoenzyme is inactive, it can be assayed in the presence of $80 \mu\text{M}$ ZnCl_2 . Therefore activity decay, determined under these conditions, represents the transition from the reversibly to irreversibly inactivated apoenzyme form.

Inactivation followed first-order kinetics under any adopted condition, showing that autolytic cleavage was not responsible for the inactivation, but that it was more a case of thermal unfolding. Accordingly, no proteolysed form of the enzyme was detected in either native PAGE and or SDS/PAGE (results not shown). Removal of the bivalent cation dramatically destabilized the carboxypeptidase, as shown by the increase in inactivation rates. In fact, the holoenzyme form was completely stable up to 80°C , whereas at the same temperature the apoenzyme had a half-life of 14 min (Figure 1). Arrhenius graphs were plotted

from the respective inactivation rate constants (Figure 2), and activation energy values of 494.4 and $205.6 \text{ kJ} \cdot \text{mol}^{-1}$ were determined for holo- and apo-enzyme forms respectively. Activation free-energies ($\Delta G^\ddagger$), enthalpies ($\Delta H^\ddagger$) and entropies ($\Delta S^\ddagger$) were also calculated at each temperature (see the Experimental section). Table 1 shows that $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values of both enzyme forms are not significantly affected by temperature; thus, thermal inactivation only resulted from an enhanced entropic contribution to $\Delta G^\ddagger$, which in turn reflected the increased structural fluctuations of the polypeptide chain. Furthermore, metal removal led to a marked reduction of the E_a value and, consequently, of $\Delta H^\ddagger$ (Table 1), suggesting that major intramolecular stabilizing forces are associated with the presence of the bivalent cation in the holoenzyme. A much lower $\Delta S^\ddagger$ value for zinc-depleted carboxypeptidase, compared with that of the holoenzyme form, was also evident from Table 1, suggesting that the molecule adopts a more extended conformation as a result of metal removal.

Figure 1 Thermal inactivation of *S. solfataricus* carboxypeptidase

The holoenzyme (+Zn) and the apoenzyme (-Zn) were incubated at the indicated temperatures in 50 mM triethanolamine/HCl buffer, pH 7.5 (measured at 25°C), brought to 0.1 M with NaCl. At predetermined times aliquots were withdrawn, residual activity was determined and expressed relative to zero-time incubation.

Figure 2 Arrhenius plots for thermal inactivation of *S. solfataricus* carboxypeptidase

Inactivation rate constants k (min^{-1}) were determined for both holo- (+Zn) and apo-enzyme (-Zn).

Table 1 Thermodynamic activation parameters for thermal inactivation of *S. solfataricus* carboxypeptidase

Enzyme form	<i>T</i> (°C)	$\Delta G^\ddagger$ (kJ·mol ⁻¹)	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$\Delta S^\ddagger$ (kJ·mol ⁻¹ ·K ⁻¹)
Holoenzyme (+Zn)	85	116.0	491.4	1.05
	87.5	113.7	491.4	1.05
	90	110.8	491.4	1.05
	92.5	108.2	491.4	1.05
Apoenzyme (-Zn)	65	111.8	202.7	0.27
	70	110.8	202.7	0.27
	75	109.2	202.7	0.27
	80	107.9	202.6	0.27

Figure 3 Effect of lyotropic salts on thermal inactivation of *S. solfataricus* carboxypeptidase (holoenzyme) at 86 °C

The enzyme was incubated in 50 mM triethanolamine/HCl buffer, pH 7.5, containing sodium salts of the indicated anions at a 1 M *I*. At predetermined times samples were withdrawn, residual activity determined and expressed relative to zero-time incubation. ▽, control; ■, sulphate; ▲, acetate; ●, chloride; □, nitrate; ○, thiocyanate; △, perchlorate.

Effect of lyotropic anions, other chemical agents and pH on carboxypeptidase stability

The effect of lyotropic salts on time-dependent inactivation of the holoenzyme was assessed at 86 °C (Figure 3). It was shown that chaotropic anions, such as nitrate, perchlorate and thiocyanate, were strong destabilizers. This result was expected on the basis of the ions' well-known ability to weaken the hydrophobic interactions which generally play a major stabilizing role in the native conformation of proteins [19,20]. However, our data did not permit assessment of whether this contribution to the intrinsic kinetic thermal stability of our enzyme is significantly different from that of other thermophilic or mesophilic proteins. A comparative evaluation of the effect of chaotropic salts on several proteins would be required to determine this. On the other hand, sulphate, acetate and chloride ions also enhanced inactivation, though to a lesser extent (Figure 3). Sulphate and acetate ions are kosmotropes, i.e. they stabilize proteins via their ability to strengthen hydrophobic interactions, whereas chloride ions have neither a stabilizing nor a destabilizing effect [19,20]. Thus it should be expected that these ions either do not affect

Figure 4 Effect of increasing ionic strength (*I*) of sodium sulphate and sodium chloride on thermal inactivation of *S. solfataricus* carboxypeptidase (holoenzyme) at 86 °C

The enzyme was incubated in 50 mM triethanolamine/HCl buffer, pH 7.5, containing sodium sulphate (■) or sodium chloride (●) at the indicated *I* values. Residual activity was determined after a 30-min incubation period and expressed relative to zero-time incubation.

protein stability at all or they increase it: this was indeed their effect on alkaline serine proteinase from *Thermomonospora fusca*, as shown by Kristjansson and Kinsella [21]. Therefore, the effect of these anions on *S. solfataricus* carboxypeptidase cannot be the result of interference with hydrophobic interactions, but suggests that intramolecular ionic bonds (i.e. salt bridges) are involved in maintaining the active form of the enzyme. Profiles of residual activity, as a function of ionic strength constructed using sodium sulphate and sodium chloride, also support this theory. Figure 4 shows that residual activity first decreased on incubation with sodium sulphate at increasing ionic strengths, then it partially recovered, which suggests two combined effects: the above-mentioned kosmotropic effect of the anion and the general capacity of ions to interfere with salt bridges. Consistently, NaCl, which is neither chaotrope nor kosmotrope, only caused progressive destabilization (Figure 4). The effect of phosphate, which is regarded as the best kosmotrope, could not be assayed as it strongly inhibited the enzyme.

Interestingly, NaCl also had a denaturing effect on a serine proteinase from *S. solfataricus*, previously isolated in our laboratory [22]: this suggests that the salt bridges might represent a more general strategy of thermostabilization in this micro-organism. It should be noted that most proteins from this source possess quaternary structures [7,16,22–26], suggesting a possible relationship between salt bridges and oligomeric structures in these molecules. For example, amino acid residues located on different polypeptide chains of the oligomer might concur to form ionic bonds.

The holoenzyme was also incubated at 50 °C and at pH values corresponding to the extremes of its pH-stability curve (9.0 and 5.1). Although at pH 9.0 thermal stability was dramatically reduced, NaCl did not stimulate activity decay further (Figure 5). This again supports the theory that ionic intramolecular interactions have a major stabilizing role, as a pH-dependent decline in stability is likely to occur if one or more salt bridges involved in maintaining the native conformation are broken. In turn, this event should result in loss of sensitivity to the denaturing effect of ionic compounds. On the other hand, NaCl still stimulated inactivation at the lower pH value (Figure 5), which implies that

Figure 5 Effect of NaCl on thermal inactivation of *S. solfataricus* carboxypeptidase (holo- and apo-forms) at different pH values

The holoenzyme (+ Zn) was incubated at 50 °C and pH 5.1 or pH 9.0. The apoenzyme (− Zn) was incubated at 40 °C and pH 4.1 or 9.0. Incubations were done in the presence (●) or the absence (○) of 1 M NaCl. Buffers used: at pH 4.1 and 5.1, 50 mM sodium acetate buffer; at pH 9.0, 50 mM 2-amino-2-methyl-1,3-propanediol/HCl. At predetermined times, samples were withdrawn, residual activity determined and expressed relative to zero-time incubation.

Figure 6 pH-dependence of thermal inactivation rate constants of *S. solfataricus* carboxypeptidase at 70 °C

The holoenzyme (closed symbols) and the apoenzyme (open symbols) were incubated at the indicated pH values. Based on determinations of residual activities performed at predetermined times, the respective first-order inactivation rate constants (k) were calculated. The following buffers, at a concentration of 50 mM, were used: (△) sodium acetate; (●, ○) sodium Mes; (■, □) triethanolamine/HCl; (▼, ▽) 2-amino-2-methyl-1,3-propanediol/HCl. All buffers were brought to 0.1 M with NaCl.

the stability loss caused by pH decrease is not caused by salt-bridge breakage. Kester and Matthews [27] showed that bovine carboxypeptidase A and thermolysin, a metallo-endopeptidase from the eubacterium *Bacillus thermoproteolyticus*, despite the lack of any sequence similarity, both carry, at the active site, a Zn^{2+} ion which is co-ordinated to two histidine side chains, one glutamate side chain and one water molecule; thus it is possible that *S. solfataricus* carboxypeptidase shares the same catalytic

device. If this were the case, a pH drop to a suitably acidic value would also lead to protonation of histidine residues close to the bivalent cation, resulting in repulsion and destabilization of the holoenzyme. To confirm the hypothesis we determined the inactivation rate constants of both holo- and apo-enzyme at 70 °C over a broad pH range. As expected, at pH values below 5.7, the metal-depleted enzyme was substantially more stable than the native form (Figure 6), a probable consequence of a reduction in electrostatic repulsion; in contrast, at any pH value above 5.7 loss of Zn^{2+} severely impaired enzyme stability. Below pH 5 the apoenzyme was also significantly destabilized (Figure 6); furthermore, at pH 4.1 and 40 °C NaCl did not stimulate its inactivation any further (Figure 5), suggesting that such an acidic environment also caused salt-bridge breakage as a consequence of protonation of the carboxyl group involved. As expected, at pH 9.0, the apoenzyme also showed a complete loss of sensitivity to the destabilizing action of the salt (Figure 5). Based on the pH at the extremes of the pH-stability curve, we tentatively identify the groups involved in the ionic bonds as ϵ -amino of lysine residues and β - or γ -carboxyl of aspartate or glutamate residues.

Reducing agents, such as 2-mercaptoethanol or dithiothreitol, had no significant effect on carboxypeptidase stability: this allowed us to rule out the hypothesis that disulphide bridges stabilize the molecule. On the other hand, amino acid analysis shows that the enzyme has only one cysteine residue per subunit [16], which makes the occurrence of a disulphide bridge unlikely. No effect on carboxypeptidase stability was detected for Ca^{2+} concentrations up to 10 mM, even though it was thought to stabilize several proteases [28].

One of the main questions posed by this investigation is, what kind of structural changes are associated with carboxypeptidase thermal inactivation? To answer this we tried to isolate modified forms of the enzyme which may be formed during the process. However, native PAGE of incubation mixtures did not permit identification of any such product. In particular, no change in the aggregation state of the protein was detected; instead, the band intensity of the native enzyme underwent a time-dependent decrease which was roughly parallel to activity decay. At the same time, progressive clouding of the incubation mixtures during the process showed that inactive molecules produced large aggregates. This is not the inactive form which first arises from the native enzyme during the degradation process, since aggregation cannot follow first-order kinetics, as was shown for activity decay. So, the initial degradative modification of native carboxypeptidase has still to be clarified.

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