Engineering thermolysin-like proteases whose stability is largely independent of calcium

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Received 9 December 1996; revised version received 7 February 1997

Abstract Thermal stability of the thermolysin-like protease produced by *Bacillus stearothermophilus* (TLP-ste) is highly dependent on calcium at concentrations in the millimolar range. We describe the rational design and production of a fully active TLP-ste variant whose stability is only slightly dependent on calcium concentration.

Key words: Calcium binding; Thermal stability; Thermolysin; Autolysis; Unfolding pathway

1. Introduction

Thermolysin-like proteases (TLPs) are a family of homologous metalloproteases that contain a catalytically important zinc ion in their active site. The three-dimensional structure of thermolysin is known [1] and this enzyme has been shown to bind four calcium atoms which contribute to thermal stability [2,3]. Two calcium ions are bound in the so-called double-calcium-binding site (Ca1,2), that is composed of ligands that are conserved in all TLPs. The other, single binding sites (Ca3 and Ca4) are composed of ligands that are conserved only in the more stable TLPs such as thermolysin and the TLP produced by *Bacillus stearothermophilus* (TLP-ste).

At elevated temperatures, TLPs are irreversibly inactivated as a consequence of autolysis. Autolysis follows first-order kinetics because its rate is determined by local unfolding processes that render the protease susceptible to autoproteolytic cleavage [4–7]. In their studies on the contribution of calcium ions to thermolysin stability, Dahlquist et al. [3] and Roche and Voordouw [2] concluded that the initial steps in thermal inactivation are accompanied by the release of one calcium ion (Ca3 or Ca4). Extensive mutagenesis studies of the TLP-ste have shown that a region near the Ca3 site is crucial for thermal stability [8,9]. Thus, thermal inactivation seems to be dominated by one single 'weak' region, near Ca3. Considering the expected high structural similarity between thermolysin and TLP-ste (86% sequence identity) the studies on TLP-ste suggest that the critical calcium ion is Ca3 rather than Ca4.

Using detailed knowledge about the thermal inactivation mechanism of TLPs that was gathered from a long series of mutation experiments, we set out for the rational design of calcium-independent variants. The Ca3 site was deteriorated by mutating one of the main ligands (Asp-57). Subsequently, the (expected) loss in stability was compensated for by introducing stabilising mutations in the direct environment of the Ca3 site. The results confirm the importance of the Ca3 site for stability and they show the feasibility of engineering less calcium-dependent, stable TLP-ste variants.

2. Materials and methods

2.1. Production and characterisation of mutated enzymes

Cloning, sequencing, sub-cloning, and expression of the TLP-ste gene (from strain *B. stearothermophilus* CU21 [10]), as well as production, purification and subsequent characterization of wild-type and mutant TLP-ste were performed as described earlier [11,12]. Thermal stability was measured as described previously [8,9], using varying $CaCl_2$ concentrations in the standard assay buffer (20 mM Na acetate, pH 5.3, 0.01% Triton X-100, 0.5% isopropanol, 62.5 mM NaCl). T_{50} is the temperature of incubation at which 50% of the initial proteolytic activity is lost during a 30 min incubation.

The kinetic parameter k_{cat}/K_m (at 37°C) for the substrate 3-(2-furylacryloyl)-L-glycyl-L-leucine amide (FaGLa, Sigma Chemical Co., St. Louis, MI, USA) was determined according to the method of Feder [13], in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 5% DMSO, 1% isopropanol and 125 mM NaCl, using an 100 μM substrate concentration. Activities were derived from the decrease in absorption at 345 nm, using a $\Delta\varepsilon$ of -317 M^{-1} cm^{-1}.

2.2. Structure analysis

TLP-ste and thermolysin have 85% sequence identity which allowed the construction of a three-dimensional model of TLP-ste that is sufficiently reliable to predict the effects of site directed mutations [6]. The 55–69 region was expected to be highly similar in TLP-ste and thermolysin. Comparison of the two known TLP structures, thermolysin and the TLP from *B. cereus* [14] supported this: TLP-cer has lower homology to thermolysin (73% sequence identity) but, nevertheless, has a strikingly similar fold in the 55–69 region (the RMS positional difference is in the order of a few tenths of an ångström, i.e. in the order of the crystallographic error). Indeed, the TLP-ste model has been used successfully for the rational design of stabilising mutations (e.g. [15,16]). Structure analyses, three-dimensional modelling, prediction of the effects of point mutants, and data base searches were performed with the WHAT IF program [17]. The only insertion/deletion in the alignment of thermolysin (316 residues) and TLP-ste (319 residues) is a three-residue insertion between residues 25 and 30 in TLP-ste [9].This insertion was omitted in the numbering of the TLP-ste sequence, meaning that TLP-ste residues are numbered according to the corresponding residues in thermolysin.

3. Results and discussion

From a structural point of view Asp-57 seemed more important for calcium binding than Asp-59 because both Oδs of Asp-57 interact with the calcium versus only one Oδ of Asp-59 (Fig. 1). Asp-57 was replaced by Ser because in the less thermostable TLPs residue 57 is a serine. From a visual in-

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0014-5793/97/$17.00
PII S0014-5793(97)00193-2

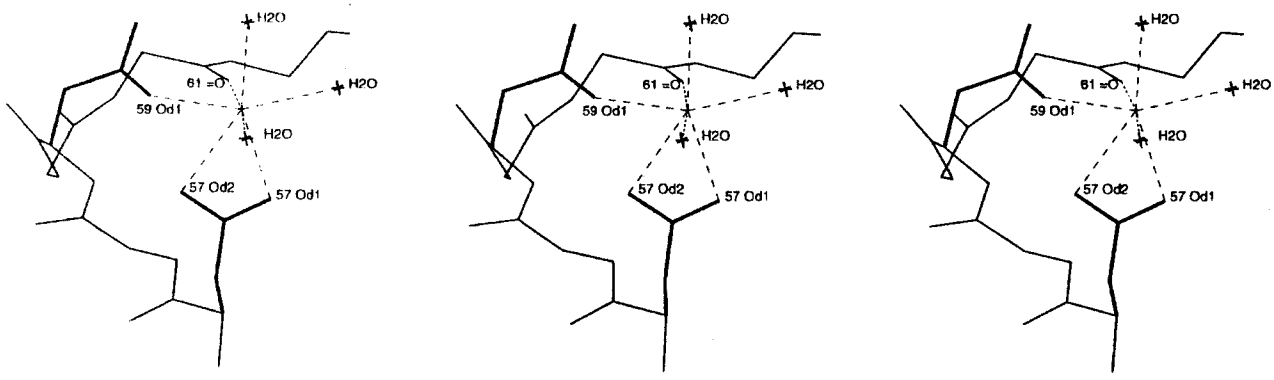

Fig. 1. Structure of calcium binding site 3 (Ca3) in thermolysin [1]. Crystal waters are indicated by crosses. Dashed lines represent contacts of the Ca3 atom with surrounding residues and crystal waters. Od1 and Od2 denote the side chain oxygen atoms of the Asp residues (Oδ1 and Oδ2, respectively). In the region depicted in this figure, the model is virtually identical to the crystal structure of thermolysin.

spection of the three-dimensional environment of residue 57 it was concluded that the D57S mutation would not have additional negative effects such as disturbance of the local hydrogen bonding network or the introduction of clashes. To compensate the expected destabilising effect of this mutation, the combined T63F-A69P mutation was chosen. The stabilising mutations had been identified in previous site-directed mutagenesis studies of differences between naturally occurring TLPs [18] and in studies concerning the design of stabilising Xxx→Pro mutations in TLP-ste [15]. The mutations are located in the direct environment of Ca3 and the double mutation had previously been shown to drastically stabilise TLP-ste. Characteristics of the various mutants, including the dependence of stability on calcium concentration are presented in Tables 1 and 2 and in Figs. 2 and 3. As shown in Table 2, the wild-type and mutant enzymes were similar with respect to their activity towards FaGLa.

The D57S mutation reduced the T_{50} of TLP-ste at 12.5 mM calcium from 77.9 to 69.4°C (Table 1). In the stable T63F-A69P mutant the effect of the D57S mutation was even more noticeable, and reduced T_{50} from 90.2 to 77.2°C. Thus, the integrity of the Ca3 site is clearly important for TLP-ste's thermal stability.

The stability of TLP-ste and the T63F-A69P mutant (which both have the Ca3 site intact) depended strongly on the calcium concentration (Fig. 2, Tables 1 and 2). Introduction of the D57S mutation reduced this calcium dependence (Fig. 2). Consequently, the destabilising effect of the D57S mutant became smaller with decreasing calcium concentration (Table 2, Fig. 3); at the lowest calcium concentration tested, the wild-type enzyme was even slightly stabilised by the D57S mutation). The stability versus calcium concentration curves of TLP-ste and T63F-A69P (Fig. 2) can be superimposed remarkably well. The same is true for D57S and D57S-T63F-A69P (Fig. 2), strongly suggesting that the observed effects on

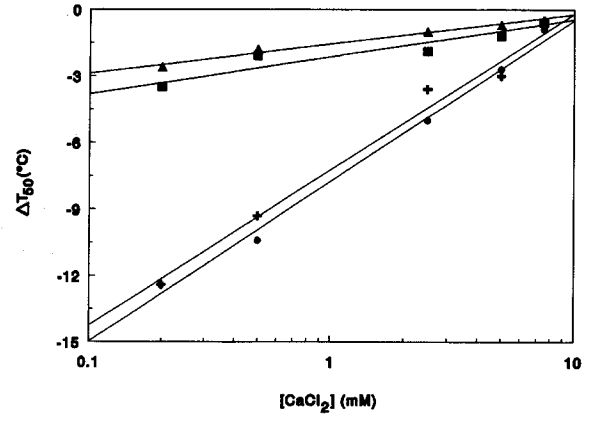

Fig. 2. Effect of calcium concentration on T_{50} for the wild-type (●), D57S mutant (■), T63F-A69P mutant (+) and D57S-T63F-A69P mutant (▲). ΔT_{50} is the change in T_{50} upon lowering the calcium concentration. The T_{50} at 12.5 mM $CaCl_2$ is used as reference value ($\Delta T_{50} = 0$).

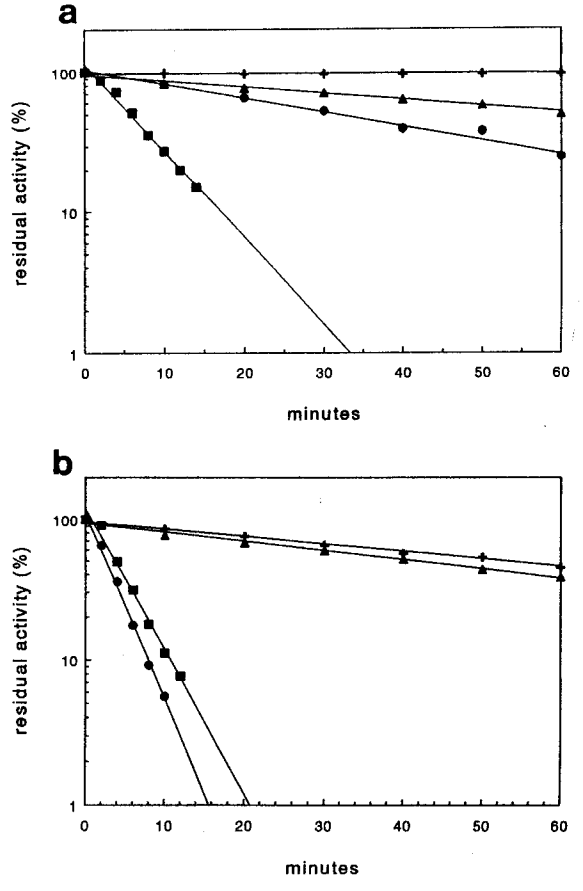

Fig. 3. First-order thermal inactivation of TLP-ste variants at 75°C; in (a) 5 mM $CaCl_2$ and (b) 0.2 mM $CaCl_2$; wild-type TLP-ste (●), D57S (■), T63F-A69P (+), D57S-T63F-A69P (▲).

Table 1
Thermal stabilities (T_{50}) at varying concentrations of $CaCl_2$ of *B. stearothermophilus* thermolysin-like protease variants

[$CaCl_2$] (mM)	TLP-ste T_{50} (°C)	D57S T_{50} (°C)	T63F-A69P T_{50} (°C)	D57S-T63F-A69P T_{50} (°C)
0.2	65.5	65.9	77.8	74.6
0.5	67.5	67.3	80.9	75.4
2.5	72.9	67.5	86.6	76.2
5.0	75.2	68.2	87.2	76.5
7.5	77.0	68.8	89.7	76.7
12.5	77.9	69.4	90.2	77.2

Error margins in the T_{50} values were in the range of 0.3–0.5°C.

Table 2
Activity and stability of TLP-ste variants

Variant	FaGLa, $k_{cat}/K_m \times 10^{-3}$ (M^{-1} s^{-1})	$t_{1/2}$ at 5 mM $CaCl_2$ (min)	$t_{1/2}$ at 0.2 mM $CaCl_2$ (min)	Half-life ratio (5 mM/0.2 mM)
TLP-ste	34	31	2.6	12
D57S	22	5.8	3.8	1.5
T63F-A69P	21	990	52	19
D57S-T63F-A69P	28	64	42	1.5

The activity against FaGLa was determined at 37°C. Half-lives ($t_{1/2}$) were calculated from the inactivation curves shown in Fig. 3a,b. The $t_{1/2}$ value of the T63F-A69P mutation was extrapolated from the data presented in Fig. 3a. The error margins in the $t_{1/2}$ values are less than 4%.

the calcium stability are indeed caused by the disturbance of the Ca3 site by the D57S mutation.

The fact that the D57S mutation largely abolishes the calcium dependence of stability confirms earlier suggestions [2,3,8] that calcium-3 is crucial in the process of thermal inactivation of TLPs. Apparently, the affinities of the other calcium sites are so high that titration effects are hardly noticeable at concentrations above 0.2 mM. We cannot entirely exclude that the remaining effects of calcium on the stability of the mutants carrying D57S reflect titration of e.g. the Ca4 site. However, the dominant role of the Ca3 region in determining stability (e.g. [9]; see also above) makes it more likely that the residual calcium dependence reflects residual binding to the impaired Ca3 site (see Fig. 1).

The D57S-T63F-A69P mutant represents a TLP-ste variant whose stability is largely independent of the calcium concentration and which, at lower calcium concentrations, is considerably more stable than the wild-type enzyme (Table 2). Combining known stabilising mutations in the Ca3 region has resulted in extremely stable TLP-ste variants [8]. Therefore, it is likely that mutants can be designed that are even less dependent on calcium than the ones described here and that are more stable. Engineering calcium independence does not necessarily need to be based on deterioration of the Ca3 site. Instead, it could be based on adding mutations that stabilise the local structure, regardless of the presence of a calcium ion. For example, preliminary analyses of a mutant in which the (intact) calcium binding site is covalently cross-linked with the N-terminal β-hairpin [16] showed that the stability of this mutant is also less calcium dependent.

Results similar to those described here have been reported by Strausberg et al. [19] who engineered a calcium-independent, stable variant of the alkaline protease subtilisin BPN'. These authors first created a labile variant by deleting the high affinity calcium-binding site. Subsequently, semi-random mutagenesis and screening methods were used to isolate calcium-independent, stable variants. In the present study, detailed knowledge about the thermal inactivation of TLP-ste permitted the identification of the stability-determining calcium-binding site and, subsequently, the design of a stable mutant in which the calcium dependency of stability is strongly reduced. Both studies show that the deleterious effect of impaired calcium binding can be overcome by compensating mutations. This leads to the important conclusion that calcium binding is a useful but certainly not unique way to obtain stable proteins.

Acknowledgements: We thank H.J.C. Berendsen, B.W. Dijkstra and C. Sander for stimulating discussions, B. van der Vinne for technical assistance, and I.F. Nes for general support.

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