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Calcium ion binding by thermitase

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Thermitase is a thermostable member of the subtilisin protease family. It was found that this enzyme binds very strongly one calcium ion. This metal ion cannot be removed without denaturation by any known method. A second binding site for calcium ions can be depleted partially by gel filtration, similar to the strong binding site of subtilisin Carlsberg. In both enzymes the calcium ion bound to this site can be removed completely using Ca^{2+} chelating reagent. In thermitase and subtilisin Carlsberg, depleting calcium ions from any binding site results in a significant decrease of stability against autolysis.

Bacterial protease; Thermitase; Subtilisin; Ca^{2+} binding; Stability

1. INTRODUCTION

It is known that Ca^{2+} decreases the denaturation and/or the autolysis rate of many proteases. Examples are trypsin, thermolysin, subtilisin and others [1–11]. In the subtilisin family, subtilisin Carlsberg (SCB), subtilisin BPN′, proteinase K and thermitase (TRM), respectively, can bind Ca^{2+}. SCB and BPN′ show stronger stabilisation by Ca^{2+} than TRM [5,11]. On the other hand, the enzymatic activity of proteinase K as well as its stability is controlled by Ca^{2+} [8]. According to X-ray analysis of subtilisin BPN′ [7] and SCB [4,6], there are one or two binding sites. In TRM, there are three binding sites [5]. One site has a dissociation constant of about 0.1 mM. The other two sites bind Ca^{2+} much stronger. To determine the role of each binding site, we first tried to remove the Ca^{2+} completely from TRM and SCB, respectively, by several methods and secondly added Ca^{2+} in defined amounts. In this study the calcium ion content was measured directly by atomic absorption. To our surprise there was no method to obtain calcium-free TRM. In contrast, SCB lost all bound Ca^{2+} through EDTA or chelating resin treatment.

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Abbreviations: pNPA, *p*-nitrophenyl acetate; SCB, subtilisin Carlsberg; TRM, thermitase

2. MATERIALS AND METHODS

TRM (EC 3.4.21.14), an extracellular protease from *Thermoactinomyces vulgaris*, was purified from concentrated culture filtrate by flat bed isoelectric focusing [12]. SCB was purchased from Sigma (St. Louis, USA). The purity of enzymes was checked by isoelectric focusing and electrophoresis [13]. The absolute protein concentration was determined by a modified biuret method [14], using bovine serum albumin as a standard, or spectrophotometrically at 280 nm using an absorption coefficient, $A[1\%,1\text{ cm}]$, of $A = 18.5$ for TRM [12] and $A = 11.7$ for SCB [15]. All other chemical reagents are from Laborchemie, Apolda, GDR, and were at least analytical grade. Solutions were prepared using bidistilled water. To remove Ca^{2+}, buffer stock solutions were prepared by chromatography with chelating resin A1 (column length, 75 cm; diameter, 2 cm; flow rate, 1 ml/min). Ammonium sulfate was recrystallized in the presence of EDTA.

The calcium concentration has been determined using an atomic absorption spectrophotometer (AAS1, Carl Zeiss Jena, GDR) calibrated in the concentration range from 10^{-6} to 2×10^{-4} M. Esterolytic activity of TRM was determined photometrically at 400 nm wavelength using *p*-nitrophenyl acetate (pNPA) as substrate (0.5 mM pNPA in 0.1 M Tris-HCl, at pH 8.0 at 25°C) [12]. The time course of the irreversible inactivation was determined by incubation in Tris-HCl buffer, pH

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8.0, 0.1 M at 65°C and measuring the residual enzyme activity at 25°C.

To remove Ca^{2+} from enzymes different methods were used. First chelating resin ion-exchange chromatography was done according to [16]. Chelating resin A1 (Serva, Heidelberg, FRG) was washed first with 0.01 M HCl and then equilibrated by 0.01 M NaOH to obtain a neutral pH value. Then the resin was equilibrated with 0.2 M Tris-HCl, pH 8.0, and acetate buffer, pH 5.5, respectively. Several experiments were carried out in batch procedure. 10 ml enzyme solution (1 mg per ml) were added to 4 ml resin. Experiments at room temperature gave identical results in comparison to experiments at 4°C. Other experiments were performed using a column (25 cm long, diameter 1.5 cm). In these experiments 10 mg protein per ml were in contact with the resin over a period of about 2 h. Gel filtration (Sephadex G 25, Pharmacia, Bromma, Sweden) in calcium-free buffer was also used to remove bound Ca^{2+} from protein too (column length, 25 cm; diameter, 1 cm). The protein concentration used was 4 mg/ml. The flow rate was about 0.2 ml/min. Repeated ammonium sulfate precipitation with 95% saturation in the presence of EDTA was successfully used to get calcium-free lactalbumin [17]. 4 mg enzyme were solved in 1 ml buffer containing 0.1 M EDTA. After stirring for 1 h the protein was precipitated by ammonium sulfate (saturation 95%). The precipitate was collected by centrifugation and after several repeats of the procedure solved in calcium-free buffer.

Table 1

Calcium ion content of different preparations of subtilisin Carlsberg and thermitase

(No.)Experimental conditions	Calcium content Ca^{2+}/protein mol.
Subtilisin Carlsberg	
(1) Freshly prepared solution (1 mg/ml)	2.11
(2) After gel filtration Sephadex G25	0.60
(3) After chromatography, batch procedure resin A1, 1 h, pH 8.0	0.14
Thermitase	
(4) After preparative isoelectric focussing according to [12] directly removed from gel bed	1.75
(5) No.4 after dialysis against 4 × 500 ml distilled water	2.03
(6) No.5 after lyophilization and solubilizing in buffer (1 mg/ml)	1.95 to 2.70
(7) After crystallization according to [18] and washing in saturated ammonium sulfate solution	1.85
(8) No.5 after gel filtration on Sephadex G25	1.50
(9) After chromatography, batch procedure with chelating resin A1, pH 8.0, 1 h	1.11
(10) After chromatography, batch procedure with chelating resin A1, pH 5.5, 1 h	1.04
(11) After chromatography, batch procedure with chelating resin A1, pH 5.5, 30 h	1.17
(12) After chromatography, column with resin A1, pH 8.0, 1 h	1.36
(13) After chromatography, column with resin A1, pH 8.0, 2 h	1.36
(14) Precipitation by ammonium sulfate in presence of 0.1 M EDTA twice	1.10

The calcium ion determination was done at least three times, the absolute error does not exceed 10%. 0.2 M Tris-HCl buffer and 0.1 M acetate buffer were used at pH 8.0 and 5.5, respectively

3. RESULTS

Table 1 gives the results of all experimental approaches used to obtain a calcium-free enzyme. From SCB one calcium ion is partially removed by gel filtration. In a short time a complete depletion of Ca^{2+} could be reached using a chelating reagent (fig.1, table 1, [11]). Fig.1 shows the change of the calcium ion content of SCB and TRM in a batch experiment using chelating resin. After 1 h SCB does not contain significant amounts of Ca^{2+} whereas TRM does. The extension of the batch procedure over a period of 30 h does not further change the calcium ion content of TRM. Yamada and Kometani [16] described ion-exchange chromatography of troponin C using a column

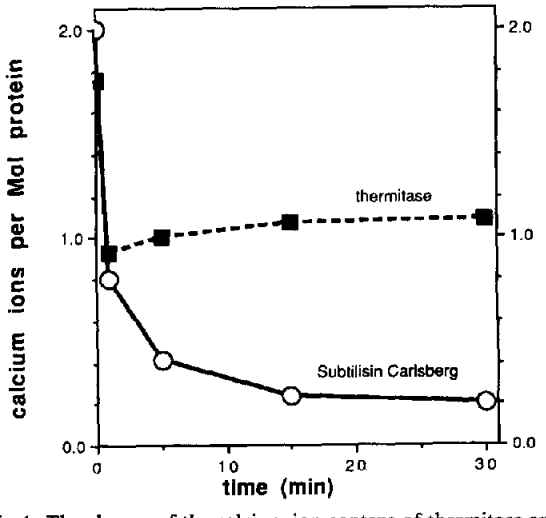

Fig.1. The change of the calcium ion content of thermitase and subtilisin Carlsberg in a batch experiment using chelating resin A1 at pH 8.0 0.2 M Tris-HCl buffer. Protein concentration about 1 mg/ml. The calcium ion content of thermitase is not changed further over a period of 30 h (see table 1).

Table 2

The influence of Ca^{2+} on the activity of thermitase

Calcium content mol/mol thermitase	Activity (μcat/l)	Significance
1	16.1	
2	A(min) 15.9	–
3	A(max) 17.6	+

The esterolytic activity (A) was measured with pNPA as substrate at pH 8.0, 25°C. The Ca^{2+} content was checked by atomic absorption. To get saturation of the third calcium-binding site, 1 mM $CaCl_2$ was added to the activity buffer. The change in activity was completely reversible in a short period after addition of EDTA and Ca^{2+}, respectively. The activity of TRM with different Ca^{2+} content is unchanged for at least 18 h. The enzyme concentration used is 0.01 mg/ml

which contains chelating resin A1. They obtained a molar relationship between Ca^{2+} and protein of 0.2. The dissociation constant for the calcium-binding site with the highest affinity was determined as 10^{-8}–10^{-10} M [17]. Surprisingly no experiment could be designed to remove the calcium ions from TRM completely (table 1). Also the precipitation method in the presence of EDTA or EGTA [17] does not result in calcium-free TRM (table 1). In lactalbumin, the dissociation constant for Ca^{2+} is between 10^{-10} and 10^{-12} M [16]. From that we concluded that the dissociation constant for the strongly bound calcium in TRM must be lower than 10^{-12} M. The second calcium ion bound to TRM can be removed partially by gel filtration in calcium-free buffer. It behaves similar to the tightly bound one of SCB. Both strongly bound Ca^{2+} were not removed from TRM during the isoelectric

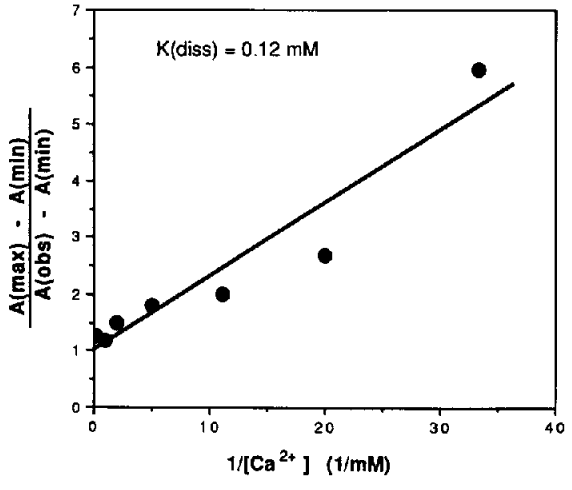

Fig.2. The influence of the calcium ion concentration on the esterolytic activity of thermitase (double reciprocal plot) activity measurements with p-NPA as substrate according to the description in section 2. Definition of A(min) and A(max) see table 2. A(obs) is the enzymatic activity at a given calcium ion concentration.

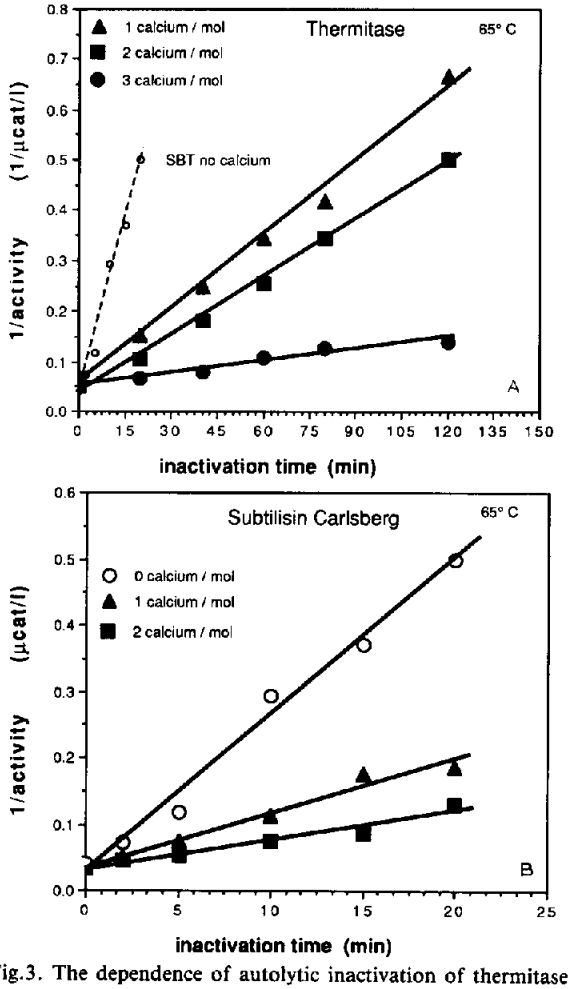

Fig.3. The dependence of autolytic inactivation of thermitase (A) and subtilisin Carlsberg (B) on calcium content of the enzymes (second order plot). (A) To maintain the content of thermitase at 1 calcium ion per molecule this experiment is performed in the presence of 1 mM EDTA. In control experiments we found no direct influence of EDTA on stability. For comparison the inactivation of Ca^{2+}-free subtilisin Carlsberg is also shown. (B) Enzyme concentration in both inactivation experiments was about 3.0×10^{-5} M, at pH 8.0 (Tris-HCl buffer 0.2 M), 65°C. To maintain Ca^{2+} content of subtilisin Carlsberg 1/molecule and of thermitase 2/molecule, the inactivation solution contains 10^{-6} M free Ca^{2+}. To saturate the weak Ca^{2+}-binding site we added 1 mM $CaCl_2$. The Ca^{2+} content of the enzymes at a given free-calcium concentration was checked by gel filtration according to [11].

focusing procedure (table 1). Also in crystallization experiments, two Ca^{2+} per molecule TRM remained bound. Due to the molar concentration of ammonium sulfate used during crystallization [19] and the low ionic product of calcium sulfate [1], all binding sites with $K_{diss} > 10^{-5}$ M for Ca^{2+} should be depleted.

In proteinase K, there is a remarkable influence of Ca^{2+} on activity. In 6 h irreversible loss of activity without autolysis was observed [8]. Such an inactivation of TRM containing 1 Ca^{2+} and of SCB with no bound calcium ions could not be observed over a period of 18 h. The weakly bound calcium ion causes a small increase (about 10%) of esterolytic activity of TRM (table 2) and SCB (not shown). This effect could be titrated resulting in a K_{diss} of about 10^{-4} M (fig.2).

The dependence of enzyme stability against autolysis on calcium ion content is shown in fig.3. The known difference between TRM and SCB [3,5] in intrinsic stability is confirmed. This difference is smaller if enzyme species are compared with identical calcium ion content. This is also true if one compares the stability at 65°C of proteinase K with 1 bound calcium ion per molecule [8] and TRM with 1 calcium ion too (fig.2). But in all comparable experiments TRM shows the highest stability of the three enzymes compared.

Table 3

The calcium-binding sites of different members of the subtilisin protease family and their protein ligands (predicted or experimentally observed)

Site	Thermitase		Subtilisin Carlsberg	Subtilisin BPN'	Proteinase K
	Predicted	Observed			
Ca1	$K_{diss} < 10^{-10}$ M (a) O Thr 64 Asp 57 Asp 60 Asp 62 Gln 66 Asn 68/70 Asp 105	 Asp 57 Asp 60 Asp 62 Gln 66			
Ca2	K_{diss} 10^{-10} M (a) Ser 9 Asp 47 O Val 82 Asn 84	 Asp 47 O Val 82 Asn 85 O Thr 87 O Ile 89	10^{-10} M (a) Gln 2 Asp 41 O Leu 74 Asn 76 O Thr 78 O Val 80	10^{-10} M (a) Gln 2 Asp 41 O Leu 75 Asn 77 O Gly 79 O Val 81	n.b.
Ca3	K_{diss} 10^{-4} M O Ala 173 O Tyr 174 O Tyr 175 O Ala 178 Asp 200		10^{-4} M (b) O Ala 168 O Tyr 170 O Val 173 (Asp 171) (Glu 196)	10^{-4} M (b) O Gly 169 O Tyr 171 O Val 174 O Glu 195	10^{-7} M (a) O Pro 175 O Val 177 Asp 200
Ca4					very low (b) O Thr 16 Asp 260
Ref.	[20]	[18,19]	[4,6]	[7]	[8,9]

The calcium-binding sites are ordered according to the sequence alignment of the 4 proteinases [20]. n.b., no binding observed. O, peptide oxygen. K_{diss} are ordered to the distinct binding site according to the reported occupancy in X-ray analysis. (a) High occupancy of the binding site by Ca^{2+} in X-ray structure, (b) low occupancy of the binding site by Ca^{2+}, calcium binding uncertain in X-ray structure

4. DISCUSSION

The results of previous direct binding studies performed by means of calcium-sensitive electrode measurements [5] are in good agreement with almost all results of this study. But one observation of the first calcium-binding study must be reinterpreted. After treatment with EDTA, dialysis, and denaturation, the amount of Ca^{2+} per mol TRM was about 0.2 as determined by means of a calcium-sensitive electrode [5]. One has to keep in mind that this method is specific for free Ca^{2+}. In contrast, the atomic absorption measures the total content of calcium of any solution. In this study we show that at least one calcium ion remains bound in TRM. This contradiction can be explained by assuming that in the presence of EDTA we get a ternary complex between protein-Ca^{2+}-EDTA. After denaturation the Ca^{2+} remains mainly complexed with EDTA. So the calcium-sensitive electrode cannot detect free Ca^{2+}. A ternary complex between protein-Ca^{2+}-EDTA is also proposed for proteinase K in the presence of EDTA [8].

There is crystallographic evidence for two binding sites in the SCB and subtilisin BPN′ structures, respectively [10] (table 3). The weaker of the two sites (binding constant 10^{-4} M, Ca easily removable by EDTA) shows many main chain oxygen atoms as ligands. This Ca^{2+}-binding site is apparently conserved in TRM. The stronger of the two sites (binding constant 10^{-8} M [10]) involves side chain oxygens from Gln 2, Asp 41, and Asn 77, and several main chain oxygens in subtilisin BPN′ and the corresponding conserved residues in SCB (table 3). Also in TRM this site appears to be well conserved, except for the replacement of Gln 2 by Ser 9.

In addition to binding sites identified in the TRM structure by homology to known sites in SCB and subtilisin BPN′, respectively (table 3), there is a third one with a strong binding of Ca^{2+}. The ligands are several oxygen atoms from amino acid side chains of the loop around residue 60 [18,19]. Two of the three Ca^{2+} in TRM are bound rather tightly. The two strongly bound Ca^{2+} in TRM give rise to 2 units higher isoelectric point [12] in comparison to SCB [15]. This shift of isoelectric point cannot be explained by the change of amino acid composition.

The removal of any Ca^{2+} from SCB and TRM leads to a significant decrease in stability. The function of the calcium ion which cannot be removed from native TRM remains unproven. In the absence of other significant additional intramolecular interactions in TRM in comparison to other members of the subtilisin family this strongly bound calcium ion is the most likely candidate for the cause of the higher stability of TRM [20].

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