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Thermostable and active phosphoenolpyruvate carboxylase from *Thermus* sp. even after proteolytic cleavage

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Abstract

Limited proteolysis of recombinant phosphoenolpyruvate carboxylase (PEPC) from *Thermus* sp. (ThPEPC) with either trypsin or chymotrypsin cleaved the enzyme into major fragments of about 70 and 20 kDa. Both proteases cleaved ThPEPC in the same chain region, which is suggested to be a flexible loop near the active site. The cleavage sites by trypsin and the site by chymotrypsin were immediately adjacent. Trypsin-treated ThPEPC remained active, whereas, the activity of chymotrypsin-treated ThPEPC was essentially not detectable. Both of the cleavages had substantially no effect on the enzyme's quaternary structure, secondary structure, and thermostability. Therefore, the effect of the cleavage on enzyme activity was dependent on the cleavage sites which are apart from each other by only one residue, while that on the tertiary structure and thermostability of ThPEPC was not.

Keywords: Phosphoenolpyruvate carboxylase; Thermostable; Limited proteolysis; Flexible loop; Main chain connectivity

1. Introduction

Enzymes from thermophiles are good candidates for a bioreactor because they are tolerant not only to severe conditions such as high temperature and chemical denaturants but also to proteolytic cleavage in general. However, once proteolytic cleavage of the enzymes from thermophiles occurs, they usually lose their activities. In this study, we found a novel phenomenon that phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) from *Thermus* sp. is thermostable and active even after proteolytic cleavage by trypsin.

PEPC catalyzes the reaction of PEP and bicarbonate to form oxaloacetate and orthophosphate in the presence of Mg^{2+} [1]. PEPC is widespread in all higher plants and many kinds of bacteria and replenishes C_4 dicarboxylic acids in the citric acid cycle. This anaplerotic role makes PEPC a key enzyme in the industrial fermentation process of metabolites such as lysine and succinate [2,3]. In addition, in C_4– and crassulacean acid metabolism plants, a specific molecular species of PEPC plays a key role in photosynthetic CO_2 assimilation [4]. PEPC is composed of four identical subunits with a molecular mass of about 100 kDa. The three-dimensional structure of PEPC has been determined for the enzyme from *Escherichia coli* (EcPEPC) [5,6]. As well as PEPC from mesophilic bacteria and higher plants, the enzyme from thermophilic organisms such as *Thermus* sp. [7–9], *Rhodothermus obamensis* [10,11], and *Methanothermus sociabilis* [12] has been characterized.

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In this study, we characterized PEPC from ThPEPC cleaved at a putative flexible loop near the active site by limited proteolysis. ThPEPC was digested into two major peptide fragments of about 70 and 20 kDa either by trypsin or by chymotrypsin. The cleavage had essentially no effect on the enzyme's quaternary structure, secondary structure, and thermostability. While the activity of chymotrypsin-treated ThPEPC was not detectable, trypsin treatment did not inactivate the enzyme. ThPEPC was cleaved at four adjacent sites including Arg686–His687 by trypsin and was cleaved at Tyr685–Arg686 by chymotrypsin. All the cleavage sites occurred in a putative flexible loop near the active site.

2. Experimental

2.1. Materials

Recombinant ThPEPC was purified as previously described [8]. *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-trypsin and immobilized TPCK-trypsin attached to beaded agarose were purchased from Pierce (IL, USA). Chymotrypsin and immobilized chymotrypsin attached to beaded agarose were from Sigma (MO, USA). All other chemicals were from Wako Pure Chemical (Tokyo, Japan).

2.2. Proteolysis of ThPEPC

ThPEPC (500 μg/ml) was digested at 30 °C in 10 mM Tris–H_2SO_4 (pH 8.0) with 10 μg/ml of trypsin or chymotrypsin. The reaction was terminated by the addition of 0.1 M sodium acetate (pH 4.0) and subjected to activity measurements and SDS-PAGE. No further digestion was observed after lowering the pH to 4.0. For gel-filtration and CD experiments (described later), immobilized trypsin or chymotrypsin was used. After confirming the disappearance of the intact polypeptide by SDS-PAGE, the reaction was terminated by removal of the immobilized proteases by filtration.

2.3. Enzyme assay

The activity of ThPEPC was measured at 30 °C in the standard reaction mixture containing, in a total volume of 1.0 ml, 100 mM Tris–H_2SO_4 (pH 8.5), 10 mM $KHCO_3$, 10 mM $MgSO_4$, 10 mM potassium PEP, 1 mM acetyl-CoA, an allosteric activator, 0.1 mM NADH, and 1 U malate dehydrogenase. The rate of NADH oxidation was followed by absorbance at 340 nm. One unit of enzyme activity was defined as that producing 1 μmol oxaloacetate per min. Protein concentrations were determined by the Bradford method [13] with bovine serum albumin as the standard.

2.4. Other methods

The N-terminal amino acid sequence was determined by Edman degradation. Gel-filtration chromatography was carried out using a Superose 6 column (ϕ: 10 mm × 300 mm, Amersham Pharmacia, Uppsala, Sweden) with 50 mM potassium phosphate (pH 7.4) and 1 mM DTT at a flow rate of 0.5 ml/min. Circular dichroism (CD) spectra were obtained at 15 °C with a J-720W spectrometer (Jasco, Tokyo, Japan) using a 2-mm cuvette. The protein concentration was 50 μg/ml and the buffer was 50 mM potassium phosphate (pH 7.4) and 1 mM DTT. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF-MS) was carried out using a Voyager DE-STR instrument (Applied Biosystems, CA, USA).

3. Results

3.1. Protease treatment of ThPEPC

We previously reported that ThPEPC is far more tolerant than EcPEPC to heat and chemical denaturants [8]. When 500 μg/ml of EcPEPC was treated with 10 μg/ml of trypsin or chymotrypsin at 30 °C, the polypeptide was almost completely digested within 30 min so that no polypeptide band was visible in SDS-PAGE (data not shown). On the other hand, when ThPEPC was treated with trypsin or chymotrypsin under the same conditions, two principal fragments of about 70 and 20 kDa were obtained and this mixture remained stable to further digestion for more than 20 h as described later. Therefore, ThPEPC was shown to be also far more tolerant to proteolytic cleavage than EcPEPC.

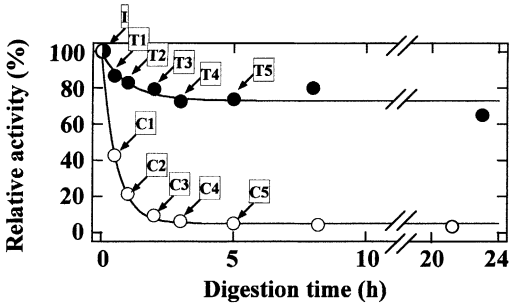

Fig. 1. Protease treatment of ThPEPC. Relative activities (%) of ThPEPC treated with trypsin (closed circles) and chymotrypsin (open circles) are plotted vs. the digestion time. Control value (100%) is the activity of the intact enzyme. For the characters at the individual data points, see the legend for Fig. 2A.

When ThPEPC was subjected to proteolytic cleavage, the residual activity changed depending on the protease used (Fig. 1). When ThPEPC was treated with chymotrypsin, the activity was largely reduced. Residual activity of 5%, detected after chymotrypsin treatment for 5 h (Fig. 1, point C5), was attributed to that of the undigested polypeptide as judged by densitometry of the SDS-PAGE band (data not shown). When the intact polypeptide was completely removed by further digestion with fresh chymotrypsin, the activity of chymotrypsin-treated ThPEPC was not detectable (>0.2% of the original activity). Therefore, chymotrypsin treatment almost completely inactivated ThPEPC. However, treatment with trypsin partially inactivated ThPEPC and the activity became constant at around 70% of the original activity. Substrate saturation experiments revealed that the maximum velocity of ThPEPC was not affected by trypsin treatment (Table 1). In contrast, the K_m values for PEP and bicarbonate were increased by 5.4- and 2.0-fold, respectively. Thus, it was due to the increase in the K_m values that the observed activity of ThPEPC was reduced to around 70% by trypsin treatment (Fig. 1).

Table 1
Catalytic property of ThPEPC

Enzyme	PEP		$KHCO_3$	
	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)
Intact	41 ± 2[a]	0.36 ± 0.05	41 ± 5	0.53 ± 0.17
Trypsin-treated	41 ± 3	1.4 ± 0.7	38 ± 3	1.0 ± 0.4

[a] Average ± S.D. from three independent measurements.

3.2. Determination of the cleavage sites

Although trypsin and chymotrypsin treatments had different effects on the activity of ThPEPC, the fragmentation patterns of ThPEPC with these proteases were very similar as judged by SDS-PAGE (Fig. 2A). During trypsin treatment, a band of around 70 kDa (band b) and three discrete bands of around 20 kDa (bands c–e) appeared in place of the intact ThPEPC band (band a, 96 kDa). Similarly, with chymotrypsin treatment, bands of around 70 kDa (band f) and around 20 kDa (band g) were observed along with the disappearance of band a. Therefore, both the trypsin and chymotrypsin treatments cleaved ThPEPC into major fragments of around 70 and 20 kDa.

The cleavage sites by protease treatment were determined by N-terminal sequence analysis of the polypeptides separated by SDS-PAGE (Fig. 2B). The N-terminal sequence of the intact ThPEPC (Fig. 2A, band a) revealed that the methionine residue of the translation initiation was processed during accumulation in the *E. coli* cells. The N-terminal sequences of bands b and f were identical to that of the intact polypeptide. The sequences of bands c–e, and g indicated that the cleavage sites were located around residue 690: the sites by trypsin were Arg689–Val690 (Fig. 2C, arrow 3), Arg691–Asp692 (arrow 4), and Arg694–Asp695 (arrow 5), and the site by chymotrypsin was Tyr685–Arg686 (arrow 1).

In order to examine whether or not cleavages at other sites occurred during the protease treatment, immobilized protease-treated ThPEPC was then completely digested by *Achromobacter* protease I (API) and subjected to MALDI TOF-MS analysis (Fig. 3). The peak, which is present in the API-digests of the protease-treated ThPEPC but is absent in those of the intact enzyme, should represent the peptides neighboring the cleavage sites (Fig. 3B, a hatched box). Most of the possible fragments indicated in Fig. 3B and two new peaks (Fig. 3A, peaks T1 and C1) were found among the API-digests of the protease-treated ThPEPC. By comparing the measured and calculated masses, peaks T1 and C1 were assigned to the peptides from Ala635 to Arg686 (M_{meas}, 6029.0 Da; M_{calc}, 6028.9 Da) and from Ala635 to

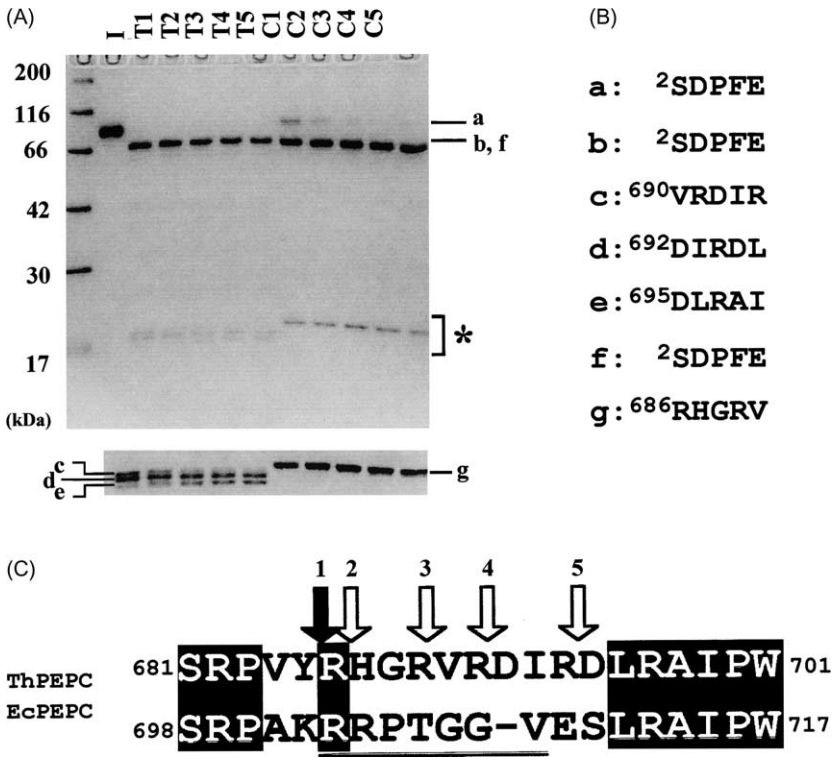

Fig. 2. Fragmentation of ThPEPC by trypsin and chymotrypsin. (A) Intact and digested ThPEPCs indicated in Fig. 1 (2.5 μg of total protein) were subjected to SDS-PAGE in a 10–20% gradient gel (upper panel). The bands around 20 kDa (indicated by an asterisk) were resolved by the use of a 15–25% gradient gel and are shown in the lower panel. Molecular masses of the standard proteins are indicated in the left column. Seven kinds of bands are indicated by characters from a to g, where band b is from lanes T1 to T5 and band f is from lanes C1 to C5. (B) Polypeptides of the bands in panel A were recovered from the gel and the N-terminal sequences were determined. Numbers at the first residue indicate the positions from the methionine residue of the translation initiation of ThPEPC. (C) Amino acid sequence alignment of ThPEPC and EcPEPC around the cleavage sites is shown. Cleavage site of ThPEPC by chymotrypsin is indicated by solid arrow 1, and those by trypsin are indicated by open arrows 2–5. Underlined residues are thought to form a flexible loop near the active site based on the crystal structure of EcPEPC [5] (see text).

Tyr685 (M_{meas}, 5872.7 Da; M_{calc}, 5872.7 Da). Therefore, ThPEPC was found to be cleaved by trypsin at Arg686–His687 (Fig. 2C, arrow 2), in addition to the cleavage sites revealed by N-terminal sequencing (arrows 3–5). Among the API-digests of the trypsin- or chymotrypsin-treated ThPEPC, peaks for the C-terminal peptide (black boxes in Fig. 3B, M_{calc}, 6260.2 Da) were found (Fig. 3A, peaks T2 (M_{meas}, 6260.4 Da) and C2 (M_{meas}, 6260.9 Da)). Therefore, the 20 kDa fragment by trypsin or chymotrypsin (Fig. 2A, bands c–e, and g) was not processed at the C-terminal end. This was also confirmed by direct mass measurements of the 20 kDa fragments produced by trypsin or chymotrypsin. During trypsin treatment, peptides from Val690, Asp692, and Asp695 to the C-terminal end (Gly857) were detected, and during chymotrypsin treatment, a peptide from Arg686 to Gly857 was detected (data not shown). Furthermore, from the time course and the mass measurements of the trypsin-treated ThPEPC, it was suggested that cleavages at Arg686–His687 (Fig. 2C, arrow 2) and Arg689–Val690 (arrow 3) preceded those at Arg691–Asp692 (arrow 4) and Arg694–Asp695 (arrow 5) (data not shown).

In summary, the cleavage sites by trypsin or chymotrypsin are shown in Fig. 2C. Cleavages at

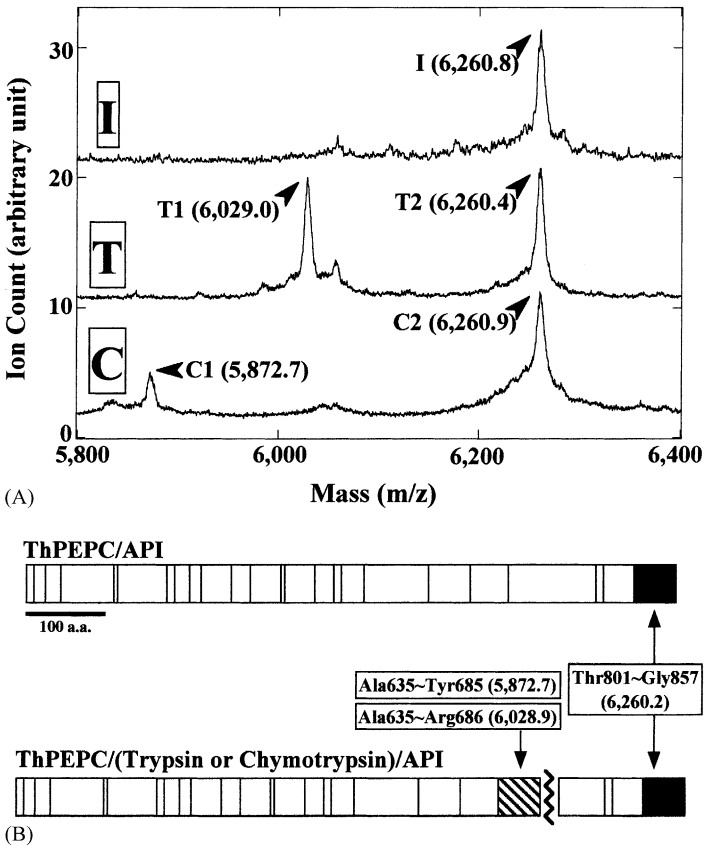

Fig. 3. Assignment of the cleavage sites by MALDI TOF-MS. (A) API-digests of intact (I), trypsin-treated (T), and chymotrypsin-treated (C) ThPEPC were subjected to MALDI TOF-MS, and the spectra around 6 kDa are shown. Measured mass values are shown in the parentheses following the peak names. (B) Possible API fragments of intact and protease-treated ThPEPC are shown. Calculated mass values are shown in parentheses. Black boxes indicate the C-terminal peptide of ThPEPC. A hatched box indicates the C-terminal peptide of the 70 kDa fragment by protease treatment, which is not found in the API-digests of intact ThPEPC. All mass values are of $[M + H]^+$ ions.

Arg686–His687 (Fig. 2C, arrow 2), Arg689–Val690 (arrow 3), Arg691–Asp692 (arrow 4), and Arg694–Asp695 (arrow 5) did not significantly inactivate ThPEPC, whereas, cleavage at Tyr685–Arg686 (arrow 1) inactivated the enzyme almost completely.

3.3. Property of ThPEPC treated with trypsin or chymotrypsin

Intact ThPEPC, as well as many other PEPCs from various organisms, forms a tetrameric complex whose molecular mass is about 400 kDa in the native state. As shown in Fig. 4, both protease-treated enzymes were eluted from a superose 6 column in a similar manner to that of the intact enzyme, indicating that the quaternary structure of the enzyme was not affected by the proteolytic cleavage. Far-UV CD spectra of the trypsin- and chymotrypsin-treated ThPEPCs were also essentially identical to that of the intact enzyme (data not shown). All the results obtained here supported the view that the trypsin and chymotrypsin treatments do not cause a significant change in the three-dimensional structure of ThPEPC.

Furthermore, the thermostability of trypsin-treated ThPEPC was evaluated by activity measurements of the heat-treated enzymes (Fig. 5). When intact and trypsin-treated ThPEPCs were incubated at 90 or 95 °C, inactivation occurred in a biphasic manner in

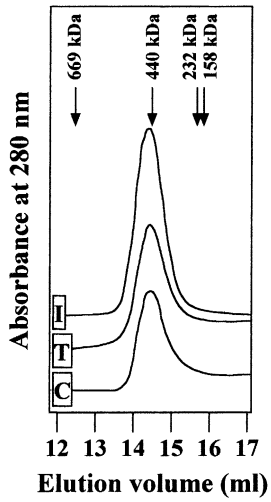

Fig. 4. Gel-filtration chromatography of intact (I), trypsin-treated (T), and chymotrypsin-treated (C) ThPEPC. Arrows indicate the elution volumes of the standard proteins.

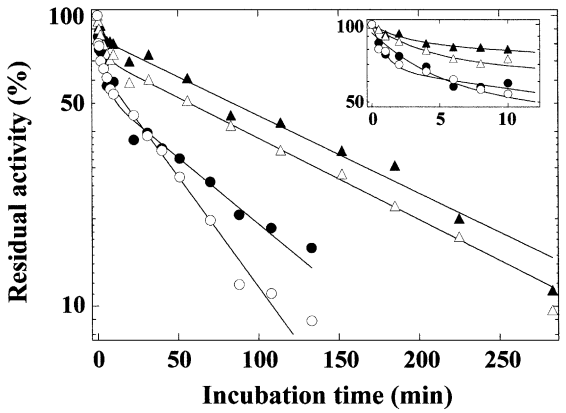

Fig. 5. ThPEPC (50 μg/ml) in 50 mM potassium phosphate buffer (pH 7.4) was incubated at 90 or 95 °C. Aliquots were withdrawn at the indicated times and chilled on ice and were assayed for activity at 30 °C. Residual activity of intact (closed symbols) and trypsin-treated (open symbols) ThPEPC at 30 °C are plotted vs. the time of incubation at 90 (triangles) or 95 °C (circles). Control values (100%) are the activities of the enzymes before heat treatment. The data were fitted to double exponential equations. The rate constants for the slower phases are as follows: (▲) $(6.2 \pm 0.7) \times 10^{-3}\,\mathrm{min}^{-1}$, (△) $(6.5 \pm 0.6) \times 10^{-3}\,\mathrm{min}^{-1}$, (●) $(1.1 \pm 0.3) \times 10^{-2}\,\mathrm{min}^{-1}$, and (○) $(1.8 \pm 0.2) \times 10^{-2}\,\mathrm{min}^{-1}$, where the errors are 95% confidence limits.

which the rapid phase was completed within 10 min (Fig. 5, inset). In the rapid phase during incubation at 90 or 95 °C, roughly 25 and 35% of the activities of both intact and trypsin-treated ThPEPCs were lost, respectively. Further inactivation proceeded in the following slower phase. The inactivation rate constants for the slower phase of intact and trypsin-treated ThPEPCs coincided well with each other, especially at 90 °C (Fig. 5, legend).

Heat denaturation of intact and protease-treated ThPEPCs was followed by monitoring CD signals at 222 nm at 90 or 95 °C. The results showed that the denaturation rates of trypsin- and chymotrypsin-treated ThPEPCs were not significantly different from that of the intact enzyme (data not shown). In summary, the thermostability of ThPEPC was not markedly altered by trypsin or chymotrypsin treatment.

4. Discussion

Several proteins are known to remain active even though they are fragmented into two pieces by limited proteolysis or chemical cleavage [14–17]. The minimum requirement for tolerance to a single cleavage is that the active site is not damaged by the cleavage. Even though the cleavage occurs within the active site, if the connectivity is not essential, the cleaved protein remains active. By discriminating the sites where the main chain needs to be continuous for activity and ones where it does not, the role of main chains forming the active site can be revealed. The importance of main chain connectivity can be studied through main chain cleavage rather than by amino acid substitutions. There have been several attempts to introduce cleavage of the main chain of a protein by procedures such as dissection [18–24] and circular permutation [25–28] of the coding gene. Different from these genetic procedures, the method using limited proteolysis has an advantage in that the folding process can be neglected so that the role of the peptide bond in a native state protein can be analyzed. On the other hand, the disadvantage of limited proteolysis is that it is difficult to precisely control the cleavage site. In this study, we obtained protein samples cleaved at different sites in the same region of a protein by limited proteolysis. By studying functional and structural features of the cleaved proteins,

we could discriminate the importance of local peptide bonds.

In this paper, we described that ThPEPC was almost completely inactivated by chymotrypsin treatment but not by trypsin treatment. One can find novelty in that fragmented ThPEPC retained a comparable level of thermostability as that of the intact enzyme, although fragmentation of enzymes even from thermophiles generally decreases the thermostability [19,20]. The cleavage sites of ThPEPC (arrows in Fig. 2C) occurred in a flexible region as suggested from the crystal structure of the homologous PEPC from *E. coli*, in which seven residues in this region (indicated by an underline, Fig. 2C) were not traced [5]. This flexible region is located in the place where catalytically important residues are concentrated. Therefore, the peptide region around the cleavage sites of ThPEPC is very likely to form a flexible loop that participates in enzyme activity. The cleavage sites by trypsin and chymotrypsin were located side by side; one of the cleavage sites by trypsin was Arg686–His687 and that by chymotrypsin was Try685–Arg686 (Fig. 2C). The results obtained in this study revealed that the main chain connectivity in the putative flexible loop is not critical for maintaining the tertiary structure and stability of ThPEPC. In other words, the fragmented ThPEPC can exist as "an octameric protein ($\alpha_4\beta_4$)" that has a structure and stability similar to those of the intact protein. As expected from the cleavage at the putative flexible loop near the active site, chymotrypsin treatment almost completely inactivated ThPEPC. However, ThPEPC was not inactivatedby trypsin treatment which caused cleavage at the same chain region, although the affinity for its substrates was slightly reduced. Based on the structural features of the fragmented enzymes, the difference between the activities of the protease-treated ThPEPCs is due to the local difference between the cleavage sites by trypsin and chymotrypsin rather than to an overall structural change. From the activities of the protease-treated ThPEPCs, the connectivity at Tyr685–Arg686 (Fig. 2C, arrow 1) was crucial to the catalytic activity of ThPEPC, whereas, those at Arg686–His687 (arrow 2) Arg689–Val690 (arrow 3), Arg691–Asp692 (arrow 4), and Arg694–Asp695 (arrow 5) were not. In other words, Arg686 needs to be connected to the N-terminal region by a peptide bond for catalytic activity of ThPEPC. This shows that proteolytic cleavage at a putative flexible loop near the active site does not always inactivate the enzyme and that the effect of fragmentation depends on the local cleavage site. In this way, for a single flexible loop near the active site, the site where the main chain needs to be continuous for catalysis and the site where it does not were distinguished.

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