Rapid and Efficient Resynthesis of Proteolyzed Triose Phosphate Isomerase

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The use of proteases for peptide bond formation in a mixed aqueous−organic milieu has a long history.[1] Laskowski and co-workers were the first to exploit this methodology for the selective formation of an amide bond between the noncovalent complex of the ribonuclease S-peptide and S-protein.[2] A mixture of ribonuclease A and des-Ser21-ribonuclease A was obtained in 50% yield after a 2 week reaction with subtilisin as the catalyst in 90% glycerol. Protease-catalyzed amide bond formation with the addition of organic cosolvents is attributed to an increase in pK_a vlaue of the C-terminal carboxyl group, resulting in a shift of the equilibrium toward synthesis.[3] Other researchers have used proteases to form a single amide bond between two protein chains, but the lengthy reaction times and low yield obtained have generally made this method impractical for protein synthesis.[4] We now report the use of subtilisin in a mixed aqueous−organic medium to catalyze a remarkably facile and high-yielding resynthesis of triose phosphate isomerase from a mixture of its subtilisin-proteolyzed fragments.

Triose phosphate isomerase (TIM), a member of the α/β-barrel structural class of proteins, is dimeric and contains no disulfide bonds.[5] When rabbit muscle TIM was treated under limited proteolysis conditions with 0.01 equiv of subtilisin Carlsberg, approximately eight fragments were generated (Figure 1a), corresponding to cleavage at three surface-exposed loop sites within the protein (Figure 2): Ala31−Lys32, Leu93−Gly94, and Thr139−Glu140.[6,7]

Upon treating the TIM proteolysis mixture, which still contained subtilisin, with acetonitrile during HPLC separation of the fragments, we made a surprising discovery: the fragments, when analyzed by SDS−PAGE, had been converted to a band with a mobility identical to that of native TIM. That all of the native TIM had initially been proteolyzed was determined by treating the entire proteolysis reaction with loading buffer followed by electrophoresis (Figure 1a). We investigated the effects of differing percentages of acetonitrile and glycerol on this apparent resynthesis of TIM, with 60% acetonitrile and 90% glycerol giving conversion of the fragment bands to a band corresponding to TIM within 10 min (Figure 1b, lanes 3 and 4). HPLC purification of the acetonitrile-treated reaction mixture gave a 62% yield of the apparently resynthesized TIM.

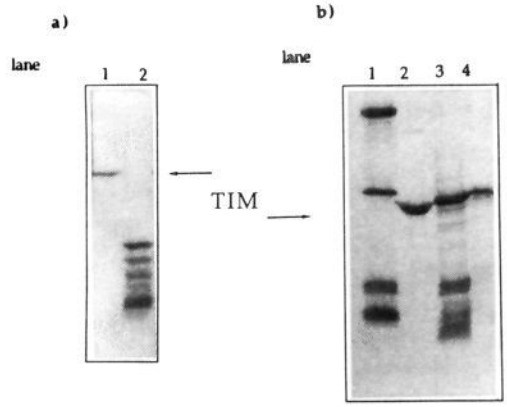

Figure 1. (a) Limited proteolysis of TIM by subtilisin. Lane 1, native TIM; lane 2, eight proteolyzed fragments of TIM. (b) Effect of organic cosolvents on the reformation of TIM. Lane 1, molecular weight markers; lane 2, native TIM; lane 3, subtilisin cleaved TIM with 60% acetonitrile; lane 4, subtilisin cleaved TIM with 90% glycerol.

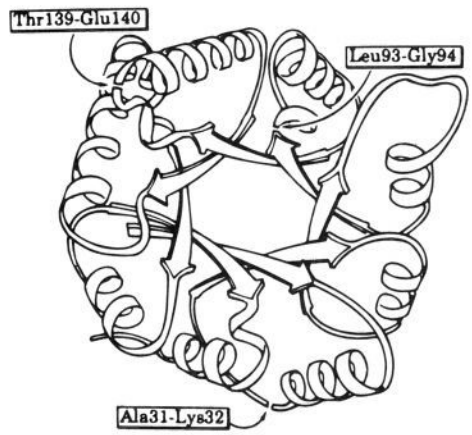

Figure 2. Ribbon diagram of TIM showing subtilisin cleavage sites: Ala31−Lys32, Leu93−Gly94, and Thr139−Glu140.

A number of methods were used to determine that covalent bonds had indeed been formed in the course of this reaction. Both native and reformed TIM (crude and purified material) had identical electrophoretic mobility when analyzed by SDS−PAGE and capillary electrophoresis.[8] The reformed TIM (crude material) was sequenced through the first 36 amino acids, confirming that the Ala31−Lys32 amide bond was intact, and, more strikingly, no N-terminal fragments corresponding to cleavage sites were observed in the sequencing, whereas sequencing of the proteolysis mixture showed all of the cleavage fragments. The native and reformed TIM (purified material) were also analyzed by electrospray mass spectrometry, and both gave an identical mass of 26 628, whereas the cleavage fragments produced a spectrum which corresponded to no one molecular weight species (Figure 3).

Circular dichroism (CD) spectroscopy, size exclusion chromatography, and triose phosphate isomerase activity were used to show that the reformed TIM had not been denatured under the reaction conditions for resynthesis. The CD spectra of both the native and the reformed TIM (purified material) were identical (Figure 4), suggesting that the α/β-barrel structure had

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(6) (a) Cleavage at the 139−140 site has been reported: Sun, A.-Q.; Yuksel, K. U.; Gracy, R. W. *Arch. Biochem. Biophys.* **1992**, *293*, 382−390. (b) Sun, A.-Q.; Yuksel, K. U.; Gracy, R. W. *J. Biol. Chem.* **1993**, *268*, 26872−26878.

(7) Limited proteolysis conditions: 0.01 equiv of subtilisin Carlsberg (Sigma) in a 50 mM triethanolamine, pH 7.5 buffer, at 37 °C for 24 h.

(8) SDS−PAGE conditions: 15% isocratic acrylamide separating gels were used with a discontinuous buffer system. Capillary electrophoresis conditions: Bio-Rad BioFocus 3000 capillary electrophoresis system with a 24 cm fused silica capillary; field strength was 7.2 kV (300 V/cm); running buffer was 50 mM glycine pH 10.

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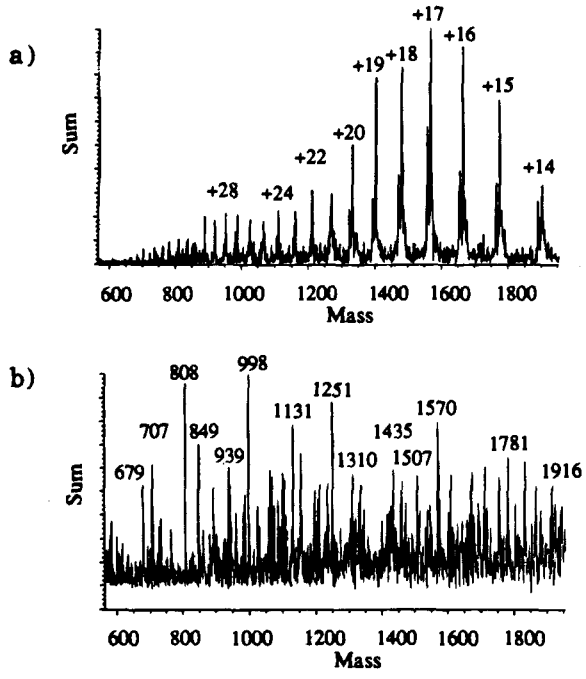

Figure 3. Electrospray mass spectra of reformed (a) and proteolysis mixture (b) of TIM. Deconvoluted spectrum of (a) gave a molecular weight of 26 628.

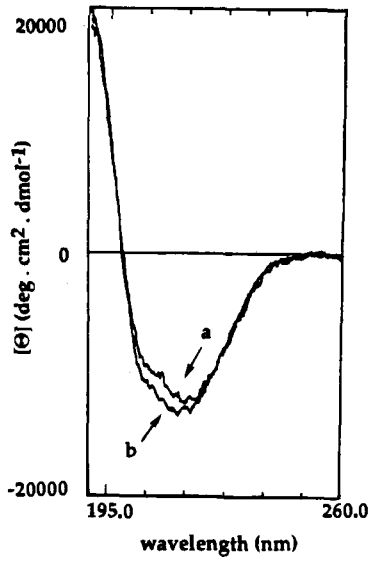

Figure 4. Circular dichroism spectra of reformed (a) and native (b) TIM at 2.5 μM in 10 mM phosphate, pH 7.0, at 25 °C. Concentrations were determined from quantitative amino acid analysis.

remained intact, and size exclusion chromatography confirmed the dimeric structure of the reformed TIM (Table 1).[9] The isomerase activity of the reformed TIM (purified material) was also 70% that of the native TIM, providing additional evidence for a native, biologically active structure in the resynthesized material (Table 1).[10]

To confirm the role of subtilisin in the resynthesis reaction, we treated the cleavage mixture with the covalent subtilisin inhibitor, phenylmethylsulfonyl fluoride, prior to the addition of acetonitrile.[11] Under these conditions, the fragments remained intact and no amide bond formation occurred. Attempts to use immobilized subtilisin, removed by filtration before the addition of the organic solvent, were inconclusive due to the dissociation of subtilisin from the solid support.

Table 1. Aggregation State and Enzymatic Activity of Native, Reformed, and Proteolyzed TIM

TIM	apparent MW	aggregation state	rate constant (s^{-1})[a]
native	48 967	1.88	4.73×10^{-3}
reformed	46 560	1.79	3.50×10^{-3}
proteolyzed	49 463	1.90	3.29×10^{-3}

[a] The rate of TIM isomerization was determined using a coupled enzyme assay where the oxidation of the cofactor NADH to NAD^+ was monitored as a decrease in absorbance at 340 nm.[10]

To test if the facile nature of the TIM resynthesis reaction was due to a stable, self-assembled form of the protein fragments, we performed nondenaturing size exclusion chromatography and assayed the rate of isomerization of triose phosphate with the proteolyzed sample. The protein fragments yielded the same apparent molecular weight as TIM under size exclusion conditions (Table 1), confirming a stable assembly of fragments.[6b] The fragments also isomerized triose phosphate at a rate similar to native that of TIM (Table 1), indicating that the fragments were assembled in a biologically-active conformation.[6b] This tendency of the proteolysis fragments to remain self-assembled may be responsible, in part, for the high efficiency of the resynthesis of TIM, as the entropic barrier of bringing together the carboxylic acid and amino groups prior to amide bond formation would be lowered.

In conclusion, we have demonstrated that fragments derived from three cleavage sites within TIM are reformed with rapid reaction times and in high yield in the presence of subtilisin and 60% acetonitrile or 90% glycerol. The TIM formed from this reaction has a structure and biological activity similar to those of native TIM. This is the first example of the use of a protease to form three specific bonds within a protein, as well as being the longest sequence prepared by this method. Due to the interest in self-assembly in *de novo* protein design, a knowledge of a limited number of proteolysis sites within a protein may provide essential information for catalyzed reassembly of protein fragments in protein synthesis, and we are currently exploring the utility of these remarkable findings with other protein systems.

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Supplementary Material Available: Figures showing capillary electrophoresis of native and reformed TIM, electrospray mass spectrum of TIM multiply-charged ions and the deconvoluted spectrum, and the deconvoluted electrospray mass spectrum of reformed TIM showing MW 26 628 (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(9) Size exclusion chromatography was performed on a Sephadex G-50 column with a 50 mM triethanolamine, pH 7.5 buffer, at 4 °C. The apparent molecular weight was determined on the basis of a standard curve generated with protein standards.

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