Conformationally driven protease-catalyzed splicing of peptide segments: V8 protease-mediated synthesis of fragments derived from thermolysin and ribonuclease A

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Abstract

We have studied the conformation as well as V8 protease-mediated synthesis of peptide fragments, namely amino acid residues 295–316 (TC-peptide) of thermolysin and residues 1–20 (S-peptide) of ribonuclease A, to examine whether “conformational trapping” of the product can facilitate reverse proteolysis. The circular dichroism study showed cosolvent-mediated cooperative helix formation in TC-peptide with attainment of about 30–35% helicity in the presence of 40% 1-propanol and 2-propanol solutions at pH 6 and 4 °C. The thermal melting profiles of TC-peptide in the above cosolvents were very similar. V8 protease catalyzed the synthesis of TC-peptide from a 1:1 mixture of the non-interacting complementary fragments (TC295–302 and TC303–316) in the presence of the above cosolvents at pH 6 and 4 °C. In contrast, V8 protease did not catalyze the ligation of S1–9 and S10–20, although S-peptide could assume helical conformation in the presence of the cosolvent used for the semisynthetic reaction. V8 protease was able to synthesize an analog of S-peptide (SA-peptide) in which residues 10–14 were substituted (RQHMD → VAAAK). While S-peptide exhibited helical conformation in the presence of aqueous propanol solutions, SA-peptide displayed predominantly β -sheet conformation. SA-peptide showed enhanced resistance to proteolysis as compared with S-peptide. Thus, failure of semisynthesis of S-peptide may be a consequence of high flexibility around the 9–10 peptide bond due to its proximity to the helix stop signal. The results suggest that protease-mediated ligations may be achieved by design and manipulation of the conformational aspects of the product.

Keywords: conformation; organic cosolvent; proteosynthesis; reverse proteolysis; semisynthesis

Recent years have seen remarkable progress in the area of synthetic protein chemistry toward developing strategies for chemical ligation of polypeptide fragments for construction of natural as well as novel proteins based on *de novo* design principles (Dawson & Kent, 1993; Dawson et al., 1994; Gaertner et al., 1994; Jackson et al., 1994; Liu & Tam, 1994; Tam et al., 1995; Wallace, 1995). The protease-catalyzed ligation of protein/peptide fragments offers the same flexibility to a selected segment as that available in modular chemical ligation strategy and may act as a bridge between chemical and genetic approaches for construction of pro-

teins. Unfortunately, the semisynthesis of proteins through reverse proteolytic condensation of protein/peptide fragments in aqueous-organic solution (Homandberg et al., 1978) is generally facilitated in special circumstances where the complementary fragments are held together by covalent or noncovalent interactions yielding appropriate stereochemistry for the reacting α -amino and α -carboxyl groups as in “fragment complementing” systems of proteins such as RNase A, nuclease T, and somatotropin (Homandberg & Laskowski Jr., 1979; Komoriya et al., 1980; Graf & Li, 1981). In situations where interaction or complexation between the complementary fragments is absent, the equilibria of a protease-catalyzed reaction may be facilitated to peptide bond synthesis by using “molecular traps” that specifically interact with the product (Homandberg et al., 1982; Nyberg, 1988).

The V8 protease-mediated stitching of Glu30–Arg31 peptide bond of α globin in a 1:1 mixture of complementary fragments, α 1–30 and α 31–141, as well as of truncated segments, α 17–30 and α 31–40, was distinct from semisynthetic reactions alluded to above (Roy et al., 1992). The α globin semisynthetic reaction occurred without the benefit of any association or interaction between the reacting fragments, proceeded smoothly in the absence of an ex-

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Abbreviations: RPHPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; CD, circular dichroism; TFE, trifluoroethanol; TC-peptide, residues 295–316 of thermolysin; RNase A, ribonuclease A; S-peptide, the peptide sequence representing the first 20 residues of RNase A where the Glu-2 residue has been substituted by Tyr to facilitate determination of the peptide's concentration; SA-peptide, an S-peptide analog containing substitutions at positions 10–14 (RQHMD → VAAAK).

ternal molecular trap, and was facilitated in helix-promoting organic cosolvents as opposed to aqueous glycerol solutions. It was suggested that the organic cosolvent-induced helix formation in the nascent ligated peptide (synthetic product) acted as a "conformational trap" for the α globin semisynthetic reaction.

The logical extension of the conformational trap hypothesis is that protease-catalyzed ligation of any two peptide fragments (compatible with the specificity of the protease) should be possible in principle, provided the contiguous peptide (product) acquires a proteolytically resistant folded conformation. Furthermore, since conformational flexibility of a peptide bond is the major determinant of proteolysis (Fontana et al., 1986), local conformation of the target peptide bond (cleavage/ligation site) in the ligated peptide (product) should be an important factor in "reverse proteolysis."

The present study has been designed to test the aforementioned aspects of the "conformational trap" idea. The fragments spanning helical regions of proteins have been chosen as test cases with the assumption that intrinsic helix-forming propensity of these sequences would be enhanced in the presence of favorable organic cosolvents (1-propanol, 2-propanol, TFE, etc.) used in protease-mediated splicing reactions and act as a conformational trap. Accordingly, studies on the conformation and V8 protease-catalyzed synthesis of carboxy terminal helical fragment of thermolysin spanning residues 295–316 (TC-peptide) has been carried out. Also, an analog of S-peptide (residues 1–20 of RNase A) containing amino acid substitution of residues 10 to 14 (RQHMD $\rightarrow$ VAAAK) has been designed and its conformation and semisynthesis studied to see whether the failure of V8 protease to catalyze the synthesis of S-peptide was a consequence of the local conformation around the 9–10 peptide bond. The overall results suggest that reverse proteolytic condensation of protein/peptide fragments may be achieved by simply manipulating the conformational aspects of the product.

Results

Choice and design of peptide fragments

The choice of carboxy terminal helical fragment of thermolysin spanning residues 295–316 (TC-peptide) for semisynthetic studies is based on the crystal structure data of thermolysin that show residues 301–312 in helical conformation (Titani et al., 1972; Holmes & Matthews, 1982). Also, CD and NMR data in aqueous and aqueous-organic solutions for the fragment 299–316 of thermolysin indicate helical structure for residues 301–311 (Jimenez et al., 1993). Therefore, it is reasonable to assume that the Glu302–Val303 peptide bond in TC-peptide (Fig. 1) would be trapped in a helical conformation once the contiguity of the complementary fragments, 295–302 and 303–316, is established through V8 protease-catalyzed reverse reaction in helix-promoting organic cosolvents. The 302–303 peptide bond has been shown to be highly resistant to proteolysis in native thermolysin as well as in the isolated 205–316 fragment (De Filippis & Fontana, 1990).

S-peptide, the N-terminal helical fragment of RNase A, has been studied extensively and shown to acquire helical conformation. However, V8 protease was not able to catalyze the synthesis of S-peptide (residues 1–20 of RNase A) from complementary segments, S1–9 and S10–20, although S-peptide (product) could assume significant helical conformation under the reaction conditions used for semisynthetic reaction (Acharya et al., 1993). Apparently, the 9–10 peptide bond was not afforded protection from proteolysis. We surmise that the cause for the failure of V8 protease to

Fig. 1. Sequence of peptides. Amino acid sequence of the carboxy terminal fragment spanning residues 295–316 of thermolysin (TC-peptide), amino terminal fragment of RNase A spanning residues 1–20 (S-peptide), and an analog of S-peptide (SA-peptide) are illustrated. Y has been substituted for E at position 2 in S-peptide and SA-peptide for facilitating concentration determination. The presence of Y at position 296 in TC-peptide serves the same purpose. The sequences representing respective complementary fragments are underlined. Note that residues 1–9 in S-peptide and SA-peptide are identical. V8 protease slicing and splicing site in each peptide is shown by an arrow pointing upward while endoproteinase lys-C or trypsin site in TC-peptide is shown by an arrow pointing downward.

facilitate the synthesis of the 9–10 peptide bond might be enhanced conformational flexibility in this region of the molecule arising from fraying of the helix as His12/Met13 act as a helix stop signal in both RNase A and isolated S-peptide (Kim & Baldwin, 1984). The NMR studies of S-peptide have suggested the persistence of a helix stop signal even in strong helix-promoting solvents such as TFE (Nelson & Kallenbach, 1989). It is therefore conceivable that restriction of conformational flexibility around the 9–10 peptide bond in S-peptide might effect reverse proteolysis. To test this possibility, we prepared an analog of S-peptide (SA-peptide) containing amino acid substitutions at positions 10–14 (Fig. 1). The amino acids RQHMD in S-peptide were substituted to VAAAK based on the AGADIR program (provided by Dr. Victor Munoz, EMBL, Germany) and were expected to confer helical stability around the 9–10 peptide bond.

Cosolvent-induced helical conformation in TC-peptide

A prior knowledge of the conformation of TC-peptide (ligation product) in the presence of helix-promoting organic cosolvent is desirable for design of experimental conditions for protease-mediated ligation. 1-Propanol was chosen as a cosolvent based on earlier semisynthetic studies of α globin (Sahni et al., 1989; Roy et al., 1992). Accordingly, the effect of the concentration of 1-propanol (cosolvent) on the conformation of TC-peptide was monitored by CD spectroscopy (Fig. 2). The TC-peptide exhibited spectra characteristic of helical conformation with minima at 222 nm and 208 nm. The peptide exhibited sigmoidal conformational transition curve with a mid-point at around 22% 1-propanol. The induction of helicity was complete in about 35–40% 1-propanol concentration. The helical content of the peptide was estimated to be about 30% (Chen et al., 1974) in the presence of 40% 1-propanol.

We also recorded a series of spectra of TC-peptide in 40% 1-propanol (pH 6, 4 °C) where the peptide concentration was varied to determine whether intermolecular interaction stabilizes the helical conformation of TC-peptide. No changes in the CD spectrum were observed for a peptide concentration range of 20–100 μ M (data not shown) suggesting a monomeric behavior under these conditions.

Fig. 2. 1-propanol concentration dependence on the CD spectra of TC-peptide. The spectra of TC-peptide was recorded in aqueous solution and in the presence of various concentrations of 1-propanol at pH 6 and 4 °C as described in Materials and methods. The peptide concentration in each sample was approximately 46 μ M. The spectra are illustrated in the order of increasing negative MRE in the presence of increasing concentration of 1-propanol (v/v): no 1-propanol, 10%, 15%, 20%, 30%, 40%, and 60%. Inset shows the dependence of MRE at 222 nm as a function of 1-propanol concentration.

Conformation of 1:1 mixture of complementary fragments

A conformational analysis of discontinuous TC-peptide (1:1 mixture of TC295–302 and TC303–316) was carried out to determine the extent of helical stabilization that may be attained by the nascent product as a consequence of the establishment of contiguity by V8 protease-mediated ligation of the 302–303 peptide bond (data not shown). The discontinuous peptide did not show significant helicity in aqueous solution. However, in the presence of 40% 1-propanol at pH 6 and 4 °C (conditions yielding maximum helix formation in TC-peptide), the discontinuous system assumed about 15% helicity as estimated from the mean residue ellipticity at 222 nm. This implies that about a two-fold increase in helicity would be realized due to enzymatic peptide ligation since contiguous TC-peptide under identical conditions exhibited 30% helicity.

V8 protease-catalyzed synthesis of TC-peptide

We investigated the ability of V8 protease to splice the 302–303 peptide to see whether a contiguity-mediated increase of helical conformation in the presence of organic cosolvent can act as a “trap” for the synthesis of TC-peptide. An equimolar mixture (2 mM) of complementary fragments (TC295–302 and TC303–316) was incubated with V8 protease in 40% 1-propanol at pH 6 and 4 °C. The RPHPLC analysis of an aliquot of the reaction mixture after 24 h showed the presence of a new peak eluting at the same position as that of standard TC-peptide indicating that peptide ligation had occurred. The ligation reaction attained equilibrium in about 48 h. The equilibrium yield of the product was around 23%.

Structural identity of the enzymically ligated product

The product was characterized as TC-peptide based on the following: V8 protease digestion followed by RPHPLC analysis yielded only two peptides that eluted at the respective positions of TC295–302 and TC303–316; RPHPLC peptide maps generated from endoprotease Lys-C digest of the enzymatic product and authentic synthetic TC-peptide were identical; 10 cycles of Edman degradation yielded the same sequence as that of the first 10 residues of TC-peptide, establishing the covalent contiguity of the 302–303 peptide bond; and the positive ion electrospray ionization mass spectrometry gave a mass of 2,284.09, which was in agreement with the calculated mass (Table 1).

Table 1. Electrospray ionization mass spectrometric characterization of peptides

Sample	Mass ^a (Da)	
	Calculated	Measured
TC-peptide	2,284.55	2,284.09
TC295–302	883.91	883.99
TC303–316	1,418.65	1,418.43
SA-peptide	1,973.21	1,973.56
S1–9	1,027.70	1,028.17
SA10–20	963.05	963.38

^a Values represent average mass (natural isotopic abundance).

V8 protease-mediated synthesis of TC-peptide in other organic cosolvents

Glycerol has been frequently used as an organic cosolvent in proteosynthetic reactions of peptide/protein systems (Chaiken, 1981). However, V8 protease was not able to synthesize TC-peptide in the presence of 50% glycerol, indicating that propanol-mediated stabilization of the structure of the product played a pivotal role in driving the synthetic reaction. If cosolvent-mediated acquisition of helical conformation by the nascent product is the structural element that facilitated the ligation reaction, the synthesis of TC-peptide should not be unique to the presence of 1-propanol and must also occur in other helix-favoring organic cosolvents. Therefore, we carried out the synthesis in the presence of 40% 2-propanol. The RPHPLC analysis revealed the formation of TC-peptide from a 1:1 mixture of complementary fragments. The yield of the product was about 25%, which was similar to that obtained in the presence of 40% 1-propanol.

Temperature dependence of the CD spectra of TC-peptide

The similar yield of product in either solvent suggested that stability of TC-peptide in 40% 1-propanol or 40% 2-propanol should be similar. To investigate stability differences, if any, the temperature dependence of CD spectra in the presence of these cosolvents was studied. The mean residue ellipticity at 222 nm in the presence of 40% of 1- and 2-propanol at 4 °C was $-10,537$ and $-11,734$, respectively, and as expected, decreased linearly with an increase in temperature, showing a loss of helicity. The isodichroic point at 203 nm was present for spectra in both the solvents, indicating a two-state helix-to-coil transition. Interestingly, almost identical slopes in the thermal melting profiles in both the solvents (Fig. 3) suggest similar stability of TC-peptide in the presence of 40% 1-propanol and 40% 2-propanol solutions.

Fig. 3. Thermal melting profile of TC-peptide. The spectra were recorded at various temperatures at a peptide concentration of $45 \mu\text{M}$ in the presence of 40% 1-propanol and 40% 2-propanol at pH 6. $[\Theta]_t/[\Theta]_4$ represents the ratio of MRE at 222 nm at the indicated temperature to MRE at 4 °C.

Absence of interaction between the complementary fragments

The above results of synthesis of TC-peptide in the presence of helix-promoting aqueous-organic solutions without the aid of an external molecular trap suggest that the protease-mediated ligation reaction was facilitated by conformational trapping of the product. However, this conclusion is valid only if the complementary fragments (TC295–302 and TC303–316) do not exhibit noncovalent interaction between them. In order to show that enzymic coupling occurs even if the two fragments do not interact to form a rather stable complex, we carried out gel filtration experiments on HPLC using a superdex peptide column that was equilibrated and run in 50 mM ammonium acetate buffer (pH 6) containing 40% 1-propanol. The chromatographic profile (Fig. 4) of a 1:1 mixture of the complementary fragments (TC295–302 and TC303–316) revealed the elution of two distinct components at 29 min and 38 min, which corresponded to the retention times of TC303–316 and TC295–302, respectively, when the individual peptides were chromatographed separately under identical conditions. The gel filtration profile did not show the presence of a 1:1 complex of the fragments, suggesting the absence of strong noncovalent interaction between the complementary fragments of TC-peptide. However, associations with low-binding constants would escape detection in the gel filtration experiments. Thus, the possibility of weak interaction between the fragments cannot be ruled out.

Conformation of S-peptide and SA-peptide

Next we turned our attention to investigate whether amino acid substitutions in the proximity of the ligation site (Glu9) can effect the V8 protease-mediated synthesis of the 9–10 peptide bond. For

Fig. 4. Gel filtration of complementary fragments of TC-peptide. The HPLC of 1:1 mixture of TC295–302 and 303–316 was carried out in 50 mM ammonium acetate buffer (pH 6) containing 40% 1-propanol using a superdex peptide column (3.2×30 mm). Twenty μL of a 2 mM solution of the complementary fragments prepared in the above buffer was loaded onto the column and eluted at a constant flow rate of $50 \mu\text{L}/\text{min}$. The two peaks were collected and their identity was established by RPHPLC.

Fig. 5. 1-Propanol concentration dependence on the CD spectra of S-peptide. The spectra of S-peptide ($44 \mu\text{M}$) at various concentrations of 1-propanol were recorded as described for Fig. 2. The numbers refer to % 1-propanol concentration (by volume).

this purpose we synthesized an analog of S-peptide (SA-peptide) in which residues 10–14 were substituted. The substitutions, RQHMD $\rightarrow$ VAAAK, made based on the AGADIR program (Munoz & Serrano, 1994), were anticipated to confer helical stability in this region of the molecule. We studied the circular dichroic behavior of the S-peptide and the SA-peptide in the presence of various concentrations of 1-propanol to gain insight into the conformational features of these peptides.

The 1-propanol concentration dependence on the CD spectra of S-peptide at pH 6 and 4°C exhibited two minima at 222 nm and 208 nm, respectively (Fig. 5) with an isodichroic point at 203 nm typical of a two-state helix-coil transition. Also, the conformational transition showed a cooperative behavior reaching saturable induction of helix formation around 40% 1-propanol. The midpoint of this transition was around 23% 1-propanol. The helical content of S-peptide in 40% 1-propanol was estimated to be around 21% based on MRE at 222 nm (Chen et al., 1974) and 23% from the CD traces employing a computer-based program (Yang et al., 1986).

In contrast to S-peptide, SA-peptide did not show any cooperativity or isodichroism indicating the presence of multiple conformational states in aqueous 1-propanol solution (Fig. 6). The CD spectra were characterized by two minima around 220 nm and 207 nm up to 60% 1-propanol. However, increasing 1-propanol concentration to 70% resulted in a dramatic shift to a single minimum at 217 nm, which is typical of β -sheet conformation. The secondary structure estimate from the CD traces showed the presence of β -sheet conformation even in the presence of lower than 70% 1-propanol solution. About 20–25% β -sheet was estimated from the spectra of SA-peptide recorded in the presence of 40–60% 1-propanol. These results show that, contrary to the expectations, the substitutions in the vicinity of the splice site (residues 10–14),

favoured the formation of β -sheet conformation in aqueous-propanol solutions. This may be a manifestation of the solvent effect.

To investigate the possibility of intermolecular structure formation in SA-peptide, we studied the concentration dependence of SA-peptide on the CD spectra in the presence of 40% 1-propanol (pH 6, 4°C). The spectra were recorded between 23 and $92 \mu\text{M}$ (data not shown). The spectra showed decreasing intensity of the negative band in the 202–208 nm region with increasing peptide concentration. The spectra up to $69 \mu\text{M}$ were characterized by two distinct negative minima, which shifted dramatically to a broad 216–218 nm negative band as the concentration of SA-peptide was increased to $92 \mu\text{M}$, suggesting that structuring of this peptide into β -sheets involved some degree of intermolecular association.

Stability of S-peptide and SA-peptide against proteolysis

We explored whether the ability of SA-peptide to assume β -sheet conformation in 40% 1-propanol could stabilize the 9–10 peptide bond against proteolysis. The proteolysis of S-peptide and SA-peptide in the presence of 40% 1-propanol (pH 6, 25°C) was monitored by RPHPLC. The results shown in Figure 7 indicate that the rate of proteolysis is much slower for SA-peptide ($t_{1/2} = 14$ min) than for S-peptide ($t_{1/2} = 5$ min). About 70% of S-peptide is hydrolyzed by V8 protease within 10 min, in contrast to only 20% hydrolysis of SA-peptide during the same period. The RQHMD $\rightarrow$ VAAAK substitution has conferred elements of resistance to the 9–10 peptide bond against proteolysis in the SA-peptide compared to that of the S-peptide. Thus, the conformational aspects of the SA-peptide (product) should facilitate the reverse proteolytic condensation of S1–9 and SA10–20.

Fig. 6. 1-Propanol concentration dependence on the CD spectra of SA-peptide. The spectra of SA-peptide at various concentrations of 1-propanol were recorded under identical conditions as that of S-peptide. The numbers refer to % 1-propanol concentration (by volume).

Fig. 7. Kinetics of proteolysis of S-peptide and SA-peptide. The V8 protease catalyzed hydrolysis of 9–10 peptide bond of the respective peptide was carried out in 50 mM ammonium acetate buffer (pH 6) containing 40% 1-propanol. The respective peptides (2 mM) were incubated with V8 protease (1:100, w/w, E:S) at 25 °C. The relative peptide concentrations were determined by RPHPLC and are plotted as a function of incubation time. Δ – Δ , SA-peptide; O–O, S-peptide.

V8 Protease-catalyzed ligation of S1–9 with SA10–20

V8 protease-mediated ligation of S1–9 and SA10–20 was carried out at pH 6 and 4 °C in the presence of 40% 1-propanol (conditions that favor β -sheet formation in SA-peptide). The RPHPLC analysis of an aliquot of the reaction mixture demonstrated the formation of a product that was identified as SA-peptide by mass spectrometry and peptide mapping. The yield of the product was about 15%. A 1:1 mixture of complementary fragments of S-peptide did not yield any product under the aforementioned reaction conditions.

Absence of interaction between S1–9 and SA10–20

The complementary fragments of S-peptide, S1–9 and S10–20, do not exhibit any interaction between them. However, the possibility of complex formation between S1–9 and SA10–20 (complementary fragments of SA-peptide) cannot be ruled out. To clarify this point, we carried out gel filtration experiments. The 1:1 mixture of S1–9 and SA10–20 eluted as one peak (retention time, 36 min) when chromatographed on a superdex peptide column run in 40% 1-propanol at pH 6 (Fig. 8). The co-elution of the complementary fragments is expected since molecular weight differences between S1–9 and SA10–20 (1027 Da and 963 Da, respectively) are not enough to effect separation between them. The retention time of the above fragments from the superdex peptide column is consistent with the elution pattern of TC295–302 (mol wt., 883; retention time, 38 min) seen earlier (Fig. 4). The two peptides, S1–9 and SA10–20, when chromatographed separately, also eluted at the same position (retention time, 36 min) as that of the 1:1 mixture.

Fig. 8. Gel filtration of SA-peptide and its complementary fragments. The peptide samples were dissolved in 50 mM ammonium acetate buffer, pH 6, containing 40% 1-propanol, and 20 μ L of the sample (2 mM) were loaded onto the superdex peptide column. The chromatographic conditions were the same as described in Fig. 4. A: SA-peptide; B: 1:1 mixture of S1-9 and SA10-20.

On the other hand, SA-peptide (mol wt., 1973) eluted much earlier when chromatographed under identical conditions. Thus, it may be concluded that strong noncovalent interaction is absent in the complementary fragments of SA-peptide.

Discussion

The proteolysis at a given site is determined by the protease specificity, stereochemistry, and flexibility of the peptide bond (Fontana et al., 1986). Accordingly, peptide bonds in the unstructured regions of polypeptide chains are generally more susceptible to proteolysis than those that are part of rigid secondary structures such as α -helices or β -sheets. Based on the same concept, "reverse proteolysis" of protein/peptide fragments should be facilitated if the target peptide bond (slicing/splicing site) in the synthetic product is trapped in a protease-resistant helical or β -sheet conformation under the employed reaction conditions. We report examples to illustrate that this is indeed true.

The enhancement of helicity in the TC-peptide due to contiguity of the 302-303 peptide bond in the presence of the organic cosolvent used for the ligation reaction, avid preference of the ligation reaction for helix-inducing cosolvents (1-propanol, 2-propanol) as

opposed to aqueous glycerol solutions, similar yield of the product in 40% 1-propanol (23%) and 40% 2-propanol (25%) under identical reaction conditions correlated to the almost identical slopes of the thermal melting profiles in these cosolvents, and the absence of noncovalent interaction between the complementary fragments, suggest that cosolvent-induced helical conformation of the nascent contiguous product drives the V8 protease-catalyzed synthesis of TC-peptide. In contrast, the successful synthesis of SA-peptide is not a consequence of increased helical conformation, as the peptide assumes a predominantly β -sheet conformation in the presence of the organic cosolvent used for the ligation reaction. However, this conformational aspect of the molecule endows resistance against proteolysis to the 9-10 peptide bond, facilitating the synthesis of SA-peptide.

The transition of a helical peptide to β -sheet conformation by removal of the helix stop signal is an interesting result. The reasons for this are not readily apparent. However, the formation of β -sheet structures are reported for peptides containing a combination of Ala, Glu, and Lys residues (Zhang et al., 1993), as are α -helical conformations for peptides of the same composition but with a different sequence (Marqusee & Baldwin, 1987). Recently, a 16-mer peptide containing 14 Ala residues flanked by lysine was shown to exhibit highly stable β -sheet conformation (Forood et al., 1995). The SA-peptide is rich in Ala and Lys residues (8 Ala and 3 Lys). Also, Val, an amino acid with the highest β -sheet propensity ($P_{\beta} = 1.64$), has been substituted at position 10 for Arg ($P_{\beta} = 0.94$) (Smith et al., 1994). Furthermore, 1-propanol has been shown to elicit biphasic effects on the phase transition of poly(L-Lysine) between α -helix and β -sheet conformations (Shibata et al., 1992). It is conceivable that combinatorial factors emanating from the primary sequence of SA-peptide and the organic cosolvent (1-propanol) are responsible for β -sheet formation in SA-peptide.

The present studies have demonstrated that proteosynthesis is possible without the use of an external molecular trap even when the "complementation" phenomenon is absent. The propensity of proteases to act in reverse may be harnessed for peptide bond synthesis if the product acquires a folded conformation, such as α -helix or β -sheet, as present in TC-peptide and SA-peptide. However, the failure of V8 protease-mediated synthesis of S-peptide, in spite of the presence of significant helical conformation (21% in 40% 1-propanol at pH 6 and 4°C), indicates the lack of a correlation between the degree of secondary structure present in the product of a protease-catalyzed reaction and the yield of that product. This observation suggests that local conformational aspects of the target peptide bond (slicing/splicing site in the product) plays an important role in reverse proteolysis. In this connection it is interesting to note that endoproteinase lys-C or trypsin was not able to splice the Lys307-Gln308 peptide bond from complementary fragments, TC295-307 and TC308-316 of TC-peptide (data not shown). On the contrary, both trypsin as well as V8 protease were able to synthesize the 30-31 and 31-32 peptide bonds from their respective complementary fragments to generate α 17-40 (Roy et al., 1992). It is conceivable that that region of 307-308 peptide bond of TC-peptide is more flexible (as in the 9-10 peptide bond of S-peptide) than the 302-303 peptide bond because the helix is expected to extend to residue 311 in thermolysin (Holmes & Matthews, 1982; Jimenez et al., 1993). Thus, the protease-specific slicing/splicing site in the product should lie away from the helical ends for facile ligation reaction since the peptide bonds in the middle of the helices are likely to be more rigid than those that are toward the termini.

Though DNA-based site-directed mutagenesis is a powerful tool for protein design and engineering, the search for innovative approaches for construction of proteins has continued (Schnolzer & Kent, 1992; Chang et al., 1994; Jackson et al., 1994; Tam et al., 1995). This is in consonance with the protein chemists' desire to expand the range of modification of the covalent structure of proteins, including incorporation of noncoded/nonstandard amino acids or other designer "fixed elements of 3D structure" (Balaram, 1992; Schnolzer & Kent, 1992), since only limited variation is possible through genetic approaches due to inherent problems of the ribosomal protein synthesizing machinery.

Protease-mediated ligation of protein/peptide fragments is an attractive approach for modular assembly of new and novel proteins. Although applications of reverse proteolysis have been limited due to paucity of knowledge about mechanisms governing such reactions, examples of protease-catalyzed splicing of proteins keep emerging. The recent demonstrations of subtilisin-mediated regeneration of triose phosphate isomerase from four fragments (Vogel & Chmielewski, 1994) and splicing of concanavalin A by asparaginyl endopeptidase (Min & Hugh Jones, 1994) are noteworthy. The accumulating examples of reverse proteolysis suggest that proteases have enormous synthetic potential, which can be fully exploited only when the structural principles governing protease-catalyzed ligation reactions are completely deciphered. In this connection, the results of the present study suggesting "conformational trap"-mediated facilitation of ligation of non-interacting peptide segments should help in designing strategies for reverse proteolytic fragment condensation.

Materials and methods

Materials

V8 protease (endoproteinase Glu-C), endoproteinase lys-C, and TPCCK-treated trypsin were purchased from Pierce Chemical Co. All other chemicals were of reagent grade and procured from standard commercial sources.

Chemical synthesis of peptides and purification

TC-peptide, S-peptide, and SA-peptide (Fig. 1) were synthesized by standard solid phase method using Fmoc chemistry. The peptides were cleaved from the resin and deprotected with appropriate volume of a mixture consisting of phenol, ethanedithiol, thioanisole, water, and TFA (5:5:5:5:80, by volume) for 2–3 h with gentle shaking at ambient temperature. The resin was removed by filtration and the crude peptides precipitated in cold anhydrous ether. The precipitate was dissolved in water and lyophilized. The peptides were purified by RPHPLC on a semi-preparative aquapore RP300 (250 × 7 mm, 7 μ m; Applied Biosystems) column using a linear gradient of 5–70% solvent B (solvent B: 80% acetonitrile containing 0.1% TFA, solvent A: 0.1% TFA in water) in 130 min. The flow rate was maintained at 2 mL/min throughout the run. The identity of the peptides was established by peptide mapping and mass spectrometry (Table 1).

Preparation of complementary fragments

The respective peptides (TC-peptide, S-peptide, and SA-peptide) were dissolved in 50 mM ammonium acetate buffer, pH 6, and digested with V8 protease (1:100, E/S) at 37°C for 3 h. The

mixture was diluted with TFA to a final concentration of 0.1% of TFA and loaded onto the column for RPHPLC. The digest of each peptide yielded only two peptide segments as expected from a single site cleavage (see Fig. 1) at Glu302 in TC-peptide and Glu9 in S-peptide and SA-peptide. The identity of the peptides was established by mass spectrometry (Table 1).

Mass spectrometry

The RPHPLC purified peptides were taken in 50% acetonitrile containing 1% formic acid and analyzed by positive ion electrospray mass spectrometry (VG Platform, Fisons). The instrument calibration was usually done with standard gramicidin S or myoglobin solutions.

Protease-catalyzed ligation of complementary fragments

V8 protease-catalyzed ligation of complementary fragments was carried out in 50 mM ammonium acetate buffer (pH 6) containing appropriate amount of organic cosolvent. An equimolar amount of respective complementary segments (2 mM) was incubated with V8 protease (1:100, w/w, E:S) at 4°C. Aliquots of the reaction mixture were analyzed by RPHPLC at various time points. The extent of synthesis was quantified from the peak areas obtained using a computer program.

Amino acid sequence analysis

The sequencing of peptides were done using an automated protein sequencer (ABI, model 470A) equipped with an on-line PTH analyzer.

Circular dichroism analysis

CD spectra were recorded on a Jasco-710 instrument equipped with a Peltier-type constant-temperature cell holder (PTC-348W). The instrument was calibrated with (+)-10-camphorsulfonic acid. In general, spectra were an average of five to 10 scans and data presented as mean residue ellipticity (MRE) expressed in deg cm² dmol⁻¹. The spectra were smoothed using a built-in algorithm of the Jasco program. Secondary structure was estimated using a SSE-338 program supplied by Jasco (Yang et al., 1986) or utilizing MRE at 222 nm, taking peptide chain length into account (Chen et al., 1974), %Helix = 100 MRE/39,500(1 – 2.57/n) where *n* is the number of residues. The peptide concentration was determined spectrophotometrically using molar extinction coefficient of tyrosine, $E_{280\text{ nm}} = 1280\text{ lit mol}^{-1}\text{ cm}^{-1}$ (Edelhoch, 1967).

Size exclusion chromatography

The gel filtration chromatography of peptides were carried out using a superdex peptide column PC 3.2/30 (3.2 × 30 mm, Pharmacia) by HPLC. The column was equilibrated and run with 50 mM ammonium acetate buffer containing 40% 1-propanol. The peptide samples (2 mM with respect to each component) were prepared in the above buffer, and 20 μ L of the sample was analyzed. The flow rate was maintained at 0.05 mL/min throughout the run.

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