Product-conformation-driven ligation of peptides by V8 protease

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Abstract

Organic co-solvent-induced secondary conformation of α_{17-40} of human hemoglobin facilitates the splicing of E₃₀-R₃₁ in a mixture of its complementary segments by V8 protease. The amino acid sequence of α_{17-40} has been conceptualized by the general structure FR^I-EALER-FR^{II} and the pentapeptide sequence EALER playing a major role in inducing the α -helical conformation. The primary structure of α_{17-40} has been engineered in multiple ways to perturb one, two, or all three regions and the influence of the organic co-solvent-induced conformation and the concomitant resistance of E₃₀-R₃₁ peptide bond to V8 protease digestion has been investigated. The central pentapeptide (EALER), referred to here as splicedon,³ appears to dictate a primary role in facilitating the splicing reaction. When the same flanking regions are used, (1) splicedons that carry amino acid residues of low α -helical potential, for example G at position 2 or 3 of the splicedon, generate a conformational trap of very low thermodynamic stability, giving an equilibrium yield of only 3%–5%; (2) splicedons with amino acid residues of good α -helical potential generate a conformational trap of medium thermodynamic stability and give an equilibrium yield of 20%–25%; (3) the splicedons with amino residues of good α -helical potential and also an amino acid that can generate an *i*, *i* + 4 side-chain carboxylate-guanidino (amino) interaction, a conformational trap of maximum thermodynamic stability is generated, giving an equilibrium yield of 45%–50%; and (4) the thermodynamic stability of the conformational trap of the spliced peptide is also influenced by the amino acid composition of the flanking regions. The V8 protease resistance of the spliced peptide bond is not a direct correlate of the amount of α -helical conformation induced into the product. The results of this study reflect the unique role of the splicedon in translating the organic co-solvent-induced product conformation as a site-specific stabilization of the spliced peptide bond. It is speculated that the splicedon with higher α -helical potential as compared to either one of the flanking regions achieves this by integrating its potential with that of the flanking region(s). Exchange of flanking regions with the products of other V8 protease-catalyzed splicing reactions will help to establish the general primary structural requirements of this class of splicing reactions and facilitate their application in modular construction of proteins.

Keywords: Reverse proteolysis; splicedon; flanking region; semisynthesis; conformational trap; cosolvent

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Abbreviations: RP-HPLC, reverse phase high performance liquid chromatography; fmoc, 9-fluorenylmethoxycarbonyl; FR, flanking region; Hb, hemoglobin; E, glutamic acid; A, alanine; G, glycine; L, leucine; R, arginine; Y, tyrosine; V, valine; K, lysine.

³Splicedon is a pentapeptide sequence (EALER) sandwiched between the two peptides with intrinsic α -helical potential and provides the resistance to the spliced peptide bond E-R by V8 protease once it is integrated as a contiguous segment and the peptide folds into an α -helical conformation.

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Although the selective coupling of unprotected peptides through reverse proteolysis is one of the early protein semisynthetic reactions documented, delineation of molecular aspects that facilitate the reverse reactions of a protease continues to be a subject of considerable interest (Homanberg and Laskowski 1979; Wallace 1995; Nacharaju and Acharya 1999). Ligation of the discontinuity site of the fragment-complementing systems catalyzed by protease in the presence of organic cosolvent is the best studied example of this class of protein semisynthetic reaction (Homanberg and Laskowski 1979; Komoriya et al. 1980; Graf

and Li 1981; Jullierat and Homandberg 1981; Homandberg et al. 1982; Iyer and Acharya 1987; Kullman 1987; De Filippis et al. 1990; Chang et al. 1994; Davey et al. 1995; Wallace 1995; Woods et al. 1996; Cerovsky et al. 1997; Nacharaju and Acharya 1999). In these systems, noncovalent interactions of two or more segments of a protein maintain a native-like folding despite the discontinuity in the polypeptide chain and provide the proximity to the ligating ends of the peptides, which is crucial for the protease-catalyzed splicing reaction. In the presence of organic cosolvent, the fragment complementation facilitates the protease-catalyzed splicing of the discontinuity site by reverse reaction. Accordingly, these protein-splicing reactions are referred to as conformationally assisted protein ligation reactions (Kullman 1987). Proteases have also been used to splice noninteracting peptides in the presence of organic cosolvents. Use of very high concentrations of one of the reactants (the amino compound) relative to the other, as well as trapping the product of the splicing reaction out of the equilibrium by using antibody (Nyberg 1988) or other proteins that strongly interact with the product, have been used as new strategies to achieve the reverse proteolytic reaction.

The α -globin semisynthetic reaction, the V8 protease-catalyzed splicing of the segments (see Table 1 for the amino acid sequence of the peptides used in this study), namely, α_{1-30} and α_{31-141} of human HbA (reformation of $E_{30}-R_{31}$ peptide bond), is a new member of the class of protease-catalyzed splicing reactions of unprotected peptides (Nacharaju and Acharya 1999). However, this splicing reaction is unique and distinguishes itself from the previously described conformationally assisted splicing reactions in that it is the conformational aspects of the product that facilitates the splicing reaction (Sahni et al. 1989; Roy et al. 1992). Delineation of the molecular aspects of the product that facilitates the organic co-solvent-induced conformation of the product which in turn stabilizes the spliced peptide bond should pave the way for the translation of this reaction as a general approach to achieve the splicing of noninteracting peptides using protease.

The thermodynamic stability of the conformational trap of the splicing reaction is sensitive to the chemical nature of the amino acid residues around the spliced junction, par-

ticularly to the presence or absence of the γ -carboxyl group of E^{27} and the guanidino group of R^{31} (Sahni et al. 1998). The segments α_{1-27} and α_{1-23} carry E at their carboxyl end and satisfy the specificity requirements of V8 protease; however, they do not undergo the splicing reaction with α_{31-40} , α_{31-47} , or α_{31-141} (Seetharam and Acharya 1986). Thus, the propensity of α_{17-30} to splice with α_{31-40} can be considered as the consequence of the ability of the sequence of α_{28-30} (i.e., ALE) to integrate the secondary structural information of α_{17-27} with that of α_{31-40} . The γ -carboxyl group of E^{27} and the guanidino group of R^{31} play a significant role in establishing the maximum thermodynamic stability for the conformational trap of the splicing reaction (Sahni et al. 1998). Therefore the thermodynamic stability of $E^{30}-R^{31}$ peptide bond of α_{17-40} can be considered as the reflection of the integration of the α -helical potential of α_{17-26} with that of α_{32-40} by the pentapeptide sequence EALER to establish the product conformation. Accordingly, the primary structure of the peptides of α -globin for the purpose of stabilizing $E^{30}-R^{31}$ peptide bond against V8 protease can be generalized by the structure FR^I -EALER- FR^{II} (Fig. 1). Here, we refer to the pentapeptide EALER as the splicedon³ region that carries the information to splice the peptides, while the segments FR^I and FR^{II} are the flanking regions with some α -helical potential. In the V8 protease-catalyzed splicing reaction, the sequence EALE of the splicedon is at the carboxyl end of the FR^I and R of the splicedon is at the amino terminal end of the FR^{II} .

In this study, we investigate the influence of replacing the amino acid residues of splicedon with residues of lower α -helical propensity on the V8 protease resistance of the $E_{30}-R_{31}$ peptide bond. The influence of the exchanging of FR^I and FR^{II} region on the product conformational trap in the presence of 30% n-propanol, and its correlation with V8 protease resistance has also been established. A list of peptide designations and their amino acid sequence are shown in Table 1. The results have led us to conceptualize that the thermodynamic stability of the V8 protease resistance of the $E^{30}-R^{31}$ peptide bond in the contiguous segment reflect the potential of the splicedon region of the spliced peptides to flex between the helical (V8 protease resistant) and nonhelical (V8 protease susceptible) conformation. The rate of the

Table 1. The amino acid sequence of amino and carboxyl components of V8-catalyzed splicing reaction

Carboxyl component		Amino component	
Peptide name	Amino acid sequence	Peptide name	Amino acid sequence
α_{17-30}	VGAHAGEYGAEALE	α_{31-40}	RMFLSFPTTK
$\alpha_{17-30}^{des_{23-26}}$	VGAHAGEALE	$\alpha_{31-40} (K^{31})$	KMFLSFPTTK
$\alpha_{17-30}^{des_{23-26}}(Y^{28}G^{29})$	VGAHAGEYGE	$\alpha_{31-40} (V^{31})$	VMFLSFPTTK
$\alpha_{17-30}^{des_{23-26}}(G^{28}A^{29})$	VGAHAGEGAE	β_{30-39}	RLLVVYPWTQ
$\beta_{18-29}(A^{22}E^{29})$	VNDVGGGEALE	$\beta_{30-39} (V^{30})$	VLLVVYPWTQ

Fig. 1. Schematic conceptualization of structural elements of the α -globin semisynthetic reaction. The complementary segments that generate the splicedon sequence EALER could be spliced. The pentapeptide EALER acts as the spacer group between two segments with α -helical propensity (regions FR^I and FR^{II}) and the contiguous peptide assumes α -helical conformation in the presence of organic cosolvent and induces a degree of resistance to the V8 protease digestion of the E-R peptide bond of the contiguous peptide.

helix-coil transition of the splicedon is apparently a correlation of its ability to integrate the α -helical propensities of the two flanking regions to lower the conformational transition around the spliced peptide bond relative to the rest of the molecule.

Results

V8 protease-catalyzed splicing of α_{17-30} des₂₃₋₂₆ with α_{31-40}

The RP-HPLC pattern of equimolar mixtures of α_{17-23} and α_{31-40} (Fig. 2A), α_{17-30} des₂₃₋₂₆ and α_{31-40} (Fig. 2B), and of α_{17-30} and α_{31-40} (Fig. 2C) incubated at 4°C in ammonium acetate buffer (pH 6) containing 30% propanol with V8 protease for 48 h is shown in Figure 2. V8 protease spliced α_{17-30} with α_{31-40} generating α_{17-40} in an overall yield of 44% (Fig. 2C). However, V8 protease failed to splice α_{17-23} , (i.e., α_{17-30} des₂₄₋₃₀ with α_{31-40}) under identical conditions (Fig. 2A). But V8 protease catalyzed the splicing of α_{17-30} des₂₃₋₂₆ with α_{31-40} to generate the contiguous segment α_{17-40} des₂₃₋₂₆ in an overall yield of 41% (Fig. 2B). The Figure 2C inset shows the kinetics of V8 protease-catalyzed splicing reaction of α_{17-30} and α_{17-30} des₂₃₋₂₆. These two systems are ligated with α_{31-40} by V8 protease at nearly comparable rates. In both of these cases the reaction reached an equilibrium in ~24 h. Continuation of the incubation up to 96 h has limited influence on the equilibrium yield.

Generation of chimeric peptides through V8 protease-catalyzed splicing reactions

The studies with α_{17-40} des₂₃₋₂₆ suggest the flexibility of the flanking regions of the splicing reaction. Accordingly, it follows that other peptides with α -helical potential comparable to that of α_{17-26} or α_{32-40} may be able to replace the FR^I and FR^{II} segments of the α_{17-40} system. This concept

has been tested with the segment β_{18-29} of Hb, which is in the α -helical region and homologous to the segment α_{17-30} . If the G at β_{29} is replaced with E, it will satisfy the V8 protease specificity, and the splicing of this segment with α_{31-40} will generate the splicedon EALER. Although the splicing reaction proceeded well when this synthetic segment (β_{18-29} [G²⁹→E]) was incubated with α_{31-40} , the secondary cleavage at β_{22} complicated the analysis of the equilibrium yield of the splicing reaction. To overcome this cleavage at E²², this residue was replaced by an A residue.

V8 protease catalyzed the splicing of β_{18-29} (A²²,E²⁹) with α_{31-40} in an overall yield of 48% (Table 2). The chi-

Fig. 2. V8 protease-catalyzed splicing of α_{17-30} and its truncated (either at its carboxyl end or internally) variants with α_{31-40} . RP-HPLC analytical pattern of an equimolar mixture of peptides after 48 h of reaction. The reaction mixture was analyzed on a protein C4 column (Vydac). The column was eluted with a linear gradient of 5%–50% acetonitrile containing 0.1% TFA in 100 min. The elution of the peptides was monitored at 210 nm. (A) Reaction mixture of α_{17-30} des₂₄₋₃₀ and α_{31-40} . No splicing reaction occurs in this incubation. The contiguous segment expected to be generated by the V8 protease-catalyzed splicing, namely α_{17-40} des₂₄₋₃₀, was chemically synthesized and its elution position is marked on the figure. (B) The reaction mixture of α_{17-30} des₂₃₋₂₆ and α_{31-40} . (C) The reaction mixture of α_{17-30} and α_{31-40} . The inset compares the kinetics of the splicing of α_{17-30} with α_{31-40} (solid circles) with that of the splicing of α_{17-30} des₂₃₋₂₆ with α_{31-40} (open circles). Both reactions reach an equilibrium in ~24 to 30 h of the reaction.

Table 2. Exchangeability of the flanking regions in the splicing reaction

Peptide-segment flanking regions ^a		Synthesis in 30% propanol	MRE at 222 nm 30% propanol
FR ^I	FR ^{II}		
α_{17-26}	α_{32-40}	44	-10300
α_{17-22}	α_{32-40}	41	-7042
$\beta_{18-25}A^{22}$	α_{32-40}	48	-11336
α_{17-26}	β_{31-39}	41	-12836
$\beta_{18-25}A^{22}$	β_{31-39}	22	-13433

^a The corresponding amino acid sequence of the peptides is given in Table 1.

meric peptide differs from α_{17-40} only in terms of the amino-terminal sequence, that is, VGAHAGEYGA (α_{17-26}) of α_{17-40} versus VNVDVGG (β_{18-25}), and the rest of the sequence is identical. Consistent with this, the exchange of the FR^{II} part of α_{17-40} with β_{31-39} (homologous to α_{31-40}) had very limited influence on the overall yield of the splicing reaction (Table 2). Thus the FR^I and FR^{II} segments of the primary structural motif could be exchanged with peptides of comparable α -helical potential without significant change in the overall thermodynamic stability of the conformational trap of the splicing reaction. In view of the exchangeability of homologous segments of the β -chain in the splicing reaction of α_{17-40} , the propensity of V8 protease to splice $\beta_{18-29}(A^{22},E^{29})$ with β_{30-39} has also been investigated. Surprisingly, the equilibrium yield of the splicing reaction of these complementary segments was only 22% (Table 2). When both flanking regions are from the β -chain, they appear to neutralize part of their intrinsic potential to stabilize the conformational trap of the splicing reaction.

Influence of the substitution of the amino acid residues of the splicedon EALER of $\alpha_{17-40}des_{23-26}$

The acceptability of other amino acid residues at various positions in the splicedon region has been studied. The fourth residue in the splicedon, namely E, is needed to satisfy the specificity requirement of V8 protease. The two important structural aspects of the pentapeptide sequence of splicedon is such that (1) the amino acid residues of this peptide are primarily α -helix-promoting residues and (2) the spatial orientation of 1 and 5 residues can engage in an ionic interaction. The residue at position 4 dictates the protease specificity and can be considered as conservative. Thus, the substitution studies have been limited to 2, 3, and 5 positions of splicedon. The conservative substitution of the positively charged R at position 5, with conservative K, has little influence on the equilibrium yields. The substitution with V (loss of positive charge) on the other hand reduces the equilibrium yields of the splicing reaction by nearly 50%, as shown in Figure 3A. The peptide $\alpha_{17-30}des_{23-26}$ can also

splice with α_{32-40} , which carries an amino-terminal M instead of V. The equilibrium yield of this splicing reaction is similar to that of the peptide $\alpha_{17-40}des_{23-26}$ (R $\rightarrow$ V) from its complementary segments. Thus, α -helix-promoting amino acid residues are acceptable at this position.

The substitution of amino acid residues at 2 and 3 positions, keeping the presence of E and R at the first and the fifth place of the splicedon with amino acid residues that have lower α -helical potential, decreased the equilibrium yield of the splicing reaction. On substituting the residues A and L at positions 2 and 3 of the splicedon by Y and G, respectively, (new splicedon EYGER), as well as by G and A, respectively, the equilibrium yields were 3% and 5% respectively (Fig. 3A). The results reflect the stringent re-

Fig. 3. Panel A shows the influence of the eight different splicedons on the equilibrium yields of the $\alpha_{17-40}des_{23-26}$. Panel B shows the influence of changing the splicedon from EALER to EALEV on the equilibrium yields of the chimeric peptides. This influence is established by changing the amino-terminal R residue of α_{31-40} and of β_{30-39} . In both cases, the respective complementary segments were incubated with V8 protease under conditions identical to that described in Fig. 2. and were analyzed by RP-HPLC after 48 h of incubation. Equilibrium yields were calculated from the integration of the peaks corresponding to the respective reactants and the products in the RP-HPLC maps.

quirement for the strong α -helix-promoting amino acid residues at 2 and 3 positions of the splicedon to endow the highest thermodynamic stability to the conformational trap of the α -globin semisynthetic reaction.

Thermodynamic stability of the conformational trap of $\alpha_{17-40}\text{des}_{23-26}$ with substitutions at positions 2, 3, and 5 of the splicedon has been evaluated with R $\rightarrow$ V substitutions of the splicedon EYGER and EGAER. In these systems the substitution at the fifth position did not affect the equilibrium yields of the splicing reaction (Fig. 3A). Thus it appears that the presence of low α -helical-potential amino acid residues at positions 2 and 3 eliminates the thermodynamic advantage of the presence of E and R at positions 1 and 5 of splicedon.

Influence of R $\rightarrow$ V substitution in the splicedon EALER of $\alpha\beta$ and $\beta\alpha$ chimeric peptides and of $\beta_{18-29}(\text{A}^{22}, \text{E}^{29})$ on the splicing reactions

The equilibrium yield of $\beta_{18-39}(\text{A}^{22}, \text{E}^{29})$ is lower than that of α_{17-40} (even though the splicedon is EALER). This could be a consequence of failure of the interaction of the positive charge of R at the fifth position and the negative charge of E at the first position of the splicedon in contributing to the overall thermodynamic stability of the conformational trap of this splicing reaction. The influence of R $\rightarrow$ V substitution can neutralize the contribution of i, i + 4 side-chain interaction has been studied. For this purpose, segments α_{31-40} and β_{30-39} with the substitution of R to V have been spliced with α_{17-30} , as well as with $\beta_{18-29}(\text{A}^{22}, \text{E}^{29})$ and the results have been compared (Fig. 3B) with that of splicedon EALER. The equilibrium yield of the splicing reaction of α_{17-40} system ($\alpha\alpha$ system) and the $\alpha_{17-30}\beta_{30-39}$ system (chimeric $\alpha\beta$ system), as well as $\beta_{18-29}(\text{A}^{22}, \text{E}^{29})\alpha_{31-40}$ (chimeric $\beta\alpha$ system) was reduced by 50%–60%. But the R $\rightarrow$ V substitution in the $\beta_{18-39}(\text{A}^{22}, \text{E}^{29})$ results in a very limited reduction in the equilibrium yield of the splicing reaction (Fig. 3B). This reflects that in two chimeric systems, the i, i + 4 side-chain interaction of the splicedon plays a role similar to that in α_{17-40} . On the other hand, the equilibrium yield of the splicing of the $\beta_{18-39}(\text{A}^{22}, \text{E}^{29})$ EALER as the splicedon does not appear to generate i, i + 4 side-chain interaction. The equilibrium yield of this peptide is only 50% of that of the α_{17-40} system and is comparable to that of α_{17-40} and the chimeric peptide systems when the splicedon is EALEV.

Influence of deletion of the segment α_{23-26} of FR^I of α_{17-40} on the propanol-induced α -helical conformation

The far UV-CD spectra of α_{17-40} and $\alpha_{17-40}\text{des}_{23-26}$ in aqueous buffer shows very little α -helical conformation in the absence of the organic cosolvent. The presence of 30% propanol in the buffer containing $\alpha_{17-40}\text{des}_{23-26}$ induces

α -helical conformation to this peptide just as for α_{17-40} (Table 2). However, the amount of α -helical conformation induced (mean residue ellipticity value) at 222 nm for $\alpha_{17-40}\text{des}_{23-26}$ is nearly 30% lower than that of α_{17-40} . The equilibrium yield of α_{17-40} and $\alpha_{17-40}\text{des}_{23-26}$ is nearly the same (Table 2) in the two semisynthetic reactions. This observation suggests that the overall α -helical conformation of contiguous peptide is not a correlate of the thermodynamic stability of the conformational trap.

Influence of the flanking regions on the induction of α -helical conformation

The influence of the exchange of only α_{17-40} of the flanking regions of the peptides (splicedon is EALER) in the presence of 30% n-propanol was studied and the results are given in Table 2. The flanking regions of α_{17-40} appear to make a strong contribution on the amount of α -helical conformation induced into the peptide. The chimeric $\beta\alpha$ peptide (with the sequence difference at the amino-terminal flanking region) showed a 10% higher α -helical conformation, whereas the $\alpha\beta$ peptide has ~25% higher α -helical conformation than that of α_{17-40} . The results establish that the contribution of the flanking region β_{32-39} (amino acid sequence LLVVYPWTQ) in developing the α -helical conformation of $\alpha\beta$ chimeric peptide is significantly higher than that of α_{32-40} (MFLSFPTTK). Similarly, the flanking region $\beta_{18-25}(\text{A}^{22})$ with the amino acid sequence VNV-DAVGG makes a slightly higher contribution to the α -helical conformation as compared to the α_{17-26} with sequence VGAHAGEYGA in the contiguous segment. However, this higher α -helical content makes limited contribution to the thermodynamic stability of the conformational trap. The α -helical conformation of $\beta_{18-39}(\text{A}^{22}, \text{E}^{29})$ is nearly 30% higher than that exhibited by α_{17-40} . This can be considered as the additive effect of the higher α -helical propensity of $\beta_{18-25}(\text{A}^{22})$ and β_{31-39} as compared to those of α_{17-26} and α_{32-40} . However, the splicedon EALER has been able to integrate the higher α -helical propensities of $\beta_{18-25}(\text{A}^{22})$ and β_{31-39} as the thermodynamic stability of the conformational trap.

Influence of the substitution in the splicedon EALER of $\alpha_{17-40}\text{des}_{23-26}$ on induced α -helical conformation

The influence of substitution of ELEAR on propanol-induced α -helical content of $\alpha_{17-40}\text{des}_{23-26}$ is shown in Table 3. The substitution of V at the fifth position resulted in 25% reduction in the α -helical content. Thirty percent n-propanol induced very little α -helical conformation into the mutant forms of $\alpha_{17-40}\text{des}_{23-26}$ when the splicedon sequence is changed from EALER to EYGER or EGAER. These substitutions considerably neutralize the propensity of α_{17-40} to adopt α -helical conformation. The propensity of the peptide

Table 3. Influence of the substitution in the splicedon EALER on the *n*-propanol induced-helical Conformation of α_{17-40} des₂₃₋₂₆

Splicedon	MRE ^a at 222 nm 30% propanol
VGAHAGE E ALERMFLSFPTTK	-7042
VGAHAGE A LE V MFLSFPTTK	-5438
VGAHAGE Y GERMFLSFPTTK	-862
VGAHAGE G AERMFLSFPTTK	-2189

The identified splicedon of the α_{17-40} des₂₃₋₂₆ is shown in bold letters. The site-specific change within this region is marked by underline of the amino acid residue.

^a MRE, mean residue ellipticity.

EYGER to assume α -helical conformation is only 12%, whereas the propensity of the peptide EGAER to assume α -helical conformation is only ~32%, compared to that of the parent peptide carrying the splicedon sequence EALER. These results clearly reflect that the substitution in the splicedon with low α -helix-inducing residues (EYGER) has very strong influence on the α -helical conformation in the presence of 30% propanol.

Correlation between V8 protease digestion and the equilibrium yields of the splicing reactions

The kinetics of the digestion of the E-R peptide bond of the splicedon EALER have been measured to reflect its thermodynamic stability. The two peptides, α_{17-40} and β_{18-39} (A²²,E²⁹) have very different susceptibility of their E-R peptide bond to V8 protease in the presence of 30% propanol. The data in Figure 4 shows that the rate of digestion of the E-R peptide bond of the β_{18-39} (A²²,E²⁹) is nearly two times faster than that of the α_{17-40} . Thus the equilibrium yield of the splicing reaction appears to be correlated inversely with the rate of digestion of the spliced peptide bond. The rate of digestion of the spliced peptide bonds of α_{17-40} , α_{17-40} des₂₃₋₂₆, α_{17-40} des₂₃₋₂₆(Y²⁸,G²⁹), and α_{17-40} des₂₃₋₂₆(G²⁸,A²⁹) has also been determined. The Figure 4 inset shows the equilibrium yields of these peptides with the rate of digestion of their E-R peptide bond in 30% *n*-propanol. Thus, the equilibrium yields of the splicing reaction and the susceptibility of peptides to V8 protease digestion seem to be dictated by the same structural aspect of the peptides. The co-solvent-induced α -helical conformation represent another structural aspect of the peptide though the correspondence between the two is not a direct one.

Discussion

The molecular basis of the reverse proteolytic splicing of peptides or proteins has been the subject of intense investigations since the early 1930s (Homandberg and Laskowski

1979; Fruton 1983; Seetharam and Acharya 1986; Kullman 1987; Roy et al. 1992). In such systems, an increase in the equilibrium yield of the products has been achieved by developing protocols to remove the reaction products from the reversible equilibrium reactions. The design of an antibody trap wherein the product could be trapped as antigen-antibody complex is exemplified by the synthesis of substance P (Chaiken et al. 1987; Nyberg 1988). The molecular aspects that facilitate the splicing of RNase₁₋₉ with RNase₁₀₋₂₀ in the presence of RNase₂₁₋₁₂₄ are distinct from those which facilitate the splicing of RNase₁₋₂₀ with RNase₂₁₋₁₂₄, the first protease-catalyzed protein-splicing reaction (Homandberg and Laskowski 1979) in that the product of the splicing reaction is removed from the reaction equilibrium as an RNase-S type of species (Acharya et al. 1993). In the semi-synthesis of RNase-A from RNase-S, the proximity of the ligating ends within the non-covalent-interacting, fragment-complementing system plays the critical role. The template-mediated chemical ligation of small peptides can be considered as the extension of the same principle (Lee et al. 1996; Severin et al. 1997; Issac et al. 2001)

The α -globin semisynthetic reaction is distinct from the rest of the protease-catalyzed reactions in that the organic cosolvent induced conformation of the contiguous peptide that removes the product from the reversible splicing reaction. This has been conceptualized as the product-conformation facilitated ligation reaction of the α_{17-40} system (Roy et al. 1992), that is, the organic co-solvent-induced folding of the ligated peptide shifts the equilibrium in favor of conformationally ordered structure. The synthesis of α_{17-40} from a mixture of its complementary fragments can be

Fig. 4. Kinetics of the V8 protease digestion at the E-R peptide bond. The kinetics of digestion of various peptides was carried out as described in Materials and Methods. The concentration of the contiguous peptide was 1 mM during the start of the digestion. The inset shows the correlation of the equilibrium yields of splicing reactions of α_{17-40} (solid squares), β_{18-39} (A²²,E²⁹) (open squares), α_{17-40} des₂₃₋₂₆ (open triangles), α_{17-40} des₂₃₋₂₆(Y²⁸,G²⁹) (open circles), and α_{17-40} des₂₃₋₂₆(G²⁸,A²⁹) (solid circles) with the rate of the digestion of these peptides.

considered as a prototype reaction to delineate the molecular aspects that facilitate such reactions and can be adopted to design new splicing reactions. In our present study the primary structure of the peptides generated from such ligation reactions has been dissected out into three units, two flanking regions and the central splicedon, to facilitate the correlation between product conformation and splicing reaction. This conceptualization of the structure of α_{17-40} has provided new insights about this splicing reaction; a degree of flexibility exists within primary structure to induce the secondary structure (product conformation) that can generate the conformational trap of the splicing reaction.

Flexibility in the flanking regions of the peptides

Our earlier studies on the generation of chimeric α -chains between the human and animal sources clearly suggested the flexibility of the flanking regions in the V8 protease-mediated splicing reactions (Roy et al. 1993; Nacharaju et al. 1997; Rao et al. 2000). The results presented here have extended these studies further and established that flanking regions could be exchanged without sacrificing the equilibrium yields of the splicing reaction. Flanking regions of comparable α -helical potential could be substituted, as long as the splicedon EALER is used for the integration of the secondary structure as the conformational trap. When the flanking regions are exchanged, the overall thermodynamic stability of the conformational trap appears to be the propensity of the splicedon to integrate the secondary structural information of the flanking regions with that of its own. The lower equilibrium yield of the $\beta\beta$ peptide ($\beta_{18-39}[A^{22},E^{29}]$) with a higher α -helical conformation despite the fact that EALER is the splicedon, shows that the splicedon dictates a unique role in the thermodynamic stability of the conformational trap.

Flexibility within the splicedon region

There are few other systems reported in literature wherein the product conformation has facilitated the V8 protease-mediated splicing reactions (De Filippis and Fontana 1990; Kumaran et al. 1997, 2000). When the same rationale applied here to dissect out the structural information of these systems, three unique splicedon sequences are identified: (1) the splicedon EALER of the α -globin system, (2) the splicedon TSQEV of the thermolysin system, and (3) the splicedon AKFER of the RNase S-peptide system. The only common feature among the three splicedons is that the amino acids of the splicedon in general are of good α -helical potential. This suggests that a combination of residues with good α -helical potential could serve as splicedon between the flanking regions of good α -helical potential to develop the conformational trap of the protease-catalyzed splicing reactions. Of these three splicedons, only EALER

developed a conformational trap of maximum thermodynamic stability with an equilibrium yield of 45%. The equilibrium yields in the other two cases, which did not have an intrinsic potential to develop an $i, i + 4$ side-chain γ -carboxylate-guanidino interaction were in the range of 15%–20%, which is <50% of α_{17-40} system.

The lower equilibrium yield of splicedon EYGER and EGAER in the $\alpha_{17-40}\text{des}_{23-26}$ system appears to be reminiscent of the observation that the presence of G either preceding or at position 2 or immediately following the E of the splice junction has a deleterious effect on the polymerization reaction of RNase₃₋₉ (Kumaran et al. 2000). On the other hand, A at any of these positions is acceptable within the structural constraints for the development of the conformational trap of the splicing reaction. However, it should be noted that in the present study the splicing reaction is observed at a level of 5%, as compared to zero in the V8 protease-catalyzed polymerization reaction of RNase₃₋₉. The α -helical potential of G as compared to A is significantly lower and it is dictated by the position of the peptide. The maximum influence is when the $A \rightarrow G$ change is in the middle of the helix (Chakrabarty et al. 1991), a situation comparable to the present study. In the peptides with the splicedons EYGER or EGAER, the $R \rightarrow V$ substitution at the splicedon had no influence on the thermodynamic stability of the conformational trap. Thus these peptides are unable to make use of the intrinsic potential of an $i, i + 4$ side-chain γ -carboxylate-guanidino interaction. The lowered α -helical conformation of the mutant peptide fails to lock in the induced α -helical conformation as a strong α -helix and fails to increase the thermodynamic stability of the conformational trap.

Correlation between V8 protease digestion and the thermodynamic stability of the conformational trap of the splicing reaction

In proteins/peptides with considerable secondary structures, the accessibility of peptide bonds to proteolysis has been suggested to correspond to the ease in the transition of an ordered structure to a disordered state (i.e., conformational dynamics). The proteolysis proceeds smoothly in the flexible or disordered state (Fontana et al. 1986; Spolaore et al. 2001). Extending this concept to present studies, the rate of digestion of the E-R peptide bond of the products could be considered as a correlate of the equilibrium between the coiled and uncoiled states of the product (i.e., the rates of the transition of the α -helical conformation around the E-R peptide bond of the splicedon in the presence of organic cosolvent). The thermodynamic stability of the conformational trap of splicing reaction could then be rationalized as a correlate of rate of helix-coil transition (Zimm and Bragg 1959; Lifson and Roig 1961; Chakrabarty and Baldwin 1995) of the spliced peptide bond (splicedon region) when

the peptide assumes the α -helical conformation. The helix-coil transition of the peptide is dictated by a unique balance of the α -helical potential of the amino acid residues of three structural units of the peptide, the flanking regions and the splicedon. A splicedon that carries amino acid(s) of low α -helical potential appears to be unable to integrate the intrinsic α -helical potential of the flanking regions. In general, a better splicedon is the one that carries only amino acid residues of higher α -helical potential. In such cases, the rate of the helix-coil transition of the spliced region could be reduced further by engineering an $i, i + 4$ side-chain interaction in the spliced region. The exchange of flanking regions of other peptides that undergo this class of product-conformation-driven reaction can shed further insight into the delicate balance of α -helical potential of three structural units that is needed to facilitate the spliced peptide bond driven reverse proteolytic synthesis of peptides/proteins.

Materials and methods

V8 protease was purchased from Pierce Chemical. All other chemicals and buffer salts were purchased from Sigma.

Design of α -helical peptides

The fragments providing the α -carboxyl group for the splicing reaction were generated by the grafting of the tetra peptide EALE to the carboxyl end of the desired FR^I region. Similarly, the amino fragment was also generated by grafting the amino acid residue R to the amino-terminal end of the desired FR^{II} region. For the flanking region exchange studies, two new carboxyl fragments, $\alpha_{17-30}^{\text{des}_{23-26}}$ and $\beta_{18-29}^{\text{(A}^{22}\text{,E}^{29})}$, and one new amino component, β_{30-39} were chosen. Different primary structural versions of the V8 protease-catalyzed protein/peptide-ligation motif FR^I-EALER-FR^{II} were generated by the V8 protease-catalyzed splicing of the respective complementary fragments.

Chemical synthesis of peptides

The various peptides with desired amino acid sequence were chemically synthesized in the Macromolecular Laboratory of Albert Einstein College of Medicine using fmoc chemistry. The synthetic peptides were purified by RP-HPLC and the authentication was done by mass spectral analysis.

V8 protease-catalyzed splicing of complementary fragments

V8 protease-catalyzed splicing of the desired complementary fragments were carried out in 50 mM ammonium acetate buffer (pH 6, 4°C) containing 30% n-propanol. An equimolar mixture (2 mM each) of the respective complementary fragments were incubated with V8 protease at an enzyme substrate ratio of 1:200 (w/w). Aliquots of the reaction mixture were withdrawn at different time intervals and the yield of splicing reaction was quantitated by RP-HPLC.

Circular dichroic spectral measurements

The circular dichroic measurements in the far UV region (260–190 nm) were recorded in a Jasco B 720 Spectropolarimeter fitted with a computer. The contiguous peptide samples were dissolved in either 10 mM ammonium acetate buffer or in 10 mM ammonium acetate buffer containing 30% n-propanol (pH 6). The measurements were made at 4°C. The peptide concentration ranged between 60 and 80 $\mu\text{g/mL}$. The concentration of the peptide was determined by using the molar extinction of the tyrosine at 280 nm (1280; Edelhoch 1967).

Kinetics of the proteolysis of E^{30} -R³¹ peptide bond by V8 protease

The contiguous peptides (1 mM) were incubated in 50 mM ammonium acetate buffer (pH 6) containing 30% n-propanol at 4°C with V8 protease at an enzyme substrate ratio of 1:200. Aliquots of the reaction mixture were analyzed by RP-HPLC after various incubation periods for the formation of the complementary fragments. The rate of the digestion of the contiguous segments were calculated from the slope of the digestion curves.

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