Product conformation driven splicing of unprotected peptides by reverse proteolysis: Influence of intrinsic and extrinsic factors

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The structural motif of 'product conformation driven V8 protease catalyzed ligation reaction' can be represented by FR

EALER-FR. The relative roles of the flanking regions (FR and FR) and of splicedon, the central penta-peptide, on the thermodynamic stability of the 'conformational trap' of the product has been now evaluated as a function of co-solvent concentration. The studies have established that the thermodynamic stability of the conformational trap of RNase, with four different splicedons (EALER, EALE, EYGER, or EGAER) that differ in the intrinsic α-helical potential of their amino acid residues and/or ability to generate i, i+4 side chain interaction is a direct correlate of the n-propanol induced α-helical conformation of the product. On the other hand, when the product is defined by only splicedon EALER, and the flanking regions are distinct; no correlation could be drawn between the stability of the trap and solvent induced α-helical conformation, even though these generally give an equilibrium yield of 45% in 30% n-propanol and is not influenced by an increased propanol concentration. However, when the splicedon EALER with given FR and FR region develops a 'conformational trap' of a lower stability in 30% propanol as seen with p12,25(A)-EALER-p11,26, the stability increases in 60% n-propanol, without significant increase in the α-helical conformation. Though, primary structure of RNase, could be presented as RNase, EAKER, RNase, and α-helical conformation is induced to this peptide both in 30 and 60% propanol, splicedon AFKER by itself does not develop the 'conformational trap' of RNase. The splicedon AKFER of RNase, fails to develop the 'conformational trap', due to an intrinsic inhibitory potential of its FR region. RNase, replacing RNase with α11,26 enables the splicedon AFKER to generate the 'conformational trap'. The studies presented here have demonstrated the primary role of flanking regions in establishing the amount of the solvent induced α-helical conformation and that of the splicedon in dictating the thermodynamic stability of its 'conformational trap' of the products, nonetheless one influences the other to some degree. We suggest that the stability of the 'conformational trap' of the product reflects the ability of the splicedon to 'recruit' the product conformation to protect the spliced peptide bond, i.e. to reduce the helix-coil transition of the spliced region which in turn imparts a degree of resistance to the spliced peptide bond.

Modular construction of proteins from synthetic peptide segments, total chemical synthesis and semisynthesis are alternative approaches to site directed mutagenesis. The unique advantage of these synthetic protein chemistry approaches is that it provides means of engineering unusual structural features (not possible through normal site directed mutagenesis) into desired protein for structure function correlation studies. The selective coupling of unprotected peptides through novel chemical strategies and/or reverse proteolysis are methods available for such modular assembly of proteins. Ligation of the discontinuity sites of the fragment complementing systems catalyzed by protease in the presence of organic co-solvents is the best example of this class of modular protein semisynthetic reaction. This approach has permitted the assembly of new chimeric proteins by exchanging segments of proteins of identical function present in different classes of living organisms (for example chimeric RNAs and chimeric cytochromes), but exhibiting multiple amino acid sequence differences. In these systems, the non-covalent interactions between the complementary fragments of the parent protein provide a proximity to the ligating ends of the system. This non-covalent interaction between the complementary fragments has been the crucial aspect of all the protease catalyzed protein splicing reactions. Chemical splicing reactions that utilize the proximity of the α-amino and α-carboxyl ends of the discontinuity present in the complementing systems to facilitate the splicing of the discontinuity have also been developed. All these splicing reactions are classified together as 'conformationally assisted protein ligation reactions'.
The α-globin semisynthetic reaction, V8 protease catalyzed splicing of the segments of α-globin of Hb, namely α1-30 and α31-141 (reformation of Glu30 Arg31 peptide bond), represents a new class of protease catalyzed protein splicing reaction. This splicing reaction is distinct from the previously described conformationally assisted protein ligation reactions in that the conformational aspects of the product generated in situ by the splicing of the two complementary fragments plays the pivotal role in facilitating the ligation reaction. As noted above, the conformational aspects of reactants are the essential element of the conformationally assisted protein ligation reactions.

The unique features of α-globin semisynthetic reaction that distinguishes it from the 'conformationally assisted protein ligation reactions' are: (i), the segments being spliced by V8 protease do not exhibit any non-covalent interactions between themselves; (ii), the splicing reaction requires the presence of 30% propanol rather than 90% glycerol. Glycerol is the prototypic solvent that has been used in all previously studied 'conformationally assisted protein ligation reactions'; (iii), organic co-solvents other than n-propanol that can induce α-helical conformation into peptides, for example, isopropanol, trifluoroethanol, 1,3-propane diol and 1,4-butane diol are also compatible with this V8 protease catalyzed splicing reaction; (iv), extensive removal of amino terminal and carboxyl terminal regions of α1-30 and α31-141 can be made without influencing the equilibrium yields of the splicing reaction. In view of the critical role of the conformation of the product to facilitate these protein/peptide ligation reactions, this class of protein ligation reactions are referred to as 'protease catalyzed, product conformation driven protein ligation reactions'.

The primary structural features of product of the 'V8 protease catalyzed, product conformation driven ligation reactions' have been recently conceptualized by us in a generalized structure FR-EALER- FR. The region FR represents the flanking region and should be a polypeptide segment of good α-helical propensity. The relative roles of the amino acid sequence of penta-peptide, EALER, around the spliced junction (ER), referred to as splicedon and the two flanking regions (FR and FR') in establishing the thermodynamic stability of the 'conformational trap' of splicing of short peptides of α-globin has been evaluated. We have demonstrated that in the presence of 30% propanol and at pH 6.0, a reaction condition that has been routinely used for the α-globin semisynthetic reaction.

A more general application of the product conformation driven protease catalyzed ligation of the unprotected peptides in peptide/protein synthetic chemistry needs a better appreciation of all the factors, such as the solution conditions (extrinsic) and the amino acid sequence (intrinsic) that can influence the splicing reaction. In our earlier studies, we have focused our attention on the structure of the spliceds. A splicedon carrying amino acid residues of good α-helical potential and with a propensity to generate an i, i+4 side chain interaction when the spliced product assumes an α-helical conformation generated a 'conformational trap' of maximum thermodynamic stability with an equilibrium yield in the range of 45%. An inability to generate an i, i+4 side chain interaction into the co-solvent induced α-helical conformation of product in a particular splicedon reduces the thermodynamic stability of the 'conformational trap', resulting in equilibrium yield of 25%, with noticeable decrease in the propanol induced α-helical conformation of the product. On the other hand, if one or more amino acid residues of low α-helical potential are present in the splicedon, most of the thermodynamic stability of the 'conformational trap' is lost and the equilibrium yield is lowered significantly, which is accompanied by significant reduction in the amount of the α-helical conformation induced into the product in the presence of 30% n-propanol. For example an A->G substitution at δ27 in α17-4δE823-26 resulted in nearly 70% reduction in n-propanol induced α-helical conformation, and a 80-90% reduction in the equilibrium yield of the splicing reaction.

The peptides (products) that were produced in lower equilibrium yields (reflecting the lower thermodynamic stability of the 'conformational trap' of the products in 30% n-propanol) were also induced with lower levels of α-helical conformation in 30% n-propanol as compared to the control peptide (α17-4δE823-26). The α-helical conformation of the peptides are generally increased on increasing the co-solvent concentration. Accordingly, we have now studied the influence of increasing the concentration of n-propanol from 30 to 60% in the splicing reaction with the hope that the co-solvent induced α-helical conformation of these peptides could also be
increased in a similar fashion. If this increase in the induced helical conformation is translated by the respective splicedons into increased thermodynamic stability of the 'conformational trap' of the product, it should be reflected as increase in the equilibrium yields. The earlier studies have shown that 30% n-propanol is the optimal concentration in terms of equilibrium yields for the α-globin semisynthetic reaction, wherein the splicedon is EALER.\textsuperscript{25,30} If the equilibrium yield of the products is indeed influenced by the organic co-solvent concentration, this provides a means to manipulate the splicing reaction by controlling the extrinsic factors.

The amino acid sequence of RNase1-20, which can be represented as RNase1-5-AKFER-RNase11-20, satisfies the primary structural motif for V8 protease catalyzed formation of ER peptide bond in a mixture of RNase1-5-AKFE and R-RNase11-20. RNase1-20 also assumes significant amount of α-helical conformation in the presence of 30% n-propanol. The structural aspect of RNase1-20 that inhibits the splicing reaction has been investigated by exchanging the complementary segments between the systems α17-40 and RNase1-20. The results establish that the splicedon AKFER facilitates the splicing when RNase11-20 is replaced by α32-40, the thermodynamic stability of the conformational trap being comparable to that of α17-40des21-26 with the splicedon EALEV. It is suggested that His-12 of RNase1-20 prevents the translation of co-solvent induced α-helical conformation into a 'conformational trap' by the splicedon AKFER by keeping the rate of helix-coil transition of the spliced peptide high. The non-covalent interaction of RNase1-20 with RNase21-124 is critical to decrease the rate of helix-coil transition and stabilize the ER peptide bond of RNase1-20. The studies presented here expose the high degree of flexibility in protease catalyzed, the product conformation driven peptide ligation reactions.

**Materials and Methods**

V8 protease was purchased from Pierce Chemical Company, Rockford, IL. All other chemicals and buffer salts were purchased from Sigma Chemical Company, St. Louis, MO. RNase A, RNase1-20, RNase21-124 were also purchased from Sigma Chemical Company.

**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 f-moc chemistry.\textsuperscript{31} The synthetic peptides were purified by RP-HPLC and the authenticity was done by mass spectral analysis.\textsuperscript{32} Complementary fragments providing the α-carboxyl group for the splicing reaction are generated by grafting tetra peptide -EALE or its mutated forms like EYGE or EGAE to the carboxyl end of the desired FR\textsuperscript{1} region. Similarly, the amino fragment is also generated by grafting the amino acid residue R at the amino terminal end of the desired FR\textsuperscript{4} region.\textsuperscript{33} Different primary structural versions of the protein/peptide ligation motif, FR\textsuperscript{3}-EALER-FR\textsuperscript{4} have been generated by the V8 protease catalyzed splicing of the respective complementary fragments.

**V8 protease catalyzed splicing of complimentary fragments**

V8 protease catalyzed splicing of the desired complementary fragments was carried out in 50 mM ammonium acetate, buffer (pH 6.0, 4°C) containing either 30% or 60% n-propanol. An equi-molar mixture (2 mM each) of the respective complimentary 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 equilibrium yields in the splicing reaction was quantitatively by RP-HPLC.

**Circular dichroic spectral measurements**

The CD spectra in the far UV region (260-190 nm) were recorded on a Jasco B 720 Spectropolarimeter fitted with a computer. The contiguous peptide samples were dissolved either in 10 mM ammonium acetate buffer or in 10 mM ammonium acetate buffer containing 30% or 60% n-propanol (pH 6.0). The measurements were made at 4°C. The peptide concentration ranged between 60 and 80 μg/ml. The concentration of the peptide was determined by using the value of 1280 for the molar extinction coefficient of tyrosine at 280 nm.\textsuperscript{33}

**Results**

**Influence of the amino acid sequence of splicedon (intrinsic factor) and the concentration of propanol (extrinsic factor) on the co-solvent induced α-helical conformation of α17-40des21-26 and the equilibrium yields of the splicing reaction**

The influence of increasing the concentration of n-propanol from 30 to 60% on the co-solvent induced
α-helical conformation of the peptide \( \alpha_{17-40}\text{des}_{23-26} \) carrying the four splicedons is presented in Fig. 1. As can be seen from the figure, the mean residue ellipticity (MRE) values at 222 nm in 60% \( n \)-propanol was higher for all the samples as compared with that in 30% \( n \)-propanol. The α-helical content of the peptide with the splicedon EAER increased by about 20%, while that of the peptide with EALEV increased only by about 10%. Maximum increase in the α-helical conformation occurred with the peptide having the splicedon EYGER, which was nearly 2.5-fold (250%) over that seen in 30% \( n \)-propanol. The increase in the helical conformation in the peptide with splicedon EGAER on increasing the concentration of propanol to 60% is about 1.8-fold. The helical content of the peptides (with defined FR^I and FR^II regions) increased in the presence of 60% \( n \)-propanol in the order of the splicedons EYGER > EGAER > EAER > EALEV.

The influence of the increasing concentration of propanol on the V8 protease catalyzed splicing of equimolar mixtures of \( \alpha_{17-30}\text{des}_{23-26} \) with \( \alpha_{31-40} \) with four different splicedons are also shown in Fig. 1. Increasing the propanol concentration in the reaction mixture from 30 to 60%, has very little influence on the peptides with the splicedons EAER and EALEV. But with the peptides carrying the splicedons, namely EYGER as well as EGAER, the equilibrium yield increased nearly 2.5 to 3 fold (5 to 15%). However, the actual equilibrium yield was still lower than that of the peptides carrying the splicedon EALEV (25%), that lacks the potential to generate the \( i, i+4 \) chain interaction when the contiguous peptide assumes the α-helical conformation. However, both the splicedons EYGER as well EGAER have an intrinsic potential to generate the \( i, i+4 \) side chain interaction once the respective contiguous segments assume the α-helical conformation, but the equilibrium yields of peptides in 60% \( n \)-propanol is only about 30 to 40% of that of the peptides with the same flanking regions and carries the splicedon EAER, a peptide with the potential to form \( i, i+4 \) side chain interaction and with amino acid residues of high α-helical potential. The results support the idea that if a given contiguous peptide (product) with its splicedon (an intrinsic factor) is unable to generate a ‘conformational trap’ of high enough thermodynamic stability, one could modulate the solution conditions to facilitate the development of a ‘conformational trap’ of higher thermodynamic stability.

The correlation of the α-helical conformation of \( \alpha_{17-40}\text{des}_{23-26} \) in the presence of \( n \)-propanol (both at 30% or at 60%) that carry different splicedons with the corresponding equilibrium yields with the peptides in the splicing reaction is shown in Fig. 2. A direct correlation between the equilibrium yields of the splicing reaction and the propanol induced α-helical conformation of the respective peptides can be noted. Therefore, under these experimental conditions, there appears to be a direct correlation between the cosolvent induced α-helical conformation of the peptide and the thermodynamic stability of the ‘conformational trap’ of the peptide generated in the product conformation driven ligation of peptides.

![Graph](image_url)

**Fig. 1** — Splicedon dependent, influence of the concentration of \( n \)-propanol on the equilibrium yields of the splicing reaction and cosolvent induced α-helical conformation of the \( \alpha_{17-30}\text{des}_{23-26} \) [The influence of increasing the concentration of \( n \)-propanol from 30 to 60% in the reaction medium on the equilibrium splicing yield of the complementary segments with four different splicedons in the \( \alpha_{17-30}\text{des}_{23-26} \) system has been measured after 48 hr of incubation. The increase in the equilibrium yield is expressed as % change in 60% propanol over that of 30% propanol (filled bars). Similarly, the induced α-helical conformation of the contiguous \( \alpha_{17-30}\text{des}_{23-26} \) with different splicedons at 222 nm was calculated as % change (striped bars)].

**Influence of increasing the concentration of \( n \)-propanol on the equilibrium yield and cosolvent induced α-helical conformation of \( \beta_{38,26}(A^{32}) - \text{EAER-β}_{38,39} \)**

The studies discussed above have established that with \( \alpha_{17-40}\text{des}_{23-26} \), the splicedon EAER gives the
Table 1 — Influence of the concentration of organic co-solvent on the thermodynamic stability of the 'conformational trap' of peptides with the spliced on EALER

<table>
<thead>
<tr>
<th>Peptides</th>
<th>30% n-propanol</th>
<th>60% n-propanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% synthesis</td>
<td>MRE at 222 nm</td>
</tr>
<tr>
<td>α_{17,40}-EALER-α_{32,40}</td>
<td>44</td>
<td>-10,300''</td>
</tr>
<tr>
<td>α_{17,40}-EALER-β_{31,39}</td>
<td>41</td>
<td>-12,836</td>
</tr>
<tr>
<td>β_{18,25}-EALER-α_{32,40}</td>
<td>48</td>
<td>-13,433</td>
</tr>
<tr>
<td>β_{14,25}-EALER-β_{31,39}</td>
<td>22</td>
<td>-11,336</td>
</tr>
</tbody>
</table>

*MRE values are taken from Roy et al.**

The equilibrium yields of the α_{17,40}, β_{18,25}(A^{22})-EALER-β_{31,39}, and the chimeric peptides generated by the exchange of the complimentary segments of α and β peptides, in 30% n-propanol and 60% n-propanol are compared in Table 1. The α peptide, and the chimeric peptide showed very little changes in the equilibrium yields, whereas the β peptide showed a significant increase in the equilibrium yield, which is now close to that of the α-peptide. The influence of the co-solvent on the induced α-helical conformation is also given in Table 1. Again, the increase in the solvent induced α-helical conformation is marginal on increasing from 30% n-propanol to 60% n-propanol, including the β-peptide. Accordingly, with the β-peptide, the increase in the co-solvent concentration has increased the efficiency of the EALER spliced on to translate the co-solvent induced α-helical conformation into a 'conformational trap', by nearly 100% on going from 30% n-propanol to 60% n-propanol, but without inducing a significant increase in the α-helical conformation of the peptide. The increased equilibrium yield in 60% implies a decreased frequency with which the contiguous peptide in the co-solvent induced β-helical conformation flexes into a protease digestible conformation as compared to that in 30% n-propanol. This is contradictory to what has been noted with the spliced on that carried one or more amino acid residues of low β-helical potential discussed in the previous section, wherein the amount of the co-solvent induced α-helical conformation also increased considerably with increase in the equilibrium yield.

**V8 protease catalyzed splicing of the complimentary segments of RNAse_{1-20} (S-peptide)

The amino acid sequence of RNAse_{1-20} could be presented schematically to corresponding to that of 'motif' of a product conformation driven peptide ligation reaction, i.e., RNAse_{1-5}-AKFER-RNAse_{10-20}. AKFER will be the spliced on to facilitate the maximum equilibrium yield in 30% n-propanol, and this yield does not change in 60% n-propanol inspite of the small increase in the α-helical conformation. However, all peptides that carry EALER as the spliced on do not give the highest amount of equilibrium yields (45% or better), though most did. The peptide β_{18,25}(A^{22})-EALER-β_{31,39}, gives an equilibrium yield of only 22% (Table 1), when its V8 protease complimentary fragments are spliced together in 30% n-propanol, even though the propanol induced α-helical conformation of this peptide under these conditions is even higher than that of α_{17,40}.

Fig. 2 — Correlation between the co-solvent induced α-helical conformation and equilibrium splicing yield of the contiguous α_{17,30} des_{23,26} with different splicedons (Correlation between the four different splicedons of the contiguous peptide α_{17,30} des_{23,26} is established by plotting the equilibrium splicing yield and the induced α-helical conformation of the peptides both in the presence of 30 and 60% n-propanol. There was a linear correlation between the synthesis and induced α-helical conformation of the peptides)
V8 protease catalyzed splicing of RNase$_{1-9}$ and RNase$_{10-20}$. As shown in Fig. 3A RNase$_{1-20}$ exhibits considerable amount of α-helical conformation in the presence of 30% n-propanol and further increases in 60% n-propanol. Accordingly, one can anticipate that V8 protease could splice the complementary segments of RNase$_{1-20}$, namely RNase$_{1-9}$ and RNase$_{10-20}$ in the presence of 30 as well as 60% n-propanol, if the splicedon AKFER could translate the co-solvent induced α-helical conformation into a conformational trap of the spliced peptide bond. However, V8 protease failed to reform the peptide bond ER in an equimolar mixture of RNase$_{1-9}$ and RNase$_{10-20}$ in the presence 30 as well as 60% n-propanol.

Though, V8- protease complementary segments of RNase$_{1-20}$, i.e. RNase$_{1-9}$ and RNase$_{10-20}$, failed to splice in 30% propanol, the contiguous segment is generated if RNase$_{11-124}$ (S-protein) is also present in the splicing mixture. V8 protease mediated splicing of the complementary segments to generate RNase$_{1-20}$ takes place in the presence of 90% glycerol as well. Accordingly, it follows that the contiguous segment RNase$_{1-20}$ can be generated in situ by V8 protease mediated splicing of the complementary fragments.

![Fig. 3](image_url)

Fig. 3—Co-solvent induced α-helical conformation of RNase S-peptide and chimeric peptide, RNase$_{1-20}$ [Panel A shows the Far UV CD-spectra of RNase A S-peptide and panel B shows the CD spectra of chimeric peptide RNase$_{1-9}$ α$_{31-40}$. The measurements were done in 10 mM acetate buffer (pH 6.0) in the presence and absence of propanol at 4°C. (a) 10 mM acetate buffer (pH 6.0), (b) 30% n-propanol and (c) 60% n-propanol].
but the conformational trap of the spliced peptide bond is generated only in the presence of RNAses_{21-124}. The presence of RNAses_{21-124} along with 30% n-propanol also facilitates the splicing reaction. Thus, the protection of the spliced peptide bond of RNAses_{1-20} needs its non-covalent interaction with RNAses_{21-124}.

The failure of spliced don AKFER to translate the co-solvent induced α-helical conformation of the nascent contiguous segment could also be a consequence of inhibitory potential of one or both flanking regions of RNAses_{1-20}. The splicing of an equimolar mixture of RNAses_{1-4} with α_{31-40} to generate RNAses_{1-5}-AKFER-α_{32-40} by V8 protease has been attempted to verify this and identify the flanking region that has the inhibitory potential. The results of these studies are shown in Fig. 4. This splicing reaction proceeded with an equilibrium yield of 23%.

The splicing reaction has also been carried out in the presence of 60% n-propanol to increase the equilibrium yields. However, the equilibrium yield of the reaction did not increase significantly (Table 2) on increasing the concentration of the co-solvent. Thus, the spliced don AKFER, behaves like the spliced don EALEV. Thus, some structural information present in RNAses_{11-20} appears to neutralize the propensity of the spliced don AKFER to translate the co-solvent induced α-helical conformation into the conformational trap of the splicing reaction.

Similarly, V8 protease catalyzed splicing of α_{17-30} with RNAses_{10-20} has also been investigated to see whether a chimeric peptide α_{17-26}-EALER-RNAses_{11-20} could be generated, i.e. whether a strong spliced don EALER, could overcome the ‘conformational trap’ development inhibitory activity of RNAses_{11-20}. However, V8 protease could not catalyze the splicing.

![Fig. 4](image_url)
reaction of these two peptides either in 30% or in 60% \( n \)-propanol (Fig. 4). Therefore, it may be suggested that even in the presence of splicedon EALER, the best splicedon that we have studied, the structural information present in RNase124 inhibits the development of a conformational trap of the splicing reaction.

Since the V8 protease catalyzed splicing of RNase2124 from the complementary fragments is facilitated in the presence of RNase2124, the influence of the presence of RNase2124 on the equilibrium yields of the chimeric peptides has also been investigated. The presence of RNase2124 neither increased the equilibrium yield of the chimeric peptide RNase15-AFKER-\( \alpha_{2-40} \) nor facilitated the generation of RNase15-\( \alpha \)-EALER-RNase1120 (Table 2). Apparently, the nascent chimeric peptides generated in situ do not conserve the ability to interact non-covalently with RNase2124 even though the chimeric peptides conserve a part of RNase120.

**Co-solvent induced \( \alpha \)-helical conformation of the chimeric peptide, RNase15-AFKER-\( \alpha_{2-40} \):**

CD spectra of the chimeric peptide, RNase15-AFKER-\( \alpha_{2-40} \), in 30 and 60% \( n \)-propanol at pH 6.0 is shown in Fig. 3B. The chimeric peptide exhibits a significant amount of \( \alpha \)-helical conformation in the presence of 30% \( n \)-propanol. We have shown earlier that neither RNase10 nor \( \alpha_{1-40} \) exhibit any helical conformation in the presence of 30% \( n \)-propanol. The chain contiguity established between the complementary segments of the chimeric peptide endowed the propensity to assume significant amounts of \( \alpha \)-helical conformation, a feature of the product conformation driven peptide ligation reaction. Besides, the propanol induced \( \alpha \)-helical conformation of the chimeric peptide has been translated as the ‘conformational trap’ of the spliced peptide as reflected by the equilibrium yields. Furthermore, when the propanol concentration is increased to 60%, a further increase in the \( \alpha \)-helical conformation of the chimeric peptide is noticed. It may also be noted that the \( \alpha \)-helical conformation of RNase120 in 60% \( n \)-propanol is lower than that of the chimeric peptide in 30% \( n \)-propanol.

**Discussion**

Reverse proteolytic splicing of the discontinuity sites of fragment complementing system, first described by Laskowski and his colleagues, has been suggested to be a consequence of the proximity of the ligating ends at the discontinuity and the protonation of the \( \alpha \)-carboxyl group of the discontinuity site in the presence of the organic co-solvent used to facilitate the protease catalyzed splicing reaction\(^19\). The ‘native-like’ non-covalent interactions between RNase S-peptide and RNase S-protein provides the proximity to the discontinuity sites and has facilitated the splicing reaction and the same principle has lead to the development of ‘conformationally assisted chemical ligation reactions’\(^1,15\). The use of molecular templates to facilitate the splicing of segments bound on such templates has also been advanced\(^15,35\). Approaches to trap the spliced nascent peptides (molecular traps) generated by reverse proteolytic splicing have also been designed, as exemplified by the ‘antibody-mediated molecular traps’ to facilitate the removal of the reaction product from the equilibrium reaction mixture\(^38,39\). The product conformation driven, protease catalyzed peptide/protein ligation reactions discussed here represents a new class of protease catalyzed peptide/protein ligation reaction. The molecular aspects of this class of reaction have some similarity with that of the approach of designing molecular traps.
to remove the reaction products from the equilibrium mixture. The molecular trap approaches depend on strong non-covalent interactions between the nascent peptide and a protein (antibody), that can effectively remove the product from equilibrium mixture. In the product conformation driven splicing reactions, the organic co-solvent used in the splicing reaction mixture induces a secondary structure and this traps the spliced species as a protease resistant molecule. Accordingly, the equilibrium between the unstructured nascent ligated peptide and the structured peptide generated by the induction of the secondary structure into the nascent peptide, dictates the equilibrium yields of the splicing reaction.

Our earlier studies of this 'product conformation driven, protease catalyzed splicing of peptides' have lead us to propose that a peptide chain with a generalized structure FR1-EALER-FR4 is endowed with the potential to undergo ligation of their V8 protease complementary segments. The spliced peptide EALER facilitates the generation of a 'conformational trap' of maximum thermodynamic stability, a degree of flexibility present in this system in terms of the amino acid sequence of the spliced peptides that can facilitate the protease catalyzed ligation reactions. The amino acid residues of the spliced peptide EALER could be substituted either at position 1 or at position 5 by other α-helix promoting amino acid residues still preserving nearly 50% of the original splicing potential of the spliced peptide. The substitution of residues 2 and/or 3 of the spliced peptide EALER with amino acid residues of low α-helical potential has a pronounced influence on the equilibrium yields of the splicing reaction.

In view of the low equilibrium yields of some peptides with new spliced peptides with a concomitant lower amounts of co-solvent induced α-helical conformation, the use of a higher concentration of n-propanol with these peptides to increase amount of the co-solvent induced α-helical conformation; and possibly thereby inducing an increased thermodynamic stability of the 'conformational trap' has been investigated. Accordingly, the influence of 60% n-propanol on the equilibrium yields of α17-40, des25-26 carrying different spliced peptides has been investigated.

The equilibrium yields of the splicing reaction of α17-40-EALER-α52-40 and of α17-40-EALEV-α52-40, from their respective complementary segments were not influenced on increasing the concentration of n-propanol from 30 to 60%. These peptides also did not show any significant increase in the amount of the co-solvent induced α-helical conformation as the propanol concentration increased from 30% to 60%. On the other hand, the equilibrium yield of the splicing of the complementary segments of α17-32-EYGER-α52-40 and of α17-32-EGAER-α52-40, increased significantly on increasing the concentration of the co-solvent to 60% (a nearly three fold increase, Fig 1). The lower thermodynamic stability of the 'conformational trap' of the peptides with splicedons EYGER and EGAER is increased in the presence of 60% n-propanol. The equilibrium yields of these peptides indeed are now closer to the values of peptides with the splicedon EALEY. The peptides, α17-32-EYGERα52-40 and α17-32-EGAERα52-40 that had increased equilibrium yields in 60% n-propanol, also increased the amount of their co-solvent induced α-helical conformation.

When the equilibrium yields (thermodynamic stability) of all splicedon variants of α17-40, des25-26 are correlated with the co-solvent induced α-helical conformation induced into them ( in 30% or 60% n-propanol) a linear correlation is seen. This suggests that peptides that take up α-helical conformation can be generated by splicing their complementary fragments using an appropriate protease in the presence of an α-helical conformation inducing organic co-solvent.

We have shown earlier that the equilibrium yield of the peptide β18-25(A22)-EALER-β31-39 is only around 25% even though its spliced peptide is EALER, and all other peptides tried so far with EALER as their spliced peptides spliced together from their complementary segments in an equilibrium yield of 45% or better. The inability of the EALER of this peptide to translate the co-solvent induced α-helical conformation of β18-25(A22)-EALER-β31-39 into a 'conformational trap' with a thermodynamic stability comparable to that of α17-40 of the spliced peptide has been attributed to the higher α-helical potential of both flanking regions of the β-peptide as compared to that of the respective flanking regions of α-peptide. The equilibrium yields with the chimeric peptides is comparable to that of the parent α-peptide, α17-40. Thus, the lowered equilibrium yield is a consequence of the sympathy of the two flanking regions to neutralize the propensity of the spliced peptide EALER to generate the 'conformational trap'. The
higher potential of either of the FR₁ or FR₃ of the β peptide could be neutralized by the complementary FR region from α₁₇-₄₀. Presumably, the information content of FR regions of the peptide from the β-chain acting together reduces the intrinsic efficiency of the spliced on to translate the propanol induced α-helical conformation of the product into a ‘conformational trap’, i.e. to efficiently ‘recruit’ the α-helical conformation of the peptide to protect the spliced peptide bond. The thermodynamic stability of peptides α₁₇-₄₀-EALER-α₃₂-₄₀, α₁₇-₄₀-EALER-β₃₁-₃₉, β₁₈-₄₅-EALER-α₃₂-₄₀, did not change significantly on increasing the concentration of n-propanol to 60% from 30%. On the other hand, the thermodynamic stability of the ‘conformational trap’ of β₁₈-₄₅(A²²)-EALER-β₃₁-₃₉ increased in 60% n-propanol, increasing the equilibrium yield from 25% to about 40% which is close to the equilibrium yield of α₁₇-₄₀ (about 45%). The increased concentration n-propanol (60%) increases the efficiency of the spliced EALER to ‘recruit’ the co-solvent induced α-helical conformation of the β-peptide into a ‘conformational trap’ of the splicing reaction.

Thus, the studies clearly distinguish the two phases in the development of the ‘conformational trap’ in the product splicing driven protease catalyzed splicing of peptides; induction of α-helical conformation into the nascent spliced product by the organic co-solvent and the translation of the co-solvent induced product conformation into a ‘conformational trap’ of the spliced peptide bond. This is modulated by the structure/composition of flanking regions, structure of the spliced and the macro-environment (concentration of the co-solvent). Each of these can down regulate the equilibrium yield from the 45% level that can be obtained with α₁₇-₄₀, both in 30% or 60% n-propanol at pH 6.0 and 4°C. An extreme situation is seen when RNase₃₁-₄₀, wherein one of the flanking region appears to have completely inhibited the translation of the co-solvent induced α-helical conformation into a ‘conformational trap’ of the spliced peptide bond.

The amino acid sequence of RNase₃₁-₄₀ can be conceptualized in the general motif of product conformation driven protease catalyzed splicing reaction, but the complementary segments of RNase₃₁-₄₀ could not be spliced by V8 protease either in 30% or in 60% n-propanol. The failure of the splicing reaction is not due to the failure of V8 protease to catalyze the splicing of Glu³ with Arg¹⁰ in a mixture of RNase₁₉ and RNase₁₀-₂₀, since RNase₁₉ is generated from such mixtures if RNase₁₁-₁₂₄ is present in the mixture. RNase₁₀-₂₀ also assumes an α-helical conformation in the presence of 30% as well as 60% n-propanol. Accordingly, we conclude that spliced on AKFER of RNase₁₀-₂₀ is not able to translate the co-solvent induced α-helical conformation into a ‘conformational trap’ to resist the hydrolysis of the spliced ER peptide bond. Though significant amount of α-helical conformation is induced into the nascent peptide, it does not influence the rate of synthesis or the rate of hydrolysis of the ER peptide which either proceed at the same rate or the latter is faster than the synthetic rate. But, in the presence of RNase₁₁-₁₂₄, the nascent RNase₁₀-₂₀ generated in situ, is trapped by RNase₁₁-₁₂₄ as RNaseS, and ER peptide bond of RNase₁₀-₂₀ in this complex is inaccessible for hydrolysis and this process of complementation facilitates the splicing reaction.

The exchange of the complementary segments of RNase₁₀-₂₀ with that of α₁₇-₄₀ has demonstrated that the structural information present in the segment RNase₁₀-₂₀ inhibits the translation of the co-solvent induced α-helical conformation into a ‘conformation trap’ of the spliced ER peptide bond of RNase₁₀-₂₀. His₁₂ of RNase₁₀-₂₀, acting as the helix stop signal is apparently responsible for this. The His₁₂ of the peptide can make the short stretch of peptide that can assume α-helical conformation in a medium containing organic co-solvent rapidly flex between the folded (helical) and unfolded (random) conformation, thereby making the ER peptide bond accessible to hydrolysis. On the other hand, in the presence of RNase S-protein, the nascent peptide is trapped as RNase S and this reduces the amount of free peptide that can unfold, since the S-peptide has to dissociate from the complex to undergo the flexing between the helical and non-helical conformation of the peptide.

Therefore, the thermodynamic stability of chimeric peptide, RNase₁₅AKFERα₃₂-₄₀ can be considered as the propensity of the contiguous peptide to have a longer residence time in the α-conformation that resists the proteolysis at the ER peptide bond. Apparently, in the presence of helix stop signal close to the splice junction, i.e., even a strong splicedon like EALER, appears to be unable to generate a
conformational trap' to facilitate the splicing of $\alpha_{17-30}$ and RNase$\text{S}_{10,20}$. These results are consistent with the studies of Kumaran et al.\textsuperscript{40}. They have re-engineered the structure of RNase$\text{S}_{10,20}$, besides eliminating the His-12 of the peptide, the stop signal and replacing it with helix promoting residue, A. Besides, R at position 10 was replaced by V, and a number of additional helix promoting residues were incorporated. The resultant peptide spliced with RNase$\text{S}_{1,9}$ in about 15% yield (Table 3). However, the co-solvent induced structure of the modified peptide has been characterized as $\beta$-sheet, rather than $\alpha$-helix. The amount of co-solvent induced secondary structure increased considerably in 60% n-propanol, however the concomitant influence on the splicing reaction has not been established. Accordingly, we suggest that the 'conformational trap' in the peptide designed by Kumaran is distinct from that of $\alpha_{17-40}$ and that is operating in the chimeric peptide RNase$\text{S}_{1,5}$-AKFER-$\alpha_{32-40}$. The V8 protease catalyzed self polymerization of RNase$\text{S}_{5,9}$ in aqueous system described by Roy and his colleagues could be a consequence of the coiled coil conformation that the oligomerized RNase$\text{S}_{4,9}$ could assume\textsuperscript{41}. This structure could act as the 'molecular trap' of the splicing reaction. This reflects the diverse ways by which the product conformation can be made to facilitate the splicing reaction of peptides.

Accordingly, the question arises as to the meaning of the 'equilibrium yield' or the 'thermodynamic stability' of the 'conformational trap', i.e. the mechanism by which the co-solvent induced $\alpha$-helical conformation of the product reduces the accessibility of the spliced peptide bond to V8-protease. The hydrolysis of the contiguous peptide can be considered as a measure of the rate of the peptide chain around spliced junction to flex in and out of the 'conformational trap' of the splicing reaction, i.e. to flex in and out of the 'conformation' that is resistant to proteolysis. The present studies suggest that $\alpha_{17-40}$ and $\alpha_{17-40}\text{des}_{52-56}$ with the spliced donor EALER, flexes in and out of the 'conformational trap' (V8 protease resistant conformation) at the slowest rate, and accordingly develops 'conformational trap' with highest thermodynamic stability. This propensity is not increased on increasing the concentration of n-propanol from 30% to 60%, even though some increase in the $\alpha$-helical conformation is seen in 60% n-propanol. The exception is the $\beta$-peptide. These results suggest that in these peptides, the rate of helix-coil transition\textsuperscript{42,43} of the co-solvent induced $\alpha$-helical conformation of these peptides (helix being the protease resistant conformation and coil being the protease digestible conformation) is not influenced by the increased concentration of the co-solvent, the rate of unfolding as well as folding are changed to the same degree. The i, i+4 side chain interaction possible within the spliced donor when the product assumes $\alpha$-helical conformation, appears to lower the rate of unfolding, as this side chain interaction keeps the folded peptide for longer periods of time in the protease resistant conformation. When this propensity to generate i, i+4 side chain interaction is eliminated as in the spliced donor EALEV, the equilibrium yield is lowered. But the maximum stability for the 'conformational trap' is still established in 30% n-propanol. Though the amount of the co-solvent induced conformation increased slightly, presumably this has no influence on the rate of helix-coil transition of the spliced region of the peptide. But, in peptides like the $\beta\beta$-peptide with EALER spliced donor, though considerably more $\alpha$-helical conformation is induced in the presence of co-solvent, the efficiency of the spliced donor EALER to 'recruit' the co-solvent induced conformation to protect the spliced peptide bond is lowered, presumably due to the higher propensity of the flanking regions to be in a helical conformation (relative to that of the flanking regions of the $\alpha$-peptide). On increasing the co-solvent concentration to 60%, the efficiency of the spliced donor EALER to 'recruit' the co-solvent induced conformation of the peptide to protect the spliced donor.

<table>
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<tr>
<th>Peptide system</th>
<th>Synthesis</th>
<th>MRE at 222 nm</th>
<th>Synthesis</th>
<th>MRE at 222 nm</th>
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<tr>
<td></td>
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<td>60% propanol</td>
<td>30% propanol</td>
<td>60% propanol</td>
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<td>na</td>
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</tr>
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</table>

*The data is taken from Kumaran et al.\textsuperscript{40}
peptide bond is increased and is now comparable to that in the \( \alpha \)-peptide.

On the other hand, the propensity of the splicedon to ‘recruit’ the co-solvent induced \( \alpha \)-helical conformation into a ‘conformational trap’ is very sensitive to the presence of the potential to generate i, i+4 side chain interaction, in their absence the thermodynamic stability of the ‘conformational trap’ is reduced by 50%. This implies that the ‘thermodynamic stability’ of the splicedon peptide bond represents the resistance of the region to get into a disordered, protease digestible structure. Since the thermodynamic stability of the ‘conformational trap’ of the splicedon peptide bond with EALEV as the splicedon is not changed by the increase in the concentration of the co-solvent, it follows that the rate of transition of the peptide between the protease resistant conformation and the protease digestible conformation is not influenced when the solvent concentration is increased to 60% from 30%. The co-solvent induced \( \alpha \)-helical conformation is also not influenced very much.

On the other hand, the equilibrium yield as well as co-solvent induced \( \alpha \)-helical conformation increased significantly in the peptides with splicedon EYGER and EGLER, when the concentration of \( \pi \)-propanol is increased to 60%. In this case, the higher concentration of the co-solvent increases the helical conformation, and these splicedons can ‘recruit’ the induced \( \alpha \)-helix into a ‘conformational trap’. The influence of amino acid residues of low \( \alpha \)-helical potential present in the splicedon is partially overcome by the presence of a higher \( \alpha \)-helical conformation. This raises another interesting question as to whether the potential to form i, i+4 side chain interaction present in the splicedons EYGER and EGAER that developed ‘conformational trap’ of increased thermodynamic stability when the propanol concentration is increased played any role in this process. Further studies will be needed to address these issues. Besides, the influence of the length of flanking region, particularly with peptides that generate ‘conformational traps’ of low thermodynamic stability, on the equilibrium yields of splicing reactions as a function of co-solvent concentration will provide ways to modulate the application of this reaction in synthetic protein/peptide chemistry.

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