Helix Formation in Enzymically Ligated Peptides as a Driving Force for the Synthetic Reaction: Example of α-Globin Semisynthetic Reaction†

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ABSTRACT: The α-globin semisynthetic reaction, namely, the ligation of the complementary fragments of α-globin, α_{1-30} and α_{31-141}, in the presence of 30% 1-propanol that is catalyzed by V8 protease is distinct as compared with the previously studied protease-catalyzed splicing of the discontinuity sites of the fragment complementing systems [Sahni et al. (1989) *Biochemistry 28*, 5456]. The complementary fragments of α-globin do not exhibit noncovalent interaction between them even in the presence of 1-propanol, the organic cosolvent used to facilitate the α-globin semisynthetic reaction. Besides, a significant portion of the fragment α_{31-141} does not contribute to the protease-catalyzed splicing reaction. α_{1-30} and α_{31-40} are ligated by V8 protease to yield α_{1-40} in much the same way as the splicing of α_{1-30} with either α_{31-141} or α_{31-47} to yield α-globin or α_{1-47}, respectively. An equimolar mixture of α_{1-30} and α_{31-40} does not show any 'complexation' in the presence of 30% 1-propanol, the medium used for the synthetic reaction. The splicing junction, i.e., Glu^{30}–Arg^{31} peptide bond, is located in the middle of the B-helix (residues 20–35) of the parent protein. Most of the residues from the A-helix of the protein could also be deleted from segment α_{1-30} without influencing the V8 protease-catalyzed splicing reaction. V8 protease catalyzed the splicing of α_{17-30} and α_{31-40} in the presence of 30% 1-propanol with an overall yield of 40% that compares well with the yield of semisynthesis of full length α-globin. Segment α_{17-40} exhibits very little α-helical conformation in the absence of the organic cosolvent. On the other hand, in the presence of 1-propanol, a significant amount of α-helical conformation is induced into α_{17-40}. However, the discontinuity at the Glu^{30}–Arg^{31} peptide bond of α_{17-40} completely abolished the α-helical conformation of the peptide even in the presence of the organic cosolvent. Therefore, we hypothesize that the increased helical conformation of the contiguous system (compared to the discontiguous system) in the presence of organic cosolvent operates as a 'conformational trap' of the semisynthetic reaction. Consistent with this hypothesis, V8 protease-catalyzed synthesis of α-globin and of α_{1-47} from the respective complementary fragments proceeds smoothly in the presence of other helix-inducing organic cosolvents, like trifluoroethanol and 2-propanol. A corollary to this conformational trap hypothesis is that the splicing reaction will be independent of the protease used as far as the specificity requirements of the enzyme used are satisfied. Trypsin indeed catalyzed the ligation of Arg^{31} of α_{17-31} with Met^{32} of α_{32-40} in the presence of 1-propanol to generate the contiguous segment α_{17-40}. The results establish that the covalent contiguity generated by enzymic catalysis in a mixture of the complementary segments represents an assembly of a nascent segment with high 'α-helical conformational propensity'. The helical conformation is induced into the nascent contiguous peptide in the presence of the organic cosolvent, 1-propanol. This induction of α-helical conformation into the semisynthetic segment in the presence of the organic cosolvent generates the conformational trap, which acts as the driving force of the synthetic reaction. Thus, the α-globin semisynthetic reaction represents a new and novel class of protease-catalyzed protein/peptide splicing reactions.

Construction of new and novel proteins by splicing their structural modules (segment condensation) is an attractive concept and has both theoretical and practical advantages in synthetic protein chemistry (Kaiser, 1989; Kent, 1989). The protease-catalyzed splicing of the discontinuity site of fragment complementing system of proteins (Chaiken, 1981; Offord, 1987) can be considered as a simple case of such a segment condensation approach. Our previous studies demonstrating the facile splicing of the complementary fragments of α-globin, namely, α_{1-30} and α_{31-141} at pH 6.0 and 4 °C in the presence of 30% 1-propanol, catalyzed by *Staphylococcus aureus* V8 protease to generate the full-length molecule (Acharya et al., 1985; Sahni et al., 1989) is a new addition to this class of enzyme-catalyzed peptide/protein splicing reactions. However, the experimental conditions that facilitate the latter semisynthetic reaction are distinct compared to the previously studied protese-catalyzed splicing reactions of the discontinuity site of the fragment complementing systems of proteins (Homandberg & Laskowski, 1979). Unlike the other protease-catalyzed semisynthetic reactions, glycerol was not a suitable solvent for the α-globin semisynthetic reaction. Instead, the helix-inducing solvents, namely, 1-propanol, trifluoroethanol, and 2-propanol, were efficient organic cosolvents for this semisynthetic reaction (Acharya et al., 1985; Sahni et al., 1989). A dual role has been invoked for the organic cosolvent, 1-propanol, used in the splicing reaction: (a) the organic cosolvent favors the synthetic chemistry by facilitating the protonation of the α-carboxyl group of the discontinuity site

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in much the same way as glycerol has been suggested to do in other protease-catalyzed protein splicing reactions and (b) the organic cosolvent also influences the energetics of the reaction indirectly by inducing native-like conformation into globin and thus traps the synthetic product in a low-energy conformation (Iyer & Acharya, 1987).

The noncovalent interaction of the fragment complementing system has been considered as one of the crucial structural elements needed to facilitate the protease-catalyzed peptide/protein splicing reactions (Homandberg & Laskowski, 1979). The V8 protease-catalyzed condensation of the complementary α-globin fragments occurs in the absence of the prosthetic group (heme). The removal of heme from the α-chain is known to result in an extensive loss of α-helical conformation of the chain. Accordingly, the possibility of noncovalent interactions between the complementing fragments of α-globin appeared to be very minimal. On the other hand, our earlier observation that 1-propanol can restore a considerable amount of helical conformation into α-globin (Iyer & Acharya, 1987) raised the possibility that propanol may also induce helical conformation into the complementary fragments (discontiguous system) which in turn could generate enough noncovalent interactions between them to form a 'native-like' structure. Such a presumptive interaction could provide the appropriate stereochemistry for the α-carboxyl group of Glu^{30} and the α-amino group of Arg^{31} of the complementary fragments α_{1-30} and α_{31-141}, respectively. This proposal confers a third role to the organic cosolvent used in the α-globin semisynthetic reaction. However, the fact that a shorter component of α-globin, namely, α_{1-47}, can be readily prepared by the ligation of α_{1-30} and α_{31-47} with V8 protease in the presence of 30% 1-propanol (Seetharam & Acharya, 1986) suggests that the principle of fragment complementation, even if assumed to be present in the presence of 1-propanol, may not be playing a pivotal role in dictating the facile protein splicing reaction. Besides, the possibility that V8 protease possesses an avid preference for ligating Glu–Arg bonds in the presence of 1-propanol is also ruled out based on the inability of α_{1-23} and α_{1-27} (both containing Glu as their carboxy terminus) to condense with either α_{31-141} or α_{31-47} (Seetharam & Acharya, 1986).

In an attempt to gain further insight into the molecular events that facilitate the α-globin splicing reaction, we have initiated the present study, specifically to address the presence or absence of complexation in an equimolar mixture of the complementary fragments of α-globin and also to establish the consequence of contiguity on the conformational aspects of an equimolar mixture of the complementary fragments of α-globin. The influence of truncation of the complementary fragments on the splicing reaction and the induction of helical conformation into the truncated segments of α-globin in the presence of 1-propanol have also been investigated. These studies have led to the demonstration of a lack of complexation between the complementary segments and have also showed that propanol-induced helical conformation of the nascent ligated B-helix of α-globin serves as the primary conformational trap of the splicing reaction. Thus, α-globin semisynthetic reaction belongs to a new and novel class of protease-catalyzed peptide/protein splicing reactions.

MATERIALS AND METHODS

HbA[1] and its *p*-hydroxymercuribenzoate (HMB) reacted chains were prepared as described earlier (Bucci & Fronticelli, 1965; Seetharam & Acharya, 1986). α-Globin (α-chain without heme) was obtained by acid–acetone precipitation of the α-chain (Rossi-Fanelli et al., 1958) unless otherwise stated. V8 protease (endoproteinase Glu-C), endoproteinase Lys-C, and L-1-(tosylamino)-2-phenylethyl chloromethyl ketone trypsin were from Pierce Chemical Co., Boehringer Mannheim, and Sigma Chemical Co., respectively. Sephacryl S100 was obtained from Pharmacia.

Preparation of α_{1-30} and α_{31-141}. α-Globin was digested with V8 protease (1:200 w/w) at a concentration of 0.5 mg/mL in 10 mM ammonium acetate buffer (pH 4.0, 37 °C) for 3 h. The digest was lyophilized, dissolved in 0.1% TFA, and fractionated by RPHPLC. The identity of the isolated α_{1-30} and α_{31-141} was established by tryptic peptide mapping and amino acid analysis.

Preparation of α_{17-40}. α_{17-40} was isolated from an endoproteinase Lys-C digest of α-globin. Due to the insolubility of α-globin at higher pH values, the globin was digested at pH 6.0. The digestion was carried out at 37 °C for 18 h at a protein concentration of 1 mg/mL (E:S ratio of 1:400 w/w). The digest was lyophilized and analyzed by RPHPLC. Peptide α_{17-40} was located in the RPHPLC map of the Lys-C digest by comparison with the RPHPLC map of tryptic digests of α-globin. The identity of α_{17-40} was confirmed by amino acid analysis and the HPLC map of its tryptic digest. Only two peptides, namely, αT4 (α_{17-31}) and αT5 (α_{32-40}) were generated on tryptic digestion of α_{17-40}.

Preparation of α_{17-30}, α_{17-31}, α_{31-40}, and α_{32-40}. α_{17-40} (1 mg/mL) was digested either with V8 protease (1:200 w/w) in 0.01 M ammonium acetate at pH 4.0 or with trypsin (1:100 w/w) in 0.1 M ammonium bicarbonate at pH 7.8 at 37 °C for 2 h. The respective digests were diluted with TFA to a final concentration of 0.1% and loaded directly onto the column for RPHPLC. Each digest yielded only two components. The peptides from each of the digests were isolated by RPHPLC, and the identity of α_{17-30} and α_{31-40} (V8 protease digestion products) and α_{17-31} and α_{32-40} (tryptic digestion products) was established by amino acid analysis.

Protease-Catalyzed Splicing of Complementary Fragments of α_{17-40}. V8 protease splicing of α_{17-30} and α_{31-40} and trypsin-catalyzed splicing of α_{17-31} and α_{32-40} were carried out in 0.05 M ammonium acetate (pH 6.0, 4 or 23 °C) containing 30% 1-propanol. An equimolar mixture of the respective complementary fragments (2 mM) was incubated with the respective enzymes (1:200). Aliquots of the reaction mixture were withdrawn at different time intervals, and the extent of synthesis was quantitated by RPHPLC analysis.

Circular Dichroic Spectral Measurements. The CD spectra were recorded in an AVIV 60DS spectropolarimeter fitted with a computer (model 1600-1) from Columbia Data Products. Ellipticity values were converted to mean residue ellipticity as described previously (Iyer & Acharya, 1987). The concentration of the peptides was determined either by quantitative amino acid analysis or by utilization of the molar extinction coefficient of tyrosine (E_{280} = 1280 lit mol^{-1} cm^{-1}) (Edelhoch, 1967).

Fluorescence Measurements. Fluorescence spectra were recorded in a Hitachi MPR 600 spectrofluorimeter fitted with a temperature control bath. The spectra of the samples were recorded in 50 mM ammonium acetate buffer, pH 6.0 at 4 °C, either in the presence or in the absence of 30% 1-propanol. The samples were excited at 295 nm, and the fluorescence emission spectra were recorded from 310 to 420 nm.

[1] Abbreviations: TFA, trifluoroacetic acid; RPHPLC, reverse-phase high-performance liquid chromatography; HbA, hemoglobin A.

Gel Filtration of a Mixture of Complementary Fragments. A 1:1 mixture of the complementary fragments (α_{1-30} and α_{31-141} or α_{1-30} and α_{31-40}) was subjected to gel permeation chromatography on a Sephacryl S100 column (2.5 × 40 cm) equilibrated and eluted with 50 mM ammonium acetate (pH 6.0) containing 30% 1-propanol.

RESULTS

Influence of Discontiguity at Peptide Bond Glu^{30}–Arg^{31} of α-Globin on the Fluorescence of Its Tryptophan Residue. α-Globin has a single tryptophan residue at position 14 of the polypeptide chain. Hence, it could serve as a good conformational probe of the propanol-induced conformation and also conceivably as a reporter of the noncovalent interactions of α_{1-30} and α_{31-141}. In the absence of 1-propanol, Trp^{14} of α-globin exhibits a strong fluorescence with an emission maximum at 355 nm at pH 6.0 and 4 °C. The presence of 30% 1-propanol blue shifts the emission maximum of α-globin by 5 nm (to 350 nm) and increases the fluorescence intensity of the molecule by 20%. Thus, the microenvironment of Trp^{14} in α-globin is sensitive to the presence of 1-propanol. Our earlier studies (Iyer & Acharya, 1987) have shown that the α-helical conformation of α-globin also increases in the presence of 1-propanol. It was suggested that this induction of helicity results in the packing of the helical segments of the polypeptide chain in a 'native-like' format (Iyer & Acharya, 1987). Apparently, these molecular events have also led to the observed blue shift of the Trp fluorescence as well as the increase in its fluorescence intensity. Moreover, introduction of the discontinuity at the peptide bond of Glu^{30} and Arg^{31} of α-globin red shifts the fluorescence emission maximum in the aqueous medium to 360 nm. This is a clear reflection of the sensitivity of the microenvironment of Trp^{14} to the presence of discontinuity of the polypeptide chain at the Glu^{30}–Arg^{31} peptide bond of the chain.

The fluorescence intensity as well as the emission maximum of Trp^{14} of the isolated α_{1-30} in the aqueous buffer is not influenced by the presence of an equimolar amount of α_{31-141}. In the presence of 30% 1-propanol, the fluorescence intensity of the isolated α_{1-30} increased by about 15% and the emission maxima blue shifted to 353 nm (360 nm in the aqueous buffer). However, this emission maximum is distinct compared to 350 nm seen with α-globin in 30% 1-propanol. On the other hand, the fluorescence emission spectra of α-globin with discontinuity at the Glu^{30}–Arg^{31} peptide bond (an equimolar mixture of α_{1-30} and α_{31-141}) in the presence of 30% 1-propanol is similar to that of isolated α_{1-30} in the presence of 30% 1-propanol in terms of both fluorescence intensity and emission maximum. These results suggest that the noncovalent interaction between the complementary segments α_{1-30} and α_{31-141}, if present, are minimal even in the presence of 30% 1-propanol. Thus, it is clear that distinct structural differences exist between the contiguous and the discontiguous globin systems in terms of the microenvironment of Trp^{14} both in the presence and in the absence of 1-propanol.

Influence of Discontiguity in α-Globin on Its Far UV CD Spectra. 1-Propanol induces a significant amount of α-helical conformation into α-globin (Iyer & Acharya, 1987). Influence of discontinuity at the Glu^{30}–Arg^{31} peptide bond on the propanol-induced α-helicity of α-globin as well as the α-helical conformation of the isolated segments (α_{1-30} and α_{31-141}) has now been investigated to establish whether the discontinuity influences the induction of α-helical conformation into the protein. The discontinuity at the peptide bond 30–31 of α-globin decreased its θ_{222} both in aqueous buffer and in the presence of 30% 1-propanol (Figure 1). However, the decrease in the θ_{222} is nearly the same in the presence and absence of 1-propanol, a decrease of about −3350 and −3916, respectively.

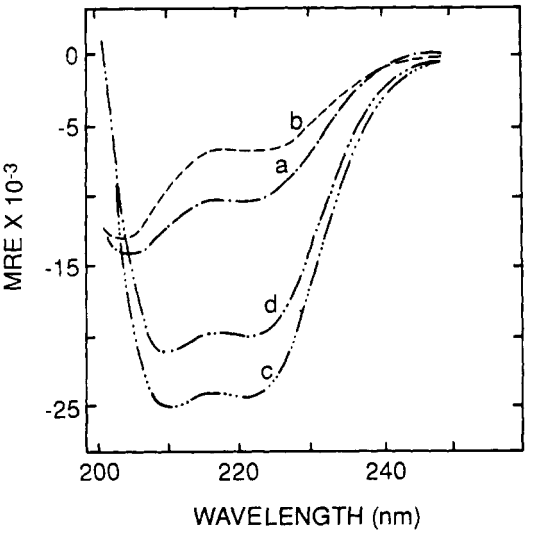

FIGURE 1: Far-UV circular dichroic spectra of α-globin: CD spectra of contiguous and discontiguous α-globin at pH 6.0 (10 mM ammonium acetate buffer) and 4 °C, either in the absence or in the presence of 30% 1-propanol. Curves a and b represent CD spectra of α-globin and discontiguous α-globin in the buffer that did not contain 1-propanol. Curves c and d are the CD spectra of contiguous and discontiguous α-globin in 10 mM ammonium acetate buffer pH 6.0 containing 30% 1-propanol.

The α-helical conformation of the isolated fragments α_{1-30} and α_{31-141} was also sensitive to the presence of 1-propanol. The α-helical conformation of α_{31-141} in 1-propanol is significantly higher as compared to that of α-globin. In contrast, the induction of α-helical conformation into α_{1-30} by 1-propanol was marginal (data not shown). The calculated α-helical content of an equimolar mixture of α_{1-30} and α_{31-141} is nearly the same as that of the experimentally determined value for α-globin with a discontinuity at the Glu^{30}–Arg^{31} peptide bond. The α-helical conformation of the discontiguous α-globin system appears to be additive. This can be interpreted as the absence of strong noncovalent interactions between the two complementary fragments of α-globin and is consistent with the results of the fluorescence studies discussed above.

Lack of Complexation between Complementary Segments of α-Globin. The conformational analysis of α-globin with discontinuity at the Glu^{30}–Arg^{31} peptide bond has established that distinct differences exist between the contiguous and the discontiguous α-globin systems and implicated the absence of strong noncovalent interaction between the complementary fragments. Direct evidence for this was sought by gel filtration of the discontiguous α-globin system. Gel filtration of an equimolar mixture of α_{1-30} and α_{31-141} on a Sephacryl S100 column equilibrated and eluted with 50 mM ammonium acetate buffer, pH 6.0, containing 30% 1-propanol yielded α_{1-30} and α_{31-141} as distinct components. The gel filtration pattern of the mixture did not demonstrate any indication of the noncovalent complexation between α_{1-30} and α_{31-141}. Therefore, it is concluded that strong fragment complementation between the two complementary segments of α-globin is absent even in the presence of 30% 1-propanol. These results are thus consistent with the fluorescence and CD data.

V8 protease is able to generate the Glu^{30}–Arg^{31} peptide bond in a mixture of α_{1-30} and α_{31-47} with an overall yield comparable to that obtained for the ligaton of α_{1-30} and α_{31-141} to generate α-globin. The results suggest that the segment

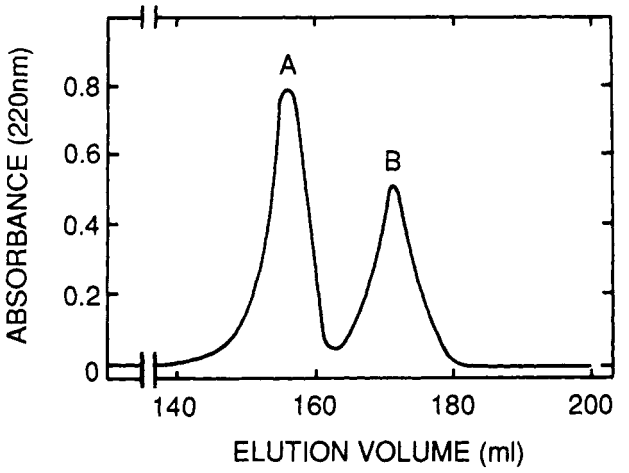

FIGURE 2: Gel filtration of an equimolar mixture of α_{1-30} and α_{31-40}. The 1:1 mixture of α_{1-30} and α_{31-40} dissolved in 50 mM ammonium acetate buffer pH 6.0, containing 30% 1-propanol, was loaded onto a Sephacryl S100 column (2.5 × 40 cm) preequilibrated with the same buffer. The chromatography was carried out using the same buffer. Fractions of 1 mL were collected at a flow rate of 10 mL/h, and their absorbance was monitored at 220 nm. Components A and B were isolated and characterized by RPHPLC as α_{1-30} and α_{31-40}, respectively.

representing the amino acid residues 48–141 of α-globin do not contribute significantly to facilitate the splicing reaction. Extending this observation further we have now shown that α_{1-30} and α_{31-40} can be ligated to generate α_{1-40} (data not shown). The absence of complex formation in the truncated system has also been established by gel filtration studies. An equimolar mixture of α_{1-30} and α_{31-40} was subjected to gel filtration under conditions used for the semisynthetic reaction. The two globin fragments eluted as chromatographically distinct components from the gel filtration column (Figure 2), and no component representing a 1:1 complex between α_{1-30} and α_{31-40} was detected. Thus, the phenomenon of fragment complementation is absent in the mixture of the complementary fragments of both the full-length and the truncated α-globin system.

V8 Protease-Catalyzed Splicing of α_{17-30} and α_{31-40}, Complementary Fragments of the B-Helix of α-Chain. The discontinuity site, Glu^{30}–Arg^{31} peptide bond of α-chain that could be spliced by V8 protease, is located in the middle of the B-helix of the chain. Therefore, it is conceivable that establishing the continuity of the B-helix and the concomitant induction of α-helical conformation to this segment is a primary molecular event that provides the driving force for the semisynthetic reaction. A consequence of this hypothesis is that V8 protease should be able to catalyze the splicing of the complementary segments of the B-helix of α-globin. The α-globin segment α_{1-40}, discussed above, contains the A-helix, a part of the C-helix, and the entire length of B-helix of the chain. Accordingly, in an attempt to decipher whether the amino acid residues of the A-helix of the chain play any role in facilitating the protease-catalyzed splicing reaction, the segment α_{17-40}, which encompasses the entire length of the B-helix of α-globin, was isolated (see Materials and Methods for details). The V8 protease-catalyzed splicing of its complementary segments, namely, α_{17-30} and α_{31-40}, was investigated. RPHPLC of an equimolar mixture of α_{17-30} and α_{31-40} incubated at pH 6.0 and 4 °C in the presence of 30% 1-propanol for 48 h with V8 protease indeed revealed the formation of a new peak corresponding to that of α_{17-40}. The generation of this new component is dependent on the presence of V8 protease since a sample incubated in the absence of the enzyme did not generate this component. These studies thus demonstrate that V8 protease can catalyze the splicing of the complementary segments of the B-helix alone and that the residues of A-helix are not essential for facilitating this splicing reaction.

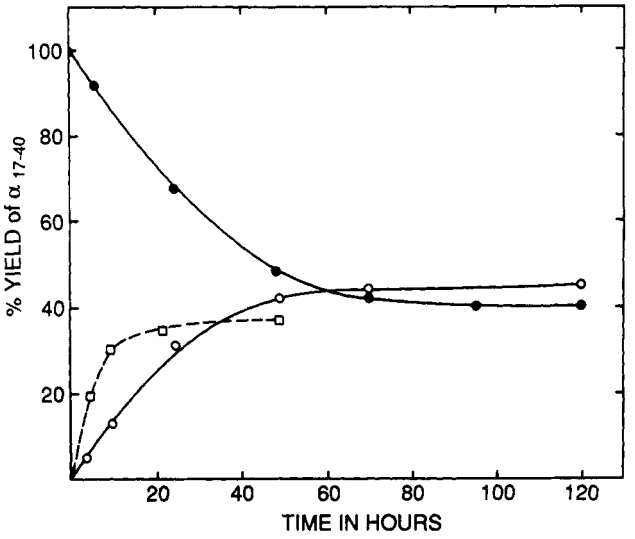

FIGURE 3: Kinetics of V8 protease-catalyzed splicing of α_{17-30} and α_{31-40}, as well as slicing of α_{17-40}. Protease-catalyzed synthesis was carried out as described under Materials and Methods. Aliquots of the reaction mixture were withdrawn at various time intervals and the synthetic yield of α_{17-40} was quantitated by RPHPLC. Synthesis of α_{17-40} at 4 °C is given by open circles with solid lines (O–O). Synthesis of α_{17-40} at 23 °C is given by open circles with broken lines (O- - -O). The kinetics of slicing of α_{17-40} (2 mM) at pH 6.0, and 4 °C by endoproteinase Glu-C in the presence of 30% 1-propanol is shown by the filled circles and solid lines (●–●). The hydrolysis was followed by the disappearance of α_{17-40} in the RPHPLC maps.

The kinetics of the V8 protease-catalyzed splicing of α_{17-30} and α_{31-40} is shown in Figure 3. The synthetic reaction reached an equilibrium in about 60 h with an overall yield of nearly 40%. Nearly the same equilibrium yield was obtained when approached from the hydrolytic side, i.e., by digesting α_{17-40} with V8 protease under identical conditions used for synthesis. Thus, the yield of 40% of the synthetic product represents the true equilibrium of the synthetic and the hydrolytic reactions catalyzed by V8 protease and compares reasonably well with the yield of 45–50% seen with the splicing of the α-globin and α_{1-47} from their respective complementary fragments (Sahni et al., 1989; Seetharam & Acharya, 1986). Thus, the molecular aspects that facilitated the splicing reaction of α_{1-30} and α_{31-141} appear to be conserved in the complementary segments of the B-helix, namely, α_{17-30} and α_{31-40}.

Induction of α-Helical Conformation into α_{17-40} in the Presence and Absence of 1-Propanol. Far-UV CD spectra of α_{17-40} in aqueous buffer at pH 6.0 and the buffer containing 30% 1-propanol are shown in Figure 4A (curves a and c, respectively). This 24-residue segment of α-globin exhibited about 10–12% helical content in the aqueous buffer (curve a). The α-helical content increased to nearly 40% in 30% 1-propanol (curve c). Thus, α_{17-40}, the isolated B-helix of α-globin has a high potential to assume α-helical conformation in the presence of 1-propanol.

Contiguity at Glu^{30}–Arg^{31}-Peptide Bond of α_{17-40} Is Essential for Induction of α-Helical Conformation into It by 1-Propanol. α-Helical conformation of discontiguous α_{17-40} has also been investigated to see whether contiguity is an essential feature for the induction of α-helical conformation (Figure 4A). An equimolar mixture of α_{17-30} and α_{31-40} did not show any helical conformation either in the aqueous

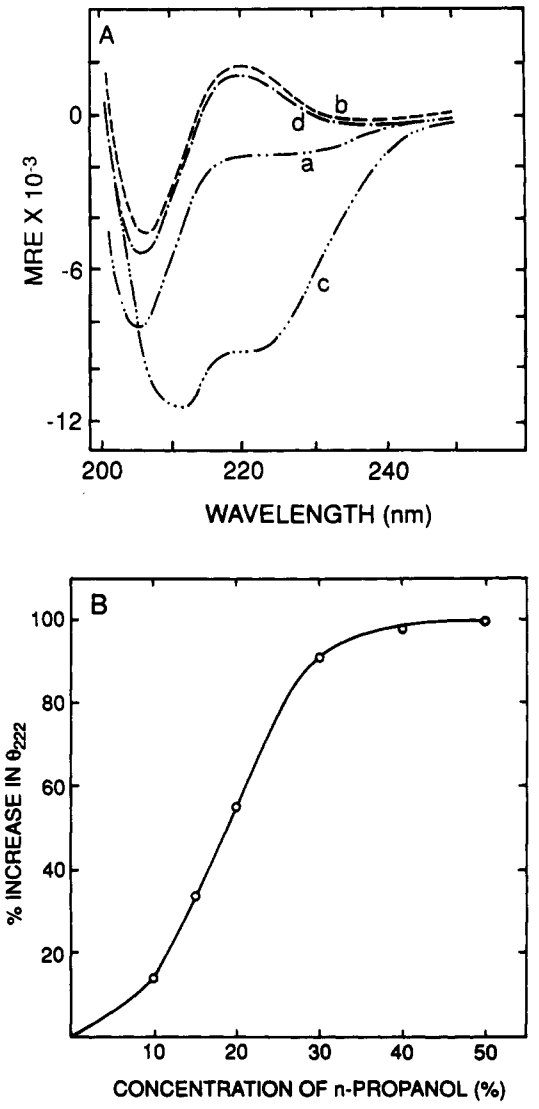

FIGURE 4: Influence of 1-propanol on the α-helical conformation of α_{17-40}. (Panel A) CD spectra of contiguous and discontiguous α_{17-40} in the presence and absence of 1-propanol. (Spectra a) Contiguous α_{17-40} in 10 mM ammonium acetate buffer. (Spectra c) Contiguous α_{17-40} in the same buffer containing 30% 1-propanol. (Spectra b) Discontiguous α_{17-40} (tryptic digest of α_{17-40}) in the buffer without 1-propanol. (Spectra d) Discontiguous α_{17-40} in the same buffer containing 30% 1-propanol. (Panel B) α-Helical conformation of α_{17-40} as a function of 1-propanol concentration. CD spectra of α_{17-40} (81 μg/mL) were taken as described in Figure 1 in 10 mM ammonium acetate buffer at pH 6.0 containing various concentrations of 1-propanol. The increase in θ_{222} value of each sample in the presence of 50% 1-propanol was taken as 100%. The respective increase in θ_{222} of values of each sample at other concentrations of 1-propanol is expressed as percent of the total increase of the respective samples in 50% 1-propanol.

medium or in the presence 1-propanol (curves b and d, respectively). Therefore, the induction of α-helical conformation into α_{17-40} is a property unique to the contiguous segment encompassing the full length of the B-helix.

α-Helical Conformation of α_{17-40} as a Function of 1-Propanol Concentration. The sensitivity of α_{17-40} (B-helix) to the concentration of 1-propanol is distinct compared to α-globin (Iyer & Acharya, 1987). Very little induction of α-helicity occurs into α_{17-40} when the concentration of 1-propanol is 10% or less (Figure 4B). On the other hand, in 30% 1-propanol, the induction of α-helical conformation is nearly complete. Thus, the isolated B-helix of α-globin, α_{17-40}, can be considered a 'micro domain' of α-globin that exhibits high propensity to translate its structural information in the presence of low concentration of 1-propanol. Most of the induction of α-helical conformation occurs in a narrow range of 15–30% 1-propanol.

It should be noted here that with α-globin, the helix induction reaches nearly 75% level in the presence of 15% 1-propanol. However, the semisynthetic yield of α-globin at this stage is very low (Sahni et al., 1989). Thus, the induction of α-helicity does not directly correlate with the α-globin semisynthetic reaction. On the other hand, α_{17-40} exhibits very little α-helical conformation in the presence of 10% 1-propanol. The induction of the helical conformation is nearly complete in the presence of 30% 1-propanol. It is of interest to note here that the optimum concentration of 1-propanol for the V8 protease-catalyzed α-globin semisynthetic reaction is around 30%. Thus with the α_{17-40} system, the sensitivity of induction of helical conformation appears to correlate better with the synthetic yield of the contiguous segment obtained at the corresponding concentration of organic cosolvent.

Influence of Temperature on Semisynthetic Reaction. The propanol-induced α-helical conformation of α_{17-40} was also studied as a function of temperature. The overall helicity in 30% 1-propanol remained reasonably constant over the temperature range of 4–37 °C (data not shown). Therefore, if the propensity of the nascent contiguous segment, i.e., α_{17-40}, to assume helical conformation in the presence of 1-propanol is a primary molecular event or a prelude to the generation of the conformational trap in situ, it follows that the splicing reaction would proceed at higher temperatures as well. As can be seen in Figure 3, V8 protease catalyzed the condensation of α_{17-30} and α_{31-40} at room temperature. The semisynthetic reaction was faster at 23 °C as compared to that at the lower temperature of 4 °C. The overall yield of the product is 38% and is again comparable to that at 4 °C.

Trypsin-Catalyzed Condensation of α_{17-31} and α_{32-40}. A corollary to the hypothesis that 1-propanol-induced α-helicity of α_{17-40} acts as a conformational trap of the protein/peptide splicing reaction is that the potential of the complementary fragments of α_{17-40} to condense should be independent of the catalyst used in the splicing reaction. Any protease should be able to catalyze this splicing reaction as far as the specificity requirement of the protease used is satisfied. The Arg^{31}–Met^{32} peptide bond present in segment α_{17-40} of α-globin satisfies the specificity requirement of trypsin. Therefore, it is expected that trypsin will be able to catalyze the synthesis of α_{17-40} from its complementary fragments, α_{17-31} and α_{32-40}, under the same conditions in which V8 protease catalyzed the condensation of α_{17-30} and α_{31-40}.

Trypsin indeed catalyzed the synthesis of α_{17-40} from its complementary fragments α_{17-31} and α_{32-40} (Figure 5). The splicing reaction, unlike that of V8 protease, was sluggish at 4 °C. However, considerable acceleration of the splicing reaction is achieved when it is carried out at room temperature. The trypsin-catalyzed reaction was also studied as a function of 1-propanol concentration (Figure 5, inset) to determine the optimum concentration of 1-propanol that is required for the trypsin-catalyzed splicing of the peptides. The trypsin-catalyzed reaction also showed optimal synthesis in the presence of 30% 1-propanol. The same concentration of 1-propanol has been found to be optimal for the V8 protease-catalyzed α-globin semisynthetic reaction. The maximum induction of α-helical conformation into α_{17-40} also occurs at 30% 1-propanol. Thus, it can be concluded that the 1-propanol-induced α-helical conformation of the nascent contiguous segment is the primary molecular event that generates the conformational trap of the proteosynthetic reaction.

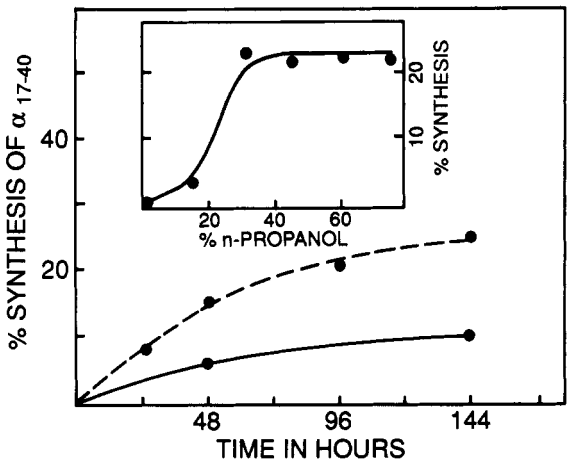

FIGURE 5: Trypsin-catalyzed splicing of α_{17-31} and α_{32-40}. An equimolar mixture of α_{17-31} and α_{32-40} (2 mM each) in 50 mM ammonium acetate buffer, pH 6.0, containing 30% 1-propanol was incubated with trypsin (1:200 w/w) either at 4 °C by (solid line) or at 23 °C by (broken line). Aliquots were withdrawn at various time intervals, and the extent of synthesis of α_{17-40} was quantitated by RPHPLC as described under Materials and Methods. Inset shows the influence of the concentration of 1-propanol on the semisynthetic yield of α_{17-40} at 23 °C, after 144 h of incubation.

DISCUSSION

The results presented here establish that the molecular basis of the V8 protease-catalyzed efficient and facile splicing of the complementary fragments of α–globin, α_{1-30} and α_{31-141}, α_{1-30} and α_{31-47}, or α_{17-30} and α_{31-40} is distinct from the previously reported splicing of the discontinuity sites of noncovalently interacting complementing systems of proteins. The fluorescence, ultraviolet CD, and gel filtration studies of an equimolar mixture of the complementary fragments of α-globin demonstrate the absence of complexation under the conditions of the semisynthetic reaction. Segment α_{17-40} is able to assume a significant amount of helical conformation under the conditions of the semisynthetic reaction. In contrast, helical conformation is completely absent in the discontiguous system (a 1:1 mixture of α_{17-30} and α_{31-40}). The results establish that the induction of helical conformation by 1-propanol is a property unique to the contiguous system (α_{17-40}). Therefore, it follows that once the contiguity is generated in situ by enzymatic ligation, the nascent contiguous segment acquires a thermodynamically stable helical conformation induced by the organic cosolvent. This organic cosolvent has been incorporated into the semisynthetic mixture to facilitate the protonation of the α-carboxyl group of the discontinuity site. This organic solvent-induced structure acts as a conformational trap, the force for driving the synthetic reaction. Thus, the molecular aspects that facilitate the α-globin semisynthetic reaction are distinct as compared with the protease-catalyzed splicing of the discontiguity sites of fragment complementing systems and hence belongs to a new and novel class of protein/peptide splicing reactions.

The use of proteases to catalyze the peptide bond formation has been a subject of considerable interest in the synthetic peptide/protein chemistry (Chaiken et al., 1987; Fruton, 1983). Proteases have been successfully used for both stepwise elongation as well as condensation of the polypeptide fragments. The recent demonstration of the in vivo protein splicing reactions as a posttranslational molecular process of generating some mature functional proteins lends considerable biological significance as well to these protein-splicing reactions (Kane et al., 1990; Carrington et al., 1985; Bowles et al., 1988). It is of interest to note here that the splicing junction in the α-globin semisynthetic reaction is at or near the junction of the products of the exon-1 and exon-2 of the α-globin gene (Craik et al., 1980, 1981; Eaton, 1980; Leder et al., 1978). The biological or the structural significance of this observation is not readily apparent at this stage.

Two distinct approaches have been adopted for successful protease-catalyzed splicing of the polypeptide segments. The first one involves the protease-mediated ligation of the discontinuity sites of the noncovalently associated fragments (fragment complementing systems). The subtilisin-mediated splicing of the discontinuity site of RNase S to generate RNase A is the first example of this class of reactions (Homandberg & Laskowski, 1979). The protease-catalyzed splicing at the discontinuity site of nuclease T (Komoriya et al., 1980), cytochrome *c* (Juillerat & Homandberg, 1981), and human somatotropin (Graf & Li, 1981) have been since demonstrated by making use of the positive effect of the organic solvent (90% glycerol) on the condensation reaction. Various proteases like trypsin, clostripain, and thrombin have been shown to catalyze the splicing reaction. Thus, the protease-catalyzed ligation of peptides/proteins can be considered a general phenomenon as far as a fragment complementing system of a protein could be developed (Kullmann, 1987). The appropriate stereochemistry of the α-amino and α-carboxyl group at the discontinuity site has been considered to play a pivotal role in dictating the selectivity of the ligation reactions. The conformation-assisted semisynthesis of cytochrome *c* analogues (Wallace et al., 1989) can be considered as a further manipulation of the principle of fragment complementation.

The protease-catalyzed splicing of non-complex-forming fragments can be facilitated by the design of a molecular trap. Such traps facilitate the splicing reaction by a selective and continuous removal of the nascent spliced material from the chemical equilibrium. In the clostripain-catalyzed formation of RNase A_{1-15} from RNase A_{1-10} and RNase A_{11-15}, the product RNase A_{1-15} was trapped by the externally added S-protein in order to facilitate the splicing reaction (Homandberg et al., 1982). The noncovalent interactions of S-protein and the nascent contiguous segment (RNase A_{1-15}) acts as the molecular trap of the clostripain-catalyzed splicing reaction. The possible use of product-directed antibodies as molecular trap in protease-catalyzed splicing reactions has been proposed by Chaiken and his associates (1987). The protease-catalyzed synthesis of substance P from its complementary fragments has been shown to be facilitated by trapping the product using an antibody (Nyberg, 1988).

The results presented here have led to the recognition of a novel molecular trap mechanism that facilitates the protease-catalyzed splicing of non-complex-forming peptide/protein segments. We have now demonstrated that 1-propanol-induced α-helical conformation of the contiguous segment serves as a conformational trap of the splicing reaction. The earlier observation that synthesis of α-globin proceeds smoothly in other α-helix-inducing solvents like trifluoroethanol and 2-propanol (Sahni et al., 1989) is consistent with this interpretation. Further, we have recently observed a comparable amount of synthesis of α-globin and α_{1-47} in the presence of other helix-inducing solvents like propanediol and butanediol (G. Sahni and A. S. Acharya, unpublished data).

The present study raises an interesting prospect that the organic cosolvent-induced secondary structure of the nascent contiguous segment could be modulated to design an in situ conformational trap to facilitate the protease-catalyzed splicing of segments of proteins even when a complex formation is

absent. Increase in the α-helical conformation of the peptides as a function of chain length has been demonstrated previously by Lau et al. (1984). It will be of interest to establish the generality of the molecular trap principle seen with the fragments of α-globin and to determine whether this principle could indeed be translated to the design of new in vitro protein/peptide splicing reactions. Delineation of such secondary structural principles that could be manipulated to achieve the enzyme-catalyzed protein-splicing reactions should facilitate the design of new strategies for the modular construction of proteins.

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