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Synthetic Potential of *Staphylococcus aureus* V8-Protease: An Approach Toward Semisynthesis of Covalent Analogs of α-chain of Hemoglobin S

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Enzyme-catalyzed reformation of peptide bonds in the noncovalent fragment systems of proteins has been emerging as a convenient procedure for the semisynthesis of covalent analogs of the respective proteins. Limited proteolysis of the α-chain of hemoglobin S with *Staphylococcus aureus* V8-protease converts the chain into a fragment-complementing system by hydrolyzing the peptide bond Glu(30)-Arg(31) of the chain. Therefore, it is conceivable that semisynthesis of covalent analogs of α-chain could be achieved if conditions for the V8-protease catalyzed formation of peptide bonds could be established. The synthetic potential of V8-protease has been now investigated by incubating V8-protease-derived fragments of α-chain, namely α_{1-30} and α_{31-47} with the enzyme at pH 6.0 in the presence of n-propanol as the organic cosolvent. RP high performance liquid chromatography analysis showed that a new chromatographically distinct component is generated on incubation, and this has been identified as α_{1-47} by amino acid analysis, redigestion with V8-protease (in the absence of n-propanol), and tryptic peptide mapping. Optimal conditions for the synthesis of α_{1-47} is at pH 6.0, 4°C, and 24 hr of incubation with 25% n-propanol as organic cosolvent. This stereospecific condensation of the fragments proceeded to a high level of about 50% in 24 hr. Further incubation up to 72 hr did not increase the yield of α_{1-47}, suggesting that an equilibration of synthesis and hydrolysis reactions has been attained. The demonstration of the synthetic potential of V8-protease and the fact that α_{1-30} and α_{31-141} interact to form a native-like complex, opens up an approach for the semisynthesis of covalent analogs of α-chain of hemoglobin S.

Key words: semisynthesis, V8-protease, gelation

Abbreviation used: HbS, hemoglobin from patients with sickle cell disease.

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A decisive way of investigating the structure-function relationships of a protein is to change the amino acid sequence at will and determine the influence of the alteration (mutation) on the tertiary and/or quaternary structure of the protein and the consequent changes in the functional properties of the molecule. However, the exponentially lowering yields of the peptides during the chemical synthesis of large polypeptides makes the task of changing the amino acid sequence of proteins at will difficult, if not impossible [1]. The recent advances in genetic engineering techniques have rendered the manipulation of the amino acid sequence of the protein relatively simple. These methods, however, still do not provide the flexibility to introduce an unnatural amino acid into the sequence or to incorporate at specific sites an isotopically enriched natural amino acid (for example, ^{13}C or ^{15}N labeled). Semisynthesis of proteins appears to provide this flexibility to protein chemists.

The semisynthesis of proteins can be defined simply as the rebuilding of proteins from two or more components, at least one of which is obtained by chemical synthesis [2]. The product of chemical synthesis is mixed with the natural counterpart, and the two fragments are stitched together by enzymic or nonenzymic methods. The essential operational steps of methodology are depicted in Figure 1. The preparation of the noncovalent analog of a protein, one of the initial steps of this methodology, makes it necessary that the limited cleavage introduced by a protease into the protein be in a

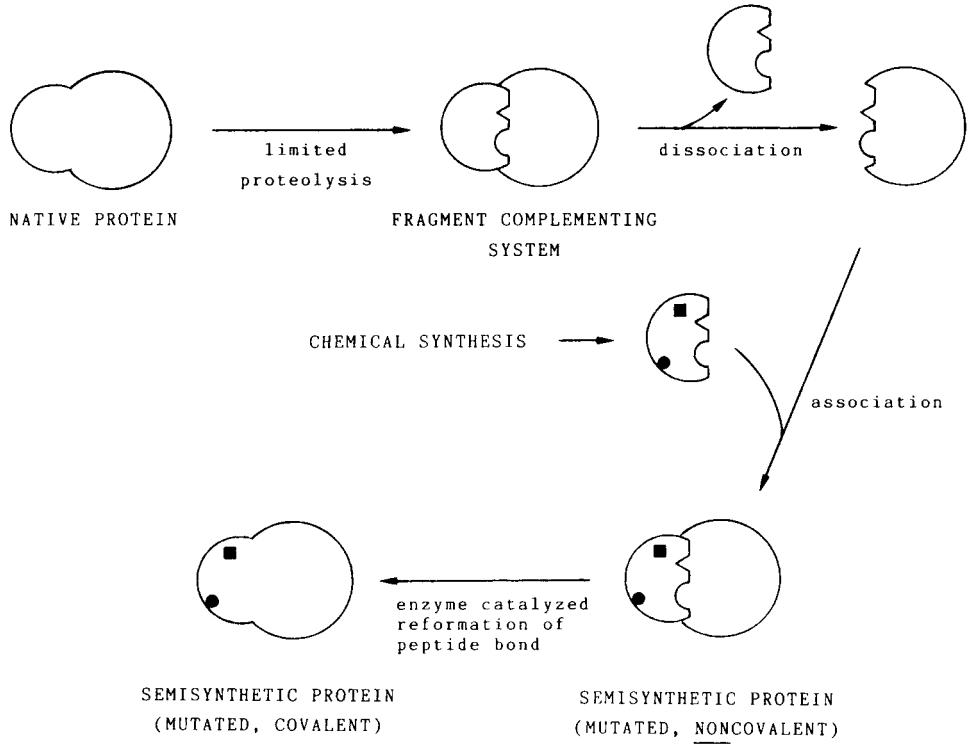

Fig. 1. Schematic representation of the steps involved in the proposed protease-catalyzed semisynthesis proteins. (●, ■) represent the chemical mutations or isotopically enriched (^{13}C or ^{15}N) amino acid introduced by chemical synthesis.

region of the peptide chain wherein a discontinuity does not perturb the overall "native-like" conformation [3,4]. Such a system, generally referred to as "fragment complementing system," has been developed for proteins such as RNase-A [5], nuclease [6], cytochrome C [7], etc. In these systems, the two polypeptide fragments derived from the single chain of the parent protein are held together by strong noncovalent interactions. The constituent polypeptide fragments can be dissociated under denaturing conditions and isolated in pure form. However, when mixed together again under renaturing conditions, they readily generate the ordered complex and biological function of the parent protein. The possibility of substituting one of two fragments with a chemically synthesized analog in the reconstitution studies has opened up the way for exploration of protein structure by manipulation of their chemical structure, namely by preparing new *semisynthetic noncovalent analogs* of proteins [1].

The demonstration that noncovalent fragment complementing systems of a protein can be converted to covalent forms by protease catalyzed reformation of the peptide bond between the discontinuity region of the complex [8] has given considerable impetus to studies of semisynthesis of noncovalent analogs of proteins. The protease catalyzed reformation of peptide bonds represents a considerable advancement in the semisynthesis of proteins, since the stereospecificity of the enzymes prevents the formation of undesirable side products so frequently generated during the conventional chemical synthesis of peptides [1].

We have been interested in developing semisynthetic procedures for the α- and β^S-chains of hemoglobin S, specifically as a methodology to prepare chains with two or more mutations in them, as well as to introduce ^{13}C or ^{15}N probes at selected intermolecular contact regions, the ultimate objective being the delineation of the mechanistic aspects of the polymerization of deoxy HbS. Our recent studies with the α-chain of HbS has demonstrated that the region of α-chain, corresponding to the junction of the translation products of Exon-1 and Exon-2 of α-globin gene is a "permissible discontinuity region" of the polypeptide chain within its tertiary interactions [9]. *Staphylococcus aureus* V8-protease introduces the discontinuity at the peptide bond Glu(30)-Arg(31) of the α-chain [9]. Therefore, it is conceivable that this noncovalent fragment system of the α-chain as well as the semisynthetic noncovalent analogs of the chain could be converted to the respective covalent form by the V8-protease-mediated reformation of peptide bond 30-31 in these analogs. V8-protease represents a unique case compared to the other proteases in that it has a double pH optima for the hydrolytic reactions: one around pH 4.0, and the other around 7.0 [10]. It is not clear whether the studies with other proteases to induce the synthesis of peptide bonds could be readily translated to achieve the reformation of a glutamyl peptide bond employing V8-protease. In the present study, we have examined the potential of V8-protease to induce the formation of glutamyl peptide bonds in a mixture of V8-protease-derived fragments of α-chain. Conditions for the V8-protease induced reformation of a glutamyl peptide bond has been determined.

METHODS

HbA- and p-hydroxymercuibenzoate (HMB)-reacted α-chain of HbA were prepared as described previously [11]. Staphylococcus aureus V8-protease was obtained from either Miles Laboratories (Naperville, IL) or Worthington Biochemicals (Free-

hold, NJ). Sep-pak cartridges were obtained from Water Associates. Sephadex was obtained from Pharmacia. Centricon 10 microconcentrators were from Amicon.

Digestion of α-Chain by V8-Protease

HMB-α-chain in the carbonmonoxy form (0.04 mM in 10 mM KPO_4, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 6.0 or in NH_4Ac, pH 6.0) was incubated with V8-protease (enzyme to α-chain ratio 1:250) at 37°C. After the desired period of incubation, the digests were subjected to reverse phase high performance liquid chromatography (RPHPLC) either directly or after concentrating the digest with centricon 10 microconcentrators.

HPLC

The HPLC system used was assembled in the laboratory from commercially available components [12]. The samples were desalted by passing them through Sep-pak cartridges previously equilibrated with 0.1% TFA. Globin, heme, and the peptides bind to Sep-pak under these conditions. The column was washed with about 10 ml 0.1% TFA. The 0.1% TFA eluate contained only the buffer salts. The material bound (globin, heme, and peptides) to the Sep-pak cartridge was eluted with 2.0 ml of 70% acetonitrile containing 0.1% TFA and isolated by lyophilization. The dried material was dissolved in 400 μl of 0.5% TFA and loaded on a Whatman Partisil 10 ODS-3 column. This procedure employing Sep-pak gave consistently high yields (>90%) of globin, globin fragments, and heme, free of buffer salts. The procedures for the preparation of globin, amino acid and sequence analyses have been described earlier [13].

Ultrafiltration

These were carried either with a regular ultrafiltration cell or centricon 10 microconcentrators depending on the volume to be concentrated.

Enzymatic Condensation of Fragment α_{1-30} and α_{31-47}

The optimal conditions used for the condensation were 0.8 mM in α_{1-30} (carboxyl component) and 1.2 mM in α_{31-47} (amino component). The components were taken in 50 mM NH_4Ac, pH 6.0. V8-protease taken in 50 mM ammonium acetate buffer pH 6.0 was added to this to get an appropriate enzyme:substrate ratio (W/W 1:1,000, or 1:500 or 1:250). n-Propanol or glycerol was then added to get final required concentration of the organic cosolvent. After incubation at 4°C, 23°C, or 37°C for various periods of time, the reformation of peptide bond was determined by RPHPLC.

RESULTS

Proteolysis of α-Chain With V8-Protease

Our previous studies [9] have shown that digestion of the α-chain by V8-protease is limited to four sites, namely to the carboxyl side of Glu(23), Glu(27), Glu(30), and Asp(47). Much more interesting is the observation that fragments α_{1-30} and α_{31-141} exhibit strong noncovalent interactions between them, thus permitting the isolation of the noncovalent fragment system by gel filtration. Thus, the region of peptide bond of Glu(30) and Arg(31) is a "permissible discontinuity region" within

the tertiary interactions of the α-chain. Besides, our recent studies have shown this bond exhibits a very high selectivity for proteolysis by this enzyme. When the α-chain is digested at 37°C and pH 6.0 at a concentration of 0.6 mg/ml employing an enzyme-substrate ratio of 1:250 for 2 hr, this peptide bond is selectively hydrolyzed as seen by the RPHPLC of the digest (Fig. 2). Quantitation of the digestion indicated that more than 80% of the α-chain has been digested by the enzyme. When this digest is concentrated (about 25 fold) by centricon, more than 90% of the fragment α_{1-30} was retained in the centricon cup, further confirming the strong noncovalent interaction of this fragment with the complementary fragment α_{31-141}.

Though high selectivity is seen during the early phases of digestion to the peptide bond 30-31, when the digestion is continued for longer periods, for example, for 24 hr, three other peptide bonds, namely those of Glu-23, Glu-27, and Asp-47, are also hydrolyzed (Fig. 3). The chromatogram (Fig. 3) shows that peptides α_{1-23}, α_{1-27}, α_{1-30}, and α_{31-47} are present as the low molecular weight peptides. These peptides were isolated and were used as substrates in the evaluation of the synthetic potential of V8-protease.

Synthetic Potential of V8-Protease

Homandberg et al [14] have shown that addition of large concentrations of organic cosolvents in proteolysis mixtures significantly raises the value of the equilibrium constant for the synthesis of peptide bonds, without abolishing the catalytic activity of proteases such as trypsin and chymotrypsin. The organic cosolvent increases the apparent pK_1 of the substrate (ionization of the α-carboxyl group of the peptide, specifically that of the carboxyl component), leading to a decrease in the free energy change associated with hydrolysis and thereby favoring the synthesis. Unfortunately, addition of organic cosolvents generally decreases the solubility of the

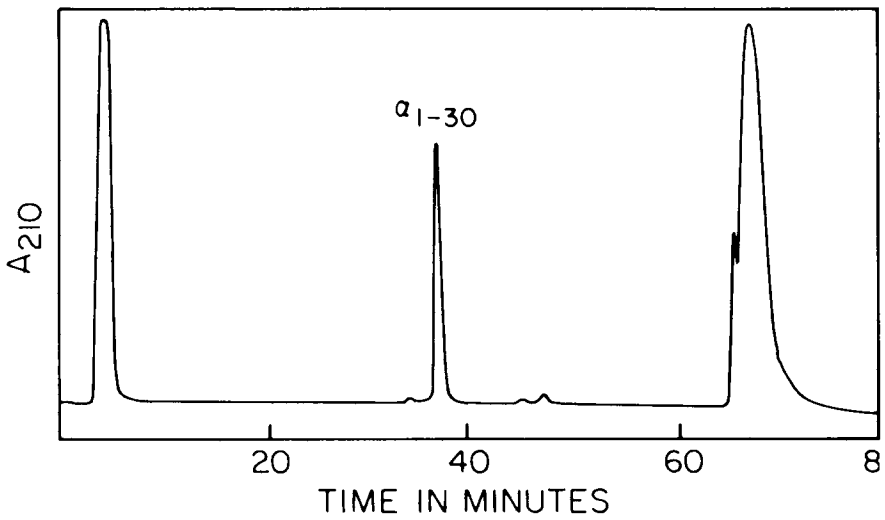

Fig. 2. HPLC analysis of a 2-hr V8-protease digest of α-chain of HbS. The digests were lyophized and then taken in 0.1 TFA and loaded onto a Whatman 10-ODS-3 column (4.6 × 250 mm). The peptides were eluted with a linear gradient of 5–70% acetonitrile both containing 0.1 TFA. Flow rate was 60 ml/hr.

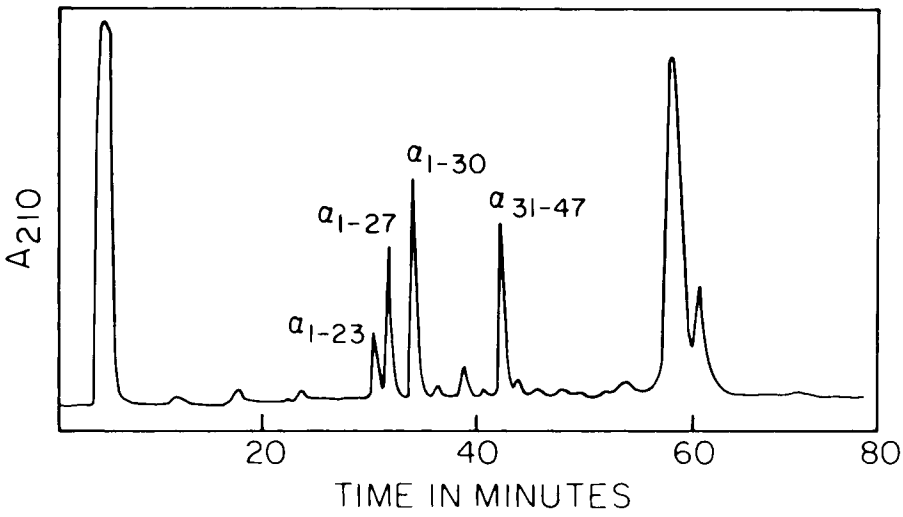

Fig. 3. HPLC of a 24-hr V8-protease digest of α-chain of HbS. The conditions of chromatography are the same as that described for Figure 2.

reactants (peptides). More importantly, a decrease in the solubility and catalytic efficiency of the enzyme required for the attainment of the equilibrium may also occur. Nonetheless, significant success has been achieved in the semisynthesis of RNase-A [8], nuclease-A [15], and cytochrome C [16] from their respective fragment complementing system employing 90% (V/V) glycerol as the organic cosolvent.

Though it has been now demonstrated that a number of proteases catalyze the reformation of peptide bonds in the presence of organic cosolvents such as glycerol, the synthetic potential of V8-protease has not been studied so far. We have now investigated the V8-protease-catalyzed synthesis of the peptide bond Glu(30)-Arg(31) in the presence of organic cosolvents employing a mixture of V8-protease-derived fragments of α-chain. The low solubility of many of the globin fragments and the fact that n-propanol is a favored solvent for the RPHPLC of many of these fragments has prompted us to investigate the use of n-propanol as the organic cosolvent for the V8-protease-catalyzed reformation of glutamyl peptide bonds.

The V8-protease-catalyzed reformation of the peptide bond was analyzed by the RPHPLC of the reaction mixtures. A mixture of α_{1-30} and α_{31-47} kept at pH 6.0 for 24 hr at 4°C in the presence of 25% n-propanol without the enzyme contained only the expected two fragments (Fig. 4A). However, when V8-protease (1:250: W/W) was included, a new component was generated (Fig. 4B). This component is conceivably α_{1-47}. However, α_{1-30} incubated alone with V8-protease under the same conditions showed the formation of only a small amount of α_{1-27} as the new component, and there is no evidence for the V8-protease-catalyzed dimerization of α_{1-30}. α_{31-47} was also stable to proteolysis and dimerization under these conditions. Thus, the new peak around 75 min in Figure 4B is not a product generated by the polymerization of α_{1-30} or α_{31-47}; both the fragments α_{1-30} and α_{31-47} as well as V8-protease need to be present in order to generate this new peptide. A small amount of this component was generated when the fragments are incubated with V8-protease in the absence of n-propanol as well.

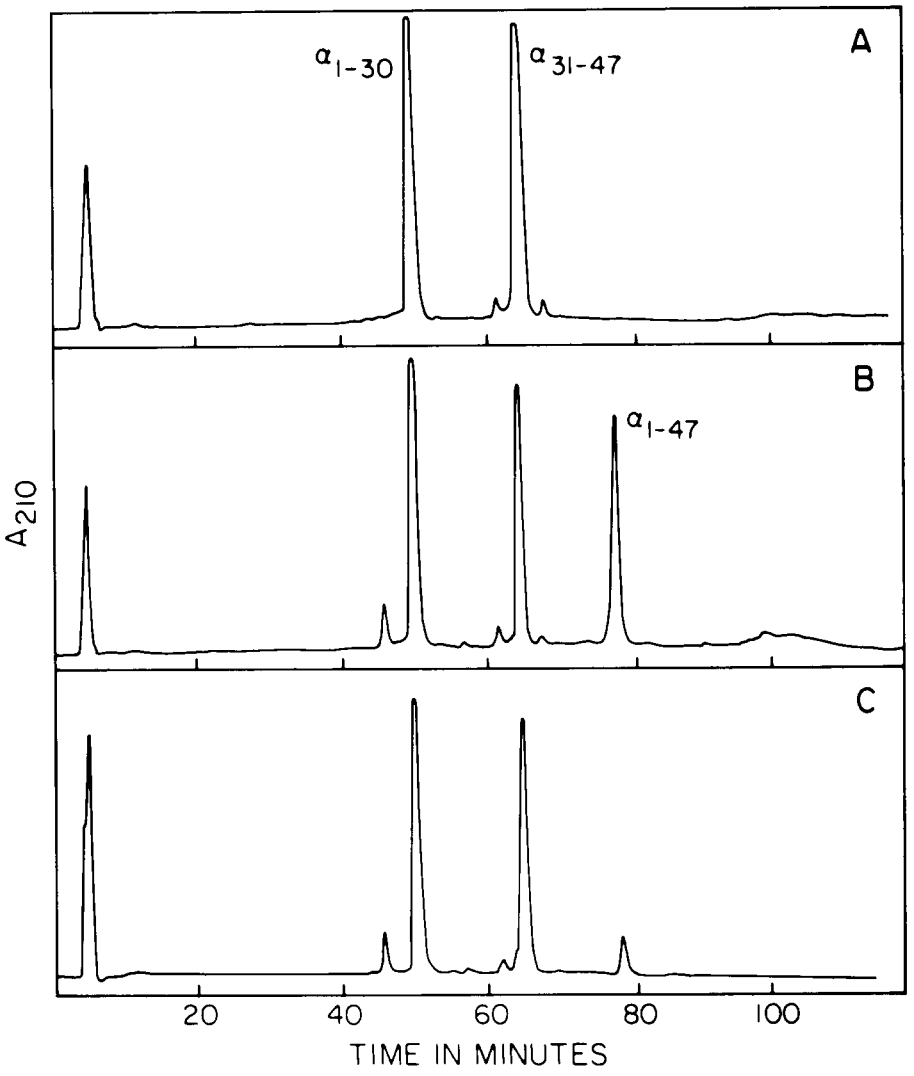

Fig. 4. V8-protease-catalyzed reformation of Glu(30)-Arg(31) peptide bond. RPHPLC analysis of the peptide mixtures on ODS-3 column. A) Mixture of α_{1-30} and α_{31-47} incubated in the presence of organic cosolvent without V8-protease for 24 hr at 4°C. B) Mixture of α_{1-30} and α_{31-47} incubated with V8-protease (enzyme:substrate::1:250) in the presence of 25% n-propanol for 24 hr at 4°C. C) Mixture of α_{1-30} and α_{31-47} incubated with V8-protease (enzyme:substrate::1:250) in the presence of 25% glycerol. The gradient used for elution of the peptides is the same as that used in Figures 2 and 3 except that the flow rate was 45 ml/hr instead of the 60 ml/hr used earlier.

Characterization of the Synthetic Product

The new component formed on incubation of α_{1-30} and α_{31-47} with V8-protease in the presence of 25% n-propanol was isolated, and its amino acid composition was determined (Table I). The amino acid composition corresponded well with that expected for α_{1-47}. V8-protease digestion (Fig. 5A) yielded α_{31-47} and predominantly α_{1-30}, and small amounts of α_{1-27}, and α_{1-23}, the secondary digestion product of

TABLE I. Amino Acid Composition of α_{1-30}, α_{31-47}, and Synthetic α_{1-47}

Amino acid	α_{1-30}		α_{31-47}		α_{1-47}	
	Found[a]	Expected	Found[b]	Expected	Found[c]	Expected
Asp	2.2	2	1.1	1	2.9	3
Thr	1.1	1	3.1	3	3.6	4
Ser	1.1	1	1.0	1	1.8	2
Glu	3.1	3	—	—	3.1	3
Pro	0.8	1	1.8	2	2.6	3
Gly	4.1	4	—	—	3.9	4
Ala	6.8	7	—	—	6.9	7
Val	3.1	3	—	—	3.0	3
Met	—	—	0.9	1	0.9	1
Ila	—	—	—	—	—	—
Leu	2.0	2	0.9	1	2.9	3
Tyr	1.1	1	1.1	1	1.9	2
Phe	—	—	4.1	4	4.0	4
His	0.9	1	0.9	1	0.9	2
Lys	3.0	3	1.0	1	4.0	4
Arg	—	—	0.9	1	0.9	1

[a] Calculated assuming a value of 3 for Lys.
[b] Calculated assuming a value of 1 for Lys.
[c] Calculated assuming a value of 4 for Lys.

α_{1-30}. Direct evidence for the formation of the peptide bond Glu(30)-Arg(31) comes from the tryptic peptide mapping of this synthetic product (Fig. 5B). Reformation of the peptide 30-31 should yield α-T_4 corresponding to the segment 17–31 of α-chain [12]. As seen in Figure 5B, the tryptic map of synthetic material contains this peptide. The identity of this peptide with α-T_4 is confirmed by amino acid analysis (Table II). The presence of α-T_4 (corresponding to segment 17–31 of α-chain) in this synthetic peptide clearly demonstrates that the V8-protease catalyzed the condensation of α_{1-30} and α_{31-47} by reforming the peptide bond Glu(30)-Arg(31).

Factors Influencing the Yield of the Synthetic α_{1-47}

The experimental protocol employed for the V8-protease-catalyzed reformation of the peptide bond Glu(30)-Arg(31) is 25% η-propanol and pH 6.0, 4°C, and 24 hr and an enzyme substrate ratio of 1:250. The yield of α_{1-47} during this period is about 50% based on the amount of the carboxyl component. A kinetic analysis of the formation of α_{1-47} indicated that the yield of the peptide increased rather linearly with time up to about 24 hr. By this time the equilibrium situation appears to have been reached and on further incubation up to 72 hr does not increase the overall yield of α_{1-47}. Therefore, a 24-hr reaction period was chosen as the optimal time. The low temperature used in the initial experiments appears to be optimal. For the synthesis, the yield of α_{1-47} at room temperature, as well as at 37°C, is much lower than that obtained at 4°C. An enzyme substrate ratio of 1:250 was also found to be optimal. When this ratio was lowered to 1:500 or 1:1,000, the yield in 24 hr decreased to 38% and 30%, respectively.

The influence of the concentration of n-propanol on the overall yield of α_{1-47} has also been investigated by carrying out the synthesis in 6%, 12%, 25%, 50%, and 75% n-propanol. At 6% and 12% n-propanol, a very negligible amount of α_{1-47} was synthesized and is close to the control value (in the absence of n-propanol).

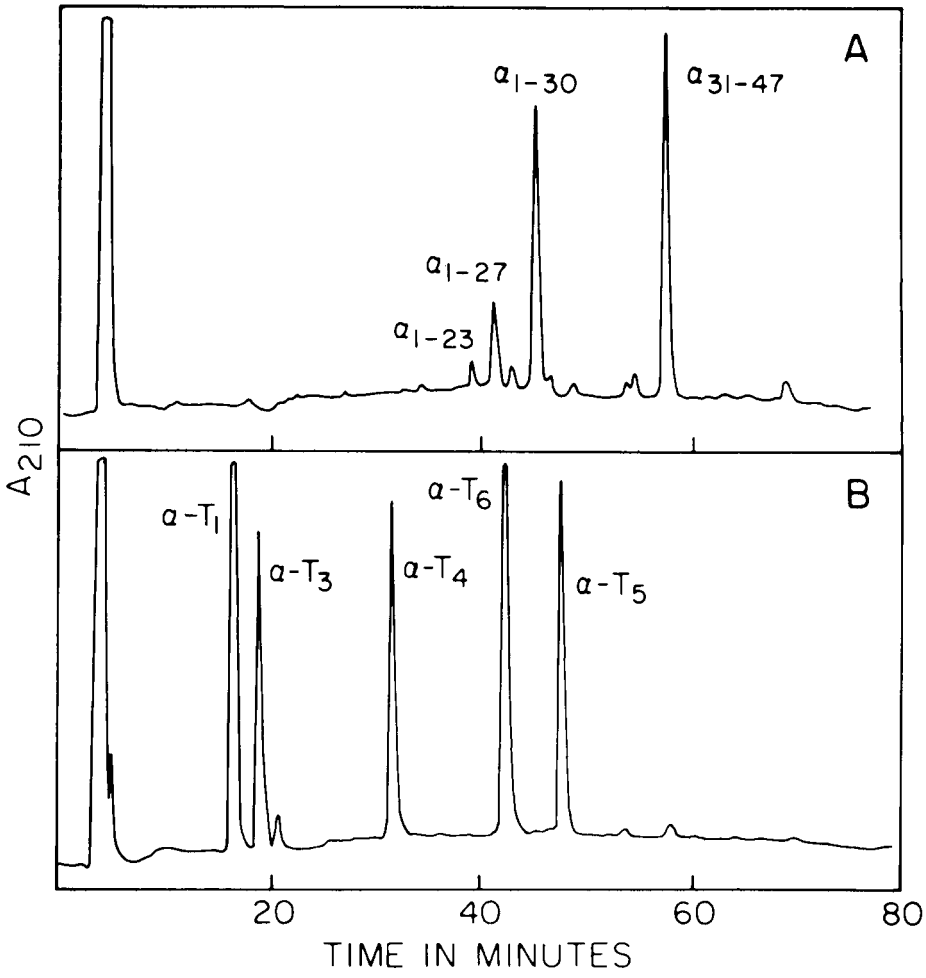

Fig. 5. V8-protease and tryptic peptide mapping of the synthetic peptide. A) V8-protease peptide mapping of the synthetic peptide. B) Tryptic peptide mapping of the synthetic peptide.

When the concentration of n-propanol increased to 50% and 75% n-propanol, the yield of α_{1-47} decreased considerably to about 15% and 10%, respectively. Thus, 25% n-propanol appears to be the best condition for the V8-protease catalyzed reformation of the glutamyl peptide bond. Assuming that the synthetic activity observed at 24 hr corresponds to the attainment of $\alpha_{1-47} \rightleftharpoons \alpha_{1-30} + \alpha_{31-47}$ equilibria, the observed values for synthesis could be converted by writing:

$$K_{Syn} = \frac{\%\ \text{maximum synthesis}}{100 - \text{maximum synthesis}}. \quad (1)$$

It has been calculated that the value of K_{syn} of α_{1-47} increases from 0.02 in water to 1 in 25% n-propanol.

TABLE II. Amino Acid Composition of αT_4 Derived from Synthetic α_{1-47}

Amino acid	Found[a]	Expected
Asp	—	—
Thr	—	—
Ser	—	—
Glu	3.2	3
Pro	—	—
Gly	3.1	3
Ala	4.2	4
Val	1.0	1
Met	—	—
Ile	—	—
Leu	1.1	1
Tyr	0.9	1
Phe	—	—
His	0.8	1
Lys	—	—
Arg	1.0	1

[a] Calculated assuming a value of 1 for Arg.

As pointed out earlier, glycerol has been the most successful organic cosolvent [8] for the protease catalyzed reformation of peptide bonds in a fragment-complementing system. In these studies, the yield of the synthetic product increased almost linearly as the concentration of the organic cosolvent (glycerol) increased up to the 90% glycerol studied. In the present study, on the contrary, the 25% n-propanol has been found to be the best concentration of organic cosolvent for the synthesis of Glu(30)-Arg(31) peptide bond. A further increase in the concentration of n-propanol resulted in the reduction of yield of α_{1-47}.

As discussed earlier, in the studies of Homandberg and Laskowski [8] 90% glycerol was found to be optimal. However, in the present study, n-propanol has been used as the organic cosolvent, in view of the low solubility of globin peptides. Nonetheless, in an attempt to compare the relative efficiency of glycerol and n-propanol to induce V8-protease to catalyze the formation of α_{1-47}, the synthesis of α_{1-47} has also been determined employing 25% glycerol as the organic cosolvent (Fig. C). Though synthesis of α_{1-47} does take place in 25% glycerol, the yield of α_{1-47} is only about 8%. The value of K_{syn} is increased only by about five-fold in 25% glycerol, whereas the increase is about 40-fold when 25% n-propanol is used as the organic cosolvent. At 40% glycerol, the yield of α_{1-47} was about 12–15%. At this concentration of glycerol, the peptides appeared to be at their lowest solubility. At higher concentrations, the peptides were nearly insoluble. Hence, no studies were carried out at higher concentrations of glycerol. Thus, of the two solvents, 25% n-propanol appears to be a better organic cosolvent for the V8-protease-catalyzed formation of peptide bond Glu(30)-Arg(31).

DISCUSSION

Two crucial steps in the enzyme catalyzed semisynthesis (Fig. 1) of covalent analogs of proteins are (a) the identification of the "permissible discontinuity region" of a protein within its tertiary interaction that could be introduced by a specific

protease, and (b) the demonstration of the synthetic potential of this protease in an appropriate organic cosolvent. It is very critical that the conditions selected for the protease-catalyzed reformation of peptide bonds are such that the integrity of the noncovalent fragment system of the protein is least perturbed.

Proteolysis of α-chain by V8-protease is limited to four peptide bonds, namely, to those of Glu(30), Asp(47), Glu(27), and Glu(23). The very high selectivity of the peptide bond Glu(30)-Arg(31) of the α-chain is surprising in view of the fact that this peptide bond is in the middle of B-helix of the chain. The ready susceptibility of this peptide bond in the isolated chain in contrast to its resistance to its hydrolysis in the tetramer suggests that in the isolated chain this region of the B-helix is in a more flexible conformation than that in the native tetrameric structure. The peptide bond Asp(47)-Leu(48), which is also accessible to V8-protease digestion though not as readily as Glu(30)-Arg(31), is in a nonhelical CD segment of the chain. The region of peptide bond Glu(30)-Arg(31) represents the "permissible discontinuity region" of α-chain.

The synthetic potential of many intracellular proteases has been pointed out by Frutton and his colleagues during the late 1930's and early 1940's [17]. However, it is the observation by Homandberg et al [14,8] that has rekindled the interest in this area for structure-function relationship studies of proteins, specifically for the enzyme-catalyzed semisynthesis of proteins. They have demonstrated that in the presence of organic cosolvents such as glycerol, ethylene glycol, 1:3-propane diol, 1:4-butanediol, 1:5-pentane diol, and triethylene glycol, the value for the equilibrium constant for peptide bond synthesis is significantly increased without abolishing the catalytic activity of the proteases [14]. Subsequently, Homandberg and Laskowski [8] have demonstrated the subtilisin-catalyzed resynthesis of peptide bond 20-21 in RNase-S to regenerate RNase-A employing 90% glycerol as the organic cosolvent. However, other organic cosolvents that were effective in catalyzing the peptide bond formation with simple peptides were ineffective with the protein systems. Use of 90% glycerol as the organic cosolvent was also effective in the trypsin-catalyzed semisynthesis of nuclease [15] and the clostripain-catalyzed semisynthesis of cytochrome C [16]. This approach for enzyme-catalyzed semisynthesis of proteins should be distinguished from the other enzyme-catalyzed syntheses of smaller peptides [17]. In this type of semisynthesis, the noncovalent interaction of the two fragments is an essential element for the success of the protease-catalyzed reformation of the selected peptide bond. The stereochemistry (and/or geometry) of the α-carboxyl and the α-amino groups that are to form the peptide bond is apparently maintained by the noncovalent interactions of the fragments. This aspect appears to play a dominant role for an efficient resynthesis of the peptide bond [1].

The results presented here clearly demonstrate that V8-protease-catalyzed reformation of peptide bond Glu(30)-Arg(31) when a mixture of α_{1-30} and α_{31-47} is incubated with the protease in the presence of organic cosolvents (n-propanol or glycerol). The synthetic potential could be demonstrated in both the solvents studied, namely glycerol and n-propanol. Better results were obtained with n-propanol as the cosolvent. n-Propanol is a good solvent for proteins and peptides and is one of the generally used solvents for the RPHPLC of proteins and peptides. Whether propanol will serve as a good organic cosolvent for the other protease catalyzed reformation of peptide bonds needs to be investigated. With glycerol as the cosolvent, the synthetic potential of subtilisin, trypsin, and clostripain increased rather linearly as the concentration of glycerol in the mixture increased from 20% to 90%. However,

with V8-protease, 25% n-propanol appears to be optimal for the resynthesis of the glutamyl peptide bonds. The factors that contribute to the decrease in the synthetic efficiency of V8-protease when the concentration of the n-propanol is increased are not clear; it could either be related to loss of activity of V8-protease at higher concentrations of n-propanol or to the possible changes in the secondary and/or tertiary interaction of the two peptides. Whether other enzymes would also behave the same way in this solvent system needs to be investigated further before n-propanol could be advocated as a general solvent system.

Enzyme-catalyzed fragment condensation has many advantages over chemical condensation. It is stereospecific, generally has a high yield (up to about 50%), does not require the blocking of side chain groups of peptides, and the reaction proceeds under gentle conditions [1]. However, one of the major limitations of this procedure is that it depends heavily on the noncovalent association of the fragments to form a complex. This complexation apparently orients the α-carboxyl and α-amino groups that are to be coupled so that they are close to one another as well as accessible to the protease. Homandberg et al [18] have demonstrated that the enzymic condensation of the nonassociated peptide fragments could be achieved with the aid of a molecular trap. Thus, clostripain-catalyzed stereospecific condensation of the two subfragments of bovine pancreatic RNase S-peptide, namely the fragments 1–10 and 11–15, could be enhanced by adding RNase-S-protein (residues 21–124) [18]. The facile stereospecific condensation of the fragments α_{1-30} and α_{31-47} seen in the present study suggests that under the conditions used for peptide bond synthesis, strong noncovalent interaction exists between the fragment α_{1-30} and α_{31-47} to provide the appropriate geometry for the carboxyl group of α_{1-30} and α-amino group of α_{31-47}.

V8-protease-derived fragments α_{1-23}, and α_{1-27}, both of which also have carboxy terminal glutamic acid just as the α_{1-30}, did not generate Glu-Arg bond when these two peptides are mixed with α_{31-47} and incubated with V8-protease in the presence of n-propanol. Thus, the conditions permissible for the reformation of the bond Glu(30)-Arg(31) are not effective for the formation of the peptide bond Glu(23)-Arg(31) or Glu(27)-Arg(31). Shortening of the carboxyl component (α_{1-30}) by either three or seven residues apparently perturbs the geometry of the carboxyl and amino groups needed for the facile reformation by peptide bonds. This appears to be reminiscent of the observation of Homandberg et al [8] that on shortening of the S-peptide by two residues at the carboxyl terminal, peptide bond formation between S-peptide and S-protein could not be demonstrated. The high selectivity in the condensation of α_{1-30} and α_{31-47} and the absence of dimerization of α_{1-30} could also be explained by the stereospecific orientation of the two fragments. This explanation further encourages the hopes of achieving the V8-protease-catalyzed restitching of the peptide bond at 30-31 in the semisynthetic noncovalent analogs of α-chain. The synthesis of α_{1-47} demonstrated in the present study also opens up an opportunity to study the noncovalent interactions of α_{1-47} with α_{48-141} in order to determine whether a discontinuity at the peptide bond 47-48 of α-chain is also permissible within the tertiary interactions of α-chain.

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