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Semisynthetic Hemoglobin A: Reconstitution of Functional Tetramer from Semisynthetic α-Globin†

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ABSTRACT: The optimal conditions for the semisynthesis of α-globin through *Staphylococcus aureus* V8 protease condensation of a synthetic fragment (α_{1-30}) with the complementary apo fragment (α_{31-141}) in the presence of structure-inducing organic cosolvents and the reconstitution of the functional tetramer from semisynthetic α-globin have been investigated. The protease-catalyzed ligation of the complementary apo fragments α_{1-30} and α_{31-141} proceeds with very high selectivity at pH 6.0 and 4 °C in the presence of 1-propanol as the organic cosolvent. A 30% 1-propanol solution was optimal for the semisynthetic reaction, and the synthetic reaction attained an equilibrium (approximately 50%) in 72 h. The synthetic reaction proceeds smoothly over a wide pH range (pH 5-8). Besides, the semisynthetic system is flexible, and it also proceeded well if trifluoroethanol or 2-propanol was used instead of 1-propanol. However, glycerol, a versatile organic cosolvent used in all other proteosynthetic reactions reported in the literature, was not very efficient as an organic cosolvent in the present synthetic reaction. The semisynthetic α-globin prepared with 1-propanol as the organic cosolvent has been reconstituted into HbA. The semisynthetic HbA was then purified by CM-cellulose chromatography. The semisynthetic HbA is indistinguishable from native HbA, in terms of its structural and functional properties. The semisynthetic approach provides the flexibility in protein engineering studies for the incorporation of spectroscopic labels (^{13}C- and/or ^{15}N-labeled amino acids), noncoded amino acids, or unnatural bond functionalities, which at present is not possible with genetic approaches.

Preparation of structural variants of a protein is a direct and powerful method that permits the delineation of the role(s) of specific amino acid residues in the structure/function of a protein. Recent advances in oligonucleotide-directed site-specific mutagenesis have made the generation of molecular variants much simpler (Gerlt, 1987; Ward & Fersht, 1988; Knowles, 1987) compared to the total chemical synthesis of proteins (Gutte & Merrifield, 1969; Clark-Lewis et al., 1986). Nonetheless, the semisynthesis of proteins still remains a potentially powerful alternative to site-directed mutagenesis in protein chemistry, especially since it provides a flexibility to introduce ^{13}C- or ^{15}N-labeled amino acids as well as noncoded amino acids at selected sites (Offord, 1985, 1987; Dimarchi et al., 1986). In these semisynthetic approaches, a synthetic polypeptide segment of a protein is ligated with the complementary fragment derived from the native protein either by enzymic or by nonenzymic methods (Chaiken, 1981).

The protease-catalyzed peptide bond formation (reverse proteolysis) has gained considerable interest in synthetic peptide chemistry in recent years (Bodansky, 1985; Kullman, 1985; Fruton, 1983). At first sight, the potential of proteases to hydrolyze preexisting susceptible peptide bonds in the fragments during condensation would seem to limit the general applicability of proteases in semisynthesis of covalent analogues of proteins (Kullman, 1985). However, the protease-catalyzed re-formation of the peptide bond at the discontinuity regions of "fragment complementing systems" is an exception in that respect. The specific noncovalent interactions of the complementary fragments of the complex maintaining a "nativelike" conformation generally limit the nonspecific digestion of the fragment complement systems by the protease (Chaiken, 1981; Kullman, 1985; Homandberg & Laskowski, 1979; Homandberg & Chaiken, 1980; Komariya et al., 1980).

The region of α-chain of hemoglobin A (HbA)[1] corresponding to the junction of the translation products of exon 1 and exon 2 of the α-globin gene has been recently identified as a "permissible discontinuity region" of the polypeptide chain

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[1] Abbreviations: Hb, hemoglobin; DPG, 2,3-diphosphoglycerate; HMB, *p*-(hydroxymercuri)benzoate.

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within its tertiary interactions (Seetharam et al., 1986). A discontinuity at the peptide bond Glu(30)–Arg(31) of the region could be readily introduced by *Staphylococcus aureus* V8 protease (Seetharam et al., 1986). These studies prompted us to investigate the feasibility of re-forming the peptide bond Glu(30)–Arg(31) in a mixture of the apo fragments α_{1-30} and α_{31-141} using V8 protease. The major emphasis in the present study was to condense the unprotected fragments of the apoprotein, i.e., in the absence of the prosthetic group (heme).

In a preliminary study, we (Acharya et al., 1985) have demonstrated the V8 protease catalyzed incorporation of synthetic ^{14}C α_{1-30} into the globin fraction of a V8 protease digest, suggesting the formation of full-length α-globin. We have now developed procedures for the preparation of homogeneous α_{31-141}, and several pertinent/newer aspects of semisynthetic reaction have been studied in greater detail to evaluate the adaptability of this semisynthetic procedure as a route for the preparation of semisynthetic Hb. Chemical evidence for the formation of the Glu(30)–Arg(31) peptide bond and the influence of organic solvents on this proteosynthetic reaction as well as the reconstitution of the functional tetramer from semisynthetic α-globin has been investigated. The results of these studies are presented here, and novel aspects of the present semisynthetic reaction have been compared with the other previously reported proteosynthetic systems that proceed only in 90% glycerol.

Materials and Methods

HbA and HMB α- and β-chains were prepared as described previously (Seetharama et al., 1986). *Staphylococcus aureus* V8 protease was from Miles Laboratory.

Preparation of α_{1-30} and α_{31-141}. HMB α-chain was digested with V8 protease (1:200 w/w) at a concentration of 1 mg/mL at pH 4.0 and 37 °C for 2 h. After this digestion period the reaction mixture was lyophilized. The lyophilized material was taken up in 0.1% TFA (approximately 0.2 mg/mL) and subjected to concentration by use of an Amicon microconcentrator (membrane: YM10). The filtrate contains most of the peptide α_{1-30}. The concentrate, containing mostly the α_{31-141} with bound heme, was diluted with 0.1% TFA to the original volume and subjected to the concentration step again. The second filtrate was pooled with the first filtrate and lyophilized to get pure α_{1-30}. The concentrate was lyophilized and then subjected to acid–acetone precipitation to get heme-free α_{31-141}. The precipitated α_{31-141} was dissolved in 0.1 M acetic acid, lyophilized, and further purified by RPHPLC. Tryptic peptide mapping and amino acid sequencing confirmed the identity of the purified samples as α_{1-30} and α_{31-141}. [^{3}H]-Leu$_2$-α_{1-30} was synthesized by the solid-phase method (Barany & Merrifield, 1981), essentially as described earlier (Acharya et al., 1985).

V8 Protease Catalyzed Condensation of α_{1-30} and α_{31-141}. The optimal concentrations of the apo fragments for the condensation reactions were 1.0 mM in [^{3}H]-α_{1-30} (carboxyl component) and 0.8 mM in α_{31-141} (amino component). The condensation reaction was carried out in 50 mM NH_4OAc, pH 6.0, containing 30% (v/v) 1-propanol. An enzyme/protein ratio of 1:200 was used for the ligation studies. The synthetic reaction was generally allowed to proceed for 48 h at 4 °C and then lyophilized. The lyophilized material was taken up in 0.1% TFA and analyzed by RPHPLC. Incorporation of the ^{3}H label of α_{1-30} into the globin fraction reflected the extent of semisynthesis.

CD Spectra. The CD spectra were measured in an Aviv 60DS spectrophotometer fitted with a thermostated cell holder interfaced with computer model 1600-1 from Columbia Data Products. Ellipticity values at 222 nm were converted to mean residue ellipticity values by using the equation $\theta_{M_r} = \theta_{222}\text{obs}(M_r/100LC)$, where θ is in degrees, L = cell path length (dm), C = concentration (g/mL), and M_r = mean residue molecular weight.

Reconstitution of Tetramer from Semisynthetic α-Globin. The reconstitution was carried out by the "alloplex" pathway essentially as described by Yip et al. (1972). In a typical experiment, approximately 60 mg of lyophilized globin (semisynthetic material, an equimolar mixture of α_{1-30} and α_{31-141}, or PMB-α-globin) was taken up in 5 mL of 8 M urea in 50 mM bis-Tris buffer, pH 7.0, containing 1 mM EDTA and was then kept in an ice bath for 10 min. The protein solution was diluted with 200 mL of the buffer (without urea) containing 3 mM DTT. After 30 min, HMB-β-subunits (8 mL of an 8 mg/mL solution) were added to this solution with constant stirring to form the half-filled tetramers (alloplex intermediates). After 30 min, a 1.2-fold molar excess of hemin dicyanide over α-globin was added. The solution was gently stirred on ice for 1 h and then dialyzed against several changes of bis-Tris buffer, pH 7.0, for 48 h. Insoluble protein was removed by centrifugation, and the supernatant fraction was concentrated to 2 mL by ultrafiltration. Reduction and conversion of the reconstituted material to the carbonmonoxy form was carried out by a brief treatment with dithionite in the presence of carbon monoxide on a Sephadex G-25 column (2.5 × 40 cm) preequilibrated with 10 mM potassium phosphate buffer, pH 6.0, saturated with carbon monoxide. The pooled protein fractions were then purified by CM-52 chromatography. The overall yield of reconstituted HbA from α-globin (untreated) was about 50%.

Results

Semisynthesis of α-Globin. We have previously established the synthetic potential of V8 protease by demonstrating the re-formation of the Glu(30)–Arg(31) peptide bond in an equimolar mixture of α_{1-30} and α_{31-47} (Seetharam & Acharya, 1986). In a preliminary study using a crude preparation of α_{31-141}, V8 protease catalyzed incorporation of synthetic ^{14}C α_{1-30} into the globin fraction was also demonstrated, suggesting the generation of the full-length polypeptide chain (Acharya et al., 1985). The α_{31-141} sample used in that study was generated by the V8 protease digestion of α-chain at pH 6.0 and 37 °C, followed by RPHPLC of the digest. Though the specificity of the proteolysis at pH 6.0 is very high, it is neither absolute nor complete (Seetharam et al., 1986). Thus, the digest will contain α_{48-141} and the full-length polypeptide chain (α_{1-141}) besides the predominant α_{31-141}. The full-length polypeptide chain (α_{1-141}) and the apo fragments α_{31-141} and α_{48-141} do not separate during RPHPLC under the experimental conditions used and coelute as a single peak. Given the chemical heterogenicity of the amino component used, the incorporation of the label of synthetic α_{1-30} cannot therefore be considered as the absolute chemical proof for the formation of the full-length polypeptide chain.

As detailed under Materials and Methods, the proteolysis of α-chain at pH 4.0 and 37 °C by V8 protease is both quantitative and absolute and has thus permitted the preparation of homogeneous α_{31-141}. incubation of an equimolar mixture of synthetic [^{3}H]-Leu$_2$-α_{1-30} and pure α_{31-141} with V8 protease at pH 6.0 in the presence of 30% 1-propanol for 48 h has now been carried out, and this resulted in a significant incorporation of the label into the material eluting at the globin position (data not shown).

In order to establish the V8 protease catalyzed re-formation of the peptide bond between Glu(30) of synthetic α_{1-30} and

Table I: Amino Acid Composition of α-T_4 Isolated from the Tryptic Digest of Semisynthetic α-Globin

amino acid	found[a]	expected for α-T_4	expected for α-$T_{4'}$
Asp			
Thr			
Ser			
Glu	2.8	3	3
Pro			
Gly	3.2	3	3
Ala	3.9	4	4
Val	0.9	1	1
Met			
Ile			
Leu	1.0	1	1
Tyr	0.9	1	1
Phe			
His	0.8	1	1
Lys			
Arg	0.8	1	

[a] Calculated with Leu as one residue.

Arg(31) of α_{31-141}, the semisynthetic α-globin was subjected to tryptic peptide mapping. A peptide eluting at the position of α-T_4, was present in the semisynthetic sample, and this was absent in the tryptic map of α_{31-141}. The material eluting at the position of α-T_4 has been isolated, and its amino acid composition has been determined. The composition (Table I) corresponds well with that of α-T_4 isolated by tryptic digestion of native α_{1-141}. The expected composition of α-$T_{4'}$ (des-Arg-α-T_4), which is formed on tryptic digestion of α_{1-30}, is also given in Table I for comparison. α-T_4 could be generated on tryptic digestion of the semisynthetic material only if the peptide bond Glu(30)–Arg(31) is re-formed.

Equilibration of the Semisynthetic Reaction. A kinetic analysis of the yield of the semisynthetic α-globin has been made to determine the optimal conditions for the synthesis of α-globin. The yield of semisynthetic α-globin increased with time up to about 72 h (approximately 55%), and further incubation up to 168 h (7 days) did not result in any significant increase (58%). Therefore, after about 48–72 h of incubation, an equilibrium in the protease-catalyzed synthetic and the hydrolytic reaction is attained.

Influence of pH on the Semisynthesis of α-Globin. The protease-catalyzed condensation reactions are generally optimal around pH 6.0 and are lowest around the neutral pH region where the hydrolytic activity of most of the neutral proteases is the highest (Homandberg & Laskowski, 1979). The pH optimum for the condensation reaction was investigated as a function of pH to establish the optimal condition for this proteosynthetic reaction (Figure 1). Surprisingly, the V8 protease catalyzed condensation reaction exhibited broad pH optima (from 5 to 8). The maximum activity was around pH 5.0–6.0. Nonetheless, a significantly high level of synthetic activity (nearly 80% of that of pH 6.0) was present even at pH 7 and 8. In all the glycerol systems the synthetic activity was optimum at pH 6.0. Very low (less than 10% of that at pH 6.0) synthetic activity was observed around pH 8.0 in all these cases, whereas in the present system the proteosynthetic activity at pH 8.0 is nearly 85% of that at pH 5 or 6.

Semisynthesis of α-Globin as a Function of 1-Propanol Concentration. Propanol has a very high propensity to induce α-helical conformation into α-globin, and the helical content is a function of propanol concentration (Iyer & Acharya, 1987). This suggested that the propensity of the alcohol to induce α-helical conformation into α-globin may be a factor responsible for making this solvent an excellent organic cosolvent for the ligation of α_{1-30} and α_{31-141}. Accordingly, the

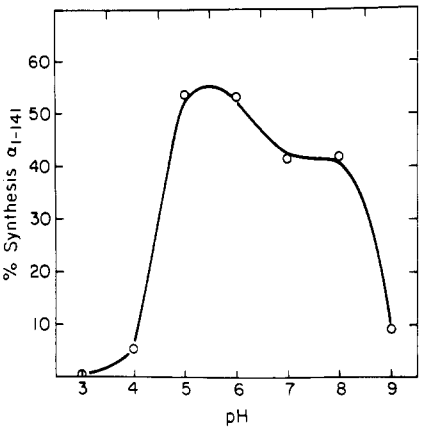

FIGURE 1: Influence of pH on the semisynthesis of α-globin. An equimolar mixture of [^{3}H]-Leu_2-α_{1-30} and α_{31-141} (at a concentration of 10 mg/mL) was incubated at 4 °C at different pHs for 48 h in the presene of 30% 1-propanol and V8 protease (enzyme to substrate ratio 1:200). Incorporation of [^{3}H]-Leu_2-α_{1-30} (percent semisynthesis) into the α-globin was determined by RPHPLC of the lyophilized samples.

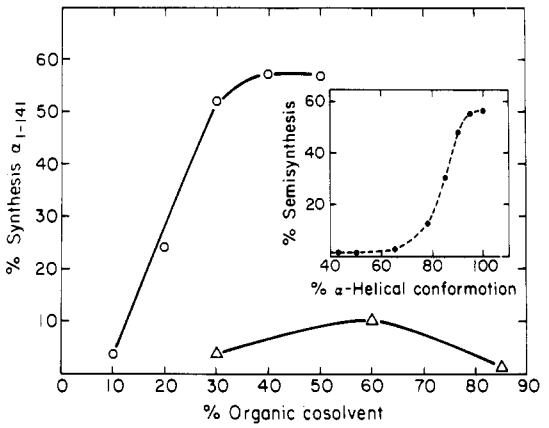

FIGURE 2: Influence of concentration of organic cosolvents on the semisynthesis of α-globin. An equimolar mixture of the complementary fragments was incubated at pH 6.0 and 40 °C for 48 h in the presence of varying concentrations of organic solvents (1-propanol or glycerol). Incorporation of [^{3}H]-Leu_2-α_{1-30} forming semisynthetic α-globin (percent semisynthesis) was determined by RPHPLC of the lyophilized samples as described in Figure 3. (○) 1-propanol; (△) glycerol. The inset shows the semisynthesis of α_{1-141} as a function of the 1-propanol-induced α-helical conformation of α-globin. The α-helical conformation of α-globin at a given concentration of 1-propanol is presented as the percent of the α-helical content of α-globin in 50% 1-propanol. The percent semisynthesis is the percent incorporation of [^{3}H]-Leu_2-α_{1-30} into α-globin.

semisynthetic activity of V8 protease has been studied as a function of 1-propanol concentration at pH 6.0 (Figure 2). The yield of semisynthetic α-globin is very low at concentrations of 1-propanol up to 15% and reaches an optimal value at 25–30% 1-propanol. This proteosynthetic activity remains nearly constant up to 50% 1-propanol. In contrast, very little synthesis occurred in the presence of 25% glycerol. However, at about 75% glycerol, 10–15% semisynthesis did occur, and the synthetic yield decreased as the glycerol concentration was increased to 90%.

The semisynthetic yield of α-globin has been correlated with α-helical content of α-globin at a given concentration of 1-propanol at 4 °C (Figure 2, inset). Semisynthesis occurred only when the α-helical content was about 75% (approximately 75% of the maximum that can be seen in 50% propanol) and above. Besides, the increase in synthesis appeared to show a cooperativity, as did the α-helical content of the protein, suggesting a close correlation between the overall synthesis

Table II: Structural and Functional Characteristics of Semisynthetic Hemoglobin A

	native HbA	HbA reconstituted from α-globin	HbA reconstituted from semisynthetic α-globin
Soret band max (nm)	419	419	419
mean residue ellipticity at 222 nm (mdeg)	-2.57×10^4	-2.5×10^4	-2.52×10^4
O_2 affinity[a] (P_{50}) in mmHg	8.5	8.4	8.4
Hill coeff	2.7 ± 0.05	2.65 ± 0.05	2.65 ± 0.05
O_2 affinity in the presence of 1 mM DPG[b] (P_{50}) in mmHg	17.0	17.0	17.0

[a] Oxygen affinity was measured with a Hem-O-Scan at pH 7.4 and 37 °C. The concentration of HbA was 0.5 mM. The buffer used in all these measurements was 50 mM bis-Tris, pH 7.4. [b] DPG (2,3-diphosphoglycerate) used represents a 2-fold molar excess over HbA.

and propanol-induced α-helical conformation.

Semisynthesis of α-Globin in the Presence of Other α-Helix-Inducing Organic Cosolvents. The V8 protease catalyzed semisynthesis of α_{1-141} in 30% trifluoroethanol has also been investigated. Trifluoroethanol is another organic solvent known to induce α-helical conformation into polypeptides and proteins (Murphy et al., 1986). Interestingly, the semisynthesis proceeded smoothly in the presence of 30% trifluoroethanol as well. The overall yield of the condensation reaction was 44% (48 h of reaction) compared to 55% in 30% 1-propanol. The ligation reaction was also carried out in the presence of 30% 2-propanol. Again, the semisynthesis of α_{1-141} proceeded smoothly, and the yield was comparable to that in 1-propanol (approximately 40%). Thus, several α-helix-inducing solvents appear to function as good cosolvents in the present semisynthetic reaction.

Reconstitution of HbA from Semisynthetic α-Globin. Semisynthetic α-globin was hybridized with the complementary subunit (β-chain) to prepare the alloplex intermediate and then reconstituted with hemin to regenerate HbA in order to determine whether semisynthetic α-globin could be reconstituted to the tetrameric structure. The reconstituted material was then chromatographed on CM-cellulose. The results of reconstitution using α-globin and α_{31-141} are shown for comparison (Figure 3). Upon reconstitution of α-globin with β-chain, and hemin, the generation of HbA occurred with an overall yield of 50% (Figure 3A). The HbA fraction was isolated and was designated as reconstituted HbA. The yield of HbA is comparable to that reported by Yip et al. (1972). On the other hand, when an equimolar mixture of α_{1-30} and α_{31-141} was used in the reconstitution experiments instead of α-globin, no HbA was generated (Figure 3B). This is consistent with the observation that the V8 protease digestion of α-chain at Glu(30) at pH 4.0 and 37 °C is quantitative. In contrast, when the semisynthetic material (protein recovered from RPHPLC) was used in the reconstitution system, a significant amount of material eluting at the position of HbA was generated (Figure 3C). This further establishes the formation of the full-length chain (α_{1-141}) through the protease-catalyzed ligation of the apo fragments α_{1-30} and α_{31-141} and the propensity of the semisynthetic α_{1-141} to reconstitute the tetrameric structure. The material eluting at the position of HbA generated from this reconstitution experiment using semisynthetic globin was also isolated and was designated as semisynthetic HbA.

The structural and functional properties of the semisynthetic HbA have been determined and compared with that of native HbA to establish the structural identity of semisynthetic HbA with native HbA (Table II). The semmisynthetic HbA is virtually indistinguishable from the native as well as reconstituted HbA in terms of its structural and functional properties. The visible absorption spectrum of semisynthetic HbA, as well as the overall α-helical conformation of the protein, is nearly the same as that of HbA (native). The semisynthetic

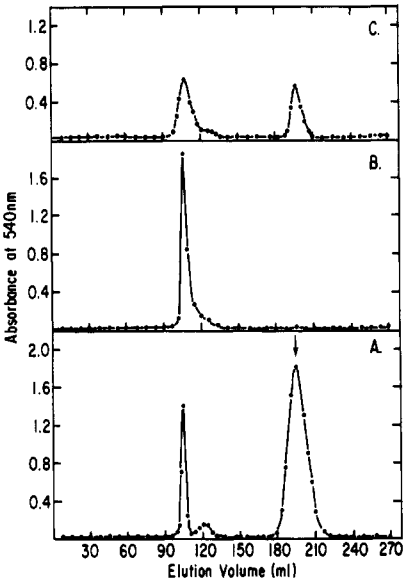

FIGURE 3: Reconstitution of semisynthetic α-globin. α-Globin (60 mg) or an equimolar mixture of α_{1-30} and α_{31-141} (60 mg) or semisynthetic α-globin (60 mg) was reconstituted as described under Materials and Methods and subjected to chromatography on a 2.2 × 30 cm column of CM-cellulose, equilibrated with 10 mM phosphate buffer, pH 6.0. A linear gradient of 10 mM phosphate buffer, pH 6.0, to 15 mM phosphate, pH 8.2, was used to elute the protein. Panel A shows the chromatogram of the sample generated on reconstituted of native α-globin, while panel C represents the pattern from the sample generated on reconstitution of semisynthetic α-globin. A sample of an equimolar mixture of α_{1-30} and α_{31-141} was also reconstituted, processed in the the same fashion, and chromatographed (panel B) to determine whether any material chromatographing in the position of HbA is formed from a mixture of unligated α_{31-141} and α_{1-30}. Note the absence of HbA in this sample. The position of native HbA is shown by (↓) in panel A. The material eluting around 105 mL represents the excess (unhybridized) β-chain present in the reconstituted sample (identified by RPHPLC; results not shown).

material exhibited O_2 affinity and Hill coefficient values virtually identical with those of native HbA. Besides, the O_2 affinity was modulated by DPG to the same degree as that of the native protein. The physicochemical properties of semisynthetic HbA establish that the native tertiary and quaternary interactions of HbA are fully restored when semisynthetic α-globin is used in the preparation of tetrameric protein.

Discussion

Semisynthesis of α-globin discussed here is novel and distinct from the previously reported protease-catalyzed protein semisynthetic reactions in that it uses structure-inducing organic cosolvents to facilitate the synthetic reaction of unprotected fragments. It should be noticed here that heme, which is crucial for maintaining the proper folding of the polypeptide chain of globins, is absent in the present protease-catalyzed condensation reaction. Glycerol, an organic cosolvent that was

used to favor the proteosynthetic activity of proteases in all the protein semisynthetic reactions previously reported, was not efficient in the present system. In the presence of 30% 1-propanol, however, the condensation of unprotected apo fragments proceeded smoothly.

At equilibrium, the yield of semisynthetic α-globin is nearly 55%. This equilibrium is attained at a relatively faster rate (approximately 48 h) compared to the slower rate seen with other protease-catalyzed proteosynthetic reactions that proceeded in 90% glycerol (Homamdberg & Laskowski, 1979; Komariya et al., 1980; Jullierat & Homandberg, 1981). The rate at which the equilibrium is attained for the semisynthesis of α-globin is, however, slower that that for the V8 protease catalyzed condensation of α_{1-30} and α_{31-47} (Seetharam & Acharya, 1986). Nonetheless, in both systems at equilibrium nearly 50% of the incubated sample is present as a contiguous segment. On the other hand, in the protease-catalyzed proteosynthetic reactions that proceed in glycerol, only with the RNase S system the yield of the synthetic product at equilibrium was about 50% (Homandberg & Laskowski, 1979) and comparable to that seen with the present system. In the case of nuclease, the synthetic yield was only 20% (Komariya et al., 1980). In the cytochrome *c* system an intermediate yield of 30% was obtained (Jullierat & Homandberg, 1981). The various molecular aspects that may contribute to the yield of the semisynthetic material at equilibrium in these systems are not readily apparent. It is conceivable that it represents the differential stabilization of the semisynthetic material once the continuity in the polypeptide chain is established. Given the fact that the overall conformations of RNase A and RNase S are nearly the same (Richards & Wykoff, 1971), the additional stabilization of the molecule in RNase S gained once the continuity is established is expected to be very minimal. Hence, as suggested by Homandberg and Laskowski (1979), the protonation of the α-carboxyl group (S-peptide) in the presence of organic cosolvent further facilitated by the lowering of the pH should be a major determinant of the synthetic reaction.

The presence of a signicant amount of synthetic activity around pH 8.0 (as compared to that at pH 6.0) in the present globin semisynthetic system suggests that the solvent-induced stabilization of the semisynthetic material should be a major determinant in the present proteosynthetic reaction. The fact that other α-helix-inducing solvents also promote the V8 protease catalyzed α-globin semisynthesis supports such a hypothesis. Further studies of this system with other protein semisynthetic reactions should unravel the flexibility and the variability that is available in such systems that one could use to manipulate the protease-catalyzed proteosynthetic reactions.

The semisynthetic α-globin could be readily reconstituted to the tetrameric $\alpha_2\beta_2$ structure through the alloplex intermediate pathway. The semisynthetic HbA is virtually indistinguishable from native HbA, in terms of its chromatographic behavior, Soret absorption, circular dichroic spectra, O_2 affinity, and the modulation of the O_2 affinity by DPG. Thus, this semisynthetic procedure is readily amenable to introduce isotopically enriched amino acids into selected sites of Hb. The general utility of site-specific isotopic enrichment of amino acids achieved through the semisynthetic approach in delineating the structure/function relationships of large allosteric protein systems has been recently demonstrated in the elegant studies of Gurd and his colleagues (Dimarchi et al., 1986; Hefta et al., 1988). Nagai et al. (1985, 1988) have developed the genetic approach for the preparation of structural variants of β- and α-globin. The present study is expected to complement the genetic approaches as it permits the site-specific incorporation of ^{13}C- or ^{15}N-labeled amino acids. It is conceivable that new structural variants of HbA and/or HbS could be prepared by the combination of these two approaches, which is difficult, if not impossible, to prepare by either of these approaches alone. Larger complementary fragments could be prepared by the genetic approach and the shorter ones by the chemical method, and the two could then be fused together by the enzymic approach. Thus, the advantages of protein engineering by chemical methods (Offord, 1987) and by genetic approaches could be appropriately coupled to generate novel anlogues of choice.

The enzymic approach developed here for the design and construction of the structural variants of α-globin by ligating the appropriate complementary segments of the apoprotein is novel, in that its success appears to be due to the dual role played by the organic cosolvent used to favor proteosynthesis. Thus, the present study clearly demonstrates that once the permissible discontinuity region(s) of a protein is (are) de lineated, one could readily translate this information to achieve the recombination of the polypeptide segments by the judicious choice of structure-inducing organic cosolvents. With homologous proteins this approach should be adaptable to construct hybrid mutant proteins. For example, double mutants of the α-chain of hemoglobin S could be prepared by using appropiate V8 protease complementary fragments from mutant α-globins. Naturally occurring α-chain mutants (with single mutation) have been employed by Benesch et al. (1979) to map the various intermolecular contact regions of deoxy-HbS. Construction of new α-globin variants containing two or more mutations (using the mutant V8 fragments of the mutant α-chains) should permit the study of additivity/synergy of the influence of the α-chain intermolecular contact regions of deoxyhemoglobin S.

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Resonance Raman Studies of *Escherichia coli* Sulfite Reductase Hemoprotein. 1. Siroheme Vibrational Modes†

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ABSTRACT: Resonance Raman (RR) spectra are reported for the hemoprotein subunit (SiR-HP) of *Escherichia coli* NADPH–sulfite reductase (EC 1.8.1.2) in various ligation and redox states. Comparison of the RR spectra of extracted siroheme and the μ-oxo Fe^{III} dimer of octaethylisobacteriochlorin with those of μ-oxo Fe^{III} octaethylchlorin dimer and μ-oxo Fe^{III} octaethylporphyrin dimer demonstrates that many siroheme bands can be correlated with established porphyrin skeletal modes. Depolarization measurements are a powerful tool in this correlation, since the 45° rotation of the C_2 symmetry axis of the isobacteriochlorin ring relative to the chlorin system results in reversal of the polarization properties (polarized vs anomalously polarized) of bands correlating with B_{1g} and B_{2g} modes of porphyrin. Various SiR-HP adducts (CO, NO, CN^-, SO_3^{2-}) show upshifted high-frequency bands, characteristic of the low-spin state and consistent with the expected core size sensitivity of the skeletal modes. Fully reduced unliganded SiR-HP (both siroheme and Fe_4S_4 cluster reduced) in liquid solution displays RR features comparable to those of high-spin ferrous porphyrins; on freezing, the RR spectrum changes, reflecting an apparent mixture of siroheme spin states. At intermediate reduction levels in solution a RR species is observed whose high-frequency bands are upshifted relative to oxidized and fully reduced SiR-HP. This spectrum, thought to arise from the "one-electron" state of SiR-HP (siroheme reduced, cluster oxidized), may be due to $S = 1$ Fe^{II} siroheme.

Escherichia coli NADPH–sulfite reductase (EC 1.8.1.2) is a multimeric hemoflavoprotein that catalyzes the six-electron reduction of sulfite to sulfide (Siegel et al., 1973, 1982; Janick et al., 1983). The hemoprotein subunit of the enzyme (SiR-HP), which contains the site of sulfite binding, possesses a novel catalytic center composed of siroheme (Murphy et al., 1973), an iron isobacteriochlorin (see Figure 1), closely interacting with a Fe_4S_4 cluster (Janick & Siegel, 1983). Interaction of the two centers may be of functional significance in facilitating the rapid transfer of multiple reducing equivalents to the bound substrate. The existence of a chemical linkage between the siroheme and cluster is strikingly manifested in the form of magnetic exchange coupling between the two prosthetic groups, which on the basis of Mössbauer (Christner et al., 1981,) and electron paramagnetic resonance spectroscopy (EPR) (Janick & Siegel, 1982) has been shown to persist in various redox states of the enzyme, both in the presence and in the absence of exogenous ligands. The detailed structural basis for the interaction, however, remains to be determined. A proposed model (Janick & Siegel, 1982) (see Figure 2) envisions a bridged structure in which one of the

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