Permissible Discontinuity Region of the α-Chain of Hemoglobin: Noncovalent Interaction of Heme and the Complementary Fragments α_{1-30} and α_{31-141}†

Ramnath Seetharam, Andy Dean, K. Subramonia Iyer, and A. Seetharama Acharya*

The Rockefeller University, New York, New York 10021

Received December 5, 1985; Revised Manuscript Received March 26, 1986

ABSTRACT: Generation of a fragment-complementing system of the α-chain on limited proteolysis with *Staphylococcus aureus* V8 protease has been investigated. Digestion of the α-chain (0.4 mM) of hemoglobin with V8 protease in phosphate buffer at pH 6.0 and 37 °C is limited to the peptide bonds of Glu-23, Glu-27, Glu-30, and Asp-47. Gel filtration of a V8 protease digest of the α-chain on a Sephadex G-50 column did not release any heme to the low molecular weight region, though some peptides were released from the protein. The filtration studies revealed the presence of two heme-containing components in the digest, the major one eluting at the α-chain position and the minor one eluting slightly ahead of the α-chain position. Reversed-phase high-performance liquid chromatography and amino-terminal sequence analysis demonstrated that the component eluting at the α-chain position contains species generated by the noncovalent interactions of heme and the complementary fragments α_{1-30} and α_{31-141}. In dilute solutions (0.04 mM) the V8 protease digestion occurred exclusively on the carboxyl side of Glu-30(α). This high selectivity was also observed at pH 4.0 and pH 7.8. The visible spectra and the ultraviolet circular dichroic spectra of the digest reflect the nativelike structure of the noncovalent fragment system. The dissociation constant of α_{1-30} appears to be in the range of 10^{-8} M. In tetrameric hemoglobin A the peptide bond of Glu-30–Arg-31 of the α-chain is not accessible to V8 protease digestion. The quaternary interactions of the α-chain and β-chain lead to the stabilization of this region, which represents a part of the $\alpha_1\beta_1$ contact region of the tetramer.

The amino acid sequence of a protein generally contains all the necessary structural information to determine its unique, biologically active three-dimensional structure (Anfinsen, 1973). The integrity of the structural information encoded in the amino acid sequence of the polypeptide chain that is crucial for the folding process, as well as for maintaining a stable folded conformation, has been studied after limited cleavage of the peptide bonds of the polypeptide chain (i.e., introduction of a discontinuity in the translated genetic formation). As a result of many such studies, it is now recognized that limited proteolysis of certain globular proteins generates polypeptide fragments, which interact noncovalently to maintain nativelike structures (Anfinsen & Scheraga, 1975). These are generally referred to as fragment-complementing systems. The first, and one of the best studied examples of such a fragment-complementing system, is RNase S (Richards & Vithayathil, 1959). Since the demonstration of the formation of RNase S, the generation of fragment-complementing systems of many other proteins has been reported. These include staphylococcal nuclease A (Taniuchi et al., 1967), cytochrome *c* (Hantagan & Taniuchi, 1977), prolactin (Birkand & Li, 1978), somatotropin (Li et al., 1978), and bacteriorhodopsin (Liao et al., 1983, 1984). The most recent addition to this list is thermolysin (Vita et al., 1985). The studies with all of these proteins have lead to the recognition of the "principle of fragment complementation". Although the polypeptide segments of a protein have a limited amount of ordered structure by themselves, a mixture of such complementary fragments is able to integrate the pieces of "structural memory" (information) encoded within their respective amino acid sequences to generate a nativelike structure. However, no generalization can be made at this stage about the region(s) of a given protein wherein a discontinuity is permissible. Nonetheless, it is quite clear that there are only a very limited number of such permissible regions for a given polypeptide chain, and generally the limited proteolysis of the native protein has been the method of choice for identifying these permissible discontinuity regions of a protein.

We have now undertaken the study of identifying permissible discontinuity region(s) of α-chains of hemoglobin within its tertiary interactions. Limited proteolysis of the chain has been the method of choice. Since the α-chain contains two internal arginine residues and four glutamic acid residues (Fermi & Perutz, 1981), clostripain and *Staphylococcus aureus* V8 protease (Drapeu & Houmard, 1972) appeared to be the enzymes of choice for the initial investigation. In view of the observation by Beychok and his associates (1981) that the native α- and β-chains of HbA[1] are not easily digested by the arginine-specific clostripain, we focused our attempts on limited proteolysis of the α-chain at glutamic acid residues, using *Staphylococcus aureus* V8 protease. This enzyme cleaves specifically on the carboxyl side of acidic amino acids, namely, glutamic and aspartic acids, the selectivity being very high for glutamic acid residues (Drapeu & Houmard, 1972). The α-chain contains four glutamic acid residues located at positions 23, 27, 30, and 116 of polypeptide chains (Fermi & Perutz, 1981). The results presented here have led to the identification of the region around the junction of the products of exon 1 and exon 2 of the α-chain gene as a permissible discontinuity region of the α-chain, and the peptide bond

†This work is supported in part by National Institutes of Health Grant HL-27183 and funds from Cetus Corp. The Aviv 60 DS CD spectrometer was purchased with funds from NSF PCM 84-00268. A.S.A. is an Established Fellow of the New York Heart Association.

[1] Abbreviations: HbA, hemoglobin A; HMB, *p*-hydroxymercuribenzoate; EDTA, ethylenediaminetetraacetic acid; RPHPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

0006-2960/86/0425-5949$01.50/0

(30–31) of α-chain is readily accessible for digestion with V8 protease.

Materials and Methods

HbA and the *p*-hydroxymercuribenzoate (HMB) reacted α-chain of HbA were prepared as described previously (Acharya & Manning, 1980). *Staphylococcus aureus* V8 protease was obtained from either Miles Laboratories or Worthington Biochemicals. Seppak cartridges were procured from Water Associates. Sephadex was purchased from Pharmacia. Centricon 10 microconcentrators were obtained from Amicon.

Digestion of α-Chain by V8 Protease. HMB-α-chain in the carbonmonoxy form (0.4 mM in 10 mM KH_2PO_4, 1 mM EDTA, pH 6.0) was incubated with V8 protease (enzyme:α-chain ratio, 1:250) at 37, 25, or 4 °C. After the desired period of incubation, the digests were either subjected to RPHPLC or Sephadex G-50 gel filtration.

HPLC. The HPLC system used was assembled in the laboratory from commecially available components (Acharya et al., 1981). The samples were desalted by passing them through Seppak cartridges previously equilibrated with 0.1% TFA. Globin, heme, and the peptides were bound to Seppak under these conditions. The column was washed with 10 mL of 0.1% TFA. The 0.1% TFA eluate contained only the buffer salts. The material bound (globin, heme, and peptides) to the Seppak cartridge was eluted with about 1.5–2.0 mL of 70% acetonitrile containing 0.1% TFA and was 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 using Seppak 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 analysis have been described earlier (Seetharam, 1983; Acharya & Seetharam, 1985).

Sephadex G-50 Gel Filtration. A Sephadex G-50 column (2.0 × 60 cm) equilibrated with 10 mM KH_2PO_4 and 1 mM EDTA, pH 6.0, was used in these studies.

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

Results

Proteolysis of α-Chain with Staphylococcus aureus V8 Protease. V8 protease has two pH optima, one around pH 4.0 and the other around pH 8.0. Around pH 6.0, the specific activity of the enzyme is the lowest. We have chosen this pH for the digestion of the α-chain with the objective of limiting the cleavage to one of the glutamic acid residues of the α-chain. A kinetic analysis of the digestion of the α-chain by protease at 37 °C (enzyme:substrate ratio, 1:250) as revealed by RPHPLC analysis is shown in Figure 1. The chromatograms shown were obtained with samples of the α-chain that had been digested with V8 protease for $^1/_2$, 2, and 3 h, respectively, and are qualitatively similar. Each of them contains at least five chromatographically distinct components, designated, in the order of their elution, αV_1, αV_2, αV_3, αV_4, and αV_5, respectively. Chromatography of the undigested α-chain showed that the αV_5 region corresponds to the elution position of heme and the undigested α-globin. It may be seen that with the progress of digestion, a shoulder appears in the 3-h digest and a component separates partially from the α-globin peak region.

Of the other four components (i.e., αV_1–αV_4), the αV_1 is present in relatively small amounts during all the periods of digestion studied. On the other hand, αV_4 appears to accumulate continuously with time. The relative distribution of components αV_2 and αV_3 shows an inverse relationship with

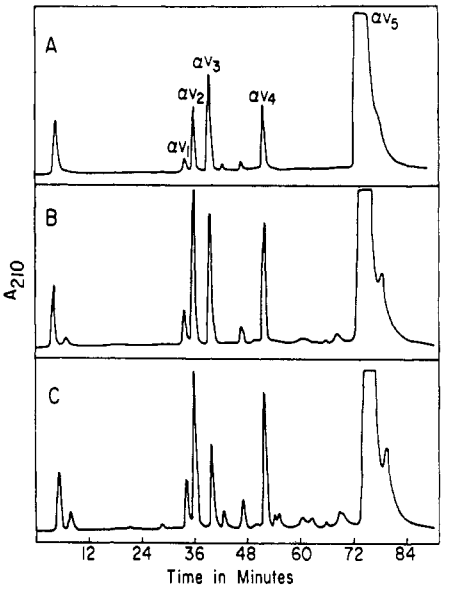

FIGURE 1: Analysis of the V8 protease digest of the α-chain of HbA by reversed-phase HPLC. The digests were desalted on Seppak, as described under Materials and Methods, and then analyzed on Whatman Partisil 10-ODS-3 column (4.6 × 250 mm). The peptides and the larger fragments were eluted with a linear gradient of 5–70% acetonitrile (70 g of each), each containing 0.1% TFA. Flow rate was 60 mL/h, and 1-mL fractions were collected. (A), (B), and (C) are $^1/_2$-, 2-, and 3-h digests of the α-chain, respectively.

the time of digestion. After $^1/_2$ h of digestion, the relative amount of αV_3 is higher than αV_2. As the digestion is continued, the fragment αV_2 starts accumulating with a concomitant decrease in the relative amounts of αV_3. A small increase in the relative amounts of αV_1 was also noticed as the digestion proceeded. This dependence of the elution pattern of components αV_1, αV_2, and αV_3 on the time of digestion suggested that these three may be overlapping fragments. Tryptic peptide mapping of the components αV_1, αV_2, and αV_3 revealed (results not shown) that all of them contain the segments corresponding to α-T_1 (residues 1–7 of α-chain), α-T_2 (residues 8–11), α-T_3 (residues 12–16), and another new tryptic peptide (the position of which was different with each component). This peptide could not be identified from the known positions of tryptic peptides of the α-chain. Thus, the peptides αV_1, αV_2, and αV_3 are derived from the amino-terminal region of the α-chain. Amino acid analysis (Table I) identified the components αV_1, αV_2, and αV_3 as peptides corresponding to the segments α_{1-23}, α_{1-27}, and α_{1-30} of α-chain, respectively. As pointed out earlier, the glutamic acid residues are present at positions 23, 27, and 30 of the α-chain, respectively. Therefore, the formation of α_{1-23}, α_{1-27}, and α_{1-30} are consistent with the specificity of the V8 protease. Amino acid analysis of component αV_4 (Table I) showed that this fragment corresponds to the segment 31–47 of the α-chain. The residue 47 in the α-chain is an aspartic acid. Thus, the V8 protease digestion of the α-chain of HbA appears to be limited to four sites on the α-chain, namely, Glu-23, Glu-27, Glu-30, and Asp-47.

Sephadex G-50 Gel Filtration of the V8 Protease Digest of α-Chain. Gel filtration of a 2-h V8 protease digest of the α-chain shows four components in this sample (Figure 2), and these have been designated A–D in the order of their elution from the column. The gel filtration yielded two heme-containing components, designated components A and B, and two low molecular weight fractions, designated components C and D. The component B elutes at a position corresponding to that of the α-chain (apparent molecular weight of 16 000). The

Table I: Amino Acid Composition of V8 Protease Fragments of α-Chain

	αV_1		αV_2		αV_3		αV_4	
amino acid	found	expected for α_{1-23}	found	expected for α_{1-27}	found	expected for α_{1-30}	found	expected for α_{31-47}
Asp	2.0	2	2.0	2	2.2	2	1.1	1
Thr	1.1	1	1.1	1	1.1	1	3.1	3
Ser	1.1	1	1.1	1	1.1	1	1.0	1
Glu	1.0	1	2.1	2	3.1	3		
Pro	1.1	1	1.1	1	0.8	1	1.8	2
Gly	3.1	3	4.1	4	4.1	4.		
Ala	4.9	5	5.9	6	6.8	7		
Val	3.0	3	3.0	3	3.1	3		
Met							0.9	1
Ile							0.9	1
Leu	0.9	1	0.9	1	2.0	2	0.9	1
Tyr			0.9	1	1.1	1	1.1	1
Phe							4.1	4
His	0.9	1	0.9	1	0.9	1	0.9	1
Lys	3.0	3	3.0	3	3.0	3	1.0	1
Arg							0.9	1

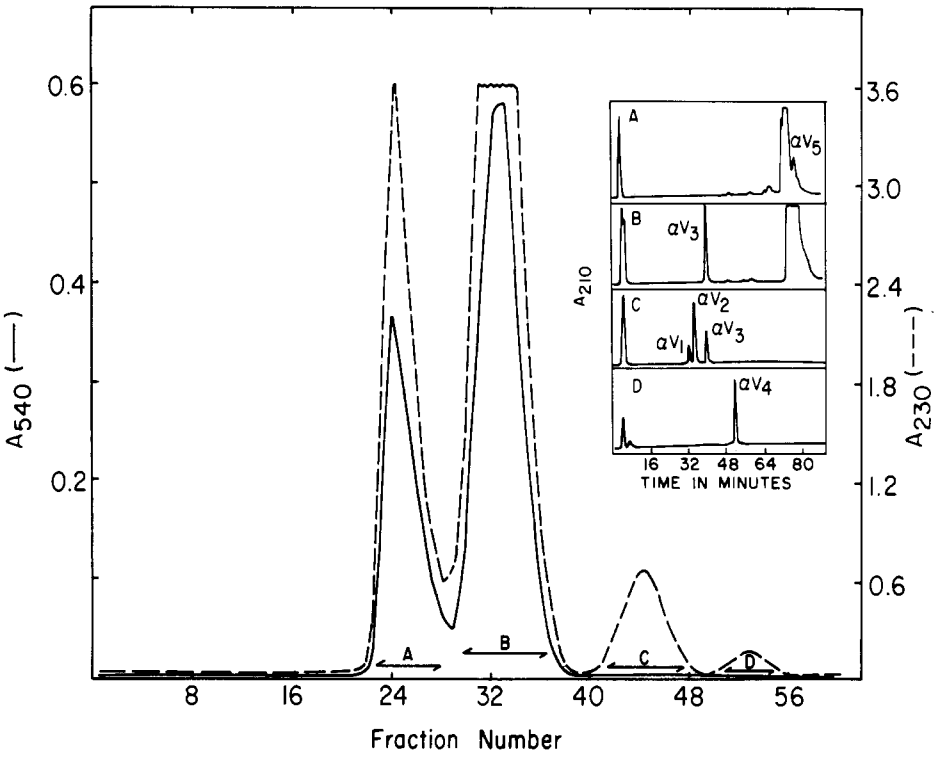

FIGURE 2: Gel filtration of the V8 protease digest of the α-chain of HbA. α-Chain was digested at 37 °C, pH 6.0, for 2 h and then gel filtered through a column (2.2 × 60 cm) of Sephadex G-50 equilibrated with 10 mM potassium phosphate buffer, pH 6.0, containing 1 mM EDTA. Fractions (3.5 mL) were collected, and the elution of the heme and heme binding fragments was determined by measuring the absorbance of the fractions at 540 nm; the elution of the peptides that do not bind heme was determined by measuring the absorbance at 230 nm. Fractions A–D were pooled and analyzed as indicated in the text. The inset shows the reversed-phase HPLC analysis of fractions A–D. The HPLC analysis was carried out as described under Figure 1. The designations of the peptides are also the same as that shown in Figure 1.

component A appears to be comprised of the aggregation products of α-chain and fragmented α-chain. Control experiments showed that incubation of unmodified α-chain at 37 °C also results in the generation of some amount of polymeric products, eluting at a position corresponding to that of component A. No heme is released to the low molecular weight region on gel filtration of the V8 protease digest, demonstrating the strong noncovalent interaction of heme with the larger fragments of the α-chain generated by V8 protease digestion.

Components A–D have been analyzed by RPHPLC to determine their molecular composition (Figure 2, inset). HPLC analysis of component D (Figure 2, inset) identified it to be α_{31-47}, the smallest of the V8 protease fragments. Component C contains the peptides α_{1-23}, α_{1-27}, and α_{1-30}, respectively. Analysis of the heme-containing component A showed that it does not contain any of the lower molecular weight fragments αV_1–αV_4. On the other hand, HPLC analysis of component B revealed the presence of the fragments α_{1-30} and αV_5. The latter component shows the presence of a shoulder, indicating that it is heterogeneous. The expected molecular weight of α_{1-30} is about 3300. The presence of α_{1-30} in this major heme-containing component B, eluting at a position corresponding to that of unmodified α-chain (a molecular weight range of about 16 000), suggests noncovalent interactions between the peptide α_{1-30} and one or more of the molecular species present in this component B.

The globin from component B was prepared by acid acetone precipitation and subjected to sequential Edman degradation to identify the large V8 protease fragment(s) of the α-chain. This analysis showed one major and one minor amino acid sequence. The major sequence, Val-Leu-Ser, corresponds to

the amino-terminal sequence of the α-chain. The minor sequence was Arg-Met-Phe. This corresponds to the residues 31–33 of the α-chain (Fermi & Perutz, 1981), demonstrating that the larger fragment in this sample is α_{31-141}. Thus, the studies clearly reflect the strong noncovalent interaction of heme and the two complementary fragments of the α-chain, namely, α_{1-30} and α_{31-141}.

The noncovalent association of the peptide α_{1-30} and α_{31-141} appears to be very specific. Of the three amino-terminal peptides, α_{1-23}, α_{1-27}, and α_{1-30}, only α_{1-30} is present in component B in significant amounts. In an attempt to determine whether the peptide α_{1-30} has any potential to polymerize to species with an apparent molecular weight of 16 000, thus causing its elution at the position of α-chain, component C was isolated and subjected to a second gel filtration on Sephadex G-50. Component C rechromatographed quantitatively in its original position, demonstrating that α_{1-30} does not polymerize. The possibility of noncovalent interaction between the peptide α_{1-30} and the unmodified α-chain has also been investigated. On gel filtration of a mixture of fraction C and the unmodified α-chain on Sephadex G-50, the component C is well separated from the α-chain peak. The α-chain thus isolated did not contain any α_{1-30} (judged by RPHPLC). Thus, it is clear that the undigested α-chain that is present in component B is not responsible for the elution of α_{1-30} in this higher molecular weight region corresponding to that of the α-chain.

Selectivity of Glu-30–Arg-31 Peptide Bond of α-Chain to V8 Protease Digestion. The kinetics of V8 protease digestion of the α-chain (protein concentration, 0.4 mM; enzyme:substrate ratio, (1:250) suggests that the peptide bond 30–31 is the most susceptible bond (Figure 1). Therefore, attempts were made to increase the selectivity of the hydrolysis of this peptide bond by changing the digestion conditions. Lowering the digestion temperature to 23 °C considerably increased the selectivity of digestion at the Glu-30–Arg-31 peptide bond by V8 protease. At 4 °C, the V8 protease digestion occurred exclusively at Glu-30–Arg-31 bond. However, the rate of digestion was extremely slow, and even after 72 h, only about 20% of the α-chain was digested.

On the other hand, selective and nearly quantitative cleavage of the peptide bond 30–31 of the α-chain occurred in about 2 h (enzyme:substrate ratio, 1:100), when the V8 protease digestion was carried out at 37 °C by using a 10-fold lower concentration of the α-chain (0.04 mM) (Figure 3A). Quantitation of the α_{1-30} formed demonstrated that more than 85% of the α-chain has been digested by 2 h of digestion. Ultrafiltration of such a digest, followed by gel filtration on Sephadex G-50, showed that a very high amount of the segment α^{1-30} elutes in the region of fraction B. Similarly, when such a digest is concentrated nearly 40-fold by using Centricon 10 microconcentrators, nearly 60–70% of α_{1-30} was retained in the Centricon tubes (Figure 3B). In the absence of noncovalent interaction, only about 2.5% of α_{1-30} should have been retained in the Centricon tubes after a 40-fold concentration. If the pH of the digest is adjusted to 2.0 before concentration, less than 2% of α_{1-30} was retained inside the Centricon tube, showing that the noncovalent association of α_{1-30} with α_{31-141} is specific for the "nativelike" structure. The amount of the fragment α_{1-30} associated with the protein after concentration indicated that the dissociation constant of the peptide is in the range of 10^{-8} M.

Influence of Cleavage of Glu-30–Arg-31 Peptide Bond on the Overall Conformation of α-Chain. Glu-30 is in the middle of B-helix; therefore, it was of interest to determine the influence of this discontinuity at Glu-30 on the overall conformation of the chain. The circular dichroic spectra of the α-chain and the 1-h V8 protease digested product at 25 °C is shown in Figure 4A. It may be seen that the overall shape of circular dichroic spectra of V8 protease digested α-chain is nearly the same as that of the native chain. However, the mean residue ellipticity of the digested material is only about 75% of that of the undigested material. Given the fact that nearly 90% of the α-chain in this sample of V8 protease digest has discontinuity at the peptide bond 30–31, the results suggest that the V8 protease digested α-chain has an overall conformation nearly the same as that of the α-chain. The slightly lower helical content is presumably a result of local perturbation of the B-helix due to the discontinuity introduced.

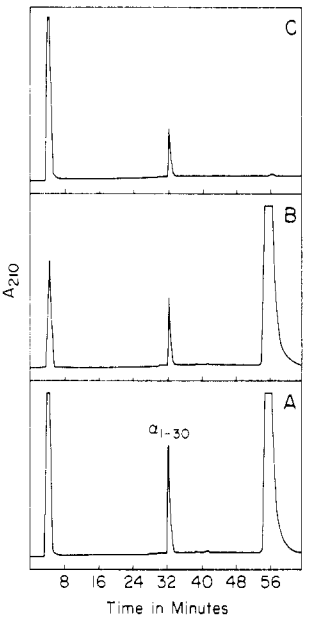

FIGURE 3: Noncovalent association α_{1-30} with α_{31-141}. α-Chain of HbA was digested at pH 6.0, 37 °C, at a concentration of 0.6 mg/mL with an enzyme:substrate ratio of 1:100 for 2 h. The digests were lyophilized and then taken in 0.1% TFA and chromatographed as described in Figure 1. Fifty percent of the digest was lyophilized directly and then chromatographed on Whatman ODS-3 column (A) as described. The remaining 50% of the digest was concentrated 40-fold by using Centricon tubes. The sample inside the Centricon tube was lyophilized and then taken in 0.1% TFA and chromatographed on the same column (B). The filtrate from the Centricon concentration is also lyophilized, taken in 0.1% TFA, and chromatographed on the same reversed-phase column (C).

In order to determine whether the permissibility seen with the carbonmonoxy α-chain is a special case of this liganded form or true of oxy form, oxy α-chain was digested with V8 protease. A 2-h V8 protease digestion of oxy α-chain gave nearly the same HPLC pattern, suggesting that V8 protease recognizes this permissible discontinuity site in the oxy form. The visible spectra of oxy α-chain and its 2-h V8 protease digested product are shown in Figure 4B. The intensity of the Soret absorption band, and the peak position of the V8 protease digested α-chain, is nearly the same as that of the unmodified α-chain. Though the spectra show small differences between the digested and the native sample, they clearly reflect the overall similarity in the conformation of environment of the heme pocket in the native and the digested α-chain. These spectral studies thus suggest that the integrity of the tertiary interactions of the α-chain is maintained to a considerable degree even after a discontinuity is introduced at the peptide bond 30–31.

Influence of pH on the Selectivity of Glu-30–Arg-31 Peptide Bond to V8 Protease Digestion. In an attempt to determine whether the facile hydrolysis of this peptide bond at pH 6.0

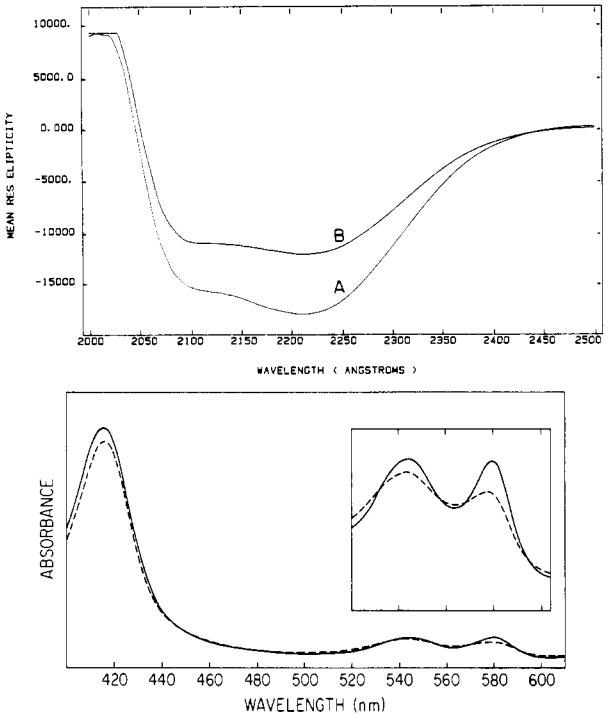

FIGURE 4: Circular dichroic spectra of the α-chain and the fragment-complementing system. The spectra were recorded in a Aviv CD spectrophotometer at room temperature in NH_4Ac buffer, pH 6.0, at a protein concentration of 0.1 mg/mL: (A) α-chain; (B) α-chain digested with V8 protease for 1 h at 37 °C at protein concentration of 1.0 mg/mL. Visible spectra of the α-chain and the fragment-complementing system. Oxy α-chain was digested with V8 protease for 2 h at a protein concentration of 0.6 mg/mL in ammonium acetate buffer at pH 6.0. The same was concentrated in Centricon tubes, and the spectra were recorded in a DW-2 spectrophotometer: α-chain (—); V8 protease digested α-chain (- - -).

and 37 °C is also a reflection of loosening of some amount of tertiary interactions of the α-chain, the V8 protease digestion of the chain at pH 4.0 and pH 7.8 has been compared with that at pH 6.0. An enzyme:substrate ratio of 1:250 and a protein concentration of 0.6 mg/mL was used in all the studies. At pH 4.0, a rapid digestion of the α-chain at the peptide bond 30–31 occurred and in about 1 h nearly 85% of the chain was digested; the yield did not increase when the incubation is continued for 3 h. On the other hand, at pH 7.8 the digestion was extremely slow. Only about 30% digestion occurred in 1 h of digestion. Thus, the peptide bond 30–31 of the α-chain appears to be accessible for a facile and reasonably selective hydrolysis by V8 protease in a broad pH region of 4–8.0.

V8 Protease Digestion of HbA. The selectivity in the digestion of the α-chain by V8 protease and the strong noncovalent association of α_{1-30} with α_{31-141} prompted us to investigate whether the peptide bond 30–31 of the α-chain is susceptible to V8 protease digestion in the tetrameric structure. Interestingly, hemoglobin A showed absolute resistance to proteolysis by V8 protease, even after 24 h at 37 °C. Thus, it is clear that the interaction of α-chains with β-chains (quaternary interaction) to generate the native $\alpha_2\beta_2$ structure stabilizes the region around the peptide bond 30–31 of the α-chain making it resistant to the V8 protease digestion.

Discussion

The results of the present study demonstrate the high selectivity of V8 protease to hydrolyses of the peptide bond of Glu-30–Arg-31 of the α-chain of HbA. This selectivity is seen in a wide pH region of 4.0–8.0. Much more interesting is the observation that the introduction of a discontinuity at this peptide bond Glu-30–Arg-31 does not significantly influence the overall noncovalent interactions of the α-chain. The complex of α_{1-30}, α_{31-141}, and heme elutes at the α-chain position on gel filtration due to specific and strong noncovalent interaction between the various components.

All the V8 protease susceptible sites of the α-chain are present in the amino-terminal region of the polypeptide. No cleavage occurred at either Glu-116 or the other aspartic acid residues of the carboxy-terminal region of the α-chain. Thus, the carboxy-terminal half of the α-chain appears to be relatively more resistant to proteolysis with V8 protease. This also appears to be the case in the proteolysis of *p*-chloromercuribenzoate-dissociated HbA by trypsin (Kimura et al., 1977). All the trypsin-susceptible sites of the α-chain are present in the A–E helices of the α-chain and none toward the carboxy-terminal end of the molecule. In view of the extensive digestion of urea-denatured or acid-denatured α-globin by trypsin as well as by V8 protease, the limited digestion of the α-chain (heme-containing globin) by trypsin and V8 protease should be considered as a reflection of the influence of the stabilizing noncovalent interactions of heme on the carboxy-terminal two-thirds of α-globin. This is consistent with the fact that the majority of the heme contacts of both the α- and β-chains are also located towards the C-terminal half of the globin chains.

From the structural point of view, it is of interest to note that the hydrolysis of the peptide bond 30–31 of α-chain introduces a break in the middle of its B-helix (Fermi & Perutz, 1981) (Figure 5). Thus, the susceptibility of this bond to proteolysis is rather unexpected. The accessibility of the Glu-30–Arg-31 peptide bond for digestion appears to be lower at pH 7.8 compared to that at pH 6.0. This may be a reflection of the destabilization of B-helix as the pH is lowered. Though the peptide bond 30–31 is susceptible to the V8 protease digestion in the isolated α-chain, the same peptide bond shows complete resistance to proteolysis in the tetrameric ($\alpha_2\beta_2$) structure of HbA. The differential susceptibility of the peptide bond of the B-helix of the α-chain (B_{11} and B_{12}) is probably suggestive of the fact that the B-helix of α-chain is in a more flexible conformation in the isolated chain, while in the tetramer it takes up a more rigid structure. The fact

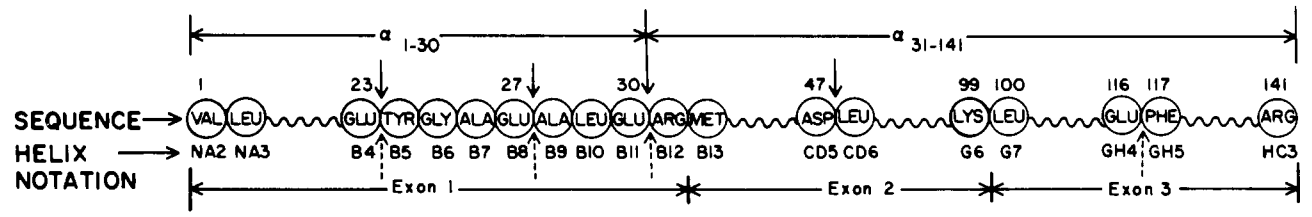

FIGURE 5: V8 protease cleavage points of the α-chain of HbA. Sequences are listed from amino terminal to carboxy terminal. Arrows (↓) indicate the observed cleavage points. The amino acid residues are also identified by helix notations (Fermi & Perutz, 1981). The four glutamyl peptide bonds are indicated by (↑). The regions of the molecule corresponding to the translation products of exons 1, 2, and 3 are also indicated. (〰) represents the regions of the α-chain for which amino acid sequence is not shown. The sequences presented are identified by the position number.

that in the tetramer this region represents the $\alpha_1\beta_1$ interface and contains a number of stabilizing noncovalent interactions (Fermi & Perutz, 1981) is consistent with this observation. Thus, the resistance of Glu-30–Arg-31 of α-chain in the $\alpha_2\beta_2$ structure can be considered as a consequence of the stabilization of this interface by the quaternary interactions. However, it should be added here that the stabilizing influence of the tetramer formation is not only limited to this region of HbA; the quaternary interactions of the tetramer appear to be propagated throughout the molecule, making both the α- and the β-chains resistant to V8 protease digestion.

The noncovalent association of α_{1-30} and α_{31-141} to maintain the nativelike structure of the α-chain appears to be reminiscent of the observation of Craik et al. (1980) that the product of central exon of β-globin gene, namely, β_{31-104}, interacts with heme to generate a cyanomet-like complex. However, this central exon peptide of β-chain appears to be unable to maintain the heme in a ferrous dioxygen complex (Craik et al., 1981). The intensity of the Soret absorption band of a mixture of heme and β_{31-104} is enhanced by the presence of the noncovalently associated side fragments, namely, the translation products of exon 1 and exon 3 of β-globin gene. It has been suggested that these may be adding precision to fit the heme pocket.

In the present study, unlike the experiments of Craik et al. (1980), the digestion by V8 protease has been carried out with heme-containing α-chain in the oxy conformation. The fragment-complementing system of the α-chain formed by V8 protease digestion appears to maintain the heme in nearly the same native environment as that in the α-chain and in the ferrous state. The visible spectra of the fragment-complementing system of the α-chain generated in the present study is nearly the same as that of the native chain. Circular dichroism studies indicated the α-helical content of the fragment-complementing system of the α-chain is about 75% of that of native protein. Since the fragment α_{1-30} represents nearly the complete translation product of exon 1 of α-globin gene (α_{1-31}), it may be concluded that the region corresponding to the junction of translation products of exon 1 and exon 2 of α-globin gene appears to be a permissible discontinuity region. Therefore, the fragment-complementing system described in the present study may be considered as representing the noncovalent interactions of product of exon 1 with a contigous segment corresponding to the products of exons 2 and 3.

The demonstration that a permissible discontinuity could be introduced at the peptide bond 30–31 of the α-chain by V8 protease opens up a new avenue to study the intramolecular noncovalent interactions of the α-chain. Analogues of α_{1-30} could now be synthesized to prepare semisynthetic noncovalent analogues of α-chain as has been done previously with other protein systems like RNase S and nuclease T (Chaiken, 1981). Recently, we have demonstrated the synthetic potential of V8 protease (Seetharam & Acharya, 1986; Acharya et al., 1985). This would permit the conversion of the semisynthetic noncovalent analogues of the fragment-complementing system (α_{1-30} and α_{31-141}) to the covalent forms (Acharya et al., 1985).

Anfinsen and his associates (Hagenmier et al., 1979) have demonstrated the noncovalent reconstitution of a myoglobin-like structure from the overlapping fragments of apomyoglobin. Fragments 1–55 and 32–139 of myoglobin generated a myoglobin-like structure in the presence of hemin. These results suggest a discontinuity is permissible in the region of the residues 32–55 of myoglobin. Given the structural homology between myoglobin and the α- and β-chains of hemoglobin, the results of the present study suggest that the carboxy-terminal region of B-helix of the β-chain may also represent a permissible discontinuity region. It may be added here further that the location of Arg-30(β) in the β-chain is nearly analogous to that of the Arg-31 of the α-chain. Therefore, it will be of interest to investigate the limited proteolysis of β-chains and determine whether a selective cleavage on the carboxyl side of Arg-30 of β-chain can be obtained by clostripain or submaxillary protease to generate a fragment-complementing system of β-chain.

ACKNOWLEDGMENTS

We thank Dr. James M. Manning for the facilities provided and Rita Quinn and Judith A. Gallea for helping in the preparation of the manuscript. We especially thank Sheenah Mische, Donna Atherton, and Dr. B. N. Manjula for the analysis and interpretations of amino acid sequence data and Y. J. Cho for the technical assistance.

Registry No. HbA, 9034-51-9; heme, 14875-96-8; *Staphylococcus aureus* V8 protease, 66676-43-5.

REFERENCES

Acharya, A. S., & Manning, J. M. (1980) *J. Biol. Chem. 255*, 1406–1412.

Acharya, A. S., & Seetharam, R. (1985) *Biochemistry 24*, 4885–4890.

Acharya, A. S., Di Donato, A., Manjula, B. N., Fischetti, V. A., & Manning, J. M. (1983) *Int. J. Pept. Protein Res. 22*, 78–82.

Acharya, A. S., Khan, S. A., & Seetharam, R. (1985) *Proc. Am. Pept. Symp., 9th, 1985*, 335–338.

Anfinsen, C. B. (1973) *Science (Washington, D.C.) 181*, 223–230.

Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205–294.

Birk, Y., & Li, C. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2155–2159.

Chaiken, I. M. (1981) *CRC Crit. Rev. Biochem. 11*, 255–298.

Craik, C. S., Buchman, S. R., & Beychok, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1384–1388.

Craik, C. S., Buchman, S. R., & Beychok, S. (1981) *Nature (London) 291*, 87–90.

Drapeau, G. R., & Houmard, J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3506–3509.

Fermi, G., & Perutz, M. F. (1981) in *Hemoglobin and Myoglobin, Atlas of Molecular Structure in Biology* (Phillips, D. C., & Richards, F. M., Eds.) p 4, Clarendon, Oxford.

Gilbert, W. (1978) *Nature (London) 271*, 501.

Hagenmier, H., Ohms, J. P., Johns, J., & Anfinsen, C. B. (1979) in *Semisynthetic Proteins and Peptides, Papers Presented at the International Meeting on Protein Semisynthesis, 1977* (Offord, R. E., & DiBello, C., Eds.) pp 23–39, Academic, London.

Hantgan, R. R., & Taniuchi, H. (1977) *J. Biol. Chem. 252*, 1367–1374.

Kimura, H., Takahashi, N., Murata, H., & Uematsu, H. (1979) *J. Biochem. (Tokyo) 85*, 1047–1051.

Li, C. H., Blake, J., & Hagashida, T. (1978) *Biochem. Biophys. Res. Commun. 82*, 2117–2122.

Liao, M. J., London, E., & Khorana, H. G. (1983) *J. Biol. Chem. 258*, 9949–9955.

Liao, M. J., Huang, K. S., & Khorana, H. G. (1984) *J. Biol. Chem. 259*, 4200–4204.

Richards, F. M., & Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459–1465.

Seetharam, R., & Acharya, A. S. (1986) *J. Cell. Biochem.*, 87–99.
Seetharam, R., Manning, J. M., & Acharya, A. S. (1983) *J. Biol. Chem. 258*, 14810–14815.
Taniuchi, H., Anfinsen, C. B., & Sodja, A. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1235–1242.
Vita, C., Dalzoppo, D., & Fontana, A. (1985) *Biochemistry 24*, 1798–1806.

Effects of Bovine Pancreatic Ribonuclease A, S Protein, and S Peptide on Activation of Purified Rat Hepatic Glucocorticoid–Receptor Complexes†

Thomas J. Schmidt,* E. E. Diehl, C. J. Davidson, and Michael J. Puk
Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242
M. L. Webb‡ and Gerald Litwack
Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140
Received April 15, 1986; Revised Manuscript Received June 16, 1986

ABSTRACT: Bovine pancreatic ribonuclease (RNase) A and S protein (enzymatically inactive proteolytic fragment of RNase A which contains RNA binding site) stimulate the activation, as evidenced by increasing DNA–cellulose binding, of highly purified rat hepatic glucocorticoid–receptor complexes. These effects are dose dependent with maximal stimulation of DNA–cellulose binding being detected at approximately 500 μg (50 units of RNase A/mL). RNase A and S protein do not enhance DNA–cellulose binding via their ability to interact directly with DNA or to increase nonspecific binding of receptors to cellulose. Neither S peptide (enzymatically inactive proteolytic fragment which lacks RNA binding site) nor cytochrome *c*, a nonspecific basic DNA binding protein, mimics these effects. RNase A and S protein do not stimulate the conformational change which is associated with activation and is reflected in a shift in the elution profile of receptor complexes from DEAE-cellulose. In contrast, these two proteins interact with previously heat-activated receptor complexes to further enhance their DNA–cellulose binding capacity and thus mimic the effects of an endogenous heat-stable cytoplasmic protein(s) which also function(s) during step 2 of in vitro activation [Schmidt, T. J., Miller-Diener, A., Webb, M. L., & Litwack, G. (1985) *J. Biol. Chem. 260*, 16255–16262]. Preadsorption of RNase A and S protein to an RNase affinity resin containing an inhibitory RNA analogue, or trypsin digestion of the RNA binding site within S protein, eliminates the subsequent ability of these two proteins to stimulate DNA–cellulose binding of the purified receptors. These data indicate that the effects of RNase A on activation do not require RNA hydrolysis but do require an intact RNA binding site. This conclusion is consistent with numerous observations which suggest that a small RNA molecule(s) may be an integral component of the glucocorticoid receptor and may influence activation.

Once glucocorticoid molecules bind with high affinity and specificity to target cell intracellular receptors, these steroid–receptor complexes must undergo "activation" or "transformation" before they can bind to nuclear acceptor sites and ultimately modulate gene expression. Activation is a temperature-dependent process which is thought to involve a conformational change in the glucocorticoid–receptor complex resulting in the exposure of positively charged amino acid residues on the surface of the protein (Milgrom et al., 1973; DiSorbo et al., 1980) and hence an increased affinity for nuclei and polyanions such as DNA and DNA–cellulose (Milgrom et al., 1973; Baxter et al., 1972; Kalimi et al., 1975; LeFevre et al., 1979). This conformational change is also reflected in an altered elution profile of the activated glucocorticoid–receptor complexes from anion-exchange resins such as DEAE-cellulose (Sakaue & Thompson, 1977). Despite the fact that activation occurs in vivo under physiological conditions and appears to be rate limiting for nuclear binding (Munck & Foley, 1979; Markovic & Litwack, 1980; Miyabe & Harrison, 1983), its underlying biochemical mechanism has not been elucidated. Although numerous theories have been proposed [reviewed by Schmidt & Litwack (1982)], recent studies have implicated subunit dissociation as a mechanism of glucocorticoid–receptor activation (Vedeckis, 1983; Raaka & Samuels, 1983). However, reconstitution experiments with purified unactivated glucocorticoid–receptor complexes have also demonstrated that maximal DNA–cellulose binding of thermally activated complexes requires the additional involvement of an endogenous heat-stable cytoplasmic protein(s) (Schmidt et al., 1985).

Just as the precise mechanism of activation requires further clarification, the exact molecular composition of the unactivated glucocorticoid receptor itself has not been ascertained. Numerous studies have suggested a homotetramer–homodimer–monomer model for the unactivated receptor (Raaka & Samuels, 1983; Vedeckis, 1983; Holbrook et al., 1983;

† At The University of Iowa, this work was supported by a Helmar Maage Memorial Grant for Cancer Research from the American Cancer Society (BC 464) and by Research Grant 1 R01 AM 34490-01 PC from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. T.J.S. is a Scholar of the Leukemia Society of America, Inc. At the Temple University School of Medicine, this work was supported by Research Grants AM-13531 and AM-32870 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health.
‡ Present address: Department of Pharmacology, Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033.

0006-2960/86/0425-5955$01.50/0