Polymerization of Hemoglobin S Amidated at the Contact Residue Glu 43(β)

A. Seetharama Acharya, Lakshmi Khandke[1], Brian T. Chait and M. Janardhan Rao

Division of Hematology, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA
and

Reprinted from

Molecular Conformation and Biological Interactions

pp 269-280

Editors
P. Balaram and S. Ramaseshan

1991
Indian Academy of Sciences, Bangalore

Polymerization of Hemoglobin S Amidated at the Contact Residue Glu 43(β)

A. Seetharama Acharya, Lakshmi Khandke[1], Brian T. Chait and M. Janardhan Rao

Division of Hematology, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA
and
[1]The Rockefeller University, 1230 York Avenue, New York 10021, USA

Introduction

The polymerization of hemoglobin S in the deoxy conformation is a direct consequence of the mutation of glutamic acid residue at the sixth position of the β-chain of HbA to valine (1–3). A high degree of specificity exists in the mutation that endows this segment (A-helix) of β-chain a 'new quinary structural memory'. Mutation of Glu 6 to glutamine, or alanine, or lysine is unable to encode the segment with this structural memory (4). Recent site directed mutagenetic studies have demonstrated that the specificity to encode the 'new quinary structural memory' is in the hydrophobicity of the amino acid residue at the sixth position rather than being unique to the Val residue. Mutants of HbA with Ile or Leu at this position also polymerize, their propensity to polymerize is even higher than that of HbS (5, 6). Early studies have demonstrated that the polymerization of deoxy HbS involves hydrophobic interactions (7). Subsequent studies have, however, established that electrostatic interactions also play a crucial role in the polymerization reaction (8, 9). Thus the polymerization of deoxy HbS is a complex phenomenon involving various types of noncovalent interactions of intermolecular regions that apparently generate the crucial stabilization energy to facilitate the polymerization reaction.

X-ray diffraction studies of deoxy HbS crystals have suggested that the carboxyl groups of Glu 22(β), Glu 121(β), Glu 23(α), and Asp 73(β) are present at or near one or more intermolecular contact regions. More recently Padlan and Love (10, 11) suggested that Glu 43(β) is also present at a contact region. The participation of these carboxylates in intermolecular interactions could be a consequence of a higher pK_a of these carboxylates compared to those of the others. A study of the chemical reactivity of carboxylates of HbS could provide an insight into this aspect of the structure.

Carbodiimide activated coupling of nucleophiles to the carboxyl groups of proteins

---

Abbreviations: HbS-sickle cell hemoglobin; DPG-2:3 diphosphoglycerate; EDC-1-ethyl-3[3'-dimethylamino propyl]carbodiimide; GEE-glycine ethyl ester.

is a relatively simple and mild procedure for the derivatization of β-and/or γ-carboxyl groups of proteins (12). The derivatization of carboxylates of proteins is very efficient at moderately acidic pH, and is generally carried out at pH 4·0, a region in which most of the carboxylates of the protein are protonated. However, in view of the possible complications associated with the dissociation of Hb tetramers as well as precipitation of the protein in this low pH region of 4·0, an intermediate pH of 6·0 was selected for the derivatization of the carboxyl groups. The initial studies demonstrated that in the presence of water soluble carbodiimide, 1-ethyl-3[-3′-(dimethylamino propyl)] carbodiimide (EDC), a selective coupling of the nucleophiles to the carboxyl groups of HbS occurs around pH 6·0 (13, 14). The selectivity of the amidation reaction was very distinct with the two nucleophiles investigated. When glycine ethyl ester (GEE) was used as the nucleophile, more than 75% of the amidation was on the γ-carboxyl group of Glu 43(β). On the other hand, with glucosamine as the nucleophile, the extent of amidation was about 50% lower, as compared with that obtained using glycine ethyl ester. Besides, with glucosamine, amidation also occurred at Glu 22(β), besides at Glu 43(β). The extent of reaction at these two carboxylates were nearly the same.

Though extent of amidation with glucosamine is significantly lower than that seen with glycine ethyl ester, the solubilities of the two amidated products (unfractionated) are nearly the same (14). This was rather surprising, and casts some doubt on the implication of Glu 43(β) as the contact residue. Therefore, preparation of a homogeneous derivative of HbS amidated only at Glu 43(β) seemed essential to unequivocally establish the role of Glu 43(β) in the polymerization reaction. Accordingly the preparation of homogeneous amidated HbS has been undertaken. The isolation and characterization of Di-GEE-HbS, the oxygen affinity and solubility behaviour of this amidated derivative of HbS, has been studied now and presented here.

Materials and Methods

Modification of Carboxyl Groups of HbS

HbS was purified from erythrocyte lysate from patients with sickle cell disease using chromatography on DE-52 (15). The amidation reaction was carried out essentially by the method described earlier (13). HbS was dialyzed against 0·1 M KCl, adjusted to pH 6·0 and concentrated by ultrafiltration. [^{14}C] GEE (New England Nuclear, specific activity 52·2 mCi/mmol) was mixed with cold GEE to give the desired specific activity. The protein sample was incubated with 100 mM [^{14}C] GEE at 23 °C in a vessel attached to a Radiometer pH stat. The coupling reaction was initiated by adding appropriate amounts of 1-ethyl-3(-3′-dimethylamino propyl) carbodiimide (EDC). The reaction was maintained at pH 6·0 by automatic titration with 0·01 M HCl. After 1 hour the reaction mixture was passed through a column of Sephadex G-25 equilibrated with 10 mM phosphate buffer, pH 6·0, containing 1 mM EDTA.

Purification of Derivatized HbS

After the removal of excess reagents by gel filtration the derivatized protein was chromatographed on carboxymethyl cellulose (CM-52) equilibrated to pH 6·0 with

10 mM potassium phosphate buffer containing 1 mM EDTA. The protein was eluted using a linear gradient of 10 mM potassium phosphate buffer pH 6·0 to 50 mM phosphate buffer pH 8·3. Both buffers contained 1 mM EDTA.

Characterization of the Amidated Derivative

The procedures used for the chemical analysis of the derivative, namely, tryptic peptide mapping, amino acid analysis, and amino acid sequencing were essentially as described earlier (13, 14)

Mass Spectrometry

Mass spectrometric measurements were performed on the 252 Cf fission fragment ionization time-of-flight mass spectrometer described previously (16–18). Samples were prepared for measurement by absorption of 1 nmole of peptide from solution (0·2 nmole/μl in 0·1% TFA) on a thin nitrocellulose film, as previously described (18). The accuracy of the mass measurements was generally better than 200 pm.

Oxygen Equilibrium Measurements

The oxygenation curves of HbS and derivatives of HbS were recorded using an Amicon Hem-O-Scan, at pH 7·4 and 37 °C as described by Benesch *et al.* (19).

Solubility Studies

Solubility measurements were performed by the ultracentrifugation procedure as discussed by Benesch *et al.* (20). The carbon monoxide in the HbS and its amidated derivatives were removed by photolysis (21). The protein was concentrated by ultrafiltration to about 35 g/dl and this solution was used for the solubility measurements. The samples were deoxygenated, mixed well, and incubated for 2 hours after which the Hb concentration in the supernatant was measured according to the method of Van Assendelft (22).

Results

Isolation and Characterization of Di-GEE-HbS

Glu 43(β) is the major reactive site for amidation with glycine ethyl ester (13). After some preliminary analysis, reaction of HbS (0·5 mM) with 10 mM EDC in the presence of 100 mM GEE for 1 h at pH 6·0, 23 °C was chosen for derivatization and the subsequent isolation of Di-GEE-HbS (figure 1). The Di-GEE-HbS isolated was further purified by rechromatographing it on a CM-52 column (figure 1 inset). The repurified Di-GEE-HbS contained two moles of GEE per mole of tetramer.

Tryptic peptide mapping of Di-GEE-HbS showed that all the radioactivity (GEE incorporated) eluted at two positions (figure 2). One peptide eluting around 64 minutes was designated as Peak A and the other eluting around 72 minutes was designated as Peak B. Peaks A and B were isolated and rechromatographed on HPLC using an aquapore RP-300 column at pH 6·0 using an acetonitrile gradient (5 to 50%) (14).

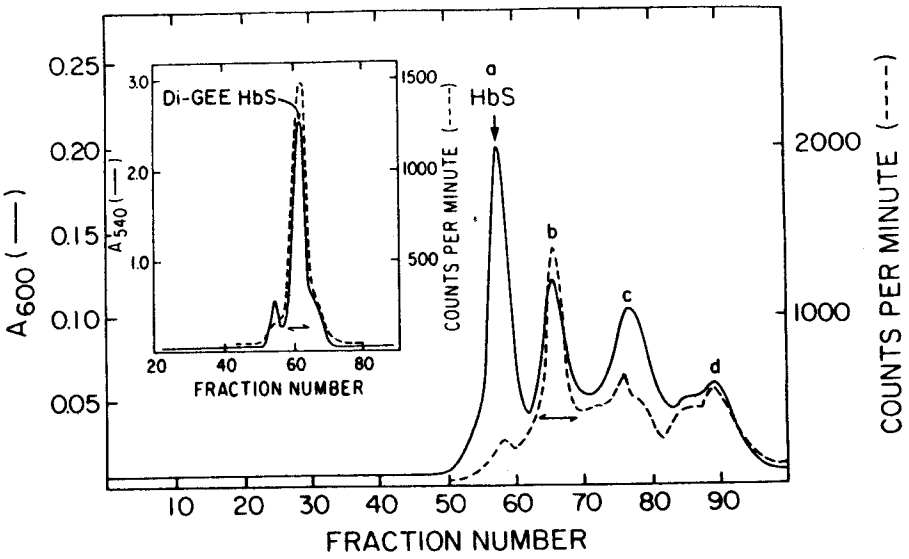

Figure 1. Preparation of Di-GEE-HbS: The excess reagents were removed from amidated HbS by passing through a Sephadex G-25 column (2·5 × 40 cm) eluted with 10 mM potassium phosphate buffer, pH 6·0. Amidated HbS was then chromatographed on CM-52 as described under materials and methods. The major radioactive fraction, eluting after HbS position and marked was pooled and concentrated. It was then dialyzed against the starting buffer, (10 mM potassium phosphate 1 mM EDTA) and rechromatographed on C-52 (0·9 × 30 mM) under the same conditions (insert). The main component marked 'Di-GEE-HbS' was pooled to isolate Di-GEE-HbS.

The amino acid composition of both the purified radioactive peptides corresponded very well with that of βT5 representing the segment 41 to 59 of β-chain, (table 1). except for the presence of an extra residue of glycine. In addition, the quantitation of the peptide using the specific activity of GEE and the amino acid composition corresponded very well, further confirming the homogeneity of the peptides. These results demonstrate that both of these peptides are formed as a consequence of the amidation of only one of the side chain carboxyl groups of βT5.

Both of these peptides were subjected to amino acid sequencing to identify the amidated carboxyl group of these peptides. The amount of ^{14}C-label released during each of the 15 cycles of the Edman degradation has been quantitated. Both peptides A and B showed the same sequence analysis and from both the radioactivity was released in the third cycle. This position corresponds to Glu 43(β). Thus, both these peptides correspond to βT5 amidated at Glu 43.

The covalent modification that results in the presence of two modified forms of βT5 in the tryptic digest of Di-GEE-HbS is not apparent from the sequence analyses. In an attempt to gain an insight into the structural differences between the two amidated βT5, the purified peptides A and B were subjected to mass spectral analysis (table 2). The predominant ion species (M^+H) of peptide B has a molecular weight of 2144·2 and is consistent with the calculated molecular weight for the βT5 amidated with glycine ethyl ester at Glu 43 as 2144·4. On the other hand, the predominant ion

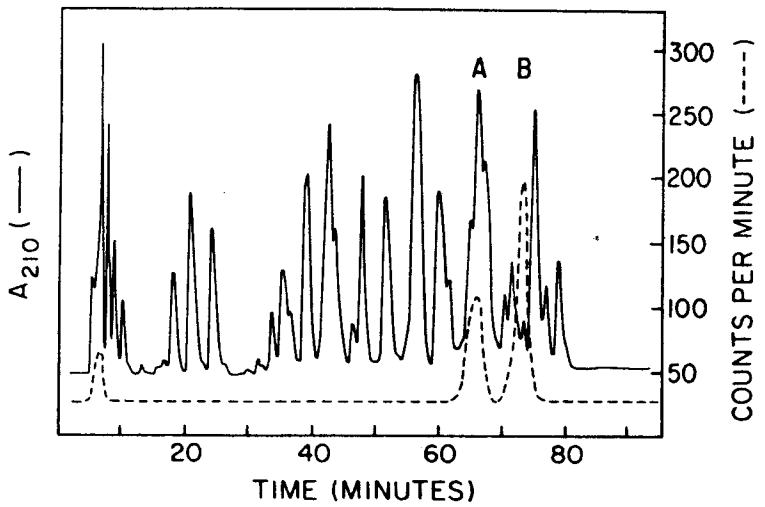

Figure 2. HPLC Analysis of the tryptic peptides of Di-GEE-HbS: The tryptic digest of Di-GEE-HbS (figure 1 insert) was taken in 500 μl of 0·1% trifluoroacetic acid and loaded on to a Partsil 10 ODS 3 column. The peptides were eluted with a linear gradient of 5–50% acetonitrile containing 0·1% trifluoroacetic acid (Acharya *et al.* 1983) over 2 h. The column was eluted at a flow rate of 1 ml/min, and 1 ml fractions were collected. The effluent was monitored at 210 nm, 100 μl of each fraction were used for measuring the radioactivity. The radioactive components labelled A and B were further purified by RPHPLC at pH 6·0 (reference 14) and identified as modified βT5 (corresponding to the segment 41 to 59 of β-chain of bS) by amino acid analysis and amino acid sequencing.

Table 1. Amino acid composition of amidated peptides A and B.

Amino Acid	Peptide A	Peptide B	Expected for BT5 (segment 41 to 59)
Asp	3·1	3·1	3
Thr	0·8	1·0	1
Ser	1·8	2·0	2
Glu	0·9	1·0	1
Pro	1·7	2·0	2
Gly-b	2·7	2·9	2
Ala	1·1	1·0	1
Val	1·2	1·1	1
Met	0·9	0·8	1
Leu	1·1	0·9	1
Lys-a	1	1	1

a. Calculated assuming Lys as one residue.
b. βT5 contains two residues of glycine, the higher value (nearly three) of glycine in the peptides A and B represents glycine ethyl ester linked to βT5 through an isopeptide linkage.

Table 2. Mass of peptides A and B.

	Ion (*a*)	Molecular weight Observed	Calculated (*b*)	M. W Observed − M. W. calculated (*c*)
Peptide A	(M +) +	2116·8	2144·4	− 27·6
	(M + Na) +	2116·3	2144·4	− 28·1
	(M + K) +	2117·7	2144·4	− 26·7
Peptide B	(M + H) +	2144·2	2144·4	− 0·2
	(M + Na) +	2144·1	2144·4	− 0·3
	(M + K) +	2144·4	2144·4	0·0

a. Only the molecular weight of the major species is given. Both peptides A and B gave another component, the mass of which was higher by 16 units than the main component. This presumably corresponds to the sulfoxide form of the peptide.
b. In calculating the molecular weight of the peptides, an average of five most abundant isotopic species is considered.
c. The net difference in the observed molecular masses of Peptide A and Peptide B appears to be about 28 mass units.

species of the peptide A indicated that this peptide has a molecular mass of 2116·8 which is nearly 28 mass units lower than that of peptide B. A mass difference of 28 between peptides A and B could arise by the hydrolysis of the ethyl ester group of glycine ethyl ester on the γ-carboxyl of Glu 43 (loss of ethyl group-CH_2CH_3 and protonation of the carboxyl). Thus the structural studies along with the mass data of peptides suggested that peptide A was generated as a consequence of hydrolysis of the ethyl group of the glycine ethyl ester of peptide B.

The distribution of the amidated βT5 of Di-GEE-HbS as peptides A and B in the tryptic peptide map showed some dependence on the duration of the tryptic digestion. During the initial stages of the digestion, peptide B was always the major radioactive component. However with time peptide A started to accumulate. This result suggested that peptide B is accumulating at the expense of peptide A during tryptic digestion. Accordingly purified peptide B was incubated at pH 8·0 (50 mM ammonium bicarbonate) at 37 °C (in the absence of trypsin) and analyzed by RPHPLC (acetonitrile–TFA system). Incubation of peptide B at pH 8·0 and 37 °C for 18 h resulted in a 40% conversion of the sample (figure 3b) to peptide A. This conversion was absent in the sample incubated at 4 °C. Mass spectral analysis of this new peptide formed on incubation of peptide B at pH 8·0 and 37 °C confirmed a loss of a group with a molecular mass of 28 mass units. Amino acid analysis and amino acid sequencing confirmed it to be peptide A. Thus, these structural studies establish that in peptide A, the ethyl group of GEE introduced onto Glu 43(β) has been hydrolyzed, and this is a trypsin independent, temperature dependent process.

Oxygen Equilibrium Properties of the Di-GEE-HbS

The oxygen affinity of HbS and the Di-GEE-HbS was determined at pH 7.4 and 37°C (figure 4). The derivatization of HbS with GEE at Glu 43(β) results in an increase in the oxygen affinity of the molecule, the P50 decreased from a control value of about 10·0 to 7·6 on derivatization of Glu 43(β). However, the Hill coefficient was not significantly altered ($n = 2·8$) and was the same as that of the control HbS. The

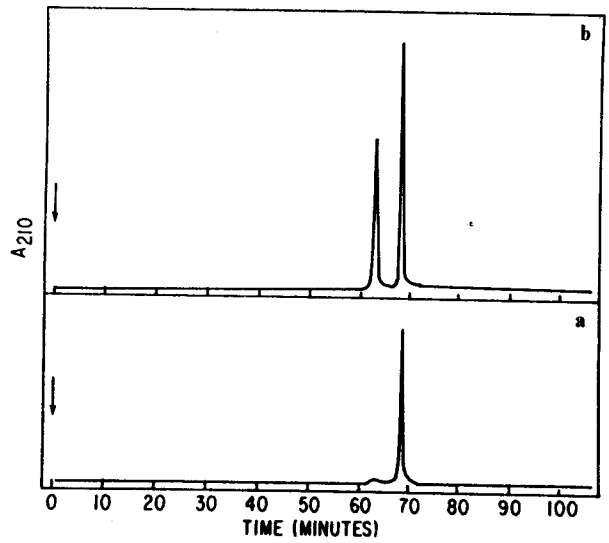

Figure 3. Hydrolysis of the ethyl ester group of amidated βT5. HPLC analysis of peptide A incubated at pH 8·0, 37 °C for 18 h. The peptide sample after incubation at pH 8·0, was lyophilized, and chromatographed on a Brownlee acquapore RP-300 column. The gradient system was the same as that used in figure 2A (control peptide A B). Peptide A incubated at pH 8·0.

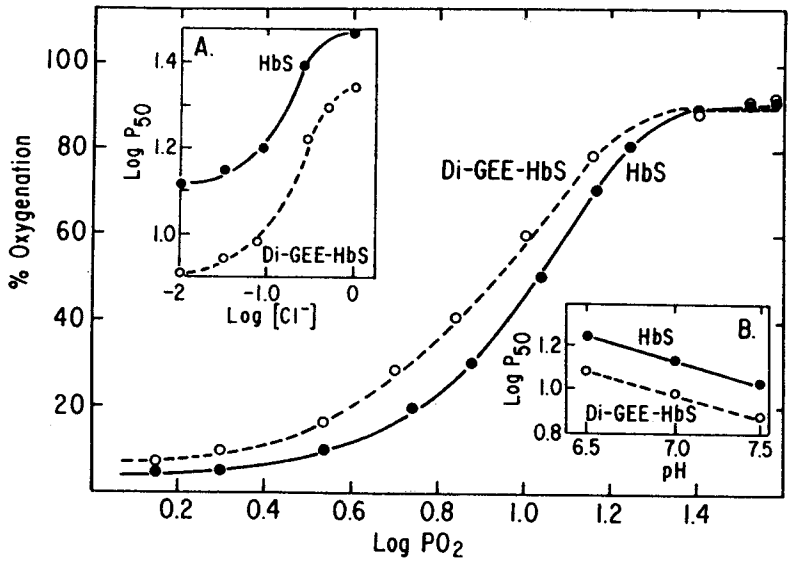

Figure 4. Oxygen affinity of Di-GEE-HbS: Oxygen equilibrium curves of HbS and amidated HbS at pH 7·4 (50 mM bis-Tris) and 37 °C. The curves were recorded in an Amicon Hem-O-Scan. A) HbS, B) HbS amidated at Glu 43(β). Insert A shows the influence of chloride ion on the oxygen equilibrium properties of Di-GEE-HbS. The samples prepared as described in the text were mixed with varying amounts of chloride and the oxygen equilibrium curves were determined in the Hem-O-Scan. The inset B presents the O_2 equilibrium properties of Di-GEE-HbS and HbS as a function of pH.

Table 3. Oxygen affinity of amidated HbS.

Modulators of O_2 affinity	O_2 affinity P50		P50 of Di-GEE P50 HbS
	HbS	Di-GEE-HbS	
None	10·0	7·6	0·76
DPG	26·5	18·5	0·70
Chloride	30·5	22·5	0·73

The oxygen affinity of hemoglobin S and Di-GEE-HbS was measured using hemoscan in 50 mM bis-Tris., pH 7·4. There was 2 fold molar excess of DPG to hemoglobin and 2000 fold excess of chloride to hemoglobin.

derivatization of Glu 43(β) does not influence the cooperativity in binding of oxygen to the protein. Besides, the binding of DPG to Di-GEE-HbS leads to a decrease in the oxygen affinity of the protein to approximately the same degree (table 3) as that seen with unmodified HbS. The Hill coefficient of the amidated HbS in the presence of DPG was also 2·8. The results suggest normal subunit interaction in this derivatized HbS in the presence of DPG as well.

The oxygen affinity of Di-GEE-HbS has also been determined as a function of chloride concentration and compared with that of unmodified HbS (figure 4, inset A). The overall shape of curves (a plot of P50 versus chloride) for HbS and Di-GEE-HbS is nearly the same. This demonstrates that modulation of quaternary interaction of HbS by the chloride ion is also not significantly influenced by the amidation of Glu 43(β).

The oxygen affinity of Di-GEE-HbS has also been studied as a function of pH in order to determine whether amidation of Glu 43(β) influences the alkaline Bohr effect of HbS. The value of log P50/pH was 0·52 for HbS, and 0·50 for the Di-GEE-HbS (figure 4, inset B).

Solubility of Di-GEE-HbS

The equilibrium solubilities of HbS, and purified Di-GEE-HbS were determined by the procedure of Benesch *et al.* (20). The solubility of the Di-GEE-HbS was nearly 25% higher than that of the control HbS (the solubility increased from a control value of 16 g/dl to 20·5 g/dl). Though the solubility of the Di-GEE-HbS was increased by nearly 25%, the concentration of the protein in the gel was still 45 g/dl, which is the same as that for the control HbS sample. This suggests a normal fiber structure is maintained even after the Glu 43(β) of HbS is derivatized. The increased solubility of Di-GEE-HbS, thus suggests the participation of the γ-carboxylate of Glu 43(β) in the polymerization process.

Discussion

The isolation of Di-GEE-HbS is consistent with the high chemical reactivity of the γ-carboxylate of Glu 43(β) (13, 14 and 23). However, the βT_5, from the Di-GEE-HbS elutes as two chromatographically distinct components from RPHPLC columns, in much the same way as we had seen earlier in the tryptic map of

unfractionated amidated HbS (13,23). This casts some doubt on the molecular homogeneity of the Di-GEE-HbS that has been isolated. This prompted us to undertake the detailed mass spectral analysis of the two samples of amidated βT_5 from Di-GEE-HbS. The results have confirmed that the hydrolysis of amidated βT_5 during the tryptic digestion is responsible for the anomalous behavior of amidated βT_5 on the RPHPLC columns. These results are also consistent with the earlier observation that when the amidation is carried out with glucosamine (14), the amidated βT_5 elutes as a single molecular component.

All the hydrolytic reaction detected by RPHPLC appears to have occurred only during the tryptic digestion of Di-GEE-HbS. When Di-GEE-HbS is maintained at pH 7·5, and 37 °C for periods up to 3 h, no detectable hydrolysis of the ethyl groups of the amidated protein occurred (CM cellulose chromatographic behavior of Di-GEE-HbS remains unchanged). It may be added here that though hydrolysis of ethyl and methyl ester groups on the side chain carboxyl groups of RNase-A is known to occur around pH 10·5 (24, 25), very little of such a hydrolysis occurs around pH 8·0. This can be considered as a reflection of the fact that, the observed hydrolysis is facilitated once the amidated sample is converted into the tryptic peptides. The observation that the pure amidated βT_5 containing the ethyl ester group, is converted to one without the ethyl ester group, in an overall yield of only 40% even after an incubation for a period of 18 h at 37 °C, demonstrates that this hydrolysis occurs at a very slow rate.

It is not clear whether this hydrolysis is a consequence of some unique structural aspects of the amino acid sequence of βT_5. Though the hydrolysis of the ethyl ester group by itself is not a surprising result, the fact that such a phenomenon has not been observed previously in protein structural studies is surprising. The nucleophiles, glycine ethyl ester and glycine methyl ester have been extensively used in the amidation studies of proteins since the introduction of this procedure for the chemical modification of the carboxyl groups of proteins by Hoare and Koshland (12). The higher resolution of the HPLC itself does not appear to be a factor for detection of this hydrolytic reaction in our studies. Huynh (26), has amidated 5-enolpyruvyl shikimate 3 phosphate synthase with EDC and GEE. However, a hydrolysis of the ethyl ester groups of the amidated protein/peptide has not been detected. Thus the present demonstration of the hydrolysis of the ethyl ester group of GEE from the amidated peptide, should be considered as a cautionary note in the chemical modification studies of carboxylates of proteins using glycine ethyl ester as a nucleophile.

The cooperative binding of oxygen to Di-GEE-HbS and the modulation of oxygen affinity by diphosphoglycerate (DPG), as well as by chloride, clearly suggests that its quaternary structure is nearly the same as that of HbS. However, the solubility of HbS is increased nearly 25% on amidation implicating a structural role for the γ-carboxyl of Glu 43(β) in the polymerization reaction. The concentration of the protein in the gel of Di-GEE-HbS is nearly the same as that of control HbS sample (45 g/dl). These results reflect the overall similarity in the polymer structure of Di-GEE-HbS to that formed from the unmodified HbS.

Glu 43(β) is a part of the CD region of β-chain that makes a portion of the heme pocket. However Glu 43(β) itself contributes little to the organization of this hydrophobic pocket that stabilizes heme in its position. The hydrophilic side chain of Glu 43(β) extends towards the surface of the hemoglobin molecule. Thus the

modification of γ-carboxylate of Glu 43(β) is unlikely to affect the heme pocket and the stability of the molecule. The limited influence of amidation on the overall quaternary structure of HbS is consistent with these structural aspects of the protein. Two mutations of Glu 43(β) have been reported. In Hb Hoshida, Glu 43(β) is mutated to Gln, (27) and in Hb G Galveston/Hb G Port Arthur/Hb G Texas Glu 43(β) is mutated to Ala (28). Both are stable and have oxygen affinities comparable to that of Hb. Thus Di-GEE-HbS is comparable to these mutant forms of Hb in terms of its structure and stability.

Fermi and Perutz (29) have indicated that Glu 43(β) could form a salt bridge with Arg 92(α). Thus mutation of Arg 92(α), could perturb the microenvironment of Glu 43(β). Two mutant forms of Hb, with a mutation of Arg 92(α) are known, namely Hb Chesapeake and Hb Capetown. In Chesapeake Arg 92(α) is mutated to a Leu residue, whereas in Hb Capetown it is replaced by a glutamine residue. The perturbation of the Glu 43(β) microenvironment in Hb Chesapeake results in an increased oxygen affinity for the protein and the loss of cooperativity ($n = 1{\cdot}3$). Hb Capetown also shows an increased oxygen affinity. However, the increase in the oxygen affinity in the case of Hb Capetown is not as large as that of Hb Chesapeake. Besides the cooperativity of Hb Capetown is normal, unlike that of Hb Chesapeake. A 2-fold increase in oxygen affinity of Hb Capetown compared to that of HbA was observed, whereas that of Hb Chesapeake is increased by about eightfold (30). The cooperativity of Di-GEE-HbS is normal, and the oxygen affinity of Di-GEE-HbS is increased only slightly suggesting that the perturbation at the microenvironment may be comparable to that occurring in Hb Capetown.

The intermolecular contact regions of deoxy HbS that stabilizes the HbS fiber have been broadly grouped into three classes (a): inter double strand (b) intra double strand axial and (c) intra double strand lateral (10, 11, 31 and 32). Edelstein (33) was the first one to propose the Glu 43(β) as an inter double strand contact residue based on his model building studies. Subsequently Padlan and Love (10, 11) have investigated the possible molecular interactions in the deoxy HbS fiber, and have suggested the molecular interactions of Glu 43(β) with Ala 53(α) and Glu 54(α). Watowich *et al.* (31) have synthesized a model for the structure of clinically relevant HbS fiber by combining the X-ray crystallographic coordinates of HbS molecules (10, 11) with the three-dimensional reconstruction of electron micrographs of HbS fibers. In their model, the inter double strand contact region of β chain appears to be concentrated along the helices D and E; and turns CD and FG. However the mutations of this region of β chain for which the influence on polymerization have been investigated, has no influence on the polymerization, i.e., an apparent discrepancy exists between the model and the solution studies (32). However, by and large, only the influence of the mutation on the *trans* position could be investigated. The exception is the residue Pro 58 for which the influence in the *cis* position has also been investigated. However, it also has no influence. Amidation of the Glu 43(β), is the first perturbation of this region that has shown a solubilizing influence.

An aspect of considerable interest, is the high reactivity of Glu 43(β) for amidation. This high reactivity is not specific for HbS, HbA also reacts in nearly the same fashion (Khandke and Acharya, unpublished results). The reactivity of Glu 43(β) is not unique for the tetrameric structure. Glu 43(β) exhibits a high reactivity even in the isolated β^s chain (23). The higher reactivity of the γ-carboxyl group Glu 43(β) for amidation at pH 6·0 reflects a higher propensity of this carboxyl to be in the protonated

form. The pK_a of this γ-carboxyl has been estimated to be 6·35 (Rao and Acharya, manuscript in preparation). Though the significance of higher pK_a of γ-carboxyl of Glu 43(β) or the structural basis for this higher reactivity is not clear at this stage, a study of the reactivity (or changes of it) of this carboxyl in the deoxy conformation and during the early phases of polymerization should be very informative. The high reactivity of Glu 43(β) should certainly prove useful to introduce other 'structural probes' into this contact region for monitoring the conformational aspects during polymerization. Photo affinity label could also be introduced at this contact site, and this could be used to map the complementary site of this contact region by the cross linking approach.

One of the inter double strand intermolecular contact residues that has been investigated in great detail is Asp 47(α). Mutation of this residue to His as in Hb Sealey, increased the solubility of HbS. Besides the gels from such a molecular variant of HbS is 'soft' and has an altered fiber structure (34, 35). Asp 47(α) is in the CD region of α-chain, in much the same way as Glu 43(β) is a residue of CD region of β chain. However the concentration of Hb in the polymer of Di-GEE-HbS is nearly the same as the control, and this suggests a normal fiber structure. In view of the fact that Glu 43(β) and Asp 47(α) represent the inter double strand contact residues, it should be of interest to generate molecular variants of HbS with these two perturbations, and establish the synergy and/or additivity (36) of the solubilizing influence of two inter double strand contact regions.

Acknowledgements

This research is supported by NIH Grant HL-27183, HL38655, and Grant-in-Aid from the American Heart Association, New York City Affiliate. ASA was an Established Fellow of the American Heart Association, New York City Affiliate. The authors wish to thank Dr. Lennette J. Benjamin for the supply of blood from patients with sickle cell anemia. The technical assistance of Youngnan J. Cho and Leslie Sussman are greatly acknowledged. We thank Dr. R. E. Benesch for her encouragement in the equilibrium measurement of the solubility and S. Kwong for her help in these measurements. Protein sequence analysis was performed by the Rockefeller University Protein Sequencing Facility (supported in part by funds provided by the U. S. Research Office for the purchase of the equipment). The mass spectrometric measurements were made by the Rockefeller University Mass Spectrometer-Biotechnology Research Resource supported by the Division of Research Resources, NIH (RR-00862).

References

1. Dean J. & Schechter A. N. (1978) *N. Engl. J. Med.* **299**, 752–763.
2. Dean J. & Schechter A. N. (1978) *N. Engl. J. Med.* **299**, 807–811.
3. Dean, J. & Schechter, A. N. (1978) *N. Engl. J. Med.* **299**, 863–870.
4. Nagel, R. L., Johnson, J., Bookchin, R. M., Garel, M. C., Rosa, J., Schiliro, G., Wajeman, M., Labie, D., Moo-Penn, W. & Castro, O. (1980) *Nature (London)* **283**, 832–834.
5. Baudin-Cheih, V., Pagnier, J., Marden, M., Bohn, B., Kister, J., Schaad, O., Edelstein, S. J. & Poyart, C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1845–1849.

6. Adachi, K., Rapport, E., Eck. H. S., Konitzer, P., Sims, J. Y. & Sussey, S. (1990) Abstracts, 15th Annual Meeting of National Sickle Cell Center, pp. 2.
7. Murayama, M. (1966) *Science* **153**, 145–149.
8. Bookchin, R. M. & Nagel, R. L. (1973) in *Sickle Cell Disease*, ed. Abramson, H., Bertles, J. F., & Weathers, D. L. (C. V. Mosby. Co., St. Louis).
9. Benesch, R. E., Kwong, S., Benesch, R. & Edalji, R. (1977) *Nature (London)* **296**, 772–775.
10. Padlan, E. A. & Love, W. E. (1985) *J. Biol. Chem.* **260**, 8272–8279.
11. Padlan, E. A. & Love, W. E. (1985) *J. Biol. Chem.* **260**, 8280–8291.
12. Hoare, D. G. & Koshland, D. E. (1967) *J. Biol. Chem.* **242**, 2447–2453.
13. Seetharam, R., Manning, J. M. & Acharya, A. S. (1983) *J. Biol. Chem.* **258**, 14810–14815.
14. Acharya, A. S. & Seetharam, R. (1985) *Biochemistry* **24**, 4885–4890.
15. Acharya, A. S. & Manning, J. M. (1980) *J. Biol. Chem.* **255**, 1406–1412.
16. Chait, B. T., Agosta, W. C. & Field, F. H. (1981) *Int. J. Mass Spectrom. Ion Phys.* **39**, 339–366.
17. Chait, B. T., Gisin, B. F. & Field, F. H. (1982) *J. Am. Chem. Soc.* **104**, 5157–5162.
18. Chait, B. T. & Field, F. H. (1986) *Biochem. Biophys. Res. Commun.* **134**, 420–426.
19. Benesch, R. E., Edalji, R., Kwong, S. & Benesch, R. (1978) *Anal. Biochem.* **89**, 162–173.
20. Benesch, R. E., Kwong, S., Edalji, R. & Benesch, R. (1979) *J. Biol. Chem.* **254**, 8169–8172.
21. Nigen, A. M., Nijikan, N., Lee, C. K. & Manning, J. M. (1974) *J. Biol. Chem.* **249**, 6611–6616.
22. Van Assendelft, O. W. (1970) *Spectrophotometry of Hemoglobin Derivatives* (Royal Vangorium Assen, The Netherlands).
23. Acharya, A. S. & Khandke, L. (1989) *J. Prot. Chem.* **8**, 231–237.
24. Acharya, A. S., Manjula, B. N. & Vithayathil, P. J. (1975) *Biochem. J.* **173**, 821–830.
25. Broomfield, C. A., Riehm, J. P. & Scheraga, H. A. (1965) *Biochemistry* **4**, 751–759.
26. Huynh, G. K. (1989) *J. Biol. Chem.* **263**, 11631–11635.
27. Iuchi, I., Veda, S., Hidaka, K. & Shibata, S. (1978) *Hemoglobin* **2**, 235–247.
28. Bowman, B. H., Oliver, C. P., Barnett, D. R. & Cunningham, J. R. (1984) *Blood* **23**, 193–199.
29. Fermi, G. & Perutz, M. F. (1981) in *Hemoglobin and Myoglobin Atlas of Molecular Structures in Biology*, eds. Phillips, D. C. & Richards, F. M. (Clarendon Press, Oxford, England), p. 101.
30. Nagel, R. L., Gibson, Q. H. & Jenkins, T. (1971) *J. Mol. Biol.* **58**, 643–650.
31. Watowich, S. J., Gross, L. J. & Josephs, R. (1989) *J. Mol. Biol.* **209**, 821–828.
32. Wishner, B. C., Ward, K. B., Lattman, E. E. & Love, W. E. (1975) *J. Mol. Biol.* **98**, 179–194.
33. Edelstein, S. J. (1981) *J. Mol. Biol.* **150**, 557–575.
34. Benesch, R. E., Kwong, S. & Benesch, R. (1982) *Nature (London)* **299**, 231.
35. Crepeau, R. H., Edelstein, S. J., Szalay, M., Benesch, R. E., Benesch, R., Kwong, S. & Edalji, R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1406–1410.
36. Acharya, A. S., Sussman, L. G. & Seetharam, R. (1985) *J. Prot. Chem.* **4**, 215–225.