Reactivity of Glu-22(β) of Hemoglobin S for Amidation with Glucosamine†

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ABSTRACT: X-ray diffraction analysis of deoxyhemoglobin S crystals has implicated that a number of carboxyl groups of the protein are present at or near the intermolecular contact regions. The reactivity of these or other carboxyl groups of hemoglobin S for the amidation with an amino sugar, i.e., glucosamine, and the influence of amidation on the oxygen affinity and polymerization have been investigated. Reaction of oxyhemoglobin S at pH 6.0 and 23 °C with 20 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and 100 mM [^{3}H]glucosamine for 1 h resulted in an incorporation of nearly two residues of glucosamine per tetramer. The amidation was very specific for the carboxyl groups of globin; the glucosamine was not incorporated into the heme carboxyls. Derivatization of hemoglobin S by glucosamine increased the O_2 affinity of the protein but had no influence on either the Hill coefficient or the Bohr effect. Amidation by glucosamine also increased the solubility of deoxyhemoglobin S by about 55%. Tryptic peptide mapping of the modified hemoglobin S indicated that the peptides β-T_3 and β-T_5 contained the glucosamine incorporated into the protein. Sequence analysis of glucosamine-modified β-T_3 and β-T_5 demonstrated that the γ-carboxyl groups of Glu-22 and Glu-43, respectively, had been derivatized with glucosamine. The residue Glu-43(β) shows a high selectivity toward glycine ethyl ester also, whereas Glu-22(β) is not reactive toward this amine. The results demonstrate that the selectivity of amidation by glucosamine is distinct from that of glycine ethyl ester and is apparently related to the differences in propensity of the carbodiimide-activated γ-carboxyl groups of Glu-22(β) and Glu-43(β) to undergo aminolysis with these two amines.

The presence of valine at the sixth position in the β-chain of hemoglobin S (HbS)[1] (Ingram, 1956), as opposed to the glutamic acid in that of HbA, is responsible for the polymerization of the protein in its deoxy conformation [reviewed by Dean & Schechter (1978a–c)]. Analysis of the deoxy-HbS crystals has shown that the basic unit of the deoxy-HbS polymer is a double-stranded fiber: each strand is formed of tetrameric protein molecules. In these double-stranded fibers, the Val-6(β) of one HbS molecule interacts with the hydrophobic side chains lying between the E and F helices of the β-chain of an adjacent tetramer (Wishner et al., 1975; Love et al., 1978, 1979). Besides the primary site of interaction involving the Val-6(β), a number of other noncovalent intermolecular interactions involving specific functional groups of the protein (quinary interactions; Edelstein, 1980) have been suggested to contribute in a cooperative way to the polymerization process as well as to the stability of the polymerized gel. Analysis of the deoxy-HbS crystals has indicated that the carboxyl groups of Glu-22(β), Asp-73(β), Glu-121(β), and Glu-23(α) are present at or near one or more of the intermolecular contact regions of deoxy-HbS gels (Wishner et al., 1975).

With an overall objective of determining the reactivity of the carboxyl groups of HbS and the role of the carboxyl groups implicated to be at or near the intermolecular contact regions in the polymerization of deoxy-HbS, we have undertaken a study of the amidation of carboxyl groups of the protein (Seetharam et al., 1983). In our initial studies, a water-soluble carbodiimide, EDC, was used to activate the carboxyl groups, and GEE was used for the aminolysis of the activated carboxyl groups, resulting in the amidation of the protein. The γ-carboxyl group of Glu-43(β) was modified with high selectivity. However, none of the carboxyl groups implicated by the crystallographic analysis to be near the intermolecular contact regions showed any detectable reactivity. The high selectivity of Glu-43(β) for amidation with glycine ethyl ester is a reflection either of the propensity of the γ-carboxyl group of Glu-43 to be activated by EDC or of the preferential aminolysis of the activated carboxyl of Glu-43 by this amine. The latter explanation for the selectivity implies that there may be other carboxyl groups of HbS that are activated by EDC but do not undergo aminolysis with GEE and hence are hydrolyzed. An answer to some of these questions could be obtained if the aminolysis of the activated carboxyl groups is carried out by using other amines. Toward this objective, in this study we have investigated the amidation of carboxyl groups (activation carried out with EDC as has been done previously) with an amino sugar, i.e., glucosamine, and the results are presented here.

Materials and Methods

The preparation of cell lysates to obtain stripped HbS, the procedure for modification of carboxyl groups of HbS, and other procedures for the chemical analysis of derivatization have been described earlier (Seetharam et al., 1983). D-Glucosamine is from Sigma Chemical Co., and ^{3}H-labeled glucosamine is from New England Nuclear. The solubility measurement of glucosamine-derivatized HbS was carried out as described by Benesch et al. (1979).

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[1] Abbreviations: Hb, hemoglobin; HbS, hemoglobin S; HbA, hemoglobin A; oxy-HbA, oxygenated HbA; deoxy-HbA, deoxygenated HbA; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GEE, glycine ethyl ester; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)-propane-1,3-diol; HPLC, high-performance liquid chromatography; RPHPLC, reverse-phase HPLC; PTH, phenylthiohydantoin.

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Amidation of HbS with Glucosamine. Carboxylate modification was carried out essentially as described earlier for amidation of HbS by using glycine ethyl ester (Seetharam et al., 1983). Stripped oxy-HbS was dialyzed against 0.1 M KCl, adjusted to pH 6.0. The HbS sample (0.6 mM tetramer) was incubated with 100 mM [^{3}H]glucosamine at 23 °C in a vessel attached to a Radiometer pH stat. The coupling reaction was initiated by adding a concentrated solution of EDC in 0.1 M KCl, pH 6.0, so that a final concentration of 20 mM EDC was obtained. The reaction mixture was maintained at pH 6.0 by automatic titration with 0.01 M HCl. After 1 h, the reaction mixture was passed through a column of Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 6.8, to separate the modified HbS from the excess reagents. The protein thus isolated was radioactive and was referred to as amidated HbS and used for the studies described. No attempt has been made in this study to purify the derivatized HbS.

Amino Acid Sequence Analysis of Glucosamine-Modified Tryptic Peptides. Edman degradation of the modified peptides was carried out with about 10 nmol of the peptide by using a Beckman 890B sequencer equipped with a Sequemat autoconverter. A modified Quadrol program (Beckman 011576) was used. Polybrene was used as a carrier to facilitate better retention of the peptide during sequencing (Klapper et al., 1978). The phenylthiohydantoin (PTH) amino acids were identified and quantitated by HPLC (Zimmerman et al., 1977) on a Du Pont Zorbax ODS column using a Hewlett-Packard Model 1084B instrument. An aliquot of the PTH-amino acid from each cycle was also counted for the presence of ^{3}H label at each step, and the counts recovered were normalized on the basis of the repetitive yield of each cycle.

Results

Modification of Carboxyl Groups of HbS by Glucosamine and Its Selectivity to Protein Carboxyls. Incubation of oxy-HbS at pH 6.0 with 20 mM EDC at 23 °C in the presence of 100 mM [^{3}H]glucosamine resulted in the incorporation of the ^{3}H label into the protein, suggesting the derivatization of the protein. Incubation for 1 h generated a product containing, on an average, 2 mol of glucosamine/mol of tetramer. The level of incorporation obtained with glucosamine after 1 h of incubation is lower than that obtained when glycine ethyl ester is used as the amine component (Seetharam et al., 1983) for the amidation reaction (nearly 3 mol per tetramer).

The presence of carboxyl groups in the heme moiety made it necessary to determine whether the glucosamine-derivatized carboxyl groups are those of heme or of globin. A preparation of glucosamine-modified HbS was subjected to acid–acetone precipitation (Rossi-Fanelli et al., 1964) to separate heme from the globin, and the amount of the ^{3}H label associated with globin (precipitate) and heme (supernatant) was estimated. More than 95% of the ^{3}H label of derivatized HbS was associated with globin. Thus, the derivatization appears to be selective for the carboxyl group of globin. Little, if any, derivatization of the heme carboxyl appears to have taken place.

Purification and Identification of the Carboxyl Group Modified Tryptic Peptides. The tryptic digest of globin obtained from glucosamine-reacted HbS was chromatographed on a reverse-phase ODS-3 column (Figure 1A). The tryptic digest contains three radioactive components, designated a, b, and c, respectively. The recovery of radioactivity from the reverse-phase column is nearly 95%. About 10% of the radioactivity (component a) eluted unadsorbed from the reverse-phase column. The rest of the label eluted as two peaks, one around 48 min (component b, ~35%) and the other around 72 min (component c, ~50% of the label), respectively.

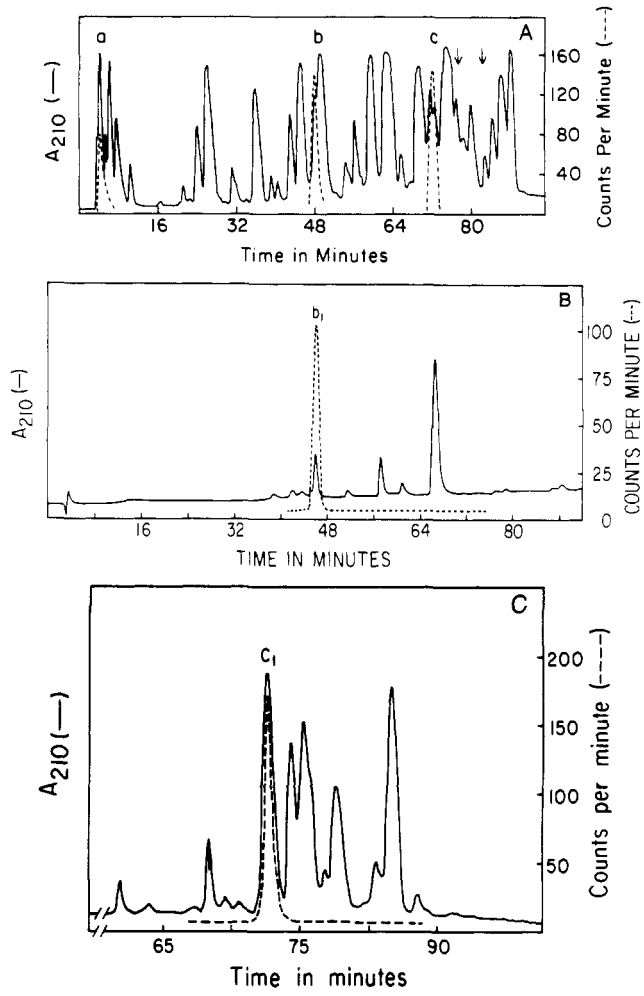

FIGURE 1: Analysis of the tryptic peptides of globin from derivatized HbS by reverse-phase HPLC. (A) Analysis of total digest. The tryptic digest was taken in about 250 μL of 5% acetonitrile containing 0.1% trifluoroacetic acid (Acharya et al., 1983) and loaded onto the Partisil-10 ODS-3 column (4.6 × 250 mm). The peptides were eluted with a linear gradient of 5–50% acetonitrile (70 g each) containing 0.1% trifluoroacetic acid (Seetharam et al., 1983). The column was eluted at a flow rate of 60 mL/h, and 1-mL fractions were collected. The effluent was monitored at 210 nm. Aliquots (100 μL) of each fraction were used for measuring the radioactivity. β-T_5 modified at Glu-43(β) by GEE eluted at two positions; this possibly reflects the oxidation of Met-55 of β-chain (Seetharam et al., 1983), and the positions are marked by vertical arrows (↓). (B) Rechromatography of peptide b by reverse-phase HPLC. Peptide b isolated by lyophilization of pooled fractions (panel A) was taken in 200 μL of 10 mM ammonium acetate, pH 6.01, and loaded onto a Partisil-10 ODS-3 column preequilibrated with the same buffer. The peptides were eluted with a linear gradient of 0% acetonitrile, 0.01 M ammonium acetate, to 40% acetonitrile, 0.06 M ammonium acetate. The radioactive peptide (b_1) was isolated and subjected to amino acid analysis as well as the sequence analysis. (C) Rechromatography of peptide c by reverse-phase HPLC. The conditions used for chromatography are the same as in panel B. The radioactive peptide c_1 was isolated and subjected to amino acid analysis and sequence analysis.

The positions at which components b and c elute from the reverse-phase column are distinct from those seen with the tryptic peptide maps of GEE-derivatized HbS. (The position of β-T_5 modified by GEE at Glu-43 is marked in the figure.)[2]

The radioactive component b was rechromatographed on an ODS-3 column at pH 6.1 by using the ammonium acetate–acetonitrile system (Seetharam et al., 1983). One radioactive component designated b_1 separated from three un-

[2] The β-T_5 modified at Glu-43(β) by GEE eluted at two positions. This probably reflects the partial oxidation of Met-55 of β-chain (Seetharam et al., 1983).

Table I: Recovery of ^{3}H-Labeled Glucosamine during Sequencing of Peptide b_1

Edman degradation cycle	residue identification: amino acid[a]	residue no.	helix notation	^{3}H label (cpm)[b]
1	Val	18	A15	8
2	Asn	19	B1	8
3	Val	20	B2	8
4	Asp	21	B3	8
5	Glu	22	B4	165
6	Val	23	B5	22
7	Gly	24	B6	8
8	Gly	25	B6	8
9	Glu	26	B8	8
10	Ala	27	B9	8

[a] Identified as the PTH-amino acid. At the fifth cycle no PTH-amino acid was detected by the HPLC, but radioactivity was released for the peptide at this cycle, demonstrating that Glu is present as the glucosamine-modified residue. [b] An aliquot of PTH-amino acid from each cycle was counted for radioactivity, and the counts recovered are normalized on the basis of the repetitive yield of each cycle. The numbers given are the normalized values.

Table II: Recovery of ^{3}H-Labeled Glucosamine during Sequencing of Peptide c_1

Edman degradation cycle	residue identification: amino acid[a]	residue no.	helix notation	^{3}H label (cpm)[b]
1	Phe	41	C7	10
2	Phe	42	CD1	10
3	Glu	43	CD2	236
4	Ser	44	CD3	70
5	Phe	45	CD4	39
6	Gly	46	CD5	20
7	Asp	47	CD6	10
8	Leu	48	CD7	10
9	Ser	49	CD8	10
10	Thr	50	D1	10
11	Pro	51	D2	10
12	Asp	52	D3	10
13	Ala	53	D4	10
14	Val	54	D5	10
15	Met	55	D6	10

[a] Identified as the PTH-amino acid. At the third cycle no PTH-amino acid could be detected by HPLC, but radioactivity was released at this third cycle, indicating that Glu-43(β) is present as the glucosamine-modified residue in the protein. [b] An aliquot of PTH-amino acid from each cycle was counted for radioactivity, and the counts recovered are normalized on the basis of the repetitive yield of each cycle. The numbers given are the normalized values.

labeled peptides (Figure 1B). The amino acid composition of this peptide agreed well with that of β-T_3 (data not shown), identifying this radioactive tryptic peptide as corresponding to the segment 18–30 of the β-chain. This identification is further confirmed by the amino acid sequence analysis (see below).

The component c was also rechromatographed on ODS-3 column, at pH 6.1, by using the ammonium acetate–acetonitrile gradient. The radioactive component is well separated from the nonradioactive component and is designated as c_1 (Figure 1C). The radioactive peptide was isolated, and its amino acid composition was determined. From the amino acid composition (data not shown) of this peptide, it is clear that the radioactive peptide is derivatized β-T_5 representing the segment 41–59 of the β-chain. The sequence analysis (see below) confirmed this identification.

Identification of Carboxyl Group Derivatized in Peptide b_1. Amino acid analysis of peptide b (Table I) identified this peptide as the derivatized β-T_3, corresponding to residues 18–30 of the β-chain. This peptide contains three carboxyl groups, the β-carboxyl group of Asp-21 and the two γ-carboxyl groups of Glu-22 and Glu-26. This peptide was subjected to Edman degradation, and the ^{3}H label released in each of the Edman degradation cycles was determined (Table I). Almost all of the radioactivity of the peptide was released in the fifth cycle, thus identifying the carboxyl group of Glu-22(β) as the residue modified in this peptide. As pointed out earlier, this peptide, b_1, accounts for nearly 35% of the derivatization by glucosamine. Thus, it is clear that the carboxyl of Glu-22(β) of HbS is reactive toward glucosamine, in the presence of EDC, whereas when glycine ethyl ester is used as the amine component, modification of this carboxyl group does not appear to occur.

Identification of Carboxyl Group Derivatized in Peptide c_1. Amino acid composition of peptide c_1 identified it as β-T_5, corresponding to the sequence 41–59 of the β-chain. This peptide also contains three carboxyl groups, namely, the γ-carboxyl group of Glu-43 and the two β-carboxyl groups of Asp-47 and Asp-52. The release of ^{3}H label (glucosamine) from the peptide during each of the 15 cycles of the Edman degradation is shown in Table II. Most of the radioactivity was released in the third cycle. These Edman degradation studies demonstrate that Glu-43(β) is the modified residue in β-T_5. Besides, no PTH-Glu could be detected by RPHPLC in the third cycle, clearly demonstrating the complete absence

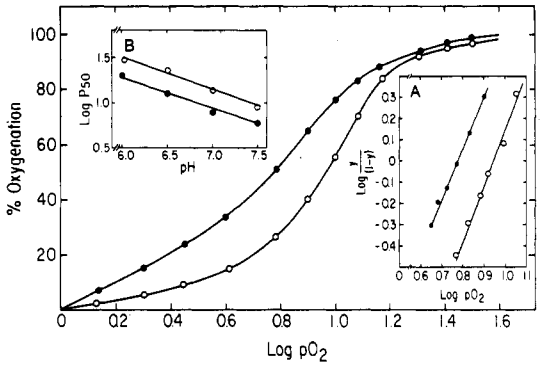

FIGURE 2: Oxygen equilibrium curve of HbS amidated with glucosamine and unmodified HbS. The oxygen equilibrium curves were recorded with an Aminco Hem-o-scan at 37 °C, pH 6.8. Inset A shows the Hill plots for (○) HbS and (●) glucosamine-reacted HbS. Inset B shows the Bohr effect of HbS and HbS amidated with glucosamine: pH dependence of oxygen affinity for (○) HbS and (●) HbS amidated with glucosamine.

of unmodified glutamic acid at the third position of peptide c_1. This reflects the high selectivity of the derivatization by glucosamine.

The identity of the peptides that elute unadsorbed during HPLC is not clear at present. In the tryptic peptide map of unmodified HbS the two small peptides that elute early on the ODS-3 column are the two carboxyl-terminal tryptic peptides β-T_{15} and α-T_{14}. It is conceivable that the small amount of label (~10%) eluting early may be due to the derivatization at one or both of the terminal carboxyl groups of the chains.

Influence of Carboxylate Derivatization by Glucosamine on the Oxygen Affinity of HbS. Since the amidation of HbS by glucosamine shows significant differences in terms of selectivity and the extent of amidation compared to that seen on amidation with GEE (Seetharam et al., 1983), it was of interest to determine the influence of amidation of the carboxyl groups by glucosamine on the functional properties of the protein. The oxygen affinity of the glucosamine-derivatized HbS was higher than that of the unmodified protein (Figure 2). The P_{50} decreased from a value of 8 for the native protein to a value around 6 for the derivatized protein at pH 7.4. This derivatization does not influence the Hill coefficient; the co-

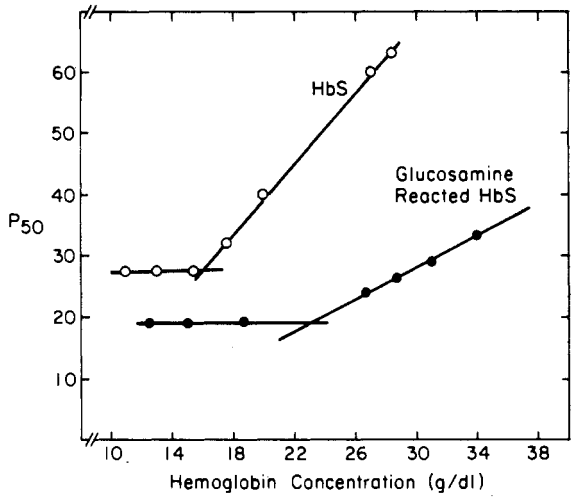

FIGURE 3: Relation between oxygen affinity and Hb concentration of HbS (○) and glucosamine-reacted HbS (●).

operativity remained essentially unaltered (n = 2.7) (Figure 2, inset A).

The oxygen affinity of the glucosamine-reacted HbS has also been studied as a function of pH (Figure 2, inset B) to determine whether the carboxyl groups derivatized contribute to the Bohr effect of the protein. The plot of log P_{50} of the glucosamine-reacted HbS against pH (6.0–8.0) runs parallel with that of the native protein, suggesting that those carboxyl groups of HbS amidated with glucosamine do not contribute to the Bohr effect.

Influence of Amidation by Glucosamine on the Polymerization of Deoxy-HbS. The polymerization behavior of glucosamine-reacted HbS has been studied by the oxygen affinity method of Benesch et al. (1978), and the results are presented in Figure 3. The concentration of protein at which the onset of polymerization occurs for the glucosamine-reacted HbS (24 g/dL) is higher than that for underivatized HbS (~16 g/dL). Thus, although the extent of amidation of HbS by glucosamine is lower than that obtained with GEE and the selectivity of amidation is different, the propensity of deoxy-HbS to polymerize is decreased to nearly the same extent.

The solubility of the glucosamine-modified deoxy-HbS has also been measured by the ultracentrifugation method as described by Benesch et al. (1979). The C_{sat} for the glucosamine-modified HbS at 30 °C is around 28 g/dL, compared with the control value of 18 g/dL. This clearly establishes that the solubilizing influence of amidation of the carboxyl groups of HbS by glucosamine is due to a reduction in the propensity of deoxy-HbS to polymerize.

DISCUSSION

The results presented in this paper demonstrate that the γ-carboxyl groups of Glu-22(β) and Glu-43(β) of HbS are activated by EDC at pH 6.0. The aminolysis of these two activated carboxyl groups by the amino sugar glucosamine proceeds to nearly the same degree. On the other hand, glycine ethyl ester shows a high selectivity for the aminolysis of the activated Glu-43(β) (Table III). Some aspects of the structure around Glu-43(β) may have relevance for this preferential aminolysis of the activated Glu-43(β) by glycine ethyl ester. Small differences in the hydrophilicity of glucosamine and glycine ethyl ester may also have contributed to the observed differences in the selectivity of amidation. It is conceivable that the ethyl groups of glycine ethyl ester may give this amine a weakly hydrophobic character.

Amidation of the carboxyl groups of oxy-HbS by glycine ethyl ester as well as by glucosamine clearly demonstrates that

Table III: Influence of Amidation of HbS on Its Functional Properties

amine used for amidation[a]	no. of carboxyl groups amidated per tetramer		O_2 affinity[b]		solubility[c] (g/dL)
	Glu-22(β)	Glu-43(β)	P_{50}	n value	
			27	2.7	16
glucosamine	0.7	1.0	20	2.7	24
glycine ethyl ester[d]		2.0	12	2.7	24

[a] Amidation was carried out at pH 6.0, 23 °C, for 1 h with 20 mM EDC and 100 mM amine. [b] Oxygen affinity values given at pH 6.9 and 37 °C. [c] Determined by the oxygen affinity method of Benesch et al. (1978). [d] These values are taken from our previous publication Seetharam et al. (1983).

the γ-carboxyl group of Glu-43(β) of the protein is one of the more reactive carboxyl groups of the protein. A simple explanation for the reactivity of Glu-43(β) is that the pK_a of this carboxyl group is higher than that of the other carboxyl groups. It has been pointed out that the carbodiimide activation of a carboxyl group involves the protonated form (Kurzer & Dougaghi-Zaceh, 1967). It may be added here that Glu-43(β) is at the $\alpha_1\beta_2$ interface. A number of hydrophobic amino acid residues, namely, Phe-41, Phe-42, and Phe-45, present in the microenvironment of Glu-43(β) should contribute to a decrease in the dielectric constant of the microenvironment of this carboxyl group. This is likely to decrease the propensity of the carboxyl group of Glu-43(β) to ionize.

The fact that Glu-22(β) is amidated by glucosamine demonstrates that this γ-carboxyl group is also activated by EDC. However, glycine ethyl ester does not appear to react with this activated carboxyl group to any significant extent. Glu-22(β) is the fourth residue in the B helix of the β-chain, it is freely exposed to the solvent in the crystal structure, and there are three histidine residues within 10 Å (Fermi & Perutz, 1981). The other stereochemical features around Glu-22(β) that may be contributory to either its carbodiimide activation and/or the subsequent aminolysis by glucosamine are not readily apparent. However, it should be pointed out here that though glucosamine reacts with Glu-22(β) and Glu-43(β) to about the same degree, the amidation of Glu-43(β) by glucosamine is only about 50% of that which occurred with glycine ethyl ester (Table III). Thus, the differential selectivity of glucosamine for amidation compared to that of glycine ethyl ester is apparently due to a combination of two aspects of the amidation reaction, namely, the increased aminolysis of activated Glu-22(β) and the decreased aminolysis of activated Glu-43(β) by this amino sugar.

The differential selectivity in the amidation of the carboxyl groups of HbS by the two amines is also reflected in their influence on the O_2 affinity of the amidated protein (Table III). Amidation of the protein with glycine ethyl ester increased the oxygen affinity. However, the increase on amidation by glucosamine is smaller than that seen earlier with glycine ethyl ester. The increase in the O_2 affinity appears to be related to the extent of amidation of Glu-43(β) (Table III). It has been pointed out by Fermi & Perutz (1981) that Glu-43(β) (CD2β_2) can form a salt bridge with Arg-92(α) (FG4α_1), even though the electron density maps do not show it clearly. It is of interest to note here that Charache et al. (1966) have shown that mutation of Arg-92(α) to Leu (Hb Chesapeake) results in an increase in the oxygen affinity and a decrease in cooperativity of the protein (Clegg et al., 1966). Hemoglobin Capetown (Greer, 1971), which has its Arg-92(α) mutated to Glu also, has an increased oxygen affinity while the cooperativity is almost normal. Judging by the raised

oxygen affinity of Hb Chesapeake, Fermi & Perutz (1981) have calculated that the FG4(92)α_1–CD2(43)β_2 salt bridge could contribute about 2 kcal of stabilization energy to the T structure. The amidation of Glu-43(β) should also result in a similar structural perturbation, since the modification eliminates the potential of Glu-43(β) to form the salt bridge with Arg-92(α).

The close correlation of the increase in O_2 affinity of Hb with the extent of amidation at Glu-43(β) (Table III) also suggests that amidation of Glu-22(β) has very little influence on the oxygen affinity of the protein. The external position of this residue and the O_2 affinity of many mutant Hbs are consistent with this interpretation. A number of mutations at this site of the protein have been documented (Villa et al., 1967; Blackwell et al., 1969; Bowman et al., 1967; Rahbar, 1973) and appear to have limited influence on the O_2 affinity of the protein. In fact, this region has been pointed out to be one of the frequently mutated areas of human Hb (Marengo-Rowe et al., 1968).

Amidation of HbS with glucosamine increases the solubility of the deoxy protein. The solubility of amidated HbS is close to that of a 1:1 mixture of HbS and HbA. This increase in solubility appears to be a direct consequence of amidation of Glu-22(β) and Glu-43(β) rather than to be due to the presence of species of HbS with random amidation. The detailed structural analysis of amidated HbS has shown that the amidation of Glu-22(β) and Glu-43(β) accounts for nearly 90% of glucosamine incorporated into the protein. If the remaining 10% of glucosamine is randomly distributed on a number of carboxyl groups of HbS, all of which increase the solubility of deoxy protein to the level of HbA, this is not enough to account for the observed increase in the solubility on amidation. It may be added here that a sample of HbS containing 25% HbA has a solubility of 20 g/dL, compared to a control value of 17.5 g/dL for pure HbS (Benesch et al., 1978). Thus, it is clear that the apparent increase in solubility of HbS on amidation with glucosamine could not be due to the low level of HbS species with random amidation.

The amidation of the γ-carboxyl group of Glu-22(β) by glucosamine is of considerable interest in terms of understanding the quinary interactions (Edelstein, 1980) of deoxy-HbS polymer. The crystallographic analysis of Wishner et al. (1975) has located the Glu-22(β) as part of one of the intermolecular contact regions of deoxy-HbS polymer. The studies of Nagel et al. (1979) have indeed demonstrated that the destabilization of this intermolecular contact site by mutation of Glu-22(β) to Ala increases the solubility of deoxy-HbS. Crystallographic analysis has also demonstrated that in the deoxy-HbS crystals Glu-22(β) of one tetramer forms an ionic interaction with the His-20(α) of another tetramer. The demonstration by Rosa and his colleagues (Rhoda et al., 1984) that the mutation of His-20(α) to Glu increases the solubility of deoxy-HbS by about 60–65% is consistent with this interpretation. Thus, the perturbation of this quinary interaction [Glu-22(β)...His-20(α)] by the amidation of Glu-22(β) would be expected to increase the solubility of deoxy-HbS. The results of our study show that though the extent of amidation of Glu-43(β) by glucosamine (Table III) is significantly lower than that seen with glycine ethyl ester, the increase in the solubility of the HbS amidated with either glucosamine or glycine ethyl ester is nearly the same. This strongly suggests the antigelation influence of amidation of Glu-22(β). Though the two samples of amidated HbS have nearly the same solubility, the O_2 affinity of glucosamine-amidated HbS is closer to the normal values than that of glycine ethyl ester amidated HbS. Thus, of the two procedures, amidation by glucosamine is preferable to achieve the desired antipolymerization influence since this reaction can bring about the same increase in the solubility with a concomitant smaller increase in O_2 affinity.

Amidation of carboxyl groups of proteins using glucosamine is expected to be of general interest to protein chemists. Amidation of β- and γ-carboxyl groups of Asp and Glu, respectively, of proteins with glycine ethyl ester results in the formation of an "isopeptide" linkage. On the other hand, a glucosamine-modified carboxyl group is a substituted amide, namely, *N*-glucosylglutamine or *N*-glucosylasparagine. The *N*-glucosylasparagine linkages are a common feature of a number of glycoproteins (Wold, 1981). Thus, amidation using amino sugars could serve as a general procedure for preparing neoglycoproteins (Waniska & Kinsella, 1984). By an appropriate choice of amino sugars, these could be covalently linked to proteins by N-glycosidic linkages.

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Registry No. HbS, 9035-22-7; Glu, 56-86-0; glucosamine, 3416-24-8; oxygen, 7782-44-7.

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Novel Arrangement of Immunoglobulin Variable Domains: X-ray Crystallographic Analysis of the λ-Chain Dimer Bence-Jones Protein Loc†

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ABSTRACT: We have characterized and crystallized a human λI light-chain dimer, Bence-Jones protein Loc, which has variable (V) region antigenic determinants characteristic for the λI subgroup and constant (C) region determinants of the $C_{\lambda I}$ gene Mcg. The crystal structure was determined to 3-Å resolution; the *R* factor is 0.27. The angle formed by the twofold axes of the V and C domains, the "elbow bend", is 97°, the smallest found so far for an antibody fragment. The antigen-binding site formed by the two V domains of the Loc light chain differs significantly from those of other immunoglobulin molecules (light-chain dimers and Fab fragments) for which X-ray crystallographic data are available. Whereas, in other antibody fragments, the V domains are related by a local twofold axis, a local twofold screw axis with a translational component of 3.5 Å relates the V domains in protein Loc. In contrast to the classic antigen binding "pocket" formed by V domain interactions in the previously characterized antibody structures, the V region associations in protein Loc result in a central protrusion in the binding site, with grooves on two sides of the protrusion. The structure of protein Loc indicates that immunoglobulins are physically capable of forming a more diverse spectrum of antigen-binding sites than has been heretofore apparent. Moreover, the unusual protruding nature of the binding site may be analogous to structures required for some anti-idiotypic antibodies. Further, the complementarity-determining residues form parts of two independent grooves. Therefore, the Loc structure might be viewed as a possible model for the T-cell receptor, which is composed of two light-chain-like polypeptides and can simultaneously bind two different proteins.

In recent years it has been determined that antibodies constitute only one element of the immunoglobulin superfamily, which includes the T-cell receptors, the polymeric immunoglobulin receptor, major histocompatibility complex antigens, and perhaps others. Immunoglobulin-type domains form the basic building blocks for all the above molecules. While it was clear from the earlier studies that Bence-Jones proteins (antibody light chains) were an effective model system for the antigen-binding fragment of an antibody molecule, it is now apparent that broader perspective is appropriate. Structures formed by antibody light chains may reveal attributes of any of the members of the immunoglobulin superfamily.

Antibody molecules consist of two heavy and two light chains; the light chains and the amino-terminal half of the heavy chains form the antigen-binding (Fab)[1] fragments.

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