Selectivity in the Modification of the α-Amino Groups of Hemoglobin on Reductive Alkylation with Aliphatic Carbonyl Compounds

INFLUENCE OF DERIVATIZATION ON THE POLYMERIZATION OF HEMOGLOBIN S*

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The reactivity of the α-amino groups of the α- and β-chains of hemoglobin toward reductive alkylation using limiting concentrations of the aliphatic carbonyl compounds, acetaldehyde (ethylation), glyoxylic acid (carboxymethylation), glycolaldehyde (hydroxyethylation), glyceraldehyde (dihydroxypropylation), and dihydroxyacetone (dihydroxyisopropylation) has been investigated. Hemoglobin A reductively ethylated at the α-amino groups eluted on hydroxyacetone (a ketone instead of an aldehyde), the dihydroxyisopropylation occurred at a slower rate and exclusively at Val-1(α). The presence of a negatively charged carboxyl group in the carbonyl component, i.e. glyoxylic acid, made this preferential reaction at Val-1(α) even more pronounced. When the reductive alkylation is carried out with dihydroxyacetone (a ketone instead of an aldehyde), the dihydroxyisopropylation occurred at a slower rate and exclusively at Val-1(β). The ethylation, hydroxyethylation, carboxymethylation, and dihydroxypropylation of the α-amino groups of hemoglobin S increased its solubility from the value of 16 g/dl for the unmodified protein to about 25 g/dl for the modified protein. Thus, the alkylation chains on the α-amino groups on the polymerization have a strong inhibitory influence. In order to determine the influence of the alkylation chains on the α-amino groups of α- and β-chains on polymerization, hybrid hemoglobin S tetramers with hydroxyethylation either at Val-1(α) or at Val-1(β) have been prepared. The solubility of each hybrid is about 26 g/dl. Thus, the hydroxyethyl group either on the α- or the β-chain appears to interfere with the polymerization of deoxygenated HbS to the same degree. The inhibitory influence of the hydroxyethyl chain at Val-1(α) on the polymerization, compared with the lack of such an influence when this α-amino group is modified by cyanate, suggests that a carbamoyl group on Val-1(α) can be accommodated in the intermolecular contact region involving this segment of the molecule without seriously perturbing the molecular fit of this contact region, whereas the hydroxyethyl group cannot be accommodated easily at this site, and hence the inhibition of polymerization.

The present work grew out of our attempts to identify the functional groups of hemoglobin S that are present at the intermolecular contact regions and accessible to chemical manipulation. The ultimate objective of these studies is to develop an antisickling agent of therapeutic value targeted to one or more critical amino acid residues at the intermolecular contact regions of deoxy-HbS. One of the early approaches to inhibit the sickling of the erythrocytes from patients with sickle cell disease was the use of cyanate (Cerami and Manning, 1971). Detailed chemical studies of HbS reacted with cyanate revealed that the major contribution for the inhibition of sickling of erythrocytes is a result of carbamoylation of the α-amino group of α-chain. This derivatization increases the oxygen affinity of HbS, thus perturbing the oxy-HbS ⇄ deoxy-HbS equilibrium, to decrease the concentration of the deoxy-HbS at a given O2 tension. This in turn influences the polymerization of HbS (Nigen et al., 1974). However, this derivatization of HbS at its α-amino group of α-chain has very little direct influence on the polymerization of deoxy-HbS (i.e. it does not increase the minimum geling concentration). On the other hand, the selective carbamoylation of the α-amino group of the β-chain of HbS slightly increased the minimum geling concentration of the protein, i.e. decreased the propensity of the deoxy-HbS to polymerize (Nigen et al., 1974). In contrast to the lack of inhibitory influence of carbamoylation of the α-amino group of α-chain on the polymerization of deoxy-HbS, Benesch et al. (1974) have shown that selective blocking of the α-amino group of

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The abbreviations used are: deoxy-HbS, deoxygenated hemoglobin S; HbS, hemoglobin S; HbA, hemoglobin A; oxy-HbS, oxygenated hemoglobin S; E, ethyl; HE, hydroxyethyl; CM, carboxymethyl; DHF, 2,3-dihydroxypropyl; DHP, 1,3-dihydroxyisopropyl; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ac, acetyl; PBS, phosphate-buffered saline; HMB, hydroxymercuribenzoate.
the α-chain of the protein by pyridoxal sulfate caused a marked increase in the minimum gelling concentration. These results suggest that the influence of substitution of α-amino group on gelation may be strongly dependent on the nature of the blocking groups. In view of the significant difference in the size of the blocking groups (carbamoyl group as opposed to pyridoxal sulfate), it was of interest to introduce small alkyl chains on the α-amino group of HbS to determine whether the inhibitory influence is dependent on the size or the nature of the substituting group introduced. In the present study we have investigated the influence of ethyl, hydroxylethyl, carboxymethyl, dihydroxypropyl, and dihydroxyisopropyl groups at the α-amino groups of HbS on the polymerization behavior of the protein.

The possibility of using reductive alkylation of hemoglobin with aliphatic aldehydes to introduce the alkyl chains on the α-amino groups has been now explored. We have previously shown that reductive alkylation of carbonmonoxyhemoglobin A at pH 7.4, 37 °C with a limiting concentration of glyceraldehyde (Acharya et al., 1983a) resulted in the selective dihydroxypropylation of the α-amino groups of carbonmonoxy-HbA. Reductive alkylation has been subsequently investigated with glycolaldehyde (hydroxyethylation) and the selectivity appears to be nearly the same as that with reductive dihydroxypropylation (Acharya and Sussman, 1983). Thus, this approach of condensation of a carbonyl compound with the α-amino groups of HbA in the presence of sodium cyanoborohydride appears to provide a general procedure to derivatize the α-amino groups of the protein with an alkyl group of choice by appropriate selection of the carbonyl compounds. By employing this procedure in the present study, we have introduced ethyl (acetaldehyde), hydroxylethyl (glycolaldehyde), carboxymethyl (glyoxylic acid), 2,3-dihydroxypropyl (glyceraldehyde), and 1,3-dihydroxyisopropyl (dihydroxyacetone) groups at the amino terminus of HbS to study their influence on gelation.* By changing the carbonyl component from acetaldehyde to glycolaldehyde to glyoxylic acid in the reductive alkylation procedure (Fig. 1), the property of the substituted alkyl group changes from being relatively hydrophobic (ethyl groups) to neutral hydrophilic (hydroxyethyl) to negatively charged hydrophilic (carboxymethyl groups). With glycolaldehyde and dihydroxyacetone, bulkier, neutral hydrophilic 3-carbon alkyl chains are introduced.

MATERIALS AND METHODS

Erythrocytes from normal adults were isolated by centrifugation and washed with carbon monoxide-saturated phosphate-buffered saline (PBS), pH 7.4. Washed cells were lysed, dialyzed first against PBS, and then dialyzed overnight against 50 mM Tris-Ac, pH 8.5. Erythrocytes from normal adults were isolated by centrifugation and washed with carbon monoxide-saturated phosphate-buffered saline (PBS), pH 7.4. Washed cells were lysed, dialyzed first against PBS, and then dialyzed overnight against 50 mM Tris-Ac, pH 8.5. HbA was isolated by chromatographing the dialyzed lysate on DE52 as described earlier (Acharya and Manning, 1980a).

Whole blood from patients homozygous for sickle cell anemia was collected into heparinized tubes by venipuncture. The erythrocytes were isolated by centrifugation and washed with phosphate-buffered saline (pH 7.4), lysed, and then dialyzed extensively against phosphate-buffered saline.

Reductive Alkylation of HbA—Purified HbA (1 mM) in the carbonmonoxy form was dialyzed against phosphate-buffered saline (pH 7.4) and treated with 10 mM aliphatic aldehyde (acetaldehyde, glycolaldehyde, glyoxylic acid, glyceraldehyde, or dihydroxyacetone) in the presence of 20 mM sodium cyanoborohydride (NaCNBH₃) at 37 °C for 30 min. After the incubation period, the excess reagents were removed by gel filtration on a Sephadex G-25 column, equilibrated and eluted with 10 mM phosphate buffer, pH 6.0, or 50 mM Tris-Ac, pH 8.5.

The preparation of p-hydroxymercuribenzoate α- and β-chains of derivatized HbA (Bucci and Fronticelli, 1965; Acharya and Manning, 1980a), the analysis of the tryptic peptides by reverse phase HPLC (Acharya et al., 1983b), and the amino acid analysis were carried out as described earlier (Acharya et al., 1983a).

Reductive Alkylation of HbS—HbS (dialyzed lysate) was reacted at a concentration of 1 mM (tetramer) with aldehydes (10 mM) as described above for HbA, except for the fact that the reaction was carried out in the oxy form of HbS. The excess reagents were removed by gel filtration on a Sephadex G-25 column equilibrated and eluted with phosphate buffer, pH 6.9.

Oxygen Equilibrium Measurement—The oxygenation curves of HbS and reductively alkylated HbS were recorded at 37 °C using an Amino Hem-O-Scan, as described by Bences et al. (1978).

Polymerization of HbS—The concentration at which the onset of polymerization both of HbS and of the reductively alkylated HbS occurs was determined by the method of Bences et al. (1978) as described earlier (Acharya et al., 1984, and Seetharam et al., 1983).

RESULTS

Reductive Ethylation of HbA—Incubation of hemoglobin A (1 mM in tetramer) with 10 mM acetaldehyde and 20 mM NaCNBH₃ for 30 min at 37 °C resulted in the incorporation of ³H label into the protein, suggesting the reductive alkylation of the protein. From the amount of the label incorporated into the protein, it is calculated that nearly four to five alkyl (ethyl) groups are introduced into the protein. Amino acid analysis of an acid hydrolysate of the labeled ethylated HbA showed that nearly 30% of the label eluted at the position corresponding to ε-N-ethyl lysine (Means and Feeney, 1988), slightly after the position of unmodified lysine on the short column of a Moore-Stein amino acid analyzer. The remainder of the label eluted near the void volume of this column and apparently is α-ethyl valine. The modification of α-amino groups is also confirmed by the tryptic peptide analysis (see below).

Reductive alkylation of the amino groups of protein in general does not significantly influence the net charge of the protein.

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![Diagram](https://example.com/diagram.png)

**Fig. 1. Schematic representation of reductive alkylation of hemoglobin with aliphatic aldehydes and ketone.**

| Aldehyde                  | Reacted with HbA                  | Formed Protein                  |
|---------------------------|-----------------------------------|---------------------------------|
| Acetaldehyde              | Hb-NH₂⁺O=CH-CH₃                  | Ethylated Protein               |
| Glycolaldehyde            | Hb-NH₂⁺O=CH-CH₃OH                 | Hydroxethylated Protein         |
| Glyoxylic Acid            | Hb-NH₂⁺O=CH-COOH                 | Carboxymethylated Protein       |
| Glyceraldehyde            | Hb-NH₂⁺O=CH₂-CH₂OH                | Dihydroxypropylated Protein     |
| Dihydroxyacetone          | Hb-NH₂⁺O=CH₂-CH₂OH                | Dihydroxyisopropylated Protein  |

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*A preliminary account of this work has been presented (Acharya et al., 1980).*

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protein, since the derivatized amino group still retains its original positive charge (Jentoft and Dearborn, 1979). Consistent with this observation, when HbA reductively ethylated, was chromatographed on DE52 around pH 8.0, the modified protein eluted at the position corresponding to that of unmodified protein. The reductively ethylated HbA has been chromatographed on CM-52 and the chromatographic behavior (Fig. 2) has been compared with that of reductively hydroxyethylated HbA. It is clear that the chromatographic behavior of the ethylated HbA (Fig. 2A) is very similar to that of hydroxyethylated HbA (Fig. 2B). In the case of the hydroxyethylated protein (as well as dihydroxypropylated HbA), we have shown previously that the two components eluting earlier than unmodified HbA (peaks A and B) are the derivatives modified at α-amino groups. The component eluting around 240-280 ml (peak A) is a derivative of HbA in which all four α-amino groups of the tetramer are modified, whereas the component eluting around 280-320 ml (peak B) is a derivative in which, on an average, two of the four α-amino groups of the tetramer are modified (Fig. 2B). The similarity in the chromatographic behavior of ethylated HbA and hydroxyethylated HbA suggests that the component eluting around 240-280 ml (peak A) in the ethylated protein is α-tetraethylated HbA, while the one eluting around 280-320 ml (peak B) is α-diethylated HbA. Consistent with this suggestion is the finding that both of these fractions contained α-ethyl valine. Furthermore, the α-ethyl valine content in fraction A (~4 mol/tetramer) is nearly twice of that in fraction B (~2 mol/tetramer). All the counts in fraction C were associated with ε-N-ethyl lysine. From these results it is clear that ethylation of α-amino groups of HbA results in an earlier elution of the protein on CM-52 compared to that of the unmodified protein, as seen earlier with the hydroxyethylated studies.

Peak A from ethylated HbA (Fig. 2B) appears to be the tetramer in which all four amino groups are ethylated. Hence, analysis of the HMB chains of peak B should provide the information about the relative reactivity of the α-amino groups of α- and β-chains of HbA. Chromatography of the HMB chains of peak B of ethylated HbA on CM-52 is shown in Fig. 3. The chromatogram shows three peaks; peak B1 is the β-chain whereas peaks B2 and B3 are α-chains. Amino acid analysis of these fractions showed that B1 and B3 contain α-ethyl valine, while B2 did not contain the α-N-ethyl valine. Tryptic peptide mapping of B1 and B3 confirmed the derivatization of the α-amino group of β- and α-chains, respectively (data not shown). Nearly 30% of the modification of B1 was present at sites other than the α-amino groups (apparently ε-amino groups, whereas with B2 almost all the modification was associated with the α-amino group. All the 3H counts of B3 are associated with ε-ethyl lysine. The chromatographic position of α-N-ethyl α-chain is close to that of α-hydroxyethyl α-chain and ahead of that of the unmodified α-chain and α-chain modified at the α-amino group. This behavior is apparently a reflection of the lowering of the pKₐ of the α-amino group as a result of ethylation. This influence is similar

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**Fig. 2. Chromatography of HbA reductively alkylated with acetaldehyde and glycolaldehyde on CM-52.** Each of the reductively alkylated HbA samples was desalted through a column of Sephadex G-25 (2.2 × 30 mm) equilibrated and eluted with 10 mM phosphate buffer, pH 5.85 (1 mM in EDTA). The desalted protein was chromatographed on a column (0.9 × 30 mm) of CM-52 equilibrated with 10 mM phosphate buffer, pH 5.85. The protein was eluted with a linear gradient from 30 ml each of 10 mM potassium phosphate buffer, pH 5.85, and 15 mM potassium phosphate buffer, pH 7.6. A, reductively ethylated HbA. B, reductively hydroxyethylated HbA.

**Fig. 3. Chromatography of p-hydroxymercuribenzoate chains of component B of reductively ethylated HbA on CM-cellulose.** The component B of reductively ethylated HbA (see Fig. 2A) was treated with p-hydroxymercuribenzoate as described earlier (Acharya and Manning, 1980a), dialyzed extensively against 10 mM potassium phosphate buffer, pH 5.85, containing 1 mM EDTA. The dialyzed sample was loaded on to a CM-cellulose column (0.9 × 30 mm) equilibrated to pH 5.85 with potassium phosphate buffer, pH 5.85. The HMB chains were eluted with a linear gradient from 150 ml each of 10 mM potassium phosphate buffer, pH 5.85 (1 mM in EDTA) and 15 mM potassium phosphate buffer, pH 7.6 (1 mM EDTA). The column is operated at 4°C and all the buffers were saturated with CO.
to that observed earlier (Acharya and Sussman, 1983) on reductive hydroxyethylation of α-amino groups of HbA.

The amount of α-ethyl α-chain, in this chromatogram (Fig. 3) accounts for about 35% of the α-chain present in fraction B. Since the rest of the α-chains are either unmodified or modified only at ε-amino groups, these did not contribute to chromatographic behavior of the tetramer (fractions from which these chains are prepared), i.e. to an early elution on CM-52 (compared with unmodified HbA). Therefore, the elution of these chains in fraction B (HbA ethylated at two of the four α-amino groups) is apparently due to their hybridization with β-chains ethylated at the α-amino groups. Thus, the distribution of ethylation at the α-amino groups of fraction B is that 65% of the β-chains are modified, while only 35% of the α-chains are modified. Thus, studies demonstrate that the α-amino group of β-chain shows a somewhat higher selectivity for reductive ethylation compared to the α-amino groups of α-chain.

**Reductive Carboxymethylation of HbS**—The reductive carboxymethylation would introduce a charge difference due to the negative charge (−COO⁻) incorporated into the protein. In view of the negative charge of the glyoxylic acid (the carboxyl component) compared with its absence in acetaldehyde and glycolaldehyde, it is of interest to determine whether the carboxylate of glyoxylic acid influences the selectivity pattern of α-amino groups of Hb for reductive alkylation. Previously we have shown that reductive carboxymethylation of the HbA is reasonably selective towards the ε-amino groups of HbA (Acharya et al., 1982, DiDonato et al., 1983). The earlier studies on carboxymethylation were, however, carried out at pH 7.2 using HEPES buffer and without chloride for 40 min. Besides, a larger excess (10-fold over the aldehyde) of sodium cyanoborohydride had been used, as compared with a 2-fold molar excess (over aldehyde) used in the studies with ethylation, hydroxyethylation, and dihydroxypropylation. Therefore, carboxymethylation of HbS has been now carried out in PBS at pH 7.4 and 37 °C for 30 min using 10 mM glyoxylic acid and 20 mM NaCNBH₃ so that the reactivity of the α-amino groups could be compared in a systematic way.

On DE52 chromatography of the carboxymethylated HbS (lysate), three chromatographically distinct components were obtained (Fig. 4). The minor component eluting around 450 ml is apparently the unmodified HbS. The two major radioactive components are the carboxymethylated derivatives. These are designated HbS-Cml and HbS-Cm2 and were isolated. Thus, the presence of the carboxylate group in the carboxyl component has made the alkylated protein more acidic and hence more retarded on the DE52 columns. Reductive alkylation with the neutral carbonyl compounds (acetaldehyde and glycolaldehyde) does not influence the chromatographic behavior of HbS on DE52.

The HbS-Cml and HbS-Cm2 contain two and four carboxymethyl groups, respectively, hence appearing to correspond to Hb and Hb, previously isolated from HbA reductively carboxymethylated using a greater excess of sodium cyanoborohydride over the aldehyde (DiDonato et al., 1983). The main difference appears to be in the amount and the distribution of carboxymethylated derivatives. The component corresponding to Hb (containing both α-N-carboxymethylated valine and ε-N-carboxymethyl lysine) was absent in the present study. Besides HbS-Cml (corresponding to Hb), the dicarboxymethylated HbS was the major carboxymethylated product in the present study, whereas Hb₃

The exact chemistry for the incorporation of tritium label into the HbS peak is not clear. It is conceivable that glycosylated HbS is reduced by NaCNBH₃ (Acharya and Sussman, 1984).

![Fig. 4. Chromatography of HbS on DEAE-cellulose after treatment with glyoxylic acid in the presence of sodium cyanoborohydride. Carbonmonoxy HbS (1 mm) was treated with 10 mM glyoxylic acid in the presence of 20 mM sodium cyanoborohydride (NaCNBH₃). The reaction mixture was then passed through a column (2.2 × 45 cm) of Sephadex G-25 and eluted with 50 mM Tris-Ac buffer, pH 8.5, to separate the carboxymethylated protein from the excess reagents. The fractions containing the protein were pooled, concentrated, and applied to a column (2.2 × 40 cm) of DE52 equilibrated with 50 mM Tris-Ac buffer, pH 8.5. The protein is eluted with a linear pH gradient of 300 ml each of 50 mM Tris-Ac buffer, pH 8.3, and 50 mM Tris-Ac buffer, pH 7.3. The position of HbS is indicated. The derivatized HbS were pooled as indicated and designated HbS-Cml and HbS-Cm2, respectively.](image-url)
HbS-The influence of the selective introduction of charged carboxymethyl groups, uncharged hydroxyethyl groups, or weakly hydrophobic ethyl groups at the α-amino groups of HbS on the polymerization of deoxy-HbS has been investigated (Fig. 6). Ethylation and hydroxyethylation increased the O₂ affinity of HbS; on the other hand, carboxymethylation decreased the O₂ affinity. However, these modifications had very little influence on the cooperativity. The Hill coefficient of the modified proteins is nearly the same as that of unmodified protein (~2.5).

The ethylation and hydroxyethylation of HbS increased the concentration of the protein needed for the onset of polymerization from the control value of 16 to about 24 g/dl for the hydroxyethylated protein, and to about 26 g/dl for the ethylated protein. The value for the carboxymethylated protein is close to that of hydroxyethylated protein. Thus, the solubilizing influence due to the presence of carboxymethyl, hy-
Droxyethyl, or ethyl groups on the α-amino groups appears to be nearly the same.

The O₂ affinity (at pH 6.9) for the ethylated and/or the hydroxyethylated HbS is nearly the same and is higher than that of unmodified HbS. The influence of ethylation and hydroxyethylation on the oxygen affinity of HbS is nearly the same. This reflects a similarity in the structural perturbations that these two modifications bring about in the molecule. On the other hand, with carboxymethylation of the α-amino groups, the P₅₀ of the molecule is increased (DiDonato et al., 1983). Thus, the propensity of the carboxyl group of the alkyl chains is to reduce the O₂ affinity of the protein. Nonetheless, this modification still increases the concentration of HbS that is needed for the onset of polymerization to nearly the same point. The nature of alkyl groups does not appear to be crucial for the inhibition of the polymerization. The substitution of the α-amino group by alkylation is sufficient to cause this inhibition of polymerization.

The influence of reductive 2,3-dihydroxypropylation of the α-amino groups of HbS, as well as that of reductive 1,3-dihydroxyisopropylation of the α-amino group of the β-chain of HbS, is shown in Fig. 7. Nearly the same influence of reductive hydroxyethylation and reductive dihydroxypropylation on the polymerization of deoxy-HbS demonstrates that the increase in the chain length of the alkyl group from 2 to 3 carbons has limited additional inhibitory influence on the propensity of the molecule to polymerize. The lower influence of dihydroxyisopropylation on the solubility of HbS, seen in the present study, is due to lower levels of derivatization (0.8 mol/tetramer as opposed to nearly 3.0 mol/tetramer for hydroxyethylation, ethylation, and dihydroxypropylation).

Polymization of HbS Selectively Hydroxyethylated at the α-Amino Group of Either α- or β-Chains—During the reductive alkylation of HbS, α-amino groups of both the α- and β-chains are derivatized. Therefore, it was of interest to determine whether the alkyl group present either on the α-amino group of the α-chain or the one on the α-amino group of the β-chain has contributed to the inhibition observed on reductive alkylation. For a detailed study, we have examined the reductive hydroxyethylation of HbS. HMB α- and β-chains of reductively hydroxyethylated HbS were prepared and mixed with HMB β- and α-chains, respectively, in the presence of β-mercaptoethanol (the procedures are similar to those used earlier for the preparation of hybrids from hydroxyethylated HbA (Acharya and Sussman, 1983). The hybrids α₂β₂(α-HE) and α₂β₂(α-HE) were purified by CM-cellulose chromatography and used for the polymerization studies. The O₂ affinity of the hybrid HbS prepared was slightly higher than that of the native HbS. The Hill coefficient of the hybrids is about 2.6, nearly the same as that of HbS. The polymerization studies (Fig. 8) have shown that hydroxyethylation of the α-amino group of either the α- or β-chain increases the concentrations of HbS at which the onset of polymerization occurs to about 26 g/dl, from a control value of 14.7 g/dl for the purified HbS. Thus, it is clear that the hydroxyethylation of the α-amino group of α-chain inhibits polymerization, as has been observed earlier on the modification of α-amino groups of α-chain by pyridoxal sulfate (Benesch et al., 1974). This influence is distinct from that seen on carbamoylation of the α-amino group of α-chain of HbS.

DISCUSSION

The studies described here were undertaken with two major objectives. The first one was to determine whether the condensation of the α-amino groups of Hb around neutral pH using limiting concentration of the aliphatic aldehydes or ketones in the presence of sodium cyanoborohydride would provide a general route for derivatizing the α-amino groups of Hb. The second objective was to determine whether reductive alkylation of the α-amino group influences the polymerization properties of HbS and, if it did, to determine whether the nature of the alkyl group introduced has any strong influence on the inhibition of polymerization.

The results of the present study clearly demonstrate that around pH 7.4 the α-amino groups of HbA show a high selectivity to form Schiff bases when a limiting concentration of the aliphatic aldehydes was used. All four aldehydes used resulted in the modification of the α-amino group of Hb. Glycolaldehyde and glyceraldehyde modified the α-amino group of both chains to nearly the same degree (Acharya and Sussman, 1983, Acharya et al., 1983a). Although acetaldehyde reacted with the α-amino groups of both the chains, it showed some preferential reaction at the Val-1(β). With glyoxylic acid, the preferential reactivity of Val-1(β) is even more pronounced. The significantly higher reactivity of Val-1(β) with glyoxylic acid is apparently a reflection of the refractory influence of the environment of Val-1(α) to accommodate the negatively charged alkyl chain of glyoxylic acid when these two aldehydes form the Schiff bases at these two sites. Prelim-
inary studies with glyceraldehyde 3-phosphate also have in-
dicated that the anionic charge of this aldehyde contributes a
higher selectivity for this reagent to Val-1(β) as opposed to
glyceraldehyde. This would imply that if lower levels of
aldehydes (over the α-amino groups) are used in the reductive
alkylation, a selective modification of Val-1(β) would occur.
Reductive alkylation using ketones which have a very low
propensity to form Schiff bases at neutral pH could be con-
sidered as equivalent to using lower levels of any of these
aldehydes. Dihydroxyisopropylation, which proceeds at a
much slower rate, indeed showed nearly exclusive reaction at
Val-1(β).

The anionic nature of the glyoxylic acid significantly influ-
ences the relative selectivity of the amino groups of the two
chains of Hb. Besides, significant differences are also seen in
the composition of the Hb species containing alkyl chains on
two of its four α-amino groups that are isolated after reductive
hydroxyethylation (or ethylation) and carboxymethylation.
On modification with the uncharged aldehydes (acetaldehyde,
glycolaldehyde, and glyceraldehyde), the Hb species, contain-
ing two of its four α-amino groups modified (material eluting
at the position corresponding to that of peak B in Fig. 2) had
the derivatizations on the α-amino groups of both α- and β-
chains. Thus, the species chromatographing in this position
could be a mixture of two different symmetrical forms of
tetramers, one form containing reductive alkylation on the α-
amino groups of Val-1(β) and the other on that of Val-1(α) of
α-chains. Alternatively, the reductive alkylation could be as-
symmetrical. Apparently these symmetrical and/or asym-
metrical forms do not readily segregate to form native Hb
(unnmodified) and Hb species with derivatization on all four
α-amino groups. Thus, the reductive alkylation with un-
charged aldehydes (i.e. ethylation, hydroxyethylation, and
dihydroxypropylation) does not significantly perturb (desta-
bilize) the intersubunit interactions.

In contrast, the tetramers of Hb containing two carbony-
methylated α-amino groups (HbS-Cm1) are species with mod-
ifications of Val-1(β). This result suggests that the symmet-
rical and/or asymmetrical carboxymethylated Hb formed
during carboxymethylation appears to segregate readily to the
native and tetracarboxymethylated derivatives. Apparently
the carboxymethyl groups on the α-amino group destabilize
the subunit interactions, permitting their ready segregation
to native and tetracarboxymethylated derivative.

Substitution of the α-amino groups of HbS by ethylation,
hydroxyethylation, carboxymethylation, and dihydroxypro-
pylation had nearly the same influence of inhibiting the
polymerization of deoxy-HbS. The ethyl group is weakly
hydrophobic, hydroxyethyl and dihydroxypropyl groups are
hydrophilic, and the carboxymethyl group is negatively
charged and also hydrophilic. The nature of these groups is
also readily reflected in the elution behavior of ε-N-ethyl
lysine, ε-N-hydroxyethyl lysine, ε-N-dihydroxypropyl lysine,
(Acharya and Manning, 1983, Geoghegan et al., 1979; Acharya
and Sussman, 1983; Acharya et al., 1984a), and the ε-N-
carboxymethyl lysine on the amino acid analyzer. It is con-
ceivable that these alkyl groups, when present at the amin-
terminal, induce similar changes around the substituted
amino group. The dihydroxypropyl group is bulkier than the
hydroxethyl groups introduced. However, in spite of these
differences in their hydrophilicity or size, the alkylation of
the α-amino group by these derivatizations appears to influ-
ence the polymerization to nearly the same degree. This would
imply that the groups introduced perturbed the stereochemi-
cal orientation of the HbS tetramer during the polymerization
around the contact regions involving Val-1(α) and/or Val-
1(β), respectively. Since the reductive alkylation by acetalde-
hyde, glycolaldehyde, glyoxylic acid, or glyceraldehyde has
nearly the same inhibitory influence, the inhibition of poly-
merization is apparently related to alkylation of α-amino groups
rather than the charge or hydrophobicity of the alkyl chain
introduced. The studies have also shown that the influence of
reductive hydroxyethylation and reductive dihydroxypro-
pylation on polymerization of HbS is nearly the same. Thus,
the small changes in size of the alkyl group also appear to
have little influence on the observed inhibition.

The above conclusion appears to be reminiscent of the
conclusions that we had drawn earlier with regard to another
intermolecular contact region of HbS, i.e. one involving Lys-
16(α) (Acharya et al., 1984b). The mutation of Lys-16(α) to
Glu there by replacing the positive charge at the intermolecu-
lar contact region with a negative charge inhibits the poly-
merization (Benesch et al., 1977). Dihydroxypropylation of
the ε-amino group of Lys-16(α), which would retain the original
positive charge of this amino group under the physiological
condition, also results in the inhibition of polymerization.
These results, as well as the results of the present study, could
be interpreted as suggesting that perturbation of the inter-
molecular contact region by the modification, which would
make the proper "fit" of the region during polymerization
difficult, rather than the changes in the charge distribution
at a particular intermolecular contact region is generally
the major influence for most of the polymerization inhib-
itors. Such a conclusion is simply a reflection of the fact that
intermolecular contact involving Val-6(β) provides the domi-
nant "force" for the polymerization process. This is also
consistent with the fact that none of the chemical modifica-
ion done so far on HbS has been able to completely neutralize
the influence of Val-6(β), i.e. to restore a solubility to HbS
similar to that of HbA.

The modification of the α-amino group of Val-1(α) as well
as that of Val-1(β) by reductive hydroxyethylation results in
reducing the propensity of HbS to polymerize. The influence
of reductive hydroxyethylation of Val-1(α) is consistent with
the influence of modification of this amino group by pyridoxal
sulfate. This influence is distinctly different than that ob-
served with the carboxylation of this amino group. On the
other hand, the influence of modification of the ε-amino
groups of Val-1(α) on the oxygen affinity is consistent with
that seen on carbamoylation of this amino group (Nigen
et al., 1974). As pointed out earlier, there is a close similarity
in the influence of reductive hydroxyethylation and carba-
moylation of α-amino groups on the oxygen affinity of HbA
(Acharya and Sussman, 1983). This is probably related to the
apparent loss of nearly one positive charge under the physio-
logical condition that occurs as a result of both hydroxyeth-
ylation and carboxylation of the α-amino group. With
hydroxyethylation, this is apparently due to the decrease in
the apparent pKₐ of the α-amino group when it is modified to a
secondary amino group. Carbamoylation of the α-amino group
results in the loss of the positive charge of this amino group.
The fact that the carbamoylation of Val-1(α) has no influence
on the propensity of the molecule to polymerize, whereas the
reductive hydroxyethylation increases the solubility of deoxy-
HbS, possibly suggests the difference in orientation of the
 carbamoyl group, and the hydroxyethyl groups from the ni-
trogen atom of the amino group of Val-1(α). The carbamoyl
group could probably be accommodated in the intermolecular
contact regions involving Val-1(α) without seriously pertur-
bting the molecular fit of this region during polymerization.

A. S. Acharya, unpublished results.
The carbamoyl group on the Val-(β) apparently perturbs the intermolecular contact region(s) involving this residue as in the case with reductive alkylation. Therefore, it would be of interest to study the influence of the substitution at Val-1(α) by a methyl group on the polymerization of deoxy-HbS to determine whether this group could be accommodated at the intermolecular contact region without significantly perturbing the molecular fit of this region.

We have previously shown that, on incubation of HbS with glyceraldehyde, the α-amino group of Val-1(β) and the ε-amino groups of Lys-16(α), Lys-82(β), Lys-59(β), and Lys-120(β) are modified (Acharya and Manning, 1980a and b). The derivatization of the α-amino group of Lys-16(α) has been shown to inhibit the polymerization (Acharya et al., 1984b). The results of the present study suggest that the derivatization of Val-1(β) by glyceraldehyde should also contribute, at least to some extent, toward the inhibition of polymerization of deoxy-HbS when the erythrocytes from sickle cell patients are treated with glyceraldehyde. Thus, the inhibition of polymerization of HbS by glyceraldehyde is a reflection of the derivatization of at least two intermolecular contact regions. Therefore, it will be of interest to prepare a doubly modified HbS, i.e., a derivative modified at Val-1(β) and also at the ε-amino group of Lys-16(α), to determine the additivity of the perturbation of these two intermolecular contact regions to inhibit the polymerization of deoxy-HbS.

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REFERENCES

Acharya, A. S., and Manning, J. M. (1980a) J. Biol. Chem. 255, 1406-1412

Acharya, A. S., and Manning, J. M. (1980b) J. Biol. Chem. 255, 7218-7224

Acharya, A. S., and Manning, J. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3590-3594

Acharya, A. S., and Sussman, L. G. (1983) J. Biol. Chem. 258, 13761-13767

Acharya, A. S., and Sussman, L. G. (1984) J. Biol. Chem. 259, 4372-4378

Acharya, A. S., DiDonato, A., and Manning, J. M. (1982) Fed. Proc. 41, 1174

Acharya, A. S., Sussman, L. G., and Manning, J. M. (1983a) J. Biol. Chem. 258, 2296-2302

Acharya, A. S., DiDonato, A., Manjula, B. N., Fischetti, V. A., and Manning, J. M. (1983b) Int. J. Pept. Protein Res. 22, 78-82

Acharya, A. S., and Sussman, L. G., and Manning, J. M. (1983c) Fed. Proc. 42, 1840

Acharya, A. S., Sussman, L. G., and Manjula, B. N. (1984a) J. Chromatogr. 297, 37-48

Acharya, A. S., Sussman, L. G., Jones, M. W., and Manning, J. M. (1984b) Anal. Biochem. 136, 101-109

Benesch, R., Benesch, R. E., Edalji, R., and Suzuki, T. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1721-1725

Benesch, R. E., King, S., Benesch, R., and Edalji, R. (1977) Nature (Lond.) 266, 772-775

Benesch, R. E., Edalji, R., Kwong, S., and Benesch, R. (1978) Anal. Biochem. 89, 162-173

Bucci, E., and Fronticelli, C. (1965) J. Biol. Chem. 240, PC 551-552

Cerami, A., and Manning, J. M. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 1189-1183

DiDonato, A., Fantl, W. J., Acharya, A. S., and Manning, J. M. (1983) J. Biol. Chem. 258, 11890-11895

Geoghegan, K. F., Ybarra, D. M., and Feeney, R. E. (1968) Biochemistry 7, 2192-2201

Meins, G. E., and Feeney, R. E. (1968) Biochemistry 7, 2192-2201

Nigen, A. M., Njikam, N., Lee, C. K., and Manning, J. M. (1974) J. Biol. Chem. 249, 6611-6616

Seetharam, R., Manning, J. M., and Acharya, A. S. (1983) J. Biol. Chem. 258, 14810-14815