The structural perturbations of the amino-terminal domains of hemoglobin A resulting from the carbamino adduct formation (a reversible in vivo modification reaction) at Val-1(α) and Val-1(β) is expected to be mimicked in the derivatives of HbA reduc-tively alkylated at its α-amino terminus with aldehydes containing a negatively charged functional group at their distal end (double-headed reagents). Derivatives of HbA with galacturonic acid linked through alkyalmine linkage either to Val-1(α) or Val-1(β) (disubstituted derivatives) as well as to both Val-1(α) and Val-1(β) (tetra-substituted derivative) have been now prepared. All three the derivatives exhibit normal cooperativity but reduced O₂ affinities. The functional consequence of the modification of HbA at its amino termini with α-galacturonic acid has been compared with that of the carbamino adduct at the amino terminus of the β-chain as well as the carboxylate of carboxymethylated group at the same site. This comparative study suggests that the stereochemistry of the carboxylate ion introduced into β-cleft of Hb dictates the level of reduction in the O₂ affinity of the molecule seen on derivatization. However such a unique stereochemistry of the carboxylate ion of the reagent does not appear to be crucial to lower oxygen affinity when the modification is at the amino terminus of the α-chain. The molecular modeling studies demonstrate that the carboxylate ion of the carbamino adduct at the α-amino terminus of the β-chain as well as the carboxylate of carboxymethylated group at the same site are in a geometrical orientation that favors the formation of an intrachain ionic interaction with the α-amino group of Lys-82(β). On the other hand the stereochemistry of a carboxylate ion of galacturonic acid on Val-1(β) appears to be appropriate to form either an intrachain salt bridge with α-amino group of Lys-82(β) of the same chain (intrachain) or alternatively an interchain salt bridge involving the α-amino group of Lys-82(β) of the trans chain. We speculate that the latter, i.e. trans configuration is favored as a result of the potential of α-galacturonic acid bound to Val-1(β) to form an additional hydrogen bond with trans His-143(β).

The amino-terminal regions of α- and β-chains of HbA play a dominant role in modulating the O₂ affinity of the molecule through their interactions with organic phosphates (DPG), anions (chloride), and carbon dioxide. The modulation of the oxygen affinity by heterotrophic effectors like DPG and chloride ions involve their noncovalent interactions with HbA (Benesch and Benesch, 1974; Perutz, 1978). On the other hand, the modulation of the O₂ affinity of HbA by carbon dioxide is through a reversible chemical modification of the protein forming the carbamino adducts at the α-amino groups of the protein (Kilmartin and Rossi-Bernardi, 1969, 1973). The overall structural consequence of the formation of the carbamino adducts at the amino terminus of the α- and β-chains is the loss of the positive charge at the α-amino group as the amino nitrogen is converted to an amide nitrogen. Concomitant with this, a negative charge is introduced at this site. Thus, with the formation of carbamino adducts, an electrostatic modification of the amino-terminal region of the molecule has been achieved. The isolation of carbamino adducts as stable products is not possible making it difficult to define the full structural and the functional consequences of this reversible chemical modification of protein in solution. One approach to gain new insights into this biochemical process is to carry out irreversible electrostatic modification of HbA and then extrapolate the structural and functional consequences of this reaction to the reversible electrostatic modification reaction (Di Donato et al., 1985).

Deciphering the molecular aspects of the carbamino adduct formation, the reversible electrostatic modification of the amino group of α- and β-chains of HbA and its functional consequences have been the subject of considerable interest. In introducing the carboxymethylation of HbA using glyoxalate as the carbonyl reagent (Acharya et al., 1981), we had hypothesized that the carboxymethylated HbA can be considered as a structural analog of the carbamino adducts of HbA. Nonetheless, it should be noted that a methylene bridge is present between the amino nitrogen and the carboxylate ion in the carboxymethylated derivative and this “spacer arm” is absent in the carbamino adduct (Fantl et al., 1987). Therefore, the stereochemistry of the carboxylate ion at the respective amino terminus of the carbamino adduct and the carboxymethylated HbA is not expected to be same. Therefore, the pertinent question to establish is the influence, if any, of the spacer arm of the alkyl chain in bringing out the carboxylate ion (electrostatic modification-mediated) modulations of the oxygen affinity of HbA. As a beginning to this line of investigation, the reductive alkylation of HbA with galacturonic acid (Fig. 1) has been now undertaken and the influence of the covalently bound galacturonic acid at Val-1(α) and Val-1(β) on the oxygen affinity of HbA has been investigated and the results have been compared with those of carboxymethylation studies.

**MATERIAL AND METHODS**

HbA and its p-hydroxymercuribenzoate (HMB)-reacted chains were prepared as described earlier (Bucci and Frongi, 1965; Seetharam and Acharya, 1986). α- and β-globin were prepared by acid-acetone precipitation of the respective chains (Rossi-Fanelli et al., 1964). Reductive Modification of HbA with α-Galacturonic Acid—DE-52-purified HbA was dialyzed extensively against phosphate-buffered saline, pH 7.4, and reacted with 100 mM α-galacturonic acid in the presence of 300 mM trisodium cyanoborohydride at 37°C for 90 min.
The concentration of HbA in the reaction mixture was 1 mM. After the incubation the excess reagents were removed by gel filtration of the reaction mixture on a 2.2 x 35 cm column of Sephadex G-25 equilibrated and eluted with Tris acetate buffer, pH 8.5. The incorporation of the tritium label into the protein demonstrates the reductive modification of HbA with D-galacturonic acid. The gel-filtered protein was purified on DE-52 column using a linear gradient of 50 mM Tris acetate buffer, pH 8.3 to 7.0. The introduction of the negative charge onto the protein as a consequence of the reductive alkylation with the aldonic acid retarded the elution of the protein based on the extent of modification of the protein by the reagent. The preparation of HMB α- and β-chains of the modified samples of HbA were carried out as described earlier (Acharya and Manning, 1980). The analysis of the tryptic peptides of the chains by reversed phase HPLC and the amino acid analysis of the of the peptides were also carried out as described earlier (Acharya et al., 1983b).

Preparation of Hybrid HbA—for the preparation of HbA specifically modified at Val-1(a), the α-chains (1 mol) are reductively alkylated with 100 mM D-galacturonic acid in the presence of 200 mM sodium cyanoborohydride. The reductive alkylation was carried out at pH 6.0 and room temperature (23 °C) to increase the specificity of the reaction at the α-amino group and decrease the precipitation of the chains respectivley. The modified α-chain is freed from the excess reagent by a Sephadex G-25 gel filtration and then hybridized with β-chains as described previously (Acharya and Sussman, 1983). The hybrid tetrascramers are purified by DE-52 chromatography.

Oxygen Affinity Studies—all the hybrid tetrascramers are prepared and purified in the carbonmonoxo form. The carbonmonoxo Hb samples were converted to the oxy form for the functional studies (Nigen et al., 1974). The oxygen affinity of the hemoglobin samples were determined in 50 mM BisTris, pH 7.4, at 37 °C using Hemoscan as described previously (Acharya and Sussman, 1983).

Molecular Modeling—the modeling studies were carried out on an Iris Crimson Workstation from Silicon Graphics interfaced with a Tektronix Phaser III printer. For graphics and structure building the Insight II software package from Biosym, Inc has been used. The starting model was the refined structure of deoxy Hb (Ten Eyck and Arnone, 1976). The hybrid tetramers were converted to the oxy form using Hemoscan as described previously (Acharya and Sussman, 1983). The starting model was the refined structure of deoxy Hb (Ten Eyck and Arnone, 1976). The coordinates (entry 2HHB, version of January 1992) were obtained from the Protein Data Bank at Brookhaven National Laboratory (Bernstein et al., 1977, Abola et al., 1987). The carbamethyl group on Val-1(f) was modeled based on the x-ray crystallographic difference maps of Fantl et al. (1986). The models for the possible orientation of D-galacturonic acid covalently linked on Val-1(f) were generated by adjusting the torsion angles ϕ and ψ of residues 1, 2, and 3 of the β-chain and free torsion angles in the proximity with the galacturonic acid ring.

RESULTS

Reductive Alkylation of HbA with Galacturonic Acid

The concentration of the galacturonic acid that is present in the reactive open chain aldehydic form in a sample of the reagent is very small. However, with glyoxalate, the reagent is present only as the reactive aldehydic form. Accordingly, in order to offset the low concentration of the reactive "aldehydic" form, the reductive alkylation of HbA with galacturonic acid was carried out using higher concentrations of the reagent. On reaction of HbA (1 mM) with 100 mM galacturonic acid in the presence of 200 mM sodium cyanoborohydride for 90 min, an average of 4 mol of galacturonic acid were Incorporated per mol of tetramer. With glyoxalate, nearly the same level of incorporation of the carbonyl reagent is obtained using only 10 mM glyoxalate (in the presence of 20 mM sodium cyanoborohydride) for 20-30 min.

Chromatography of HbA Reductively Alkylated with Galacturonic Acid

HbA reductively alkylated with galacturonic acid was chromatographed on DE-52 (Fig. 2). The chromatogram of the derivatized sample revealed the presence of at least four chromatographically distinct components in this reductively alkylated sample. One of these eluted at the position of the HbA and has not been subjected to any further characterizations. The amount of the protein eluting under this peak accounts for no more than 10% of the load on the column. The four new components present in the reductively alkylated sample represent the derivatized HbA. Their elution after the position of unmodified HbA is consistent with the introduction of negative charge into the protein. These have been designated as components A, B, C, and D, respectively, in the order of their elution. The number of moles of amino groups derivatized per mole of protein has been calculated based on the amount of the tritium label present in each of these chromatographically distinct alkylated derivatives of HbA. Component A is a disubstituted derivative, whereas both the components B and C appear to be the tetrasubstituted derivatives of HbA (Table I).

Separation of the α- and β-Chains of Components A and B

The derivatized HbA, namely the components A and B, were isolated and subjected to a second purification on a DE-52 column (Fig. 3). For the second DE-52 chromatography, a pH gradient shallower than that employed in the original chromatogram is employed in an attempt to achieve a better separation of the individual components. All three components have been characterized and only the chromatographed components were used for further structural as well as functional studies. The HMB α- and β-chains of each of these derivatives were separated on a CM-52 column (Fig. 4). In component A, the disubstituted derivative, all the radioactivity (i.e. modification) is associated with the β-chain (Fig. 4A), and this radioactive component is designated as component A1. The α-chain has remained unmodified in this disubstituted derivative.
of unmodified α-chain in it, both the α- and β-chains are modified in this derivative (Fig. 4B). The β-chain of component B has been designated as B1. The α-component of this tetrasubstituted derivative eluted in three positions, and two of these are radioactive, demonstrating that they are modified forms of the α-chain. The major α-component accounted for nearly 80% of the α-chain of this derivative, and this derivatized α-chain has been designated as B2. The second form of modified α-globin accounted for 15% of the α-component of this tetrasubstituted derivative and this product has been designated as B3.

The chromatographic pattern of the HMB chains of component C, the second tetrasubstituted derivative, is qualitatively similar to that of the HMB chains of component B and contained very little unmodified α-chain. The results suggest that both α- and β-chains are modified completely in this tetrasubstituted derivative just as in component B. The major difference in the chromatographic patterns of HMB chains of components B and C is in the relative amounts of the two modified forms of α-chain. The quantity of B2 and B3 in these two components are distinct. The yield of the modified α-chain eluting in the position of B2 is small in component B, whereas this is the major form of the α-chain in component C. The simple explanation for this observation is that the components B and C are still cross-contaminated. Accordingly, these two components were subjected to a third purification on DE-52 and much more conservative pooling was adhered to when isolating these for further studies. The chromatographic analysis of the HMB chains of repurified component B revealed that it now contains only one α-component, namely B2. Similarly, the repurified component C also contained one α-component, this corresponding to that of B3. Thus, the reductive alkylation of HbA with limiting concentrations of D-galacturonic acid leads to the formation of at least three chromatographically distinct components, one disubstituted and two tetrasubstituted derivatives. In all the components the β-chain has been derivatized. The two forms of the tetrasubstituted derivatives of HbA apparently differ with respect to the sites of modification in the α-chains.

Tryptic Peptide Mapping of the Modified α- and β-Chains

(i) Characterization of Component A—As discussed above, component A is a disubstituted derivative of HbA and all of its modification is present in the β-chain. The tryptic peptide map-

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**Table 1**

Specificity in the reductive alkylation of hemoglobin with α-galacturonic acid

| Chromatographic component | Mole of GA/mol of HbA | Amino acid residues derivatized |
|---------------------------|------------------------|-------------------------------|
| Component A               | 2.0                    | Val-1(β)                      |
| Component B               | 4.0                    | Val-1(a) and Val-1(β)         |
| Component C               | 4.0                    | Val-1(β) and ε-amino groups   |

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**Fig. 4. Chromatography of HMB chains of components A and B on CM-cellulose.** Components A and B were treated with p-hydroxymercuribenzoate as described earlier (Acharya and Manning, 1980), dialyzed extensively against 10 mM potassium phosphate buffer, pH 5.8 (1 mM in EDTA), and chromatographed on a CM-52 column (0.9 x 30 cm). The protein was eluted with a linear gradient of 150 ml each of 10 mM potassium phosphate buffer, pH 5.85, and 15 mM potassium phosphate buffer, pH 7.6 (1 mM in EDTA). The columns were operated in cold room at 4°C, and all the buffers were saturated with carbon monoxide. The elution positions of α- and β-chains are indicated in the figure. A, HMB chains of component A. The radioactive component of the sample is designated as C1, and is apparently modified β-chain. The α-chain is apparently not derivatized in this derivative. B, HMB chains of component B. This chromatogram contains three major radioactive components, two major ones and one minor one. Of the two major ones, one elutes at the position of A1. This was designated as B1. The other one eluted slightly ahead of the unmodified α-chain. This component was designated as B2. This accounts for nearly 40% of the radioactivity eluting from the column. A minor radioactive component accounting for nearly 10% of the radioactivity of the sample B eluted at position in between that of B2 and the unmodified α-chain. This component was designated as B3. When component C was subjected to HMB reaction and the chromatographic separation of the chains, it had two major radioactive components, the elution positions of which corresponded to that of B1 and B2, respectively. A minor component, the elution position of which corresponded to that of B3, was also present in this sample and this accounted for nearly 6-7% of the radioactivity of the sample (chromatography not shown). A second rechromatography and a conservative pooling of the fractions afforded the isolation of components B and C which on chain separation contained radioactive chains that corresponded in their elution position to components B1 and B2 in the sample B, and in the case of component C, to components that corresponded to that of B1 and B3, respectively.
ping of \( \text{Al} \) established that all of its radioactivity is associated with one single tryptic peptide, and modified peptide has been isolated and identified as \( \beta_1 \) (residues 1–8 of \( \beta \)-chain), in which the Val-1(\( \beta \)) has been derivatized. The results therefore establish that the component A is a disubstituted derivative of HbA in which the two \( \alpha \)-amino groups of Val-1(\( \beta \)) of HbA are reductively alkylated with galacturonic acid (Table I).

(ii) Characterization of Component B—The component B is a tetrasubstituted derivative of HbA, and the modification is equally distributed in the \( \alpha \)- and \( \beta \)-chains. The tryptic peptide map of \( \text{B}_1 \) is identical to that of the \( \beta \)-chain (\( \text{A}_1 \), component A). Thus in the component B also Val-1(\( \beta \)) has been derivatized by galacturonic acid. This was confirmed by the isolation and characterization of the radioactive tryptic peptide of \( \text{B}_1 \) as \( \beta_1 \) modified at its amino-terminal residue, valine.

The tryptic peptide mapping of \( \text{B}_2 \) from component B demonstrated that it is modified \( \alpha \)-chain. The radioactivity of this modified \( \alpha \)-chain eluted in two positions. The material isolated from the two radioactive regions of the peptide map was isolated and was purified further by a second reversed phase HPLC. The identity of the peptide has been established by amino acid analysis. The amino acid composition of the radioactive peptides corresponded to those of \( \alpha_1 \) (residues 1–7 of \( \alpha \)-globin) and \( \alpha_{1i} \) (residues 1–11 of \( \alpha \)-globin) except for the loss of 1 valine residue in each of these peptides. Besides the peptide, the composition of which corresponded to that of \( \alpha_{1i} \) and had both of the lysyl residues expected to be present in this segment. This demonstrates that the \( \alpha_{1i} \) is not derivatized in this overlapping tryptic peptide. Thus, \( \text{B}_2 \) has been identified as the modified \( \alpha \)-chain which in Val-1(\( \alpha \)) has been derivatized by the galacturonic acid. Therefore, component B represents a tetrasubstituted derivative of HbA in which all the four \( \alpha \)-amino groups of the protein have been derivatized by \( \beta \)-galacturonic acid through alkyamine linkage (Table I).

(iii) Characterization of Component C—This is also a tetrasubstituted derivative just like the component B. The difference between the two tetrasubstituted derivatives is in the site(s) at which the \( \alpha \)-chain is derivatized. The tryptic peptide mapping of the \( \text{C}_1 \) (the \( \beta \)-component of component C) is identical to that of \( \text{A}_1 \) and \( \text{B}_1 \). Isolation and the identification of the modified tryptic peptide of \( \text{C}_1 \) has confirmed the site of modification in the \( \beta \)-chain as Val-1(\( \beta \)). The tryptic peptide mapping of the \( \alpha \)-chain of this derivative revealed that the radioactivity of the \( \alpha \)-chain is not associated with either with \( \alpha_{1} \) or with \( \alpha_{1i} \). Thus the \( \alpha \)-amino group of the \( \alpha \)-chain is not derivatized in this tetrasubstituted derivative of HbA (Table I). The radioactivity associated with the \( \alpha \)-chain of component C is distributed in three to four regions of the map, suggesting a degree of heterogeneity in the reaction sites. Presumably this represents the derivatization of the \( \epsilon \)-amino groups of the internal lysyl residues of the chain. However, the identity of the sites of derivatization in the modified \( \alpha \)-chain of component \( \text{C}_1 \) has not been established.

Relative Reactivities of Val-1(\( \alpha \)) and Val-1(\( \beta \)) of HbA toward Reductive Alkylation with \( \beta \)-Galacturonic Acid

The DE-52 chromatographic studies of HbA reductively alkylated with \( \beta \)-galacturonic acid along with the characterization of the derivatives by tryptic peptide mapping has established that when nearly 90% of Val-1(\( \beta \)) is derivatized by reductive alkylation with \( \beta \)-galacturonic acid, the reaction at Val-1(\( \alpha \)) is only around 30%. The reactivity of Val-1(\( \alpha \)) and Val-1(\( \beta \)) for derivatization with acetaldehyde, glycolaldehyde, or glyceraldehyde (neutral aliphatic aldehyde) is nearly the same. The higher reactivity of Val-1(\( \beta \)) toward \( \beta \)-galacturonic acid as compared with that of Val-1(\( \alpha \)) could be a consequence of the steric factors due to the relatively larger size of galacturonic acid (compared with glycolaldehyde or glyceraldehyde) or simply the reflection of the presence of a negative charge at the distal end of the alkyl chain. Accordingly, as control for the studies with \( \beta \)-galacturonic acid, reductive alkylation of HbA with \( \beta \)-galactose (neutral analog of galacturonic acid) has also been carried out and the sites of derivatization has been determined by peptide mapping. Thus it is clear that preferential reaction of Val-1(\( \beta \)) for reductive alkylation with \( \beta \)-galacturonic acid is not a consequence of the size of the reagent. The preferential reactivity of galacturonic acid to modify the \( \alpha \)-amino groups of the \( \beta \)-chain of HbA is reminiscent of the reaction of glyoxylic acid with HbA, i.e., a "steering" effect of the carboxyl group at the distal end of the carbonyl reagent. Therefore based on these results, it could be concluded that reagents carrying a negative charge exhibit a higher propensity to derivatize the \( \alpha \)-amino group of the \( \beta \)-chain.

Preparation of Disubstituted HbA with Derivatization on Val-1(\( \alpha \))

The reductive alkylation of HbA with galacturonic acid as discussed above permits the preparation of only one of the two symmetrical isomeric forms of the disubstituted derivatives of HbA, i.e., the one in which the modification is on Val-1(\( \beta \)). For the preparation of the other symmetrical isomeric forms of the disubstituted HbA, namely the one with derivatization on Val-1(\( \alpha \)), an alternate approach had to be developed. The reaction of pHMB \( \alpha \)-chain (2 mm) with 100 mm galacturonic acid in the presence of 200 mm sodium cyanoborohydride at pH 6.0 and room temperature for 90 min at pH 6.0 and subsequently hybridized with unmodified \( \beta \)- and \( \alpha \)-chains, respectively, to generate the \( \alpha_5 \beta_2 \) type of structure. A, \( \alpha \)-chain; \( \beta \), \( \beta \)-chain. The position of HbA is marked in the chromatogram.

Fig. 5. Chromatography of \( \alpha \)- and \( \beta \)-chains that were first reductively alkylated with 100 mm galacturonic acid in the presence of 200 mm sodium cyanoborohydride at pH 6.0 and room temperature for 90 min and subsequently hybridized with unmodified \( \beta \)- and \( \alpha \)-chains, respectively, to generate the \( \alpha_5 \beta_2 \) type of structures. A, \( \alpha \)-chain; \( \beta \), \( \beta \)-chain. The position of HbA is marked in the chromatogram.
Val-1(β) could be isolated by this approach as well (Fig. 5B). The reaction of β-chain appears to generate a comparatively more heterogeneous product than that seen with α-chain.

**Influence of Reductive Alkylation of Val-1(α) and Val-1(β) of HbA with Galacturonic Acid on the Functional Properties of the Protein**

The reversible oxygenation curves of HbA, (α2β2), αGU2P’2, αβGU2, and αGUβ2GU2 have been determined. The derivatized samples, both the disubstituted derivatives and the tetrasubstituted derivative, exhibited normal cooperative oxygen binding. The Hill coefficient of the disubstituted derivative is around 2.2 and that of the tetrasubstituted derivative is around 2.1. The control value was 2.5. Thus the modification of the amino-terminal domain of HbA with galacturonic acid has not significantly influenced the oxygenation-mediated quaternary structural changes of the protein.

The oxygen affinity of the two disubstituted as well as one tetrasubstituted derivative of HbA, and the modulation of their oxygen affinity by the presence of DPG and chloride ions is presented in Table II. The intrinsic O2 affinity of HbA decreased as a consequence of the alkylation of Val-1(α) with galacturonic acid. The P50 increased to 12 from a control value of 8. Thus a nearly a 50% increase in the P50 of HbA is achieved (Table II). The alkylation of Val-1(β) also resulted in the reduction of the O2 affinity. However, the influence of reductive alkylation of Val-1(β) with galacturonic acid on the oxygen affinity of HbA is distinct compared with that seen when the modification is on Val-1(α). When both Val-1(α) and Val-1(β) are derivatized (tetrasubstituted derivative), the O2 affinity is lowered further; P50 increased to 15. The O2 affinity of the tetrasubstituted derivative suggests that the influence of the modification of the α-amino group of the second chain is additive with that of the first modification. Therefore, the oxygen affinity appears to be a direct consequence of the perturbation of the conformational aspects of the microenvironment of the modification site.

The influence of the modification on the modulation of the oxygen affinity by the heterotropic effectors has also been investigated. The P50 of the unmodified protein, α2β2, is increased nearly 3-fold in the presence of DPG. Similarly, the disubstituted HbA with the modification on Val-1(α) also exhibited an increase in its P50, by about 2.9-fold, in the presence of DPG (Table II). On the other hand, if the modification is present on Val-1(β), the sensitivity of O2 affinity of HbA to DPG is almost completely lost; P50 is increased by only about 1.3-fold instead of the 3-fold seen with the control sample. The sensitivity of the O2 affinity of the tetrasubstituted HbA to the presence of DPG is also low. The presence or absence of the modification on Val-1(β) determines the ability of the protein to interact with DPG.

The oxygen affinity of HbA is reduced in the presence of chloride ions; in the presence of 100 mM sodium chloride, the P50 of HbA is increased by nearly 60%. The sensitivity of the oxygen affinity of HbA to the presence of chloride ion is lowered when either Val-1(α) or Val-1(β) is derivatized. A 60% reduction in the sensitivity is seen on modification of Val-1(α), whereas only a 40% reduction in sensitivity is seen on derivatization of Val-1(β). The oxygen affinity of the tetrasubstituted derivative is nearly insensitive to the presence of chloride ions.

**Discussion**

The role of α-amino groups of HbA in the structure and function of the protein has been investigated by chemical modification studies. Carbamoylation of the α-amino groups of HbA represents one of the early site specific chemical modification reactions (Manning and Cerami, 1971). The modified HbA, i.e. carbamoylated product, is the amide of the carboxamino adduct. Several other chemical modification reactions of α-amino groups of HbA have also contributed to our understanding and appreciation of the structure and function of this region of molecule (Manning, 1991). More recently, we have developed reductive alkylation of the α-amino groups with aliphatic carbonyl compounds (acetaldehyde, glycolaldehyde, glyceraldehyde, dihydroxyacetone, and glyoxylic acid) as a chemical approach for a site-selective derivatization of the α-amino groups of HbA (Acharya et al., 1981, 1983a; Acharya and Sussman, 1983, 1984; Acharya et al., 1985). The resultant alkylamino derivative retains the original positive charge of the amino group. However, if the reductive alkylation is carried out with a negatively charged carbonyl reagent like glyoxalate, a negative charge is introduced to this particular environment (Acharya et al., 1981; Di Donato et al., 1983), i.e. an irreversible electrostatic modification of the region will be accomplished.

The major objective of the present studies of the reductive alkylation of HbA is to establish the influence of the nature, charge density, and/or stereochemistry of the negative charge at the distal end of an aliphatic aldehyde (double-headed reagents) on the propensity of their carbonyl group to form aldimine linkage (Schiff-base adducts) with the α-amino group of the protein and adopt this approach to prepare new and novel derivatives of HbA that could mimic the electrostatic modifications of the protein at their amino-terminal domain on the formation of the carboxamino adducts. A comparison of the results of the studies with glycolaldehyde (Acharya and Sussman, 1983; Acharya et al., 1985), and glyoxylic acid (Acharya et al., 1981; Di Donato et al., 1983) have suggested that the selectivity of the α-amino group of HbA toward reductive alkylation is significantly influenced by the presence of the negative charge at the distal end of the aldehyde used. The relative reactivity of Val-1(β) increases as compared with that at Val-1(α). The reactivity of Val-1(α) for reductive alkylation with p-galacturonic acid is also lower compared with that of Val-1(β). This reduced chemical reactivity of Val-1(α) toward p-galacturonic acid is not a consequence of the differences in the size of the carbonyl reagent. Galactose is accommodated readily at this site, and the reactivity of Val-1(α) and Val-1(β) toward reductive alkylation with galactose is nearly the same. The reactivity of galactose (molecule without the negative charge at the distal end) toward the α-amino groups of HbA is compa-

**Table II**

| Modification on | Without effectors | In the presence of DPG | In the presence of Cl- |
|-----------------|-------------------|------------------------|------------------------|
| Val-1(α)        | 12 (8)            | 36 (24)                | 15 (13)                |
| Val-1(β)        | 10 (8)            | 12 (24)                | 14 (13)                |
| Val-1 of (α) and -β | 15 (8)            | 18 (24)                | 16 (13)                |
|                 |                   |                        |                        |
|                 |                   |                        |                        |
The oxygen affinity was determined using Hemoscan. 50 mM BisTris buffer, pH 7.4, was used in these studies. Oxygenation curves were obtained at 37 °C. The oxygen affinity is expressed as mmHg of oxygen needed for 50% saturation ($P_{50}$).

| O$_2$ affinity when the alkyl group is | Carboxylate effect$^*$ |
|--------------------------------------|------------------------|
| Hydroxyethyl                         | Carboxymethyl          |
| Dihydroxypropyl                      | Carboxymethyl/ hydroxyethyl |
| Val-1(a)                             | 5                      | 12                     | 2.4                      |
| Val-1(b)                             | 11                     | 10                     | 17                      | 1.6                      |

$^*$ The carboxylate effect is expressed as the relative increase in the $P_{50}$ of the carboxymethylated sample as compared with that of respective hydroxyethyl or dihydroxypropyl derivatives of HbA.

The oxygen affinity of HbA is decreased as a consequence of the reductive alkylation of either Val-1(a) or on Val-1(b) with carbohydrate acid. In structural terms, the influence of reductive alkylation with negatively charged carbonyl reagents can be partitioned out into two components; the first component of the structural perturbation of the chemical modification comes from the introduction of the alkyl chain (galactose moiety). The derivatization of the primary amino group to a secondary amino group by reductive alkylation does not eliminate the positive charge of the site, since the change in the $pK_a$ of the $\alpha$-amino group on its alkylation is minimal. Thus the functional consequence of reductive alkylation using simple neutral aliphatic aldehydes is expected to come primarily from the presence of the alkyl chain introduced into the respective microenvironment. The second part of the structural perturbation that will occur as a result of reductive alkylation with negatively charged carbonyl reagents (double-headed reagents) is from the electrostatic modification of the microenvironment, a consequence of the introduction of the negatively charged carboxylate ion into the region.

The introduction of ethyl, hydroxyethyl, or 2,3-dihydroxypropyl group on Val-1(a) resulted in a nearly same level of increase in the oxygen affinity of the protein (Table III). The reductive alkylation of the amino groups has very little influence on the ionization behavior of the derivatized amino groups; accordingly, a significant perturbation of the "native" electrostatic interactions of this region of the molecule is not anticipated. Therefore, the structural, as well as the functional, consequences of these chemical modifications should be considered as the result of the presence of the alkyl group in the respective microenvironment. Contrary to the increase in the oxygen affinity of HbA seen on reductive alkylation of its Val-1(a) with neutral aliphatic aldehydes, the carboxymethylation of Val-1(a) results in a significant reduction in the oxygen affinity of HbA (Acharya et al., 1982; Di Donato et al., 1983). The carboxymethylation (-CH$_2$-COOH) differs from hydroxymethylation (-CH$_2$-CHOH) only in the introduction of a negatively charged carbonyl group (-COO$^-$) at the location of uncharged hydroxymethyl group. Therefore, the influence of introducing a carboxylate ion in the proximity of Val-1(a) is to increase the $P_{50}$ (decreased the oxygen affinity) by 2.4-fold (Table III).

The functional consequence of the modification of Val-1(b) by hydroxymethylation or dihydroxyethylation is to decrease the oxygen affinity of HbA. The introduction of the negatively charged carboxylate ion into the region as in the reductive carboxymethylation decreases the oxygen affinity further. The carboxylate at this site increases the $P_{50}$ by only about 1.5-fold (Table III). Thus, the intrinsic contribution of the carboxylate ion (electrostatic modification of the alkyl chain on Val-1(b) in reducing the oxygen affinity of HbA is lower compared with that at the Val-1(a). Thus, the O$_2$ affinity of HbA is more sensitive to the electrostatic modification of the amino terminal of $\alpha$-chain than that of the $\beta$-chain. The down-regulation of the oxygen affinity of HbA by the electrostatic modification correlates inversely with the propensity of the $\alpha$-amino groups of the respective chains to form aldimine adducts.

The structural consequence of carboxymethylation of the amino terminus of HbA may be anticipated to mimic the influence resulting from the formation of carboximide adducts on the $\alpha$-amino groups, an in vivo reversible electrostatic modification reaction of HbA, as this reaction introduces a negative charge to the respective microenvironments (Di Donato et al., 1983). Nonetheless, it should be noted that the stereochromy of the carboxylate group introduced on the amino nitrogen of the $\alpha$-amino groups by carboximide formation is distinct from that of carboxymethylation. Therefore, it is important to delineate the role, if any, of a unique stereochromy for the carboxylate ion of the carboximide adduct in dictating the modulation of O$_2$ affinity of HbA. It is also conceivable that the decrease in the oxygen affinity is primarily a consequence of the negative charge introduced into the region, the exact stereochromy of the charge being not very critical. A comparison of the functional consequence of the electrostatic modification of the amino-terminal domains of HbA by the carboxylate of the carboximide group with that by the carboxylate of the carbohydrate acid introduced on to the same $\alpha$-amino group will shed new insights into this structural concept.

The influence of the electrostatic modification of the Val-1(a) is nearly the same irrespective of whether the reagent used for the reductive alkylation is glyoxalate or carbohydrate acid (Table II). These results suggest that the overall perturbation of the oxygen affinity is primarily a consequence of the negative charge introduced into the region, the exact stereochromy of the carbohydrate ion being not very critical. A comparison of the functional consequence of the electrostatic modification of the amino-terminal domains of HbA by the carboxylate of the carbohydrate group introduces new insights into this structural concept.

The functional consequence of the electrostatic modification of Val-1(b) of HbA with carbohydrate acid is distinct as compared with that of carboxymethylation. The P$_{50}$ of HbA carboxymethylated at its Val-1(b) is nearly 70% higher as compared with that of HbA modified at the same site with carbohydrate acid. The lower influence of the carboxylate of carbohydrate acid on Val-1(b) as compared with that of the carboxymethyl group present at the same site does not appear to be a contribution of the differences in the length of the spacer arm itself. The influence of the reductive alkylation of Val-1(b) using galactose and glycolaldehyde are nearly the same. Thus the stereochromy of the carboxylate ion introduced on the Val-1(b), presumably its proximity to the side chain of another amino acid of the site appears to have a pronounced influence in determining the O$_2$-affinity of the derivatized HbA.

The O$_2$-affinity of the tetrasubstituted derivatives of HbA also demonstrates that the consequences of the electrostatic modification at Val-1(b) in modulating the oxygen affinity of the derivatized molecule is distinct in the carboxymethylated and carbohydrate acid modified tetrasubstituted derivatives. The carboxymethylation of the $\alpha$-amino groups of both the
Reductive Alkylation of HbA with Galacturonic Acid

Chains brings out a synergy in their propensity to reduce the oxygen affinity of the molecule. The $P_{50}$ of the tetracarboxymethylated derivative of HbA is nearly 2.5 times that of HbA with four d-galacturonic acid bound on the $\alpha$-amino groups, whereas the $P_{50}$ of HbA carboxymethylated at its Val-$\beta$ is 1.6 times that of HbA with d-galacturonic acid at the same site.

The carboxymethylated HbA may be considered as an analogue of the carbamino adduct. Indeed x-ray studies have shown that deoxy HbA-modified by carboxymethylation of Val-$1(\alpha)$ and Val-$1(\beta)$ is structurally similar to that of the corresponding carbamino adduct of deoxy HbA (Fanti et al., 1987). Of particular interest to the present study is the demonstration

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**Fig. 6.** Comparison of the possible stereochemical orientations of the carboxyl group at the distal end of the carboxymethyl group or d-galacturonic acid covalently linked to Val-$1(\beta)$ through an alkylaminine linkage. The comparison is shown as viewed in the $\beta$-cleft. The amino acid residues at the right side of the cleft are labeled with $B$s, whereas those on the left with $D$s. The carboxyl group of the carboxymethyl hemoglobin interacts with the $\epsilon$-amino group of the Lys-$82(\beta)$ of the same chain (an intrachain salt bridge). This orientation is consistent with the crystallographic data (Fig. 6A). The carboxyl group of the d-galacturonic acid on Val-$1(\beta)$ could also be accommodated to generate the intrachain salt bridge involving the $\epsilon$-amino group of Lys-$82(\beta)$ (Fig. 6B). However, the carboxyl group of the d-galacturonic acid adduct could also cross over the central cavity and form interchain salt bridge with the $\epsilon$-amino group of Lys-$82(\beta)$ of the trans $\beta$-chain (Fig. 6C). In this configuration the sugar chain forms hydrogen bonds with the side chain of His-$143(\beta)$ of the trans chain as well. Therefore the latter appears to be the more favorable configuration for the d-galacturonic acid derivative.
by crystallography that the carboxylate of the carboxymethyl group on Val-1(p) as well as that of carbamino adduct on the same site can form intrachain ionic interaction with the ε-amino group of Lys-82(p). The different levels of modulation of the oxygen affinity of HbA seen on reductive alkylation of Val-1(p) of HbA with glyoxylic acid and β-galacturonic acid is distinct and is conceivably a consequence of the distinct nature of the new electrostatic interactions engineered into the region by the two reagents. To gain further insight into the stereochemical aspects of the orientation of the carboxyl groups of the alkyl chains within the DPG cleft, molecular modeling of the alkyl chains in the respective alkylated HbA has been carried out (Fig. 6). The carboxyl group of the carbamino group on Val-1(p) interacts with the ε-amino group (Fig. 6A) of the Lys-82(p) of the same chain (an intrachain electrostatic interaction). The carboxyl group of the β-galacturonic acid on Val-1(p) is also capable of participating in a similar intrachain (cis) interactions (Fig. 6B). Interestingly, the carboxyl group of β-galacturonic acid could also cross over the β-cleft and form an interchain salt bridge involving the ε-amino group of Lys-82(p) of the second β-chain, i.e. this derivative also accounts for a trans electrostatic interaction. In this configuration with a trans interaction, the sugar chain of the β-galacturonic acid forms hydrogen bonds with the side chain of trans His-143(p) as well. Since the latter configuration involving a trans salt bridge appears to provide ample opportunity to generate additional stabilizing hydrogen bonds, we speculate that the structure involving the trans salt bridge is the favored one in the solution. Further studies involving the energy minimization of these alternate structures for HbA containing β-Galacturonic acid at Val-1(p) and the crystallographic investigations should provide further insight into these concepts.

It should also be noted here that the presence of a methylene spacer portion in the carbamino group induces a degree of localized movements in the peptide backbone which is absent in the carbamino adduct (Fantl et al., 1987). A small movement of the β-chain NH₂-terminal peptide away from central cavity is seen on carboxymethylation of Val-1(p). The imidazole of His-2(p) also moves toward the carboxyl group on Val-1(p). With these movements, the interaction of the new carboxyl terminus of the β-chain with Lys-82(p) seen on the formation of the carbamino adduct is conserved in the derivative of HbA with carboxymethylation on Val-1(p). Therefore, we speculate that the functional consequences seen on carboxymethylation of Val-1(p), particularly the synergy aspects, are conserved in the carbamino adduct formation as well. In fact, if the proximity of the microdomain of the negative charge to the amino nitrogen correlates directly with the propensity of the electrostatic modification to reduce the oxygen affinity, one would anticipate even a higher degree of reduction in the oxygen affinity of the molecule when the electrostatic modification of Val-1(p) is by the formation of carbamino adducts. At any rate, this molecular approach appears to be an adaptive mechanism evolved to ensure an efficient unloading of O₂ to the regions of the body with high levels of CO₂.

The carboxymethylation of Val-1(p) also brings out some long range structural effects mediated through the local specific interactions of the carboxylate ion with Lys-82(p). In this connection it is of interest to note that Fantl et al. (1985) have shown that carboxymethylation of Val-1(p) induces the differential ionization behavior to a group of HbA with a pKᵣ around 7.5, and this ionization behavior is particularly sensitive to ligation state of the molecule. Ho and Russo (1985) have demonstrated that the structural consequences of carboxymethylation of Val-1(p) is 2-fold. First, this electrostatic modification influences the local conformational aspects of the region as reflected by the changes in the C₁ and C₂ proton resonances of His-2(p). Besides, the long range conformational effects of carboxymethylation of Val-1(p) are centered at specific surface residues, particularly around His-116(p) and His-117(p). This conformational effect is enhanced considerably in the deoxygentated state.

An electrostatic modification of the β-cleft of HbA has been obtained by Bucci et al. (1989) using a different “double-headed reagent,” mono-(3,5-dibromosalicylic)fumarate. In this case the fumarate was linked to the ε-amino group of Lys-82(p) through an isopeptide linkage. The carboxylate of mono-(3,5-dibromosalicylic)fumarate "propels" the tagging end of the double-headed reagent toward Lys-82(p) with in the β-cleft, whereas the carboxylate of glyoxylate and β-galacturonic acid orients the reagent to facilitate the formation of aldime at Val-1(p). This clearly reflects the differences in the steering action of the distal carboxylate of these two classes of double-headed reagents. However, the oxygen affinity of the derivative of HbA generated by the reaction of mono-(3,5-dibromosalicylic)fumarate is comparable with that of HbA derivatized at its Val-1(p) by β-galacturonic acid and not to the one with carbamyl group on the Val-1(p). An interaction of the carboxylate ion of the fumarate introduced into the DPG pocket with either His-2(p) or with His 143(p) of the trans chain has been suggested and apparently this leads to the stabilization of the tetramer against dissociation around pH 5.5. The fumarolyl derivative of HbA has been referred to as “pseudo cross-linked HbA” in view of this stabilization. The molecular modeling studies of HbA containing β-galacturonic acid discussed above suggest that this derivative of HbA should also behave as the “pseudo cross-linked hemoglobins.” The lowering of the oxygen affinity of HbA on reductive alkylation of its Val-1(p) with β-galacturonic acid coupled with the fact this derivative is expected to behave as pseudo cross-linked hemoglobins makes this class of derivatized hemoglobins potential candidates as blood substitutes. The molecular modeling of the β-galacturonic acid into the DPG cleft also has provided a new dimension for the design of a new class of ββ interface directed bifunctional reagents based on the galacturonic acid chemistry.

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REFERENCES
Abola, E., Bernstein, F. C., Bryant, S. H., Koetzle, and Weng, J. (1987) The Protein Data Bank: A Computer-based Archival File for Macromolecular Structures. Crystallographic Databases-Information Content Software Systems, Scientific Applications (Allen, F. H., Bergenhoff, G., and Sievers, R., eds) pp. 107-132. Data Commission of the International Union of Crystallography, Bonn, Federal Republic of Germany
Acharya, A. S., and Manning, J. M. (1980) J. Biol. Chem. 255, 1406-1412
Acharya, A. S., and Susman, L. G. (1983) J. Biol. Chem. 258, 13761-13767
Acharya, A. S., and Susman, L. G. (1984) J. Biol. Chem. 259, 4572-4581
Acharya, A. S., Donato, A., and Manning, J. M. (1981) Fed. Proc. 41, 1174
Acharya, A. S., Susman, L. G., and Manning, J. M. (1985a) J. Biol. Chem. 260, 5296-5302
Acharya, A. S., Di Donato, A., Manjula, B. N., Fischetti, V., and Manning, J. M. (1985b) Int. J. Pept. Protein Res. 25, 76-82
Acharya, A. S., Susman, L. G., and Manning, J. M. (1985) J. Biol. Chem. 260, 6030-6046
Benesch, R. E., and Benesch, R. (1974) Adv. Protein Chem. 29, 211-237
Bernstein, F. C., Koetzle, T. F., Williams, G. J. R., Meyer, E. F., Rogers, R. R., Kennard, O., Shimanouchi, T., and Tasumi, M. (1977) J. Mol. Biol. 112, 535-542
Bucci, E., and Frongioli, C. (1965) J. Biol. Chem. 240, 551-552
Bucci, E., and Frongioli, C. (1981) Methods Enzymol. 78, 527-533
Bucci, E., Ragni, A., Urbaís, B., and Frongioli, C. (1985) J. Biol. Chem. 260, 6191-6195
Cerroni, A., and Manning, J. M. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 1180-1183
Di Donato, A., Fantl, W. J., Acharya, A. S., and Manning, J. M. (1983) J. Biol. Chem. 258, 11890-11895
Fantl, W. J., Di Donato, A., Manning, J. M., Rogers, R. H., and Arnone, A. (1987) J. Biol. Chem. 262, 12700-12707
Ho, C. (1992) Adv. Protein Chem. 43, 153-312
Reductive Alkylation of HbA with Galacturonic Acid

Ho, C., and Russu, I. M. (1985) in New Methodologies in Studies of Protein Conformation (Wu, T. T., ed) pp. 1–35, Van Nostrand Reinhold Company, New York
Kilmartin, J. V., and Rossi-Bernardi, L. (1969) Nature 222, 1243–1246
Kilmartin, J. V., and Rossi-Bernardi, L. (1973) Physiol. Rev. 53, 836–899
Manning, J. M. (1991) Adv. Enzymol. Relat. Areas Mol. Biol. 64, 55
Nigen, A. M., Njikam, N., Lee, C. K., and Manning, J. M. (1974) J. Biol. Chem. 249, 6611–6616
Rossi-Fattelli, A., Antonini, A., and Caputo, A. (1984) Adv. Protein Chem. 19, 73–222
Seetharam, R., and Acharya, A. S. (1986) J. Cell. Mol. Biochem. 30, 87–99
Ten Eyck, L. F., and Arnone, A. (1976) J. Mol. Biol. 100, 3–11