Properties of Carboxymethylated Cross-Linked Hemoglobin A†

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ABSTRACT: The selective carboxymethylation of the *N*-terminal amino groups of hemoglobin A with glyoxylic acid and sodium cyanoborohydride has been studied as a function of the state of ligation of hemoglobin. The *N*-terminal residues have been established as the primary sites of reaction by peptide mapping of the tryptic digest of each chain and subsequent amino acid analysis of the modified peptides. With oxyhemoglobin, the desired derivatives with a carboxymethyl group at the N-terminal of either or both chains amounted to 55% [Di Donato, A., Fantl, W. J., Acharya, A. S., & Manning, J. M. (1983) *J. Biol. Chem. 258*, 11890–11895]. In the present study it is shown that with deoxyhemoglobin the amount of the desired derivative is increased to 75%. The oxygen equilibrium curve of hemoglobin A carboxymethylated on its four N-terminal residues [0.5 mM as tetramer in 50 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris), pH 7.5, at 37 °C] had a P_{50} value of 30 mmHg (Hill coefficient $n = 2.8$, alkaline Bohr value = 0.4) compared to a P_{50} of 9 mmHg for unmodified hemoglobin under the same conditions ($n = 2.5$, alkaline Bohr value = 0.5). In carboxymethylated oxyhemoglobin A, cross-linked with the mild agent glycolaldehyde for 3.5 h, there was 85% of M_r 64000 species and 15% of M_r 128000 or higher species. For the former, the extent of cross-linking between two subunits was 19%. For the latter, there was 29% of two cross-linked subunits and 13% of three cross-linked subunits. Termination of cross-linking, which may be desirable in some circumstances, can be successfully achieved with isonicotinic acid hydrazide. Carboxymethylated hemoglobin after being cross-linked with glycolaldehyde for 5 h to yield about 20% of two cross-linked subunits had an average P_{50} of 14 mmHg (average $n = 2.0$) compared with an average P_{50} of 7 mmHg for cross-linked, unmodified hemoglobin A ($n = 1.6$). Thus, carboxymethylated cross-linked hemoglobin A releases its O_2 more readily than unmodified hemoglobin. This property forms the basis for further studies on the possible use of this derivative as a blood substitute.

Studies on hemoglobin-based blood substitutes have been under way in several laboratories (Bolin et al., 1983). Various derivatives of human hemoglobin have been prepared to attain an oxygen affinity of the protein compatible with efficient oxygen delivery. Within the red cell the release of oxygen from hemoglobin is facilitated by its interaction with 2,3-DPG,[1] protons, and CO_2 (Kilmartin et al., 1973). It has been known for many years that small inorganic anions such as chloride when bound to purified hemoglobin will also lead to a decreased oxygen affinity (Antonini & Brunori, 1971). This effect could be a general, nonspecific one of increased ionic strength, or alternatively, the binding of chloride could be specific for certain sites on the hemoglobin molecule. Indeed, recent studies from several laboratories with different approaches (Rollema et al., 1975; O'Donnell et al., 1979; Chiancone et al., 1975; Fermi, 1975; Nigen & Manning, 1975) have shown that there are defined sites on the hemoglobin molecule that bind chloride ions. One of these is at the N-terminus of the α-chain, and a second site is in the cleft between the two β-chains, which usually binds 2,3-DPG in deoxyhemoglobin. Furthermore, it has become evident in the past few years that any neutralization or lowering of the net positive charge within this cleft leads to a lower oxygen affinity of hemoglobin. Such an effect is produced either by binding of 2,3-DPG or of small inorganic anions or in an abnormal hemoglobin [i.e., hemoglobin Providence (Lys-82(β) → Asn or Asp) (Bonaventura et al., 1976)] or in a hemoglobin specifically modified at a residue within this cleft (Nigen & Manning, 1975). However, it is not feasible to attain a *permanent* low oxygen affinity of isolated hemoglobin simply by addition of chloride since the binding of this anion is not particularly strong, and hence, the sustained presence of a fairly large concentration would be required.

The concept of preparing a *covalent* adduct of hemoglobin to mimic the effect of chloride is one that we have considered (Di Donato et al., 1983; Acharya et al., 1982) on the basis of previous studies on the effects of the binding of chloride at specific sites on hemoglobin. To this end, we have investigated the carboxymethylation of hemoglobin at specific sites on the protein (eq 1). Other studies have been also based on

$$\mathrm{Hb\text{-}NH_2} + \underset{\mathrm{COOH}}{\mathrm{CHO}} \rightleftharpoons \underset{\mathrm{COOH}}{\mathrm{Hb\text{-}N{=}CH}} \xrightarrow{\mathrm{NaCNBH_3}} \underset{\mathrm{COOH}}{\overset{\mathrm{H}}{\mathrm{Hb\text{-}N\text{-}CH_2}}} \quad (1)$$

this strategy (Hedlund et al., 1983; Saunders & Hedlund, 1984). This approach was chosen because the carboxymethyl group is a covalent anionic moiety of small size and therefore not considered likely to interfere with the essential functions of hemoglobin to a major extent. An additional consideration in the initial choice of this derivative was that the carboxymethyl group covalently attached to the N-terminal amino groups of hemoglobin ($HbNHCH_2COO^-$) could be considered as an analogue of the CO_2–hemoglobin adduct, carbaminohemoglobin ($HbNHCOO^-$), an additional factor that could lead to a reduced oxygen affinity of the hemoglobin derivative. Indeed, this proposed analogy has received support from a

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[1] Abbreviations: Hb, hemoglobin; 2,3-DPG, 2,3-diphosphoglycerate; P_{50}, pressure of O_2 at which hemoglobin is 50% saturated with the gas; INH, isonicotinic acid hydrazide; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

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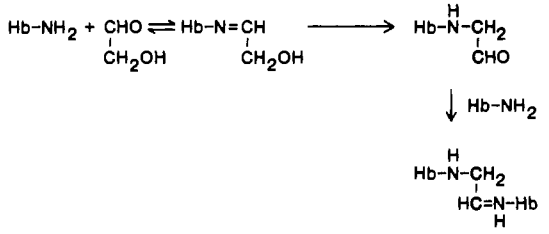
recent X-ray crystallographic comparison of the carboxymethyl and the carbamino derivatives of hemoglobin.[2]

In our initial studies, we found that purified hemoglobin hybrids with the carboxymethyl moiety at the N-terminal residues had an oxygen affinity that was lower than that of unmodified hemoglobin A (Di Donato et al., 1983). In those studies the carboxymethylation reaction was performed on hemoglobin that was fully oxygenated, and the amount of modification at the desired sites, i.e., the N-terminals of the α- or β-chains, amounted to about 50%. The other sites of carboxymethylation were the ϵ-amino groups of lysine residues, and these derivatives were removed chromatographically. In an effort to achieve more of the desired species carboxymethylated at the N-terminal residues in the initial reaction with sodium glyoxylate and sodium cyanoborohydride, we have studied the reaction as a function of the ligation state of hemoglobin. These results are reported herein.

An analogous rationale has been the basis for the use of the pyridoxal phosphate–hemoglobin adduct (Bolin & De Venuto, 1983; Sehgal et al., 1983), since some of these derivatives have been considered as covalent analogues of 2,3-DPG bound to hemoglobin (Benesch & Benesch, 1981). Some of these phosphorylated compounds share some similar binding sites on hemoglobin.

Another consideration in the successful development of a blood surrogate is that the hemoglobin derivative should not dissociate into its constituent dimers and monomers, which would be rapidly cleared by the circulation (Bunn & Jandl, 1968). Therefore, some type of covalent cross-linking of hemoglobin should be performed in order to prevent such dissociation. One approach that is currently being explored is intratetrameric cross-linking by disubstituted aspirin derivatives (Fronticelli et al., 1986; Walder et al., 1979). Other studies have utilized glutaraldehyde as the reagent for both intra- and intertetrameric covalent cross-linking (Bolin et al., 1981). This potent dialdehyde probably cross-links in a random fashion and could conceivably lead to some distortion of the hemoglobin structure and thereby affect its function. Recently, we have explored the properties of a milder cross-linking reagent, glycolaldehyde, a monoaldehyde that initially forms a Schiff base with amino groups of a protein and then undergoes the Amadori rearrangement to generate a new aldehyde function capable of cross-linking (Acharya & Manning, 1983) (eq 2). The potential advantage of this latent



cross-linking reagent is that it is a much milder agent and introduces a small cross-link into the protein and thus it is more likely to preserve the native features of the protein. The use of this cross-linking reagent with hemoglobin is explored in this paper.

Materials and Methods

Whole blood from normal individuals was centrifuged at 2000*g* for 10 min at 4 °C, and the supernatant plasma was discarded. After the cells had been washed 3 times with cold isotonic saline, they were lysed by the addition of an equal volume of distilled water. This solution was then frozen and slowly thawed. The lysate thus obtained was then centrifuged at 12000*g* for 20 min to remove membrane and particulate matter. The lysates were then dialyzed at 4 °C against 0.1 M NaCl for removal of organic phosphates (Di Donato, 1983). For some studies, the hemoglobin was saturated with CO at all steps of the preparation. For studies with deoxyhemoglobin, CO was not used until after the initial carboxymethylation reaction, as described below. Hemoglobin concentrations were determined by measurement of the absorbance at either 420 or 540 nm. Sodium glyoxylate, sodium cyanoborohydride, and glycolaldehyde were obtained from Sigma. In some experiments [^{14}C]sodium glyoxylate (Amersham) was added to unlabeled sodium glyoxylate as described previously (Di Donato et al., 1983). When necessary, the concentration of glyoxylate was determined as the 2,4-dinitrophenylhydrazone derivative (Friedemann, 1957). All other chemicals were of reagent grade or of the highest purity available.

Reductive Carboxymethylation of Hemoglobin A. Dialyzed lysates containing hemoglobin A (1.5 mM) were treated with a 10-fold molar excess of sodium glyoxylate and a 20-fold molar excess of sodium cyanoborohydride at 25 °C in 50 mM potassium phosphate, pH 7.4, for 60 min. For experiments with liganded hemoglobin, the CO form was used. For studies with deoxyhemoglobin, a specially constructed two-armed Erlenmeyer flask was used for the deoxygenation and the subsequent mixing of reactants (Manning, 1981). After the carboxymethylation reaction, the hemoglobin was saturated with CO, and the excess starting materials were removed from the protein by gel filtration on a Sephadex G-25 column (2 × 25 cm) with 50 mM Tris–acetate buffer, pH 8.3, as the eluent.

Isolation of Carboxymethylated Hemoglobin Species. The gel-filtered carboxymethylated sample was applied to a column (2.2 × 35 cm) of Whatman DE-52 (Di Donato et al., 1983). The eluant applied to this column consisted of a linear gradient of 50 mM Tris–acetate from pH 8.3 to pH 7.3 (500 mL of each). The carboxymethylated hemoglobin species were located by determination of the absorbance of each fraction at 540 nm. The designations of the various carboxymethylated hemoglobin species were the same as used previously (Di Donato et al., 1983).

Treatment of Hemoglobin with Glycolaldehyde and Estimation of the Amount of Cross-Linking. For these studies either unmodified hemoglobin or carboxymethylated hemoglobin (species Hb_1 or Hb_2 from the DE-52 column) was used. The concentration of hemoglobin was about 0.02 mM in 50 mM KPO_4 buffer, pH 7.3. Glycolaldehyde was added at a concentration of 50 mM unless otherwise indicated, and the cross-linking was performed at room temperature for varying periods of time, as described below. Samples for analysis were dialyzed extensively against 0.02 M sodium phosphate, pH 7.4.

In order to estimate the amount of cross-linking between tetramers (i.e., *inter*tetrameric), a portion of the glycolaldehyde-treated hemoglobin, dialyzed against 50 mM Tris–acetate buffer, pH 7.3, was applied to a 2 × 110 cm column of Sephadex G-100. The column was eluted with 50 mM Tris–acetate buffer, pH 7.3, and the fractions were located by their absorption at either 280, 420, or 540 nm. For estimation of the amount of subunit cross-linking, the hemoglobin sample was analyzed by SDS gel electrophoresis on a 14% cross-linked acrylamide gel. The amounts of hemoglobin loaded onto each gel were in the range of 5–10 μg. After the gel was stained with Coomassie blue for 16 h and destained

[2] W. J. Fantl et al., unpublished results.

in 30% methanol and 5% acetic acid, the amount of each cross-linked subunit was estimated by densitometry on a Gilford Model 2520 instrument. The gel scans were quantitated by weighing of the area under each peak.

Termination of Cross-Linking. In some studies it was desirable to stop or to control the cross-linking reaction. For this purpose, isonicotinic acid hydrazide (INH) was added at a concentration of 50–100 mM either at the initial time of incubation or after a certain period of time. The sample was then dialyzed prior to SDS gel electrophoresis as described above.

Determination of Oxygen Equilibrium Curves of Carboxymethylated and Cross-Linked Hemoglobin. The hemoglobin derivatives, prepared as described above, were concentrated to about 0.05 mM by ultrafiltration with an Amicon YM-10 membrane and then dialyzed against 0.05 M Bis-Tris buffer, pH 7.3. Prior to determination of the oxygen equilibrium curve on an Aminco Hem-O-Scan, each sample was converted from the CO form to the oxy form as described previously (Nigen et al., 1974) and finally concentrated to about 0.5 mM in an Amicon Centricon 10 microconcentrator. The P_{50} values were determined directly from the graphs of the Hem-O-Scan. For estimation of the Hill coefficient, the logarithmic values of the fractional saturation from 40 to 75% were plotted against logarithmic values of the oxygen tension. The slope of this straight line gave the n value.

Amino Acid Analysis. For some samples amino acid analysis was performed in order to estimate either the degree of carboxymethylation or the extent of cross-linking as reflected by the decrease in lysine content (Acharya & Manning, 1983). For such purposes the samples were dialyzed extensively against water prior to hydrolysis in 6 N HCl for 20–24 h. Estimation of the amounts of amino acids present was performed either on the amino acid analyzer of Spackman et al. (1958) or on a Dionex D-500 instrument.

RESULTS

Effects of the State of Ligation of Hemoglobin on the Profile of Its Carboxymethylation. The distribution of N-carboxymethylated species of *oxy*hemoglobin after reaction with sodium glyoxylate and sodium cyanoborohydride has been reported previously (Di Donato et al., 1983). Comparison of those results with the chromatographic profile of the products formed during the carboxymethylation of *deoxy*hemoglobin (Figure 1) indicates that although the position of each derivative is practically identical, their distribution is different. In addition, the overall extent of carboxymethylation is significantly increased when the starting material is deoxyhemoglobin as indicated by the decreased amount of unmodified hemoglobin (Hb_0) compared to that found when oxyhemoglobin was carboxymethylated (Di Donato et al., 1983).

Previously, it was demonstrated that for Hb_2 there was nearly complete carboxymethylation of N-terminal residues of the α- and β-chains of oxyhemoglobin and Hb_1 contains two carboxymethyl groups per tetramer with predominant reaction at the N-terminus of the β-chain (Di Donato et al., 1983). Hb_3 represents carboxymethylation on the ϵ-amino groups of lysine residues as well as on the N-termini of both chains. With deoxyhemoglobin as the starting material, the amounts of Hb_1 and Hb_2 are increased to nearly 75% of the total (Figure 1) compared with about 50% of the total when oxyhemoglobin is carboxymethylated. In addition, when deoxyhemoglobin is carboxymethylated, there is less of the undesirable species Hb_3.

Cross-Linking of Unmodified Hemoglobin. The initial studies on the cross-linking of hemoglobin with glycolaldehyde were performed with unmodified hemoglobin A in the liganded state. At a constant glycolaldehyde concentration of 50 mM and a hemoglobin concentration of 0.02 mM at 25 °C, aliquots were removed at several intervals during a 24-h period. The extent of reaction between hemoglobin subunits was determined by SDS–polyacrylamide gel electrophoresis, as shown in Figure 2. It is evident that the cross-linking of hemoglobin subunits by glycolaldehyde is a relatively slow process. Thus, after treatment with glycolaldehyde for 1, 3, and 5 h there was a gradual increase in the amount of two cross-linked subunits

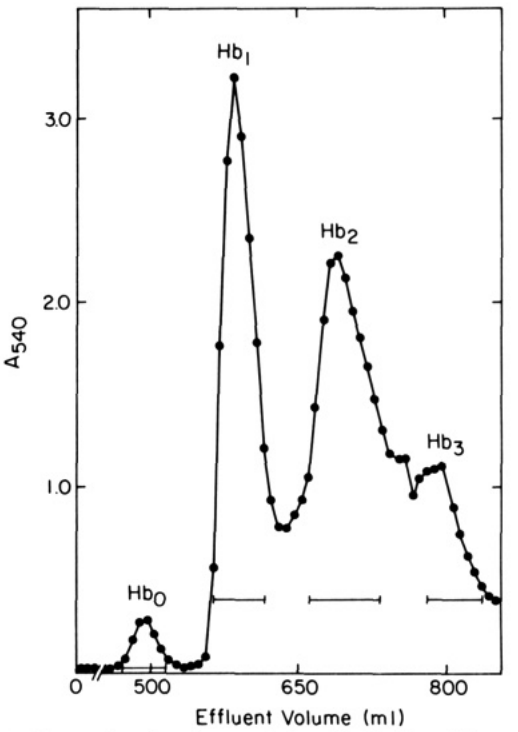


FIGURE 1: Separation of carboxymethylated derivatives of hemoglobin on DE-52. The sample of deoxyhemoglobin A was carboxymethylated as described in the text. After dialysis, the sample was applied to a column and eluted with the gradient as described in the text. The designations Hb_0, Hb_1, Hb_2, and Hb_3 are defined in the text.

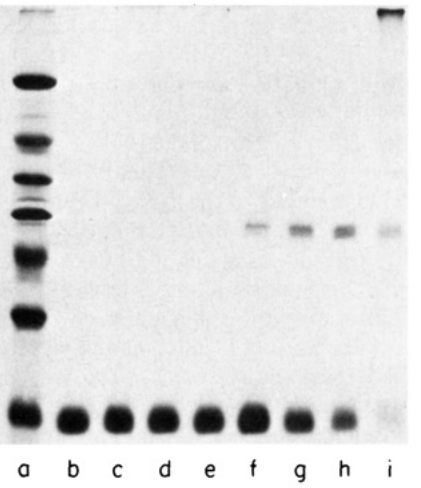


FIGURE 2: SDS gel electrophoresis of hemoglobin before and after treatment with glycolaldehyde. Lane a contains a standard mixture of proteins of defined molecular weight from 14000 (bottom) to 66000 (top). The fourth band from the bottom of the gel is carbonic anhydrase with a M_r of 29000. The hemoglobin sample was incubated in buffer alone for 1 (lane b), 3 (lane c), 5 (lane d), or 24 h (lane e). Unmodified hemoglobin A was treated with glycolaldehyde as described in the text for 1 (lane f), 3 (lane g), 5 (lane h), or 24 h (lane i).

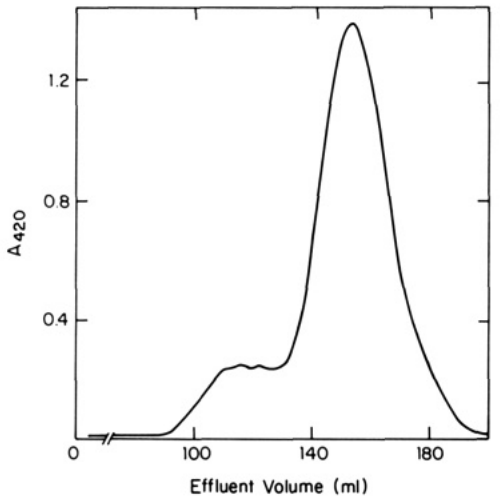


FIGURE 3: Gel filtration of cross-linked hemoglobin. A sample of carboxymethylated hemoglobin (Hb_2) was treated with 42 mM glycolaldehyde for 3.5 h. This sample was not fractionated on DE-52 but was applied to a column of Sephadex G-100 and eluted as described in the text.

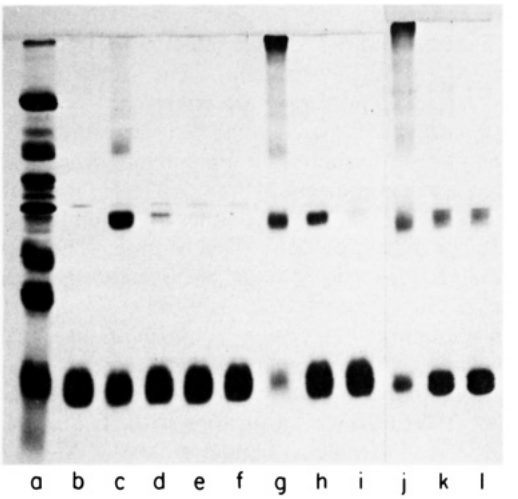


FIGURE 4: Effect of INH on cross-linking of hemoglobin by glycolaldehyde. Lane a contains a mixture of proteins of defined molecular weight as described in the legend to Figure 3. Hemoglobin A was incubated for 4.5 h in the absence (lane b) or the presence of 50 mM glycolaldehyde (lane c) or with 50 mM glycolaldehyde and 50 mM INH (lane d) or with 50 mM glycolaldehyde and 100 mM INH (lane e). The samples in lanes f–i were similar to those in lanes a–e but were incubated for 24 h. In lane j, the sample contained hemoglobin and 50 mM glycolaldehyde. In lane k, hemoglobin, 50 mM glycolaldehyde, and 50 mM INH were incubated for 24 h. The sample in lane l initially contained glycolaldehyde and INH, each at a concentration of 50 mM for 30 min. Hemoglobin was then added, and the incubation was carried out for 24 h.

of M_r 32 000 and a corresponding decrease in single hemoglobin subunits (Figure 2, lanes f–h). For the samples incubated from 1 to 5 h, there was no detectable loss in lysine content, most likely because of the large amount of unmodified, monomeric subunits present. For the sample taken at 24 h (lane i) there were a large amount of very high molecular weight aggregate and a concomitant disappearance of single subunits. Under these conditions, the lysine content was significantly decreased with no detectable effect upon the other amino acids. Thus, the Lys/Val ratio was reduced from 0.73 to 0.50, the Lys/Ala ratio was reduced from 0.56 to 0.32, and the Lys/Leu ratio was reduced from 0.55 to 0.39 (comparison of unmodified Hb with cross-linked Hb) for an average decrease in lysine content of 31%. The ratios found above for unmodified Hb are close to theoretical values of 0.74 (Lys/Val), 0.61 (Lys/Ala), and 0.61 (Lys/Leu).

In order to provide an indication of the extent of cross-linking *between* hemoglobin tetramers, gel filtration of native cross-linked hemoglobin was performed on Sephadex G-100 (Figure 3). After 3.5 h of incubation of glycolaldehyde with a sample of carboxymethylated hemoglobin, gel filtration showed a large peak of hemoglobin that eluted in the area of M_r 64 000. In addition, a significant amount of higher molecular weight species, which represented about 15% of the total material, eluted near the void volume of the column. These latter species most likely represent cross-linked hemoglobin tetramers of M_r 128 000 and larger, as judged by the slight inflections in the curve.

Although cross-linking with glycolaldehyde is a slow process, the extent of cross-linking is reproducible. Thus, after treatment of Hb (0.02 mM) with glycolaldehyde (50 mM) for 5 h, SDS gel electrophoresis of the M_r 64 000 material (from the G-100 column) indicated the presence of 24% (±1%, average of three separate experiments) of two cross-linked subunits with the remainder of the material as un-cross-linked subunits. A similar analysis for the higher molecular species from G-100 showed 13% (±3%) of three cross-linked subunits, 29% (±3%) of two cross-linked subunits, and the remainder as un-cross-linked subunits (average of three experiments).

Termination of Cross-Linking. The cross-linking by glycolaldehyde is a relatively slow process, as just described. Therefore, the *extent* of cross-linking can, to some extent, be controlled by appropriate choice of the ratios of reactants and the time of incubation. However, some preliminary studies have suggested that cross-linking of hemoglobin can continue even after removal of glycolaldehyde by dialysis. Any hemoglobin molecules that contain pendant glycolaldehyde moieties (either as a Schiff base or as the rearranged aldoamine) that had not yet cross-linked with a second amino group of a lysine residue (eq 2) could still undergo cross-linking even after removal of free glycolaldehyde. Therefore, with the aim of maintaining control of the cross-linking reaction, it seems advisable to have a means to terminate cross-linking at a definite stage. To investigate this possibility, several compounds were tested as potential terminators of the cross-linking.

The most efficient inhibitor of cross-linking that we have found thus far is isonicotinic acid hydrazide (INH). As shown in Figure 4, INH effectively hinders cross-linking of hemoglobin subunits mediated by glycolaldehyde not only after 4.5 h of incubation (lanes d and e) but also after exposure for 24 h (lanes h and i). Under these latter conditions but in the absence of INH, there is extensive formation of high molecular weight species and little remaining single subunits (lane g). Since INH can also react with carbonyls such as glycolaldehyde, there was the question whether it had reacted extensively with glycolaldehyde and thereby only *apparently* prevented cross-linking. This possibility was assessed by preincubation of glycolaldehyde and INH for 30 min. Hemoglobin was then added and the incubation continued for an additional 24 h. The extent of cross-linking to form subunit dimers was decreased only slightly (Figure 4, lanes k and l) by the preincubation. Thus, glycolaldehyde in the presence of INH is still capable of reacting with hemoglobin.

Oxygen Affinity of Carboxymethylated Hemoglobin. The oxygen equilibrium curve of a hemoglobin tetramer fully carboxymethylated at the N-terminal residues of its four chains ($\alpha_2^{Cm}\beta_2^{Cm}$) is shifted significantly to the right as reported previously (Di Donato et al., 1983). In this paper, we have extended this study to include the pH dependence of the ox-

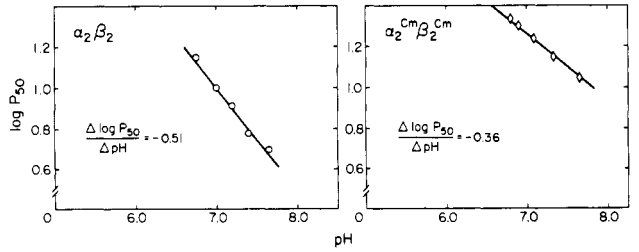


FIGURE 5: pH dependence of the oxygen affinity of unmodified and of carboxymethylated hemoglobin A. The oxygen affinity of the samples in the presence of 0.1 M chloride at each pH value was determined on an Aminco Hemo-O-Scan instrument as described in the text. This experiment was carried out at 25 °C.

ygen affinity so that the Bohr coefficient could also be calculated (Figure 5). On the left panel is shown the oxygen affinity, expressed as log P_{50} as a function of pH, for unmodified hemoglobin, $\alpha_2\beta_2$. The slope of this line, which is a measure of the Bohr coefficient, has a value of about −0.51, close to the reported values (Antonini & Brunori, 1971). The data in the right panel of Figure 5 show that the line generated as a plot of log P_{50} vs. pH is elevated. This fact indicates that the individual oxygen equilibrium curves of $\alpha_2^{Cm}\beta_2^{Cm}$ at each pH have shifted significantly to the right. The Bohr coefficient calculated from the slope of this line is lowered to a value of −0.36. These values are nearly identical with those measured for the same derivatives at pH 7.2 by the proton titration method. This moderately reduced Bohr effect is due to the presence of the carboxymethyl group on the N-termini of the β-chains since $\alpha_2\beta_2^{Cm}$ has the same lowered Bohr effect as $\alpha_2^{Cm}\beta_2^{Cm}$ but $\alpha_2^{Cm}\beta_2$ has a normal Bohr effect in 0.1 N NaCl.[2] As discussed below, the structural reasons for the lowering of the Bohr effect after carboxymethylation of the terminal amino groups may be due to the carboxymethyl group on the termini of the β-chains protruding fairly deep into the DPG cleft as assessed by X-ray diffraction studies.[2]

Oxygen Equilibrium Curves of Carboxymethylated and Cross-Linked Hemoglobin A. Treatment of unmodified hemoglobin with glycolaldehyde for intermediate periods of time (5 h) results in the formation of both intra- and intertetrameric cross-linking corresponding to 22% of two cross-linked subunits and 4% of three cross-linked subunits in an unfractionated sample (Figures 2 and 3). In order to determine whether such treatments had an adverse effect on the molecule, i.e., formation of methemoglobin or parital denaturation, the oxygen equilibrium curve of this population of hemoglobin molecules was determined. No spectral evidence of the presence of met-Hb was found. The oxygen affinity of this sample was slightly higher (P_{50} = 7 ± 0.1 mmHg, average of two determinations) than that of unmodified hemoglobin (P_{50} = 9 mmHg, Figure 6, curve A), and the Hill coefficient was lowered from 2.5 to an average n value of 1.6. Thus, the cross-linking of hemoglobin by glycolaldehyde leads to a slight increase in its oxygen affinity and a decrease in its cooperativity, which is anticipated if some subunits were not free to function independently because of the cross-linking.

The oxygen equilibrium curve of $\alpha_2^{Cm}\beta_2^{Cm}$ (Figure 6, curve C) shows that its oxygen affinity is lowered even further at 37 °C (to a value of 30 ± 0.8 mmHg, average of two determinations) than it is at 25 °C (Figure 5). The Hill coefficient for this sample is 2.8, which indicates that hemoglobin fully carboxymethylated at its N-terminal amino groups has not undergone a drastic alteration in its structure. The absorption spectrum is not affected by carboxymethylation.

Hemoglobin carboxymethylated on its four N-terminal residues and then cross-linked with glycolaldehyde had an

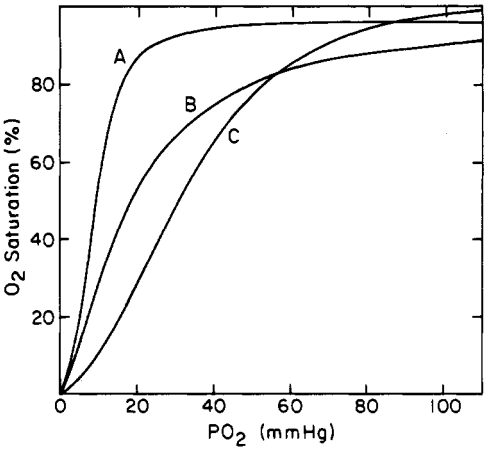


FIGURE 6: Effect of cross-linking and carboxymethylation on the oxygen equilibrium curve of hemoglobin A. The oxygen equilibrium curves of these samples was determined in 50 mM Bis-Tris, pH 7.5, and the experiments were carried out at 37 °C as described in the text on an Aminco Hem-O-Scan instrument. (Curve A) Unmodified hemoglobin A; (curve B) carboxymethylated hemoglobin A (component Hb_2 of Figure 1) treated with 50 mM glycolaldehyde for 5 h as described in the text; (curve C) carboxymethylated hemoglobin A corresponding to component Hb_2 of Figure 1.

oxygen equilibrium curve with an average P_{50} of 14 ± 1.5 mmHg (average of three determinations) and a Hill coefficient of 2.0 (Figure 6, curve B). Thus, carboxymethylated, cross-linked hemoglobin releases its oxygen more readily than unmodified hemoglobin or cross-linked, unmodified hemoglobin.

Discussion

Except for the desirable property of a low oxygen affinity, carboxymethylated hemoglobin retains many of the native properties of hemoglobin including its cooperativity, thus indicating that the conditions whereby the carboxymethyl group is introduced on the N-terminal amino groups of hemoglobin are not drastic. The nature of this carboxymethylation reaction is quite different from the carboxymethylation of amino acid side chains with iodoacetate (Crestfield et al., 1963). This latter reaction is much less specific than reductive carboxymethylation since in addition to modification of amino groups it can lead to extensive modification of sulfhydryl, imidazole, and thioether side chains of cysteine, histidine, and methionine residues, respectively, unless conditions are controlled. Reductive carboxymethylation is exclusive for amino groups because only these side chains form Schiff bases with glyoxylate prior to their subsequent reduction with cyanoborohydride. By manipulation of conditions such as pH and ratio of reactants to hemoglobin, we have selectively (but not exclusively) modified the N-terminal amino groups of the α- and β-chains of hemoglobin. This has been demonstrated by peptide mapping and subsequent amino acid analysis, and more recently, these conclusions have been corroborated by X-ray diffraction studies.[2] The carboxymethylation of proteins has been explored by King et al. (1977) for various protein allergens. In addition, the reductive carboxymethylation procedure that we described previously for hemoglobin has also been studied by Hedlund et al. (1983, 1984).

The carboxymethyl moiety, covalently bound at a specific site on hemoglobin, can be considered as a small covalently bound anion that approximates the size and charge of the chloride anion. Recent studies on the electron density maps obtained by X-ray diffraction of the specifically carboxymethylated hybrids indicate that the presence of the small covalent anion at the terminus of the α-chain has little structural effect on the protein except to expel an anion

(probably chloride) that had been bound noncovalently at this site with little change in the alkaline Bohr effect. The presence of the carboxymethyl group on the N-terminus of the β-chain does, however, lead to a lowering of the alkaline Bohr effect. The X-ray diffraction studies show that the carboxymethyl group protrudes fairly deeply into the DPG cleft. One possible interpretation for the decreased alkaline Bohr effect is that a particular residue within the DPG cleft [but not Val-1(β) itself] interacts with the carboxymethyl group such that its pK_a is slightly changed so that the *differential* in the pK_a of this residue in the oxy and the deoxy states is less than that for the same residue in unmodified hemoglobin (i.e., the basis of the Bohr effect). The present studies also clearly show that the full carboxymethylation of the terminal amino groups has not led to a large perturbation of the hemoglobin since the Hill coefficient of 2.8 was found for $\alpha_2^{Cm}\beta_2^{Cm}$.

The lowered oxygen affinity of carboxymethylated hemoglobin is most likely due to the decreased positive charge within the DPG cavity as a result of the presence of the negatively charged carboxymethyl group bound covalently. This phenomenon has been found for other chemically modified (Nigen & Manning, 1975) and mutant hemoglobins (Bonaventura et al., 1976), and a structural reason for it is currently under study. In addition, it is clear from inspection of its structure that the carboxymethyl adduct could easily be considered as an analogue of carbaminohemoglobin, in which carbon dioxide is carried on the amino groups of hemoglobin. However, in the latter case this derivative is not stable, and carbon dioxide is rapidly released. On the other hand, the carboxymethyl derivative with a methylene group introduced into the carbamate structure is a stable covalent modification of the protein and permits study of the effects of anions bound to the protein.

The previous studies on the cross-linking of proteins with the mild agent glycolaldehyde, reported earlier for RNase (Acharya & Manning, 1983), have now been successfully extended to the oligomeric protein hemoglobin. The question of the optimum molecular weight for cross-linked hemoglobin (i.e., 64 000 or 128 000) is one that is under investigation. In the present study where the majority of the cross-linked tetramers are of M_r 64 000, it is possible that there was a fair amount of $\alpha\beta$ dimer present at the low initial hemoglobin concentrations used. This factor may be subject to manipulation that could lead to the formation of a different profile of products. The relatively slow rate of cross-linking may be an advantage since more control of the reaction would be possible than with a potent cross-linking agent such as glutaraldehyde. Cross-linking by glutaraldehyde produces very high molecular weight species even after a short exposure.

It would also seem advantageous to control the extent of cross-linking by an additive (i.e., terminator) that would react with *any* existing Schiff base or aldoamine adduct of glycolaldehyde on the protein. A survey of a number of compounds indicated that a very efficient terminator is the substituted hydrazide, isonicotinic acid hydrazide (INH). Indeed, this class of reagents has been shown to derivatize, with high efficiency, the carbonyl functions at the macromolecular level of proteins whether these moieties are present as coenzymes such as pyridoxal phosphate (Wada & Snell, 1961; Yonaha et al., 1975) or in flavin-containing enzymes (Nagy et al., 1979) or present as an integral part of the native protein as in pyruvoyl enzymes (Riley & Snell, 1968; Recsei & Snell, 1984). In studies on proteins in which the carbonyl function is introduced as a result of a chemical modification, hydrazides were found to react with such adducts if they were present as the open-chain ketoamine structure. Examples include glyceraldehyde–hemoglobin adducts (Acharya & Manning, 1980), glucose–albumin adducts (Ghiggeri et al., 1986), and a glucose–collagen complex (Brownlee et al., 1986). The lack of a reaction of a substituted hydrazine with a glucose–hemoglobin adduct has been taken as evidence for a cyclic ketoamine structure (Fischer & Winterhalter, 1981). Clearly, cross-linking can come about if sufficiently high concentrations of aldoamine or ketoamine adducts are present on a protein, and any successful derivatization of such moieties should lead to efficient prevention of the cross-linking of proteins.

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Registry No. Sodium glyoxylate, 2706-75-4; glycolaldehyde, 141-46-8.

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Thermodynamics of the Quenching of Tyrosyl Fluorescence by Dithiothreitol†

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ABSTRACT: Tyrosyl fluorescence quenching by oxidized dithiothreitol (DTT_o) in *N*-acetyl-L-tyrosine *N'*-methylamide, and native bovine pancreatic ribonuclease A and its reduced, S-methylated form, in aqueous solution is studied at pH 3.0. From the temperature dependence of the fluorescence quenching, it is demonstrated that the mechanism of the quenching process is probably static (formation of a complex), and not dynamic (collisional), in origin. Although other quenching mechanisms cannot be ruled out, our proposition that the quenching of tyrosyl fluorescence in these molecules is due to the formation of a complex between the tyrosyl moieties and DTT_o is consistent with previously reported evidence indicating a strong tendency for aromatics to complex with various disulfide-containing compounds. The strength of binding is approximately the same for these three tyrosine-containing compounds, indicating that the microenvironments of their tyrosyl residues may be similar. With 1 M as the reference standard state, the following average thermodynamic parameters are established for the complexation (at 298 K): $\Delta G° = -3.32$ kcal/mol, $\Delta H° = -1.1$ kcal/mol, and $\Delta S° = 7.4$ eu. The large positive value of $\Delta S°$ suggests that hydrophobic interactions may play an important role in the stabilization of such tyrosyl–disulfide complexes; the negative value of $\Delta H°$ suggests that polar interactions may also contribute to the formation of these complexes. Some possible implications with regard to protein-folding studies are discussed.

In a study of the fluorescence of polypeptides (Cowgill, 1967), it was demonstrated that intramolecular disulfides quench tyrosyl fluorescence. The details of the quenching mechanism were not known, but it was determined (Cowgill, 1967) that the quenching was neither collisional nor a direct through-bond effect. More recently, Bodner et al. (1980) noted that, in CCl_4 solution, dimethyl disulfide exhibits a chemical shift change in the proton nuclear magnetic resonance (NMR) spectrum on addition of 1-methylnaphthalene or other aromatic compounds. These authors attributed this behavior to the formation of a complex and estimated the enthalpy of complexation to be about −1 kcal/mol. It was further noted (Morgan et al., 1978; Morgan & McAdon, 1980) that residues containing aromatic rings and sulfur atoms tend to lie near each other in the three-dimensional structures of many globular proteins, suggesting that aromatic–disulfide interactions may stabilize the native conformation. A theoretical study (Némethy & Scheraga, 1981) indicated that the lowest energy complex of dimethyl disulfide and benzene is about 0.8 kcal/mol more stable than the corresponding complex with cyclohexane.

Disulfide-containing compounds have been used extensively in oxidative regeneration of proteins from their reduced forms, e.g., oxidized glutathione (Saxena & Wetlaufer, 1970; Hantgan et al., 1974; Ahmed et al., 1975; Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982; Bouet et al., 1982), 2-mercaptoethanol (Creighton, 1979), and DTT_o[1] (Orsini et al., 1975; Creighton, 1977). In these and many other extensive

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[1] Abbreviations: DTT_o and DTT_r, oxidized and reduced forms, respectively, of dithiothreitol; Cys–SS–Cys, cystine; Cys–SH, cysteine; Ox–SS–Ox and Ox–SH, oxidized and reduced forms of a disulfide-containing redox couple; Tris, tris(hydroxymethyl)aminomethane; Gly, glycine; TFA, trifluoroacetic acid; Gdn·HCl, guanidine hydrochloride; RNase A, bovine pancreatic ribonuclease A; EDTA, ethylenediaminetetraacetic acid; 8SMe, disulfide-reduced and S-methylated RNase A; AcTyrNHMe, *N*-acetyl-L-tyrosine *N'*-methylamide.

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