Influence of Ligation State and Concentration of Hemoglobin A on Its Cross-Linking by Glycolaldehyde: Functional Properties of Cross-Linked, Carboxymethylated Hemoglobin†

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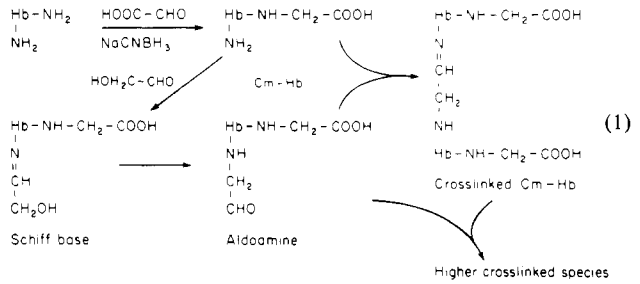
ABSTRACT: The ligation state of hemoglobin during its cross-linking by glycolaldehyde influences the ultimate oxygen affinity of the cross-linked protein. Thus, if the cross-linking is performed with carbonmonoxyhemoglobin, the oxygen affinity increases slightly to a P_{50} of 7 mmHg from a P_{50} of 9 mmHg for unmodified hemoglobin. In contrast, when deoxyhemoglobin is cross-linked with glycolaldehyde, the oxygen affinity of the product decreases (P_{50} = 15 mmHg). When deoxyhemoglobin is first carboxymethylated and then cross-linked with glycolaldehyde, an even lower oxygen affinity is achieved (P_{50} = 23 mmHg). Carboxymethylated hemoglobin is very responsive to the presence of 5% CO_2 with a P_{50} of 33 mmHg, which is lowered further to 42 mmHg when chloride (0.1 M) is also present. Hemoglobin carboxymethylated and cross-linked under anaerobic conditions is also responsive to the modulators CO_2 and chloride with a resultant oxygen affinity of 27 mmHg. The type of cross-linking of liganded hemoglobin by the mild reagent glycolaldehyde is dependent upon the initial hemoglobin concentration. Thus, with dilute hemoglobin (45 μM in tetramer), cross-linking by glycolaldehyde (50 mM) results in about 75% of 64 000 molecular weight species (some of which are cross-linked *within* tetramer) and 25% of intertetrameric cross-linked species with a range of molecular weights averaging 128 000–512 000. With hemoglobin solutions of higher concentration (360 μM), the amount of the higher molecular weight species increases to about 65% with a corresponding reduction to 35% in the 64 000 molecular weight component.

We have described previously some of the properties of carboxymethylated hemoglobin (Di Donato et al., 1983; Fantl et al., 1987a,b) and its cross-linking by glycolaldehyde (eq 1).



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The lowered oxygen affinity of carboxymethylated hemoglobin A provides the basis for studies on this derivative as a potential blood substitute. Hemoglobin (Hb)[1] A hybrids, in which either the α-chain or the β-chain was specifically carboxymethylated, each contribute separately to the overall decrease in oxygen affinity of the fully carboxymethylated hemoglobin tetramer. However, the contribution from the carboxymethyl group at the N-terminus of the β-chain is greater than that from the α-chain derivative. Both solution and X-ray diffraction studies clearly indicate that carboxymethylated hemoglobin can be considered to be an analogue of the hemoglobin–CO_2 (carbamino adduct) (Fantl et al., 1987a). Both carboxymethyl- and carbamino-Hb derivatives have lower oxygen affinities than unmodified hemoglobin, and the X-ray diffraction studies showed that both adducts present on the N-terminal residues of hemoglobin interact at similar places on the protein. However, the derivatives display very significant differences in stability. The carbamino adduct is very unstable, and this property makes hemoglobin an efficient transporter of CO_2. However, the carboxymethyl derivative is very stable, and this property lends it to studies on its potential use as a blood substitute (Fantl et al., 1987b; Hedlund et al., 1983). The stability of the carboxymethyl derivative of hemoglobin also permits a variety of studies that could help elucidate the chemistry of the interaction of CO_2 with hemoglobin. Thus, the *rate* of carboxymethylation of the N-terminal residues of hemoglobin together with competition experiments in the presence of CO_2 indicates that the N-termini of the α-chains in the deoxy state are the preferred sites for CO_2 binding, a conclusion consistent with the results of Doyle et al. (1987).

The carboxymethyl group is also considered to mimic the effect of bound chloride in terms of its charge and size and its effect on lowering the affinity of hemoglobin. The X-ray diffraction analysis lent support to this speculation. Both chloride and CO_2 when bound to hemoglobin lower its oxygen affinity under physiological conditions (Rossi-Bernardi & Roughton, 1967; Antonini & Brunori, 1971). The effects of these modulators on cross-linked, carboxymethylated hemoglobin are examined in this paper.

The initial studies on the carboxymethylation of hemoglobin with glyoxylate and sodium cyanoborohydride were done with liganded hemoglobin (Di Donato et al., 1983). Under these conditions, about 50% of the desired modification was on the N-terminal residues of either the α- or the β-chains. If the

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

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reductive carboxymethylation is performed under conditions of deoxygenation, then the amounts of the desired derivatives are increased to 75%, and there is less of the undesirable component with carboxymethylated lysine residues (Fantl et al., 1987b). This approach is developed further in this paper.

A hemoglobin-based blood surrogate should not dissociate into its constituent dimers and monomers, which would be rapidly cleared from the circulation (Bunn & Jandl, 1968). Therefore, the hemoglobin should be cross-linked or derivatized in some manner to prevent dissociation (Chang, 1987). Two bifunctional agents have been reported to lead to a tetrameric hemoglobin derivative with a low oxygen affinity when the cross-linking is performed under anaerobic conditions (Chatterjee et al., 1982; Kavanaugh et al., 1987). We have investigated the use of glycolaldehyde, a cross-linking agent of latent reactivity (Acharya & Manning, 1983), which is much milder than glutaraldehyde. The latter aldehyde reacts with a large number of lysine residues, and the resultant derivative suffers some loss of hemoglobin function. In our initial studies with glycolaldehyde and oxyhemoglobin A, we found that the cross-linking reaction took place over a period of hours with relatively few lysine residues involved (Fantl et al., 1987b). The hemoglobin derivative was a mixture of high and low molecular weight species with a substantial amount of hemoglobin function, such as its cooperativity, retained. In the present study, we have examined the effect of the ligation state of hemoglobin and its concentration on the pattern of cross-linking with glycolaldehyde. We have also studied the feasibility of performing the carboxymethylation and the cross-linking in tandem (eq 1), and we have investigated the response of the derivative to the allosteric modulators chloride and CO_2.

Materials and Methods

Whole blood from normal individuals was centrifuged at 2000*g* for 10 min at 4 °C. After removal of the supernatant plasma, the cells were washed 3 times with cold isotonic saline, lysed by addition of an equal volume of cold distilled water, and dialyzed against 0.1 N NaCl as described previously (Di Donato et al., 1983). The concentration of hemoglobin was determined by its absorbance at either 420 or 540 nm. Sodium glyoxylate and glycolaldehyde were obtained from Sigma, and sodium cyanoborohydride was from Aldrich. Sephadex G-100 and Sepharose 4B were products of Pharmacia. All other chemicals were reagent grade or of the highest purity available.

Reductive Carboxymethylation of Hemoglobin A. Dialyzed lysates containing hemoglobin A (0.5–1.5 mM) were treated with a 10-fold excess of sodium glyoxylate and a 20-fold 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 two-armed Erlenmeyer flask (Manning, 1981) was used, and deoxygenation was performed by bubbling the contents of the flask with nitrogen for 1–2 h at 25 °C before the reactants were tipped into the main chamber from the side arms. If no further reactions were to be performed, the carboxymethylated hemoglobin was saturated with CO, and the excess starting materials were removed by gel filtration on Sephadex G-25 (2 × 25 cm) with 50 mM Tris–acetate buffer, pH 7.3, as the eluent. If further reaction (i.e., cross-linking with glycolaldehyde) of deoxyhemoglobin was to be performed, CO was not used. However, the gel filtration was still carried out in order to remove $NaCNBH_3$.

Cross-Linking of Hemoglobin with Glycolaldehyde. Either unmodified or carboxymethylated hemoglobin was used for these studies. The final concentration of hemoglobin was varied from 45 to 360 μM in 50 mM potassium phosphate buffer, pH 7.3. For studies with liganded hemoglobin, the CO form was used. For experiments with deoxyhemoglobin, the oxygen was removed by flushing with nitrogen as described above. Glycolaldehyde was added to a final concentration of 50 mM unless otherwise indicated. The cross-linking was performed at room temperature for 4.5 h, and the hemoglobin derivative was then dialyzed extensively against 50 mM Tris–acetate, pH 7.3.

Separation of High and Low Molecular Weight Cross-Linked Tetramers. The amount of cross-linking *between* hemoglobin tetramers (i.e., *inter*tetrameric) was measured by application of the cross-linked hemoglobin to a 2 × 110 cm column of Sephadex G-100. The sample had been dialyzed against 50 mM Tris–acetate buffer, pH 7.3, and the column was eluted with the same buffer. Fractions (2 mL) were collected, and the amount of hemoglobin component was determined by its absorption at either 420 or 540 nm. The 64 000 molecular weight species were pooled and analyzed as described below. The fractions containing components of 128 000 or greater molecular weight were pooled, concentrated to about 150 μM, and applied to a 2 × 110 cm column of Sepharose 4B. The eluent was 50 mM Tris–acetate, pH 7.3.

Estimation of Cross-Linking between Hemoglobin Subunits. For estimation of the amount of cross-linking between hemoglobin subunits, the dialyzed samples were subjected to SDS electrophoresis by the procedure of Laemmli (1970) in 14% cross-linked gels. The amount of protein loaded onto each gel was in the range of 5–10 μg. After the gel was stained with Coomassie Blue, it was destained in 30% methanol and 5% acetic acid, and the amount of each cross-linked subunit was estimated by densitometry on a Gilford Model 2520 instrument.

Determination of Oxygen Equilibrium Curves of Hemoglobin Derivatives. The hemoglobin derivatives were dialyzed against 50 mM Bis-Tris–acetate buffer, pH 7.5. Prior to determination of the oxygen equilibrium curve at 37 °C 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 then concentrated to about 0.5 mM in an Amicon Centricon 10 microconcentrator or in an Amicon stirred cell with a YM10 membrane. The P_{50} values were determined directly from the graphs of the Hem-O-Scan as the oxygen tension corresponding to 50% of the maximal saturation attained (precision, ±1 mmHg). The Hill coefficients were estimated from the logarithmic values of the fractional saturation from 40–75% when plotted against the logarithmic values of the oxygen tension. The slope of this line gave the *n* value. In some experiments, the effects of chloride and CO_2 on the hemoglobin derivatives were determined. These modulators were added to a concentration of either 0.1 or 0.2 M (chloride) or in the appropriate tanks for the Hem-O-Scan containing an O_2/N_2 mixture and 5% CO_2 (for the latter studies, the buffer strength was increased to 0.1 M Bis-Tris–acetate, pH 7.5).

Results

Effects of the Ligation State of Hemoglobin on Its Cross-Linking by Glycolaldehyde. Liganded hemoglobin A after cross-linking with glycolaldehyde has a slightly higher oxygen affinity (P_{50} = 7 mmHg) than unmodified hemoglobin (P_{50} = 9 mmHg). These oxygen equilibrium curves are depicted in Figure 1 (curve A for unmodified Hb, curve B for cross-linked Hb). Cross-linking lowered the Hill coefficient from a value of 2.5 for unmodified hemoglobin to an average *n* value of 1.6 for cross-linked hemoglobin. When hemoglobin

Table I: Effect of Physiological Modulators on the Oxygen Affinity of Hemoglobin Derivatives

ligation state	modulator		P_{50}[g]	n
	Cl^- (M)	CO_2 (%)		
unmodified hemoglobin				
oxy	0	0	9	2.5
oxy	0	5.0	13	2.3
oxy	0.1	0	15	2.6
oxy	0.1	5.0	20	2.8
Cm-hemoglobin				
oxy[a]	0	0	30	2.2
deoxy[b]	0	0	29	2.0
deoxy[b]	0	5.0	33	2.3
deoxy[b]	0.1	0	37	2.2
deoxy[b]	0.1	5.0	42	2.4
cross-linked Hb				
oxy[c]	0	0	7	1.6
deoxy[d]	0	0	15	1.4
deoxy[d]	0	5.0	17	1.4
deoxy[d]	0.1	0	19	1.5
deoxy[d]	0.1	5.0	21	1.4
cross-linked Cm-Hb				
oxy[e]	0	0	14	1.6
deoxy[f]	0	0	23	1.3
deoxy[f]	0	5.0	24	1.4
deoxy[f]	0.1	0	25	1.4
deoxy[f]	0.1	5.0	27	1.4

[a] This sample was carboxymethylated under aerobic conditions. The measurement was performed on a chromatographically purified component that was carboxymethylated on its four NH_2-terminal residues [analogous to component Hb_2 in Fantl et al. (1987b)]. [b] These samples were carboxymethylated under anaerobic conditions. They represent a mixture of Hb tetramers, 75% of which are carboxymethylated on the NH_2-terminal residues of either or both chains [analogous to the sample described in Figure 1 of Fantl et al. (1987b)]. [c] This Hb sample (28 μM) was cross-linked with 50 mM glycolaldehyde for 5 h at 25 °C and then treated as described in the text. [d] These Hb samples (180 μM) were first deoxygenated in the side-arm flask as described in the text. Glycolaldehyde was then added to a final concentration of 50 mM and the cross-linking carried out for 4.5 h at 25 °C. [e] The sample described in footnote *a* was cross-linked with 50 mM glycolaldehyde for 5 h at 25 °C. [f] These samples were carboxymethylated as described in footnote *b* in the side-arm flask described in the text. After removal of excess reactants on Sephadex G-25, the Cm-Hb sample (180 μM) was then deoxygenated again in the side-arm flask. Glycolaldehyde was then tipped in from the side arm to a final concentration of 100 mM and the cross-linking allowed to proceed for 2 h at 25 °C. [g] Each P_{50} value (in mmHg at 37 °C) is an average of at least two and sometimes three determinations. The precision of all measurements was calculated to be ±1 mmHg.

A was treated with glycolaldehyde under conditions of complete deoxygenation, the oxygen affinity of the cross-linked hemoglobin A is significantly lowered (P_{50} = 15 mmHg) (Figure 1, curve C). The Hill coefficient of this sample was 1.4. Thus, the ligation state of hemoglobin influences the resultant oxygen affinity of the cross-linked material.

HbA first carboxymethylated and then cross-linked under anaerobic conditions (in the side-armed flask as described above) has a still lower oxygen affinity was a P_{50} value of 23 mmHg (Figure 1, curve D). These results show that cross-linking of carboxymethylated hemoglobin under anaerobic conditions increases its oxygen affinity somewhat (compared to hemoglobin that is carboxymethylated but *not* cross-linked) but the cross-linked carboxymethylated hemoglobin derivative still retains a fairly low oxygen affinity (Table I).

Effect of Various Modulators on the Oxygen Affinity of Hemoglobin Derivatives. Since any potential blood substitute will function in the presence of some plasma components, it was of interest to examine the effects of chloride and CO_2, two major components of plasma, on the functional properties of these various hemoglobin derivatives. As shown in Table I, both chloride and physiological concentrations of CO_2 lower

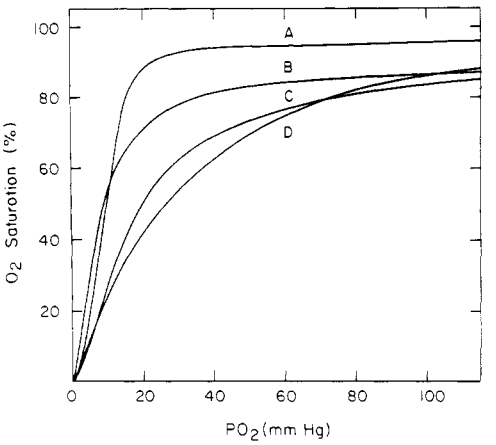

FIGURE 1: Oxygen affinity of hemoglobin after cross-linking with glycolaldehyde in either the liganded or the unliganded state. The cross-linking of Hb (45 μM) with glycolaldehyde (50 mM) was performed as described in the text. The oxygen equilibrium curves of Hb (0.5 mM) were determined in 50 mM Bis-Tris–acetate, pH 7.5, at 37 °C on an Aminco Hem-O-Scan instrument. (A) Unmodified HbA. (B) HbA; cross-linked in the liganded state. (C) HbA; cross-linked in the unliganded state. (D) HbA; carboxymethylated and then cross-linked in the unliganded state.

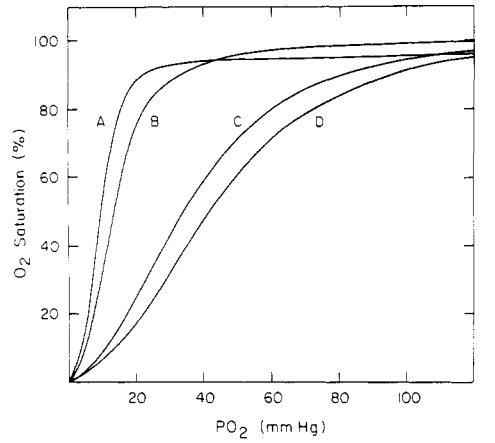

FIGURE 2: Effect of chloride and CO_2 on the oxygen affinity of unmodified or carboxymethylated hemoglobin A. The oxygen equilibrium curves were determined as described in the legend to Figure 1. (A) HbA in the absence of chloride or CO_2. (B) HbA in the presence of 5% CO_2. (C) HbA, which had been carboxymethylated under anaerobic conditions, in the presence of 5% CO_2. (D) HbA, which had been carboxymethylated under anaerobic conditions, in the presence of 5% CO_2 and 0.1 M NaCl. When present, chloride was added just before the measurement. For curves B, C, and D, the gases contained 5% CO_2.

the oxygen affinity of unmodified hemoglobin to a moderate degree, and chloride at a concentration of 0.1 M is more effective than 5% CO_2. When hemoglobin is exposed to these concentrations of *both* modulators, its oxygen affinity is lowered further such that the effect on P_{50} is additive. This result suggests that chloride and CO_2 at these concentrations act at some different sites on hemoglobin to produce these effects. However, at higher concentrations, chloride probably binds to some of the CO_2 binding sites since, in the presence of 0.2 M chloride *and* 5% CO_2, no further lowering of the oxygen affinity is found (data not shown). Thus, for unmodified Hb, these modulators result in a decrease in the oxygen affinity of over 2-fold with a limiting P_{50} value of 20 mmHg obtained under apparently saturating concentrations of each modulator.

The effects of CO_2 and of carboxymethylation on the oxygen affinity of hemoglobin A are compared in Figure 2. The effect of CO_2 alone on unmodified Hb is shown by comparison of curves A and B. The decrease in oxygen affinity is slight, with

a P_{50} increase to 13 mmHg in the presence of 5% CO_2 (curve B) from 9 mmHg in its absence (curve A). Prevously, we had shown that the P_{50} of a chromatographically purified hemoglobin derivative carboxymethylated on its four NH_2-terminal groups was in the range 30–35 mmHg (Di Donato et al., 1983). In the experiment described in Figure 2, the hemoglobin was carboxymethylated under anaerobic conditions, but it was *not* purified chromatographically. Therefore, it represents a sample consisting of about 75% of tetramers carboxymethylated on the NH_2 terminals of the α- and/or β-chains on the basis of previous analysis (Fantl et al., 1987b). For this carboxymethylated Hb sample, there is a significant shift to the right of the oxygen equilibrium curve with a P_{50} of 33 mmHg (Figure 2, curve C). Perhaps the covalent carboxymethyl (Cm) group is more effective than CO_2 alone in lowering the oxygen affinity of Hb because it is more efficient in saturating the CO_2 binding sites on Hb than is the loosely bound carbamate ($HbNHCOO^-$). Addition of 0.1 M chloride to Cm-Hb further lowers the oxygen affinity as depicted in curve D (P_{50} = 42 mmHg). Chloride binding sites not near the NH_2-terminal residues to which the carboxymethyl group is bound may be responsible for this additional effect. Full cooperativity is retained for both the unmodified and the carboxymethylated hemoglobin with n values in the normal range (Table I).

Hemoglobin A cross-linked under conditions of deoxygenation loses some cooperativity (n = 1.4) and is less responsive to chloride than unmodified hemoglobin (Table I). However, as described above, hemoglobin A cross-linked *and* carboxymethylated under anaerobic conditions has a much lower oxygen affinity than unmodified hemoglobin. Furthermore, this cross-linked carboxymethylated hemoglobin derivative is still somewhat responsive to chloride and CO_2 although less so than unmodified Hb. Nevertheless, the resultant P_{50} value of 27 mmHg for this Hb derivative in the presence of both modulators is larger than observed for unmodified Hb under the same conditions. Therefore, neither the modification by carboxymethylation nor the cross-linking by glycolaldehyde has blocked all of the binding sites for chloride or for CO_2 in these derivatives.

Effect of Hemoglobin Concentration on Its Cross-Linking by Glycolaldehyde. As reported earlier, glycolaldehyde cross-links hemoglobin both in an intermolecular and in an intramolecular fashion. The degree of this cross-linking is a function of glycolaldehyde concentration and also of time of exposure to the cross-linking agent (Fantl et al., 1987b). The concentration dependence of hemoglobin on the distribution of cross-linked components and a study of their approximate molecular weights have been investigated. With dilute hemoglobin concentrations (45 μM), most of the cross-linking is *within* a 64000 molecular weight tetramer, and there is a small amount (25%) of cross-linking between hemoglobin tetramers (Figure 3). When higher hemoglobin concentrations were employed in the cross-linking reaction, the distribution of *inter*tetrameric and *intra*tetrameric species changed (Figure 3). Thus, the higher molecular weight species are predominant if the initial hemoglobin concentration is of the order of 360 μM or about 25 mg/mL. Some of these high molecular weight species in the void volume of the Sephadex G-100 column probably contain components of greater than 128000 molecular weight. To fractionate these components further, the material that eluted in the void volume of the Sephadex G-100 column (40% of the total with the remainder as 64000 molecular weight tetramer) was concentrated and applied to a 2 × 110 cm column of Sepharose 4B. Most (85–95%) of the

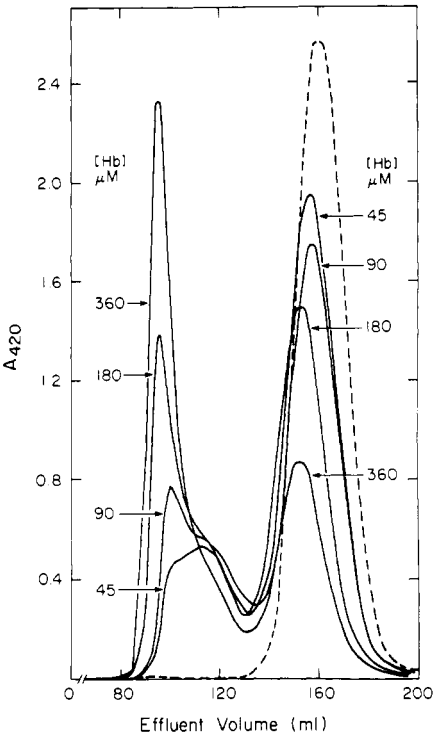

FIGURE 3: Effect of initial hemoglobin concentration on the components generated by cross-linking with glycolaldehyde. The dashed line represents unmodified HbA (90 μM).

cross-linked hemoglobin eluted between 128000 and 512000 molecular weight, corresponding to two and eight cross-linked tetramers. Lesser amounts of higher molecular weight components could have been present, but these were not readily fractionated on this column. The net P_{50} of this sample was 8 mmHg. SDS gel electrophoresis of the cross-linked Hb tetramers that were retained on the Sepharose 4B column indicated that there was 23% of two cross-linked subunits and 24% of three or more cross-linked subunits.

Discussion

The reductive carboxymethylation of hemoglobin does not adversely affect the native structure of the protein. The major change in the functional properties of carboxymethylated hemoglobin is its lower oxygen affinity, which is a desirable property for a blood substitute. Reductive carboxymethylation is directed exclusively at the amino groups of hemoglobin since these are the only amino acid side chains that form Schiff base adducts with sodium glyoxylate that are reducible by sodium cyanoborohydride. The selective modification at the N-terminal amino groups of the α- and β-chains of hemoglobin was achieved after testing a variety of conditions such as varying pH and ratios of reactants. This fact has been established by peptide mapping (Di Donato et al., 1983) and more recently by X-ray diffraction analysis (Fantl et al., 1987a).

The carboxymethyl moiety attached to hemoglobin is a small stable covalent adduct which is much more stable and hence easier to study than the carbamino adduct which forms a weak linkage with hemoglobin. Thus, the low oxygen affinity of the carboxymethyl derivative ($HbNHCH_2COOH$) mimics that of the carbamino adduct (HbNHCOOH). The X-ray diffraction maps of carboxymethylhemoglobin and of carbaminohemoglobin are strikingly similar not only with respect to the location of the adducts but also, more importantly, with respect to their interaction with other parts of the protein (Fantl et al., 1987a). It has been appreciated for some time that the CO_2–hemoglobin (carbamino) adduct has a low ox-

ygen affinity (Rossi-Bernardi & Roughton, 1967) but the molecular basis for this has been difficult to establish because of the lability of the carbamino adduct. The lower oxygen affinity of carboxymethyl-Hb is apparently due in part to the fact that the carboxymethyl group on the N-terminus of the β-chain protrudes deep into the DPG cleft as observed by X-ray diffraction analysis. This interaction leads to a neutralization of positive charge in this cleft between the two β-chains, and such an interaction is known to lead to a lower oxygen affinity. This effect has been previously observed in studies both with chemically modified (Nigen & Manning, 1975; Nigen et al., 1980) and with a mutant hemoglobin (Bonaventura et al., 1976). Thus, the introduction of the covalent carboxymethyl group at this site mimics the effects of the binding of chloride, DPG, or CO_2, which are modulators that neutralize positive charges within this site. Such neutralization of positive charge at this site stabilizes the deoxy or T structure of hemoglobin.

The low oxygen affinity of carboxymethylated hemoglobin is conducive to its potential use as a blood substitute. A successful blood surrogate must also be prevented from dissociation into its constituent subunits. Chemical cross-linking is an efficient process to accomplish this end. The cross-linking of hemoglobin by glycolaldehyde, a mild, latent cross-linking agent, has a number of unique features. Since the reaction is relatively slow, there are advantages in terms of control of the reaction regarding the degree of cross-linking. This fact has been demonstrated in the present paper since an enrichment of cross-linked species of different molecular weight was achieved as a function of the initial hemoglobin concentration. It is not clear at present whether a cross-linked 64 000 molecular weight species or a higher molecular weight species is preferable for a blood substitute. The present results which show that the reaction with glycolaldehyde can be manipulated to achieve enrichment of either species may be a desirable feature.

The finding that the cross-linking of hemoglobin in the oxy conformation stabilizes that conformation of hemoglobin and treatment of deoxyhemoglobin with glycolaldehyde stabilizes the deoxy conformation to yield a cross-linked hemoglobin with a lower oxygen affinity was unanticipated in view of the simple nature of the cross-linking agent. Therefore, it follows that the oxygen affinity obtained with other types of cross-linking agents may not be due solely to the nature of the particular cross-linking agent itself but rather, to some extent, to the ligation state of the hemoglobin used in the reaction. The lower oxygen affinity of carboxymethylated cross-linked hemoglobin is thus made up of at least two components, one of which is due to the carboxymethyl group and the second due to the ligation state of hemoglobin when it is cross-linked. Further studies are needed to define on a structural basis the reason for the high or the low oxygen affinity when hemoglobin is cross-linked in the oxy or deoxy states, respectively.

The fact that carboxymethylated, cross-linked hemoglobin still reacts with both chloride and CO_2 suggests that this potential blood surrogate would achieve an even lower oxygen affinity when mixed with plasma in which these modulators are present than it possesses intrinsically. These findings also indicate that the reactions of carboxymethylation and of cross-linking under the conditions described do not preclude the subsequent binding of either chloride or CO_2.

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