The Reaction of N-α-(Bromoacetoxy(methyl)maleimide with Hemoglobin*

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SUMMARY

The reaction of N-α-(bromoacetoxy(methyl)maleimide (AM) with human and horse hemoglobin was observed over a range of pH and ligand conditions. In the case of human hemoglobin, cys F9(93)β (PERUTZ, M. F., J. Mol. Biol., 13, 646 (1965)) was alkylated by the maleimide ring. This was followed by rapid hydrolysis of the ester bond in the reagent. With horse oxyhemoglobin, a transient covalent bridge between β chains was formed by reaction of the maleimide ring with cys F9(93)β followed by reaction of the bromoacetyl portion of the reagent with val NA1(1)β of the other β chain in the tetramer. After hydrolysis of the ester bond, which occurred spontaneously, the resulting hemoglobin derivative showed no cooperative interactions. A comparison of the dissociation behavior and rates of carboxypeptidase A digestion of horse oxy- and deoxy-AM-hemoglobin showed that the derivative resembled the oxy form of horse hemoglobin even in the absence of ligands.

The alkylation of horse oxyhemoglobin could be carried out in two steps. By first reacting the AM reagent with cyanomet(hemoglobin at pH 5.7, the cys F9(93)β sulfhydryl group was alkylated. Addition of excess carbonmonoxyhemoglobin and raising the pH to 7 resulted in alkylation of the NH2 termini of the β chains by the bromoacetyl portion of the reagent. Separation of these hemoglobin derivatives was achieved. The two-step reaction sequence was also used to examine one feature of the conformation of a mixed cyanomet-AM-deoxyhemoglobin tetramer.

In an accompanying paper we have discussed the chemical characterization and functional properties of horse hemoglobin modified with various N-substituted maleimides (1). In this report we will describe experiments with N-α-(bromoacetoxy(methyl)maleimide and hemoglobin that have led to more complete understanding of this reaction. We also present data on the physical and linked function properties of the AM-hemoglobin derivative.

Our interest in bifunctional reagents that could cross-link amino acid residues in hemoglobin was stimulated by the observation that reaction of bis(N-maleimidomethyl)ether with hemoglobin eliminated cooperative oxygen binding without introducing drastic alterations in the tertiary or quaternary structure of the molecule (2). Thus, BME-hemoglobin could be crystallized in a form isomorphous with native horse oxyhemoglobin. Furthermore, in contrast to crystals of native horse oxyhemoglobin, removal of ligand did not result in fracture of the crystals thus implying that the extensive conformational changes that occur with the native hemoglobin do not take place with BME-hemoglobin (3). The BME reagent has certain limitations as a general cross-linking reagent for proteins which we attempted to overcome by synthesis of an unsymmetrical reagent having the structure shown in Fig. 1. The choice of this reagent was based on three considerations.

First, maleimides react faster than α-haloacetyl derivatives with the cys F9(93)β (4) sulfhydryl group at pH values below 6 (5), permitting the alkylation reactions to be carried out in two steps. Second, the ester linkage makes it possible to sever any cross-link introduced into the molecule. Thus the effect of cross-linkage or monosubstitution on the functional behavior of the hemoglobin can be examined. Third, α-haloacetates react with various amino acid residues producing derivatives that can be easily identified by amino acid analysis (6).

In the previous paper it was shown that the reaction of horse hemoglobin with AM resulted in a derivative in which all functional interactions are eliminated (1). The AM-hemoglobin binds ligands reversibly, crystallizes in a form isomorphous with horse oxyhemoglobin and retains the oxy form even

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† The abbreviations used are: AM, N-α-(bromoacetoxy(methyl)maleimide; BME, bis(N-maleimidomethyl)ether; CNmet, cyanomet-; CO, carbonmonoxy-.
after removal of ligand (1). This paper describes the course of the reaction, as well as some of the physical and functional properties of AM-hemoglobin. We shall also discuss the potential of AM as a general bifunctional cross-linking reagent for proteins based on studies of its reactivity with various amino acids.

**EXPERIMENTAL PROCEDURE**

**Materials**

The "slow" component of horse hemoglobin was prepared from the blood of a single horse as previously described (1). Human blood obtained from the blood bank of the Yale-New Haven Hospital was used to prepare hemoglobin according to the method of Drabkin (7).

**Synthesis of Model Compounds**

*Reaction of N-α-(Bromoacetoxymethyl)maleimide with N-Acetylcysteine—* A 1 M solution of AM in 0.2 M sodium phosphate, pH 7.2, was reacted with a 5-fold molar excess of N-acetyl-L-cysteine for 10 hours at 25° in a nitrogen atmosphere. The reaction mixture was filtered through Sephadex G-10. The major product was characterized by paper electrophoresis at pH 6.5 and amino acid analysis after hydrolysis with 6 N HCl at 100° for 22 hours.

The rates of reaction of N-acetyl-L-cysteine with AM were studied at pH 5.5 in 0.2 M sodium acetate buffer and at pH 7.2 in 0.2 M sodium phosphate buffer. Aliquots of these reaction mixtures after separation on Sephadex G-10 were hydrolyzed with 6 N HCl, and the content of succinylcysteine and S-carboxymethylcysteine was determined by amino acid analysis.

*Preparation of N-Acetylcysteine-N-α-(Bromoacetoxymethyl)maleimide-N-α-Acetylhistidine Adduct—* The reaction product of N-acetylcysteine and AM at pH 5.5 was treated with a 10% molar excess of α-N-acetyl-L-histidine at pH 7.2 for 2 hours at 25°. The product was isolated by chromatography on Sephadex G-10 followed by paper electrophoresis. Its identity was confirmed by amino acid analysis.

**Amino Acid Analysis**

A Beckman-Spinco 120B amino acid analyzer was used with the usual buffers (8) except for the separation of succinylcysteine and S-carboxymethylcysteine where the pH 3.26 sodium citrate buffer was adjusted to pH 2.86 with 6 N HCl.

**Reaction of Human and Horse Oxyhemoglobin with N-α-(Bromoacetoxymethyl)maleimide**

The conditions for the reaction of human hemoglobin with AM were identical with those described previously for horse hemoglobin (1). The rate of alkylation by the maleimide portion of the reagent was measured by the methods given in the accompanying paper (1). The rate of release of bromide ions was measured with a bromide-specific electrode (Orion Company, Cambridge, Massachusetts). The observed changes in potential were related to bromide concentration with the use of a calibration curve determined under the conditions of ionic strength and pH used in the reaction of AM with hemoglobin.

**Osmometry**

Osmometric measurements were made with Hewlett Packard 501 High Speed Membrane osmometer at 25°. Semipermeable cellulose nitrate membranes (019) were obtained from Carl Schleicher and Schuell. They were stored at 4° in 20% EtOH and were equilibrated before use by washing in water followed by heating to 60° in buffer. The buffer used for the experiments was 0.4 M MgCl₂, 0.05 M Tris-HCl, pH 7.0, containing 100 µl of Brij-35 per 100 ml. The solutions used for the measurements contained 0.1 to 1.5% hemoglobin. The attainment of equilibrium across the membrane was monitored by the rate of change of the osmotic pressure.

**Acrylamide Gel Electrophoresis**

Aliquots of the reaction mixture of AM and hemoglobin were incubated in 0.1 M sodium phosphate buffer, pH 7.1, containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol and 25% glycerol. Aliquots containing 15 to 50 µg of protein were layered on 6-cm gels of 5% acrylamide according to the system of Shapiro, Vifuela, and Maizel (9). Electrophoresis was carried out for 2 hours at 5 volts per cm. Gels were stained with Coomassie blue, 0.25%, and destained in 7.5% acetic acid and 5% methanol (10).

**Oxygen Equilibria**

Hemoglobin solutions of 3 to 5% were deoxygenated in a tonometer modified by Simon (11) from a design of Benesch, MacDuff, and Benesch (12). The concentrations of deoxy-, oxy-, and methemoglobins were calculated with the equations of Benesch et al. (12) using the optical densities at 540, 560, and 576 µm.
normal horse oxy- and deoxyhemoglobins at pH 7.0 and at pH 4.8 in the salt solutions listed in Table I. All sedimentation coefficients were corrected for density, viscosity, and partial specific volume to water at 20° (16, 17) and are reported as $s_{20,w}$ values.

Molecular weights of the dissociated oxy-AM-hemoglobin compared to normal horse hemoglobin and myoglobin were determined by the Yphantis high speed sedimentation equilibrium method (18). The guanidine hydrochloride was prepared from guanidinium carbonate and recrystallized twice from ethanol (19). The protein solutions were dialyzed against 6 M guanidine hydrochloride-0.01 M Tris-HCl-0.1 M mercaptoethanol at pH 7.0 for 72 hours. The data were collected with the use of interference optics, recorded on Spectroscopic II-G plates, and read with the aid of a Nikon comparator. An Yphantis six-chamber centerpiece was used. Each chamber was filled with 10 µl of hydrocarbon FC43 and 100 µl of solvent or solution. The concentration of protein was kept between 0.03 to 0.05%, and runs were made at 25° and 36,000 rpm. The partial specific volume of the protein in 6 M guanidine hydrochloride was taken as about 1% lower than that found in water as determined by Tanford and others (20-25). The molecular weights were calculated from the average displacement of three fringes after depletion of the meniscus.

**Digestion of N-α-(Bromoacetoxyethyl)maleimide and Normal Hemoglobins by Carboxypeptidase A**

Carboxypeptidase A (disopropyl fluorophosphate treated) was dissolved to give a concentration of 0.1% in 10% lithium bromide, pH 10. This solution was diluted to 0.04% in enzyme by the addition of 0.02 M sodium barbitol, 0.4 M NaCl, at pH 7.65. This enzyme solution (1 ml) was incubated with 0.5 ml of a 1% hemoglobin solution at 31°. Aliquots were removed and the protein precipitated with an equal volume of 5% triehloroactic acid and centrifuged. The supernatant fractions from each tube were diluted to 2% triehloroactic acid with water and placed directly on an amino acid analyzer in order to determine the amount of histidine released. Pseudo-first order rate constants for the release of histidine were calculated from the slope of a least squares plot of the log of the change in histidine concentration versus time.

**Rate of Alkaline Denaturation of N-α-(Bromoacetoxyethyl)maleimide Hemoglobin**

The rates of alkaid denaturation were estimated by the method of Singer, Chernoff, and Singer (26).

**Ion Exchange Chromatography of Horse Hemoglobin and Its Derivatives**

The "fast" and "slow" components of horse hemoglobin (27) were separated as described previously (1). The chromatography and identification of derivatives from the reaction of AM with horse hemoglobin has been described (1). The reaction mixture of AM and human hemoglobin was chromatographed on IRC-50 resin with sodium phosphate buffer, pH 6.4, 0.14 M in sodium ion.

The reaction mixture of AM with cyanomethemoglobin (CNmet-hemoglobin) and carbonmonoxyhemoglobin (CO-hemoglobin) was chromatographed on a column (2.5 × 35 cm) of carboxymethylcellulose (CM52) with a 2-liter linear gradient of sodium phosphate buffer, pH 7.15, from 0.05 M to 0.2 M in sodium ions. The column was run at 4° with a flow rate of 40 ml per hour and the elution profile determined by measuring the absorbance at 540 µm.

The specific activity of all derivatives was determined as described previously (1).

**RESULTS AND DISCUSSION**

**Chemical Properties of N-α-(Bromoacetoxyethyl)maleimide**

The reaction of AM with N-acetylcysteine and N-α-acetylhistidine was studied to find conditions that would allow maximum discrimination between alkylation by the maleimide ring and alkylation by the bromoacetyl portion of the reagent. We also examined the stability of the ester bond in the resulting derivatives where both alkylation groups of the reagent had reacted. We hoped that studies of the behavior of the substituted reagent in model compounds would assist us in predicting and understanding the behavior found when AM was incorporated into proteins.

A simple model compound was prepared by reacting AM with an excess of N-acetylcysteine. The product released equimolar amounts of sucynylylsteine and S-carboxymethylcysteine after acid hydrolysis. Since these two cysteine derivatives were separable on the amino acid analyzer, it was possible, in subsequent experiments, to quantitatively estimate the relative rates of alkylation by each portion of the AM reagent. At pH 7.2, the relative rate of S-alkylation by the maleimide ring was thirty times faster than S-alkylation by the bromoacetyl portion of the reagent. There was little variation in the relative rates of alkylation when the temperature was varied between 4° and 30°. At pH 5.5 where both alkylation reactions proceed more slowly, a product can be isolated in which S-alkylation has occurred only at the maleimide ring. These results provide a basis for separating the reaction of the bifunctional AM reagent with hemoglobin and other proteins into two steps. Further evidence that the alkylation can be made to occur in two discrete steps was obtained in the synthesis of another model compound. To prepare this material, AM was reacted with N-acetylcysteine at pH 5.5, the product purified by chromatography and subjected to acid hydrolysis. Only succinylimine was found. When N-α-acetylhistidine was added to the pH 5.5 adduct of N-acetylcysteine and AM and the pH raised to 7.2, equimolar ratios of S-succinylimine and 1-carboxymethylhistidine were found after product isolation followed by acid hydrolysis and amino acid analysis. Not more than 5% of 3-carboxyethylhistidine would have gone undetected in this analysis.

The AM-N-acetylcysteine adduct was stable to hydrolysis between pH 1.5 and 9.5 (less than 5% of the compound amino acid or peptide derivatives could be found by paper electrophoresis after 3 hours of incubation at 25°). Ester hydrolysis, however, was appreciable, amounting to 30% at pH 10.5 and greater than 50% when nucleophilic reagents such as 0.2 M cysteine or 0.5 M NH₂OH were incubated with these compounds for 3 hours at 25°. These results indicated that the ester bond in AM should be relatively stable at neutral pH even after reaction of the maleimide portion with sulfhydryl groups, but that nucleophilic reagents might be used to sever any covalent bridge introduced into the protein.
Reaction of human oxy- or carbonmonoxyhemoglobin with an equimolar ratio of AM to α2 dimer at pH 7.15 resulted in a derivative where the reactive sulhydryl group (cys F9(93)β) was alkylated by the maleimide portion of the reagent. This was established by finding 1 mole of succinylestane per mole of α2 dimer after acid hydrolysis. When the reaction was studied with the bromide ion-specific electrode, no bromide release could be detected, indicating that alkylation by the bromoacetyl portion of the reagent had not occurred. In addition, when 14C-AM labeled in the methylol carbon atom, was reacted with human hemoglobin less than 0.2 mole of the methylol carbon remained per mole of αβ subunit. We interpret these results as indicating that alkylation by the β3 sulphydryl group went to completion but that hydrolysis of the ester bond occurred before any secondary alkylation by the bromoacetyl portion of the reagent took place. Loss of the labeled methylol carbon atom can be explained on the basis of the known instability of N-hydroxymethyl succinimides (28, 29). Thus, if the ester bond of the half-reacted reagent were hydrolyzed, it might be expected that the resulting hydroxymethyl succinimide would decompose releasing formaldehyde. When 14C-AM, labeled in the methylol carbon, was reacted with human oxyhemoglobin, 0.3 mole of 14C per mole of reacted αβ subunit was trapped as the methylene derivative of formaldehyde, thus supporting the proposed scheme of decomposition. The remaining 20% of the label found in the hemoglobin was probably due to Schiff base formation when the released formaldehyde reacted with exposed amino groups on the protein.

The reaction of AM with human hemoglobin failed to produce a cross-linked derivative, but indicated that the reagent (after reaction of the maleimide ring with cys F9(93)β) was suitably oriented with respect to a nuclease group on the hemoglobin so that hydrolysis of the ester bond occurred before any alkylation by the bromoacetyl moiety could take place.

Reaction of N-α-(Bromoacetoxy methyl)maleimide with Ligated Horse Hemoglobin; Relative Rates of Alkylation by Maleimide and Bromoacetyl Portions of Reagent

One of the advantages of an unsymmetrical reagent is the possibility of separating the alkylation reaction into two steps. We therefore studied the relative rates of alkylation by the maleimide and bromoacetyl portions of the reagent with horse oxyhemoglobin. Alkylation by the maleimide ring could be measured by the incorporation of radioactivity into hemoglobin (30). The percentage reaction of the maleimide ring of AM was measured by incorporation of radioactivity into hemoglobin (30). The percentage reaction of the maleimide ring of AM was measured by release of bromide ion (31). Amount of bromide ion released when EM-hemoglobin was reacted with AM. One hundred percent reaction was based on a ratio of 1 mole of AM per hemoglobin dimer. The theoretical curves are plotted assuming pseudo-first order rate constants of 0.24 min⁻¹ (---) for the alkylation by the maleimide ring and 0.02 min⁻¹ (——) for alkylation by the bromoacetyl group.

An interesting feature of the reaction is that the labeled methyl group was retained when carboxymethylation occurs before the ester bond in AM hydrolyzes. We have been unable to ascertain whether the methyl group remains attached to the succinimide ring or is distributed among various lysine residues in the form of Schiff base. If ceterolysis occurs before carboxymethylation, the label is lost as formaldehyde and no secondary alkylation occurs.

To understand more about the course of the reaction, horse carbonmonoxy-AM-hemoglobin, prepared at pH 7.15 and purified by column chromatography (1), was studied by osmometry under conditions that cause dissociation of the liganded tetramer into αβ dimers (0.4 mM MgCl₂, pH 7.0). The results shown in Fig. 3 demonstrated that the AM derivative of horse CO-hemoglobin dissociates in the same extent as unmodified horse CO-hemoglobin. Furthermore, when oxy-AM-hemoglobin was subjected to ultracentrifugation in 6 M guanidine hydrochloride, a molecular weight of 18,700 was obtained compared to 18,500 for horse CO-hemoglobin and 17,500 for myoglobin, all determined in the same run. Thus, the isolated AM derivative does not have a β-β' covalent bridge although it has reacted completely at the cys F9(93)β and val NAl(1)β of both β chains. The possibility that the reagent links the cys F9(93)β sulphydryl group to the NH₂-terminal valine of the same β chain is considered most unlikely because the size of the reagent is small compared to the distance between these residues. Furthermore, such a cross-link occurred, the β chain would have to be considerably distorted since a major portion of the β chain lies between the 93β sulphydryl group and the NH₂-terminal group of the same chain. That such distortion does not occur is shown by the x-ray crystallographic results of Moffat on AM-hemoglobin (30).
Properties of N-α-(Bromoacetoxyethyl)maleimide Hemoglobin

Oxygen Equilibria—Oxygen saturation curves were determined on the reaction mixture of AM and oxyhemoglobin rather than on the chromatographically purified derivatives since AM-oxyhemoglobin is easily oxidized to methemoglobin. The oxygenation curve was hyperbolic, the value of the interaction constant (n) obtained from the Hill equation\(^*\) was 1.1 to 1.2, and the logarithm of the partial pressure of oxygen at half-saturation (log \(P_{O_2}\)) was 0.4 compared with the log \(P_{O_2}\) value of 1.02 for normal hemoglobin tested under the same conditions. There was no detectable Bohr effect. Furthermore, we have shown that two products are formed in the reactions of AM with oxyhemoglobin, one having val NA1(1)β carboxymethylated and the other having val NA1(1)β free (1), but the presence or absence of the carboxymethyl group apparently does not affect the degree of cooperativity that is observed. These

\[ \log \frac{y}{1-y} = n \log P_{O_2} + \log K \]

where \(y\) is the percentage of saturation, \(n\) is the interaction coefficient, and \(K\) is a constant for the hemoglobin.

\(^*\)The Hill equation was used in the following form:
results show that cooperative interactions were almost completely eliminated when AM reacted with oxyhemoglobin.

When AM was reacted with deoxyhemoglobin, the reagent alkylated cys F9(93)β and then the ester bond in the reagent hydrolyzed. Oxygen saturation experiments carried out with this derivative showed that cooperative interactions were preserved (n = 2.0, log P05 = 0.8)4.

Dissociation Behavior—Although we have previously shown by osmometry and acrylamide gel electrophoresis that the intermolecular bridge in AM-hemoglobin is short lived, it was of interest to see if the presence or absence of ligand in AM-hemoglobin affected the equilibrium between tetramers and dimers in the same way as it does with unmodified hemoglobin (32-34). The results of sedimentation velocity ultracentrifugation of AM-hemoglobin, under a variety of conditions, with and without ligand, are presented in Table I. A comparison of the e20,3 values of AM and normal hemoglobin shows that AM-oxy and CO-hemoglobins parallel the behavior of normal horse oxyhemoglobin in their ability to dissociate into dimers under conditions known to promote splitting of the tetramers. In contrast to the behavior of normal horse deoxyhemoglobin, the unliganded form of AM-hemoglobin dissociated into aβ dimers under conditions that left normal horse deoxyhemoglobin mainly in the tetrameric form.

Since the degree of dissociation of tetramers into dimers appears to be associated with the sigmoid oxygenation equilibria in normal hemoglobin (32-34), the similarity in the dissociation properties of the oxy and deoxy forms of AM oxyhemoglobin is consistent with results of the oxygen saturation curves which indicate almost total absence of cooperativity. Furthermore, the similarity in the sedimentation velocities of oxy- and deoxyhemoglobins indicates that horse AM-hemoglobin is constrained in an oxy-like conformation.

Rate of Digestion with Carboxypeptidase A—The rates of digestion of oxy- and deoxyhemoglobins by carboxypeptidase A and B were first studied by Zito, Antonini, and Wyman (35). They were able to show that carboxypeptidase A removed tyrosine and histidine from the COOH end of the β chain three times as fast with oxyhemoglobin as compared with deoxyhemoglobin. Since these rates are dependent on the presence or absence of ligand, this provides further evidence that the cooperative interactions and the functionally important conformational changes are preserved.4

Rate of Alkali Denaturation—No difference was found between the rate of denaturation by alkali on AM- and normal horse hemoglobin indicating that no measurable alkali-sensitive instability was introduced by the modifying reagent.

Two Stage Reactions of Nα-(Bromoacetoxyenyl)maleimide with Horse Hemoglobin

The difference in reaction rates of the maleimide and bromoacetyl portions of the reagent was used to demonstrate the presence of mixed species of β chains in a tetramer and to prepare homoglobin derivatives with carboxymethyl groups on val NA1(1)β having free sulfhydryl groups at residue cys F9(93)β.

In this experiment, CNmet-hemoglobin was reacted at pH 5.7 with a 2-fold excess of AM labeled with 14C in the acetyl group. After the excess reagent had been removed by gel filtration, it could be shown that 1 mole of reagent had been incorporated into 1 mole of the hemoglobin (based on radioactivity) and therefore no esterification had occurred. A 10-fold excess of unreacted CO-hemoglobin was then added to the ---SH-reacted CNmet-hemoglobin and the pH adjusted to 7.9. No bromide ions were released at pH 5.7 but at the higher pH nearly 80% of the theoretical amount of bromide ions in the reagent were liberated. The reaction products were then chromatographed on Cm-cellulose to give a pattern shown in Fig. 6. Peak 1 contained only AM-CNmet-hemoglobin. The 14C content was equivalent to 1 molecule of reagent per aβ dimer. Peak 2 contained only CNmet-hemoglobin. Although there was no radioactivity in this peak, it contained 1 residue of succinylcysteine per β chain. This material evidently represents CNmet- part of the CNmet-CO-hybrid tetramer which had been alkylated by AM at cys F9(93)β but not carboxymethylated at val NA1(1)β. Peak 3 contained 0.85 molecule of carboxymethyl groups per β chain as measured by radioactivity and the hemoglobin was almost all in the CO- form. The presence of carboxymethylvaline was confirmed after acid hydrolysis.

**Table I**

| Conditions | Oxyhemoglobin | Deoxy- hemoglobin | Oxy- AM- hemoglobin | Deoxy- AM- hemoglobin |
|------------|---------------|------------------|---------------------|----------------------|
| 0.1 M NaCl, pH 7.0 | 3.7 | 3.7 | 3.9 | 3.8 |
| 2.0 M NaCl, pH 7.0 | 2.9 | 3.8 | 3.1 | 3.1 |
| 0.5 M MgCl2, pH 7.0 | 7.8 | 3.7 | 2.8 | 2.0 |
| 0.25 M NaOAc-HOAc, pH 4.8 | 2.4 | 2.4 | |

*All runs were carried out at hemoglobin concentrations of 5 × 10−5 M. Values are expressed at ε20,3.

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radioactivity in cpm.

No succinylcysteine was found in this peak. The distribution of radioactivity in Peak 1 and Peak 3 was 1 to 7, close to the ratio expected if there was no barrier to the rapid formation of deoxyhemoglobin in pH 7.2 (see text). The results of this hybridization experiment suggested that the reagent bridge could not be severed under mild conditions meant to test the degree of fit with respect to the distance between cys #9(93)β and val NA1(1)β' since the second alkylation occurs exclusively with the CNmet-hemoglobin tetramer.

The use of reagents that would covalently link β chains and where the reagent bridge could not be severed under mild conditions might be used to indicate the degree of fit between cato of unlike αβ dimers. We are now attempting to prepare a reagent which would be similar to AM but would have an amide instead of an ester linkage between the maleimide and the bromoacetetyl portion of the molecule.

Despite ester hydrolysis, which severs the covalent bridge, reagents similar to AM may prove useful for two-step labeling of proteins other than hemoglobin such as 3-phosphoglycerate dehydrogenase where a reactive sulphydryl group is situated near an ε amino group of lysine and a catalytically important histidine residue (37).

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