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METHYL ACETYL PHOSPHATE: A NOVEL ACETYLATING AGENT

ITS SITE-SPECIFIC MODIFICATION OF HUMAN HEMOGLOBIN A

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SUMMARY

A novel acetylating agent, methyl acetyl phosphate (MAP), has been designed to react with a nucleophile near an anion binding site of proteins. We examined the effect of MAP on hemoglobin (Hb), which has a well defined binding site for 2,3-diphosphoglycerate (DPG), to determine whether this reagent recognizes the DPG binding site. The progress of the reaction was monitored by ion-exchange high-performance liquid chromatography (HPLC) on a TSK CM-SW column. Modified Hb was initially chromatographed on CM-52 and then separated into its component chains. The α- and β-chains from modified and unmodified Hb were digested by TPCK-trypsin. The peptide mixtures were chromatographed on Whatman ODS-3 reversed-phase HPLC columns and the peptide maps of modified and unmodified chains were compared. The peaks formed by the modification with MAP were further purified on YMC ODS-S5 columns and then subjected to amino acid analysis on a Dionex D-500 instrument after acid hydrolysis. We found that the newly formed peptides are βT_1 and βT_{14+15} and that the loss of a peptide corresponding to βT_9 and βT_{10+11} is significant. No change in the α-chains was observed. The results suggest that MAP is indeed specific for the DPG binding site, as the above peptides contain the amino acid residues involved in the binding of DPG. We have assigned the acetylation sites as Val-1(β), Lys-82(β) and Lys-144(β).

INTRODUCTION

2,3-Diphosphoglycerate (DPG) is an important regulator for human hemoglobin (Hb) within the red cell. Arnone[1] has shown by X-ray crystallography that the DPG binding pocket of Hb is located in a cleft between the two β-chains. It is called an anion binding site as other anions in addition to organic phosphate, such as chloride or inorganic phosphate, also have an affinity for this pocket. Arnone's results

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suggest that the organic phosphate forms salt bridges with the basic groups in this central cavity: Val-1(β), His-2(β), Lys-82(β) and His-143(β).

Methyl acetyl phosphate (MAP) is an analogue of acetyl phosphate, a high-energy metabolic intermediate. It has been designed as a potential acetylating agent for anion binding sites, particularly of the NAD binding pocket of proteins[2]. As very few reagents modify residues at the DPG binding site of Hb, methyl acetyl phosphate has generated great interest in order to determine whether or not it interacts with basic groups in the DPG binding site of Hb. During the course of this study, there has been an incentive to monitor the progress of the reaction rapidly and quantitatively. We have developed such a method by using high-performance liquid chromatography (HPLC) with a variety of packings for analysis of the modified site by peptide mapping of the tryptic digest of the modified hemoglobin.

EXPERIMENTAL

HPLC-grade solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.) and trimethyl phosphate and acetyl chloride from Aldrich (Milwaukee, WI, U.S.A.).

Synthesis of methyl acetyl phosphate

Methyl acetyl phosphate was prepared accordingly to the method described by Kluger and Tsui[2] with minor modifications. The initial reaction of trimethyl phosphate and acetyl chloride was carried out for a longer period (about one week). Acetyl chloride was added periodically during the reaction. The product, dimethyl acetyl phosphate, was collected once by distillation.

Preparation of hemoglobin A

Hemoglobin A was prepared from erythrocytes of normal individuals. After collection of blood by venipuncture in the presence of EDTA, the red cells were collected by centrifugation at 310 *g* for 10 min and washed with an equal volume of phosphate-buffered saline (pH 7.4). Lysis of the cells was achieved by addition of distilled water to the original volume of whole blood and then freeze–thawing the suspension. After centrifugation at 7800 *g*, the red cell lysate was dialysed against 4 l of 0.1 *M* sodium chloride solution with two changes overnight and then against appropriate buffer saturated with carbon monoxide. Hemoglobin A prepared in such a way was successfully stored at −20°C without detectable conversion to methemoglobin.

Reaction of hemoglobin with methyl acetyl phosphate

Hemoglobin (0.12 m*M*) in 10 ml of 20 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) buffer (pH 7.5) and 0.1 m*M* EDTA was treated with methyl acetyl phosphate (1–10 m*M*) for 1 h at 25°C and then dialysed against 10 m*M* potassium phosphate (pH 5.85) for subsequent chromatography on CM-52 cellulose.

Monitoring of the reaction of hemoglobin with methyl acetyl phosphate by HPLC

Small aliquots (10–60 μl) of the reaction mixture were injected into a TSK IEX

535 CM HPLC column (150 × 6 mm I.D.) (Toyo Soda, Tokyo, Japan) on a Spectra-Physics (Piscataway, NJ, U.S.A.) 8000 HPLC system. The column, initially equilibrated with 90% solvent A (10 m*M* potassium phosphate buffer, pH 6.0)–10% solvent B (0.5 *M* potassium chloride in 10 m*M* potassium phosphate, pH 7.5) was eluted with a gradient of 10–100% B for 60 min at 25°C at a flow-rate of 0.5 ml/min. The elution of hemoglobin was monitored by its absorption at 540 nm by using a Perkin-Elmer Model LC-55 spectrophotometer.

Fractionation of modified hemoglobin

After treatment with methyl acetyl phosphate the hemoglobin was dialysed against 10 m*M* potassium phosphate buffer (pH 5.85) saturated with carbon monoxide. The sample was chromatographed on a CM-52 column (9 × 2 cm I.D.) (Whatman, Hillsboro, OR, U.S.A.) with a linear gradient of 150 ml each of 10 m*M* potassium phosphate (pH 5.85) and 15 m*M* potassium phosphate (pH 7.5) saturated with carbon monoxide.

Tryptic digestion of modified hemoglobin

The purified hemoglobin derivatives were separated into their chains by a modification of the method of Bucci and Fronticelli[3]. The α- and β-chains were then subjected to digestion with TPCK-treated trypsin in 0.1 *M* ammonium hydrogen carbonate solution (pH 8.0) at 37°C at an enzyme to protein ratio of 1:50. After digestion for 20 h, the material was lyophilized.

HPLC of tryptic peptides from modified hemoglobin

Reversed-phase HPLC fractionation of the tryptic peptides from methyl acetyl phosphate-modified hemoglobin was carried out on a 250 × 4.6 mm I.D. Whatman ODS-3 (Partisil-10) column, using a Beckman HPLC system (Model 110A pumps with Model 421 controller) (Beckman, Fullerton, CA, U.S.A.) equipped with a Model 770 variable-wavelength UV–VIS monitor (Kratos, Ramsey, NJ, U.S.A.) and fluorescamine post-column derivatization system, developed at Hoffmann-La Roche (Nutley, NJ, U.S.A.). A linear gradient of 5 to 75% of 1-propanol-0.1% TFA (50:50) in 0.1% TFA (in water) was used for elution at a flow-rate of 1 ml/min for 90 min. Identification of the modified tryptic peptides was achieved by selection of the newly generated peaks, which were those that eluted at positions different from the tryptic peptides derived from unmodified Hb A. The purity of each peptide was ascertained by re-chromatography on a YMC-ODS-S5 column (Yamamura Chemical, Kyoto, Japan; Pack A-312, 150 × 6 mm I.D.) with a Spectra-Physics Model 8700 HPLC system, equipped with Schoeffel Model 770 UV–VIS monitor. The elution conditions were similar to those described above. The new peaks were subjected to acid hydrolysis and their amino acid composition was determined.

Amino acid analysis

This was carried out on a Durrum D-500 amino acid analyser (Dionex, Marlton, NJ, U.S.A.) with three sodium citrate buffers as eluents, based on the method of Spackman *et al.*[4]. Peptide samples were hydrolysed in 6 *M* hydrochloric acid at 110°C for 22 h.

RESULTS

Chromatography of hemoglobin after treatment with methyl acetyl phosphate

Hemoglobin was treated with various concentrations of methyl acetyl phosphate. The reaction time was limited to 1 h because methyl acetyl phosphate is slowly hydrolysed in aqueous media[1].

The results, shown in Fig. 1A, indicate that the native hemoglobin is eluted at 35 min as a major peak. Some minor peaks were also found, which probably represent the minor hemoglobins found in a red cell lysate. Increased amounts of methyl acetyl phosphate significantly reduced the native hemoglobin peak and led to new peaks

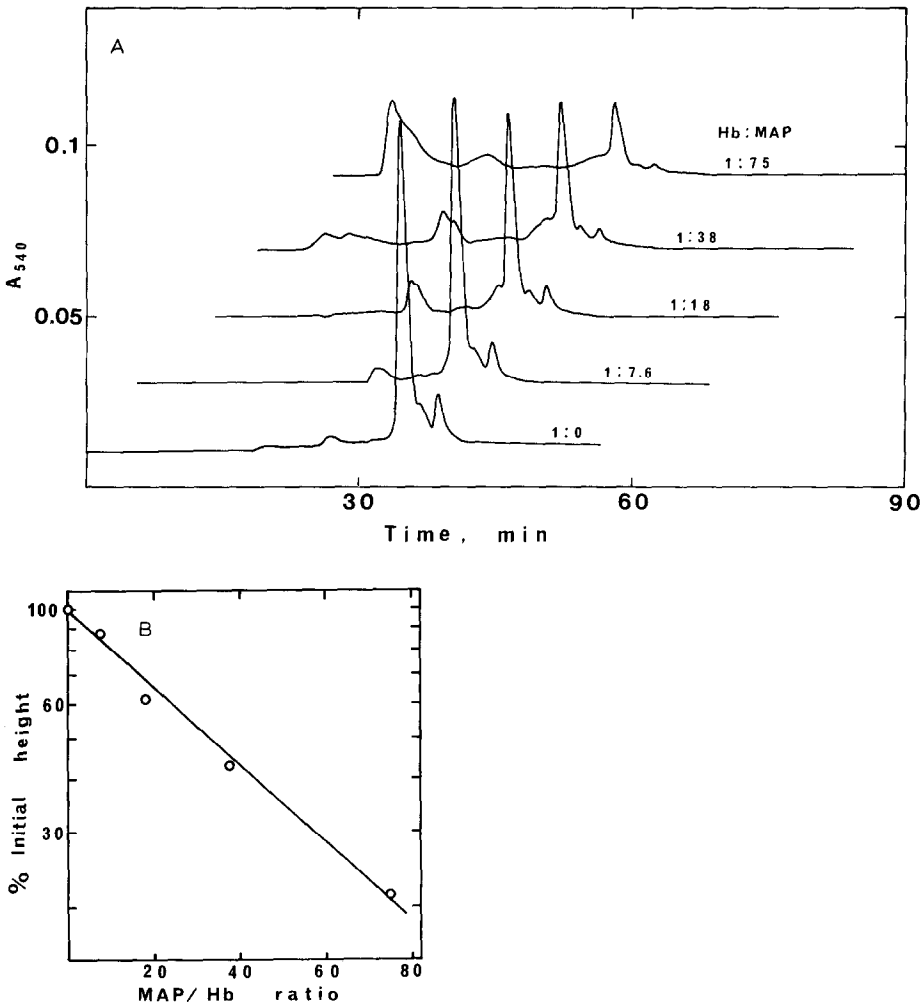

Fig. 1. Cation-exchange HPLC separation of hemoglobin acetylated by methyl acetyl phosphate (MAP). (A) Separation performed on a TSK IEX 535 CM HPLC column. Elution profiles of acetylated hemoglobin tetramer are stacked and offset. The amount of molar excess of reagent to hemoglobin is shown. Separation conditions are given under Experimental. (B) Relative peak heights of unmodified hemoglobin replotted on a semi-logarithmic scale.

around 7 min and 22 min, corresponding to products. The product at 22 min appeared predominantly when methyl acetyl phosphate was below a 20-fold molar excess over hemoglobin. When methyl acetyl phosphate was used above 20-fold molar excess, the product at 7 min became predominant. This elution pattern suggests that the modification site is at the positively charged groups of the protein, because the newly formed peaks are eluted faster than the native tetramer on CM columns. This proposal is consistent with our early results on the reaction of hemoglobin with glyceraldehyde or other aldehydes in which the modification of amino groups changes the chromatographic mobility[5].

Fig. 1B shows the relationship between the amount of unmodified hemoglobin and the molar ratio of methyl acetyl phosphate as a semi-logarithmic plot (each chromatogram in the figure is offset). The reaction progresses proportionally to the amount of reagent. Under the experimental conditions, about a 30-fold molar excess of methyl acetyl phosphate is required to complete 50% of this reaction.

For the preparation of modified hemoglobin, we chose conditions such that methyl acetyl phosphate was in 10-fold molar excess over hemoglobin. Chromatographic separation of modified hemoglobin on a preparative scale was carried out on a CM-52 cellulose column (Fig. 2). Two major fractions (peaks I and II) were pooled, and the tetramers were separated into their chains[3].

Analysis of tryptic peptides of methyl acetyl phosphate-modified hemoglobin by HPLC

Tryptic peptides obtained from the chains derived from either fraction I or II were analysed by reversed-phase HPLC (Figs. 3 and 4). The HPLC profiles of these

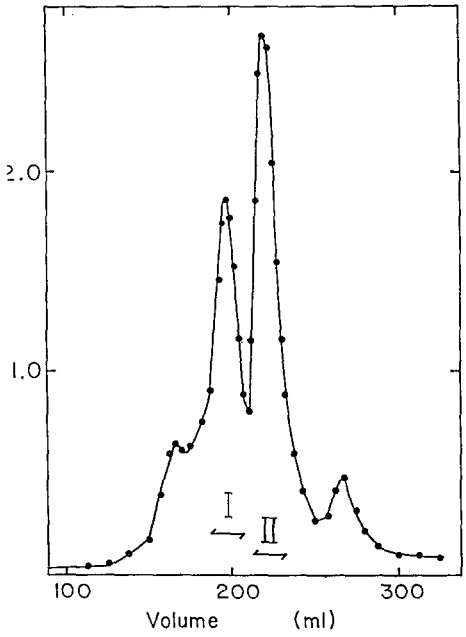

Fig. 2. Separation of acetylated hemoglobin on CM-52 cellulose. Experimental conditions are given under Experimental. Two major fractions are designated as I and II.

peptides from the chains were compared with that of unmodified hemoglobin A chains. As shown in Fig. 3, almost identical HPLC profiles are obtained for the α-chains from fraction I (C), fraction II (B) and unmodified α-chain (A). The results indicate that methyl acetyl phosphate does not react with the α-chain. On the other hand, there are significant changes in the HPLC profiles of the β-chains. The tryptic map for the β-chain from fraction I (Fig. 4C) shows three significant changes compared to that of unmodified β-chain (Fig. 4A); (1) a new peak at 30 min (labeled T_1^*), (2) a new peak at 45 min (labeled T_{14+15}^*) and (3) a decrease in peak height at 55 min (labeled T_9, T_{10+11}). The β-chain from fraction II (Fig. 4B) shows no significant change in the HPLC profile compared with unmodified β-chain.

The peak around 25 min (labeled X) showed absorption at 210 nm but did not contain any ninhydrin-positive material on amino acid analysis after acid hydrolysis. Similarly, no peak was observed in this area when the fluorescent monitor was employed in conjunction with fluorescamine post-column derivatization. We therefore

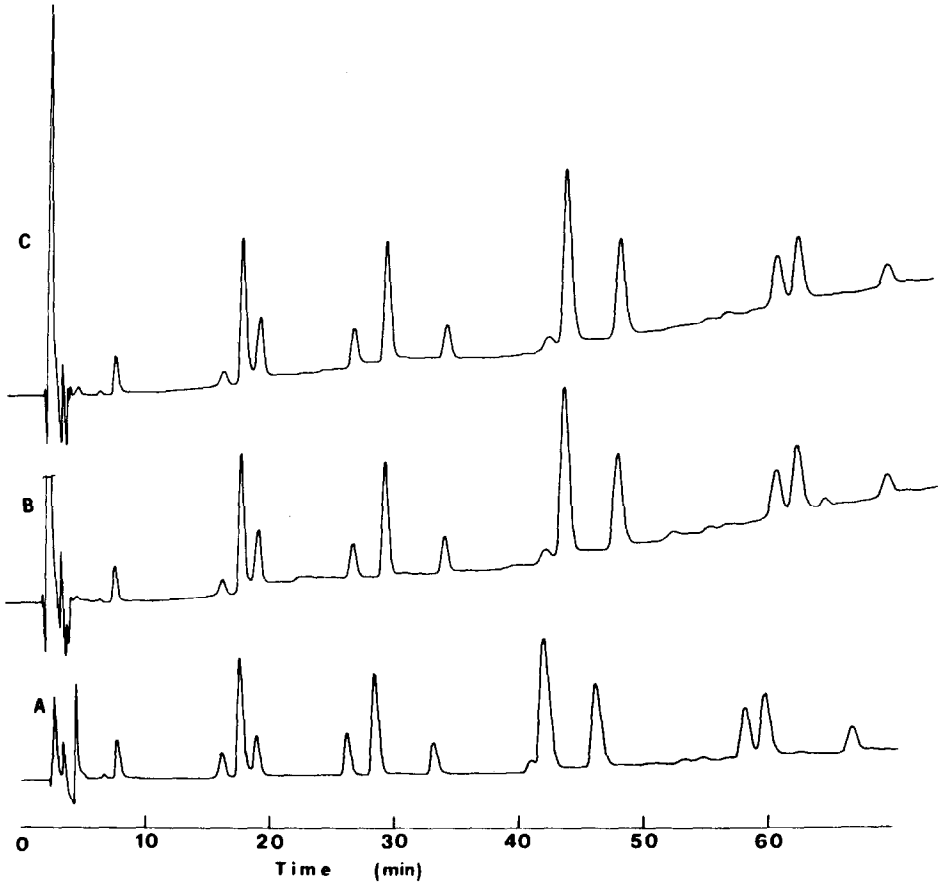

Fig. 3. Peptide map of tryptic digest of the α-chains from acetylated and unacetylated hemoglobin on a Whatman ODS-3 column. (A) Peptide map of α-chain from unmodified hemoglobin; (B) peptide map of α-chain from fraction II; (C) peptide map of α-chain from fraction I.

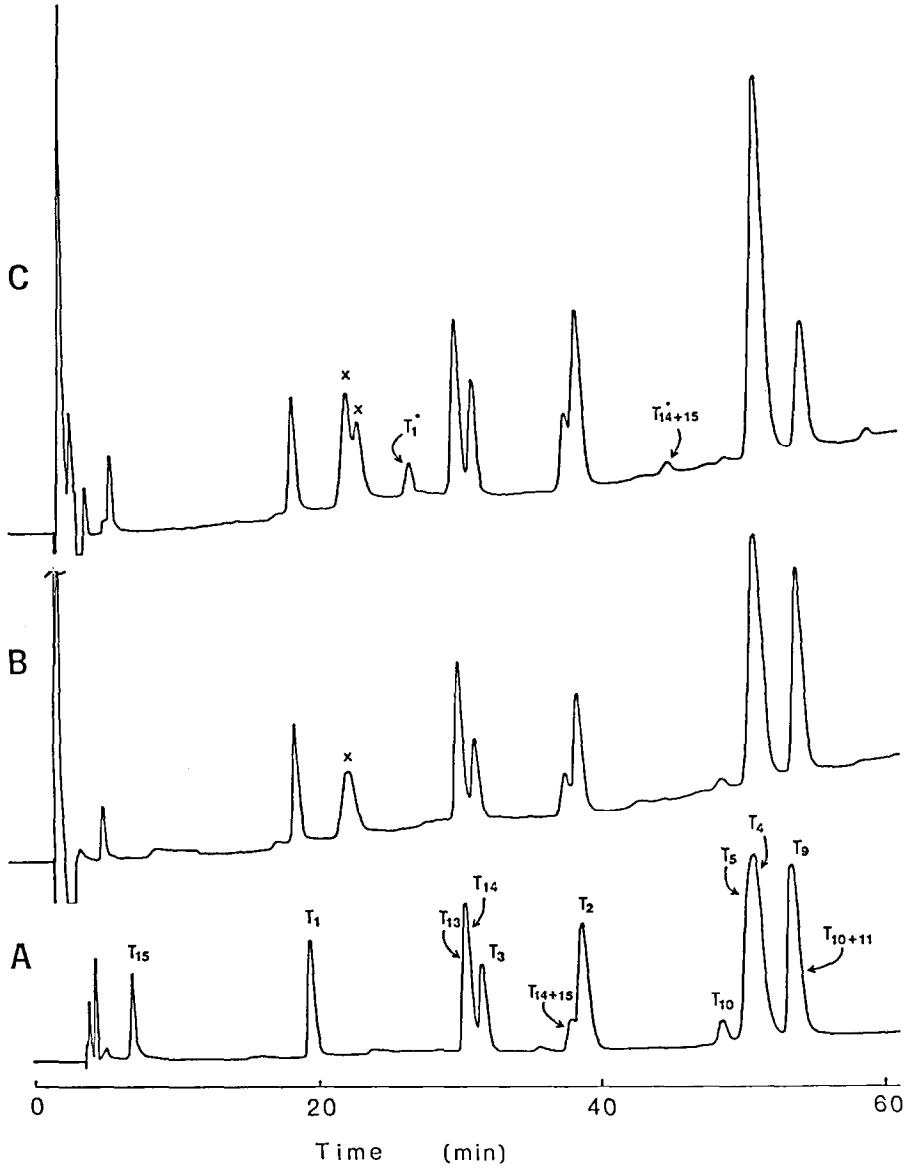

Fig. 4. Peptide map of tryptic digest of the β-chains from acetylated and unacetylated hemoglobin on a Whatman ODS-3 column. (A) Peptide map of the β-chain from unmodified hemoglobin; (B) peptide map of the β-chain from fraction II; (C) peptide map of the β-chain from fraction I.

conclude that this material is not derived from tryptic peptide fragments. Each of the newly formed peaks (at 30 and 45 min) was subjected to amino acid analysis after acid hydrolysis. Their amino acid compositions were identical with those of βT_1 and βT_{14+15}, respectively. As unmodified βT_1 and βT_{14+15} were eluted at 20 min and about 38 min, respectively, we believe that the presence of the acetyl group on these peptides makes them more hydrophobic and, therefore, retained longer on the col-

umn. The last major peak, eluted at 55 min, contains a mixture of βT_9 and βT_{10+11}. The bond between βT_{10} and βT_{11} is resistant to trypsin under our experimental conditions, probably because the presence of the *p*-hydroxymercuribenzoate group on Cys-93 prevents tryptic digestion of βT_{10+11}. There is a significant disappearance of both βT_9 and βT_{10+11} from the β-chain of fraction I. If Lys-82(β) is modified on βT_9, it is reasonable that trypsin would not cleave the bond between βT_9 and βT_{10+11}. Under the conditions employed, this uncleaved peptide is not eluted from this particular column. Nevertheless, it is clear that the modification is at βT_9 and therefore Lys-82(β) is probably acetylated.

Preliminary results indicate that the oxygen affinity of the modified hemoglobin (fraction I) is significantly reduced. In addition, the modified hemoglobin has a reduced response to DPG.

DISCUSSION

Separations of glycosylated hemoglobin or hemoglobin variants by different cation-exchange HPLC methods have also been reported[6,7]. We found that analysis of acetylated hemoglobin by HPLC is a powerful method. Peptide mapping of tryptic peptides of acetylated hemoglobin and amino acid analysis has revealed the reacted sites on peptides, βT_1, $\beta T_9 + \beta T_{10+11}$ and βT_{14+15}. Presumably, acetylation occurs on Val-1(β), Lys-82(β) and Lys-144(β). These residues were previously shown by Arnone[1] to interact with DPG.

The DPG binding domain is an important regulatory site for hemoglobin. DPG plays a role both in oxygen affinity and in the additional Bohr effect of hemoglobin. Methyl acetyl phosphate is an analogue of DPG, and it recognizes the DPG binding region of hemoglobin. Under our experimental conditions, a 10-fold molar excess of this reagent successfully modifies the functional amino groups that participate in DPG binding.

Acetylation of hemoglobin was previously demonstrated. Acetic anhydride reacts with many functional groups extensively in a non-specific fashion[8]. Aspirin (acetyl salicylate) is also an acetylating agent for hemoglobin but the reaction is slow[9]. Cross-link formation was reported between a derivative of diaspirin and the amino groups of the two β-chains of hemoglobin [Lys-82(β_1) and Lys-82(β_2)][10]. This latter compound has been used in studies with sickle cell hemoglobin. However, a monoacetylating agent such as methyl acetyl phosphate may be useful where such cross-linking would be undesirable[11].

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