The Reactions of the Sulfhydryl Groups of Human Hemoglobin $\beta_4$

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SUMMARY

The rates at which the sulfhydryl groups on each chain of hemoglobin $\beta$, react with N-ethylmaleimide and iodoacetate have been measured. The sulfhydryl group at position 93 reacts with both alkylating agents at approximately the same rate as normal liganded hemoglobin. The sulfhydryl group at position 112, which is not available in normal hemoglobin, reacts in hemoglobin $\beta_4$ and does so much more rapidly than does the sulfhydryl at position 93. The rapidly and slowly reacting groups were identified by isolation of tryptic peptides labeled with $^{14}$C-N-ethylmaleimide or $^{14}$C-iodoacetate.

Because of the different rates of reaction of the two sulfhydryl groups, it was possible to produce a derivative carboxymethylated only at position 112. This modification makes the $\beta$ chains unable to form tetramers. They have a sedimentation coefficient of 1.88 S which corresponds to monomers.

Unlike the $\alpha$ chains (1), the $\beta$ chains of human hemoglobin will form a tetramer (2). Such a molecule occurs naturally as hemoglobin H in patients with $\alpha$-thalassemia (3) or it can be made with $\beta$ chains isolated from normal adult hemoglobin (4). Despite a very tight association of the chains (5), the tetramer has no cooperative interactions. The oxygen equilibrium curve of hemoglobin $\beta_4$ is hyperbolic with an $n$ value of 1 in the Hill equation (2).1 X-ray crystallography shows no difference in the relative arrangement of the chains in liganded and unliganded hemoglobin $\beta$. In both states, the space group and the unit cell dimensions of the crystal resemble normal, unliganded horse hemoglobin (6).

The existence of such a hemoglobin raises several questions. What is the nature of the contacts among the $\beta$ chains in the $\beta_4$ tetramer? Are there differences in the conformation of individual $\beta$ chains when they are combined with each other rather than with $\alpha$ chains? One way to explore these problems is to look for functional groups which are not reactive in normal hemoglobin but which are reactive in hemoglobin $\beta_4$ or for groups which react at different rates in the two hemoglobins. Because the rate at which a given group reacts is often influenced by its environment, differences in the velocity of reaction may reflect changes in the conformation of the protein in the neighborhood of the reactive residue.

We have studied the rates of alkylation of the two $-SH$ groups in each chain of hemoglobin H derived from patients with thalassemia and in hemoglobin $\beta_4$ prepared from normal hemoglobin A. When $\beta$ chains are combined with $\alpha$ chains, only the $-SH$ group at position 93 is reactive (7). In the absence of $\alpha$ chains, the $-SH$ group at position $\beta_112$ can also be alkylated (2). The difference in reactivity between these groups is such that it has proved possible to modify the "hidden" $-SH$ group while leaving the group at position $\beta_93$ free. Our results show that substitution with iodoacetate at position $\beta_112$ profoundly affects the ability of the $\beta$ chains to aggregate with each other.

EXPERIMENTAL PROCEDURE

Materials

All solutions of hemoglobin were prepared as described by Hill et al. (8). Freshly drawn human blood was obtained through the courtesy of the Protein Foundation, Commonwealth of Massachusetts, Department of Public Health. Hemoglobin H was prepared from the blood of patients with $\alpha$-thalassemia obtained through the courtesy of Dr. David Nathan and Dr. Yuet Wai Kan of the Children's Hospital, Boston, Massachusetts.

For starch block electrophoresis, we used soluble starch for iodometry from the J. T. Baker Chemical Company (Phillipsburg, New Jersey). EM$^2$ (Mann), iodoacetic acid (Mann), PMB (Sigma), 5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich), and N-iodoacethanol (K and K Laboratories, Brooklyn, New York) were all reagent grade and were used without further purification. Guanidine hydrochloride was prepared from guanidine carbonate (Eastman) by the method of Anson (9). $^{14}$C-Iodoacetate was obtained as a water solution from Nuclear-Chicago. $^{14}$C-EM was supplied as an ethanol solution by Schwarz BioResearch.

The abbreviations used are: EM, N-ethylmaleimide; CO he- moglobin, carbonmonoxyhemoglobin; PMB, p-mercuribenzoate; Cm-cysteine, carboxymethylcysteine; Cm-histidine, carboxymethylhistidine.
Scintillation counting was done with a toluene-Liquifluor mixture purchased as the 25 times concentrated scintillator from Pilot Chemicals, Inc. (Boston, Massachusetts).

Trypsin was the three times recrystallized preparation from Worthington treated with diphenylcarbamyl chloride by the method of Erhanger and Cohen (10).

All inorganic salts were reagent grade and were not purified further.

**Methods**

Purified hemoglobin II was prepared from the blood of patients with α-thalassemia by starch block electrophoresis in 0.06 M sodium phosphate buffer, pH 7.0. The method was a modification of the procedure of Kunkel and Wallenius (11), as described by Gerald and Diamond (12). We used glass plates (20 x 45 cm) on which 400 g of starch were layered. The electrophoresis was carried out at 4°C with a constant field of 26 volts per cm. In this system, hemoglobin A, normal adult hemoglobin, stays at the origin while hemoglobin H moves toward the anode. The latter band was cut out, and the protein eluted with water or with the buffer used for electrophoresis. The composition of the eluted protein was checked by amino acid analysis on a Beckman amino acid analyzer after 24 hours of hydrolysis by 6 N HCl at 110°C. In all cases, the amino acid composition corresponded to that of pure β chains. All hemoglobin H was converted to the carbonmonoxy form.

Preparation of Isolated β Chains—We used a modification of the procedure described by Bucci and Fronticelli (13). PMB was dissolved in 0.035 M Tris-HCl buffer, pH 8.15. An aliquot of this was added to a solution of CO hemoglobin A, and this mixture was then diluted with sodium phosphate buffer to give a final solution of 0.50 M hemoglobin A, 5.4 M in PMB, 0.2 M in phosphate, and 0.1 M in NaCl, pH 6.3. After 16 to 18 hours of reaction at 4°C, the preparation was dialyzed for 24 hours against distilled water. The precipitated protein was removed by centrifugation, and 5- to 8-ml aliquots of the supernatant were placed on starch blocks which were prepared and run as described above. β\[^{PMB}\] chains moved toward the anode while the α\[^{PMB}\] chains moved toward the cathode. In every case, there was a heavy band of unseparated material which remained at the origin. α\[^{PMB}\] and β\[^{PMB}\] chains were also separated on a carboxymethyl cellulose column, as described by Bucci and Fronticelli (13). The amino acid composition of the separated protein was that of pure α or β chains.

PMB was removed from the purified chains by emulsification with N-dodecanethiol according to the method of De Renzo et al. (14). The extent of regeneration of the —SH groups was assayed with 5,5'-dithiobis(2-nitrobenzoic acid). The total increase in absorbance at 412 µm due to the formation of the thiolate anion of 5-mercapto-2-nitrobenzoate was measured in solutions which were 6.0 M in β chains, 0.39 M in 5,5'-dithiobis(2-nitrobenzoic acid) and 0.2 M in sodium phosphate, pH 7.1. An extinction coefficient of 13,600 M\(^{-1}\) cm\(^{-1}\) (15) was used to calculate the extent of reaction. In all chains used for further experiments, at least 85% of the —SH groups could be titrated with 5,5'-dithiobis(2-nitrobenzoic acid). The physical and functional properties of hemoglobin reconstituted from prepared β chains are described in the following paper (16).

Reaction of β with EM—Hemoglobin β (0.45 M in —SH groups) was allowed to react with EM (2.5 M) at 0°C in 0.2 M NaCl, pH 0.9. The reaction was followed spectrophotometrically by measuring the decrease in absorption at 300 mg of the clear supernatant resulting from precipitation of the protein with 5% trichloroacetic acid. This method has been shown by Guidotti (17) to give good agreement between the disappearance of absorption due to EM and the appearance of S-succinylcysteine on amino acid analysis after 72 hours of acid hydrolysis. As a control, CO hemoglobin A (0.50 M in available —SH groups) was caused to react with EM under the same conditions.

To determine which of the available —SH groups of hemoglobin β was reacting rapidly and which slowly, the slowly reacting group was labeled with \(^{14}\)C-EM and the radioactive tryptic peptides isolated and characterized. The experiment was set up as described above with cold EM. After 10 min of reaction, an aliquot of \(^{14}\)C-EM was added. After an additional 50 min, the protein was precipitated with a 10-fold excess (v/v) of acid acetone (500 ml of acetone, 25 ml of glacial acetic acid, and 10 ml of concentrated HCl) and washed 5 times to remove all excess reagent. The procedures for hydrolysis with trypsin, precipitation with 5% dichloroacetic acid, separation of the soluble peptides on the cation exchange resin Dowex 50-X2, have all been described (18). The radioactive peptide was hydrolyzed in 6 N HCl, 110°C, for 72 hours. The amount of S-succinylcysteine was determined by analysis on a Beckman amino acid analyzer. The radioactive peptide from the Dowex column was further purified by ascending paper chromatography with butanol-1, pyridine, acetic acid, and water (15:10:3:12). The dichloroacetic acid-insoluble peptides were taken up in 5% acetic acid and passed through a column (1 x 200 cm) of Sephadex G-50 (Pharmacia) with 5% acetic acid as a solvent.

In all experiments, radioactivity was measured by spotting samples on filter paper. When thoroughly dry, the papers were immersed in toluene-Liquifluor scintillation mixture and counted in a Beckman liquid scintillation counter.

Reaction of β\(^{-}\) with Iodoacetic Acid—The rates of reaction of the two —SH groups were estimated by measuring the specific activities of the tryptic peptides isolated after reaction with \(^{14}\)C-iodoacetate for measured times. The experiments were performed in two ways. In one procedure, the reaction was as follows: β\(^{-}\), 0.66 M in —SH groups; iodoacetate, 50 M; sodium phosphate, 0.2 M, pH 7.1; 23°C. After 60 min of reaction, \(^{14}\)C-Iodoacetate was added as well as enough guanidine hydrochloride to make a 4 M solution. After a further 4 hours of reaction, the solution was carefully dialyzed against distilled water. The methods of precipitating the protein, digesting with trypsin, isolating the peptides, and characterizing them were the same as before. The over-all specific activity of the Cm-cysteine formed during the reaction was determined by hydrolyzing an aliquot of the unfraccionated tryptic digest and chromatographing it on the 150 cm column of a Beckman amino acid analyzer. The effluent was collected during the time that Cm-cysteine was eluted; this fraction was lyophilized and redissolved in a small volume of water. The radioactivity of one part of the sample was measured while the remainder was rerun on the amino acid analyzer to determine the exact amount of Cm-cysteine collected. Specific activity of the individual —SH groups was obtained by measuring the radioactivity of the purified peptides.

The conditions for the second type of experiment were the same except that the protein concentration was 0.36 M in —SH groups. In this case, however, \(^{14}\)C-iodoacetate was added at the start. After 50 min of reaction, the concentration of iodoacetate was increased 10-fold to 0.60 M with cold iodoacetate. At the
same time, the solution was made 4 M in guanidine hydrochloride. The pH was adjusted to 7.0 and the mixture was left at room temperature for 8 hours. After the guanidine hydrochloride and excess iodoacetate were dialyzed away, the protein was precipitated with acid acetone, and a tryptic hydrolysis performed. The 5% dichloracetic acid-insoluble tryptic peptides contained most of the radioactivity, and these were purified by gel filtration on Sephadex G-50 columns (1 × 200 cm) eluted with 0.2 N acetic acid.

Preparation of Monosubstituted Carboxymethyl Derivative—The preparative reactions were all done in 0.2 M sodium phosphate, pH 7.1, at 24°. The hemoglobin concentration was 0.32 mM in -SH groups; iodoacetate was 60 M. The reaction was allowed to proceed for 50 min. It was stopped by rapid chilling of the solution to 0° followed by dialysis in the cold against large volumes of distilled water. In each preparation, the presence of at least 0.85 mole of Cm-cysteine per mole of β chain was confirmed by amino acid analysis.

Sedimentation of Monosubstituted Derivative—Sedimentation velocity experiments were performed in a Spinco model E ultracentrifuge with schlieren optics. We used a red filter and Kodak 103F spectroscopic plates. The position of the peak was measured with a Gaertner microcomparator. All experiments were done at 20°. Values for the density and viscosity of the solvents used were obtained from the International Critical Tables. The concentration of hemoglobin was 0.2 to 0.5%. In each case, reacted β chains were compared with unreacted β chains, usually in two compartments of the same cell.

RESULTS

Kinetics of Reaction of EM with Hemoglobin β—The time course of the reaction of EM with hemoglobin β is shown in Fig. 1 in which the data are plotted semilogarithmically. The curve shown is biphasic, indicating that the reaction is not a simple first order process. Because the reaction of —SH 993 of normal hemoglobin under the same conditions shows first order behavior (17), we have taken the data shown here to indicate that the two —SH groups in hemoglobin β are reacting in a simple first order fashion at two different rates. On this assumption, we calculated the half-times for the two reactions. The half-time for the faster reaction was 3.7 min, while that of the slower was 12 min. The latter was the same as that of the control reaction done with hemoglobin A and agreed with the reported values for the reaction of —SH 993 (17). The correctness of these assumptions was borne out by analysis of the partition of radioactivity between the two cysteine-containing peptides as described below.

Identification of Slowly and Rapidly Reacting —SH Groups—Fig. 2 shows the results of chromatography on Dowex 50-X2 of the dichloracetic acid-soluble tryptic peptides from hemoglobin β labeled with 14C-EM after 10 min of reaction with cold EM. The specific activity of EM in the reaction mixture was 6.4 ×
10^4 cpm per pmole. One major peak of radioactivity was found; 75% of the counts put onto the column are accounted for by this peak. After the mixture was further purified by paper chromatography, all the radioactivity in this peak was found to be associated with a peptide of the same composition as \( \beta T-10 \), the peptide containing cysteine 93 (18). The amino acid composition of this peptide was \( S \)-succinylcysteine, 0.95; aspartic acid, 0.98; threonine, 2.1; serine, 1.3; glutamic acid, 1.2; glycine, 0.98; alanine, 1; leucine, 2.01; phenylalanine, 1.3; lysine, 1.2; histidine, 0.92. The specific activity was 4.5 \( \times 10^4 \) cpm per pmole.

The dichloracetic acid-insoluble peptides were put on a Sephadex G-50 column. The results of the gel filtration are shown in Table I. The sum of the counts in the three peaks represents all the radioactivity put on the column. Peaks A and B contained undigested chains and large fragments. Peak C gave the following composition on amino acid analysis: \( S \)-succinylcysteine, 0.89; aspartic acid, 1; glycine, 1.92; alanine, 1.05; valine, 3.16; leucine, 4.05; phenylalanine, 1.02; lysine, 0.98; histidine, 1.84. This fraction, therefore, was \( \beta T-12 \), the peptide which contains cysteine 112. The specific activity of succinylcysteine in this peptide was 1.2 \( \times 10^6 \) cpm per pmole.

From the kinetic analysis in Fig. 1, we would expect that after 10 min of reaction, 58% of the slowly reacting \( -SH \) groups would be unreacted. At complete reaction, the specific activity of these groups should be 58% of the specific activity of EM or 3.7 \( \times 10^4 \) cpm per pmole. The experimentally determined value of 4.5 \( \times 10^4 \) cpm per pmole agrees with the expectation. At 10 min, only 13% of the rapidly reacting group should be left unreacted. The specific activity of this group should, therefore, be about 0.84 \( \times 10^4 \) cpm per pmole. The experimental value is 1.2 \( \times 10^4 \) cpm per pmole.

**Reaction of Iodoacetate with Hemoglobin \( \beta \)-**The results of experiments with iodoacetate are summarized in Table I. When \( ^{14} \)C-iodoacetate was added after 60 min, the peptide with high specific activity was found to be \( \beta T-10,11 \). This is the peptide which includes cysteine 93. Chromatography on Dowex 50-X2 of the dichloracetic acid-soluble peptides gave one fraction which contained most of the radioactivity put on the column. This fraction contained the peptide \( \beta T-10,11 \) in impure form. It was separated from the contaminating peptides by gel filtration on Sephadex G-50. The radioactive label was associated with a fraction with the following amino acid composition of which was Cm-cysteine, 0.97; aspartic acid, 3.2; threonine, 2.0; serine, 1.1; glutamic acid, 2.0; proline, 0.82; glycine, 1.2; alanine, 1.2; valine, 1.0; leucine, 3.2; phenylalanine, 1.9; lysine, 1.1; histidine, 2.1; arginine, 0.9. The specific activity of this peptide was 100,200 cpm per pmole.

Amino acid analysis of an aliquot of the unfractionated reaction mixture showed that there were 1.95 residues of Cm-cysteine per \( \beta \) chain. The over-all specific activity of the Cm-cysteine was 50,600 cpm per pmole. In Experiment 2, \( ^{14} \)C-iodoacetate was present at the start (131,000 cpm per pmole). After 50 min, it was diluted with a 10-fold excess of unlabeled iodoacetate. Final specific activity was 14,100 cpm per pmole.

In this case, the peptide with high specific activity contained the rapidly reacting \( -SH \). It was found in the material which precipitated immediately upon addition of dichloracetic acid to the tryptic digest. Gel filtration on Sephadex G-50 yielded one large peak of radioactivity. The amino acid composition of an aliquot of this fraction was Cm-cysteine, 0.94; aspartic acid, 1.2; glycine, 2.0; alanine, 1.1; Cm-histidine, 0.5; valine, 2.9; leucine, 3.8; phenylalanine, 1.0; lysine, 1.2; histidine, 1.5. This, therefore, is peptide \( \beta T-12 \) containing cysteine 112. The specific activity of this peptide (corrected for the number of counts present as Cm-histidine) was 97,000 cpm per pmole.

The other cysteine-containing peptide was found in the precipitate which formed when the supernatant from the dichloracetic acid precipitation was allowed to stand in the cold for 48 hours. It was purified by gel filtration on Sephadex G-50. The radioactive label was associated with a fraction with the following amino acid composition: Cm-cysteine, 1.0; aspartic acid, 3.4; threonine, 2.1; serine, 1.3; glutamic acid, 1.9; proline, 1.2; glycine, 1.4; alanine, 1.3; valine, 1.3; leucine, 3.5; phenylalanine, 2.3; Cm-histidine, 0.4; lysine, 1.4; histidine, 1.4; arginine, 0.8. This corresponds to \( \beta T-10,11 \). The specific activity of Cm-cysteine was 21,000 cpm per pmole. At 50 min, therefore, 72% of cysteine 112 and 6% of cysteine 93 had reacted.

From the amount of reaction at 50 and 60 min, we calculated an approximate half-time for the reaction of iodoacetate with \( -SH \) of 8 hours. The half-time for the reaction with \( -SH \) 112 is approximately 20 min. Guidotti (17) reported that the half-time for the reaction of \( SH \) 93 with iodoacetate in CO hemoglobin A is 5.2 hours.

Because of the great difference in velocity of reaction of the two \( -SH \) groups with iodoacetate, it was possible to prepare \( \beta \) chains in which about 90% of cysteine 112 was carboxymethylated while no more than 10% of cysteine 93 was modified. We found no more than 0.2 to 0.3 mole of Cm-histidine per mole of \( \beta \) chain. The results of sedimentation-velocity measurements for this derivative are given in Table II. Five different preparations were examined, all of which contained at least 0.85 mole of Cm-cysteine per mole of \( \beta \) chain. The sedimentation coefficient was similar to that reported for myoglobin, molecular weight 17,800 (19). This finding indicates that when cysteine 112 is reacted with iodoacetate, the chains can no longer aggregate into tetramers.

### Table I

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>Cysteine 93 (Peptide ( \beta T-10,11 ))</th>
<th>Cysteine 112 (Peptide ( \beta T-12 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td>100,200</td>
<td>1,000*</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td>21,000</td>
<td>97,000</td>
</tr>
</tbody>
</table>

*Calculated value. See text.
TABLE II
Sedimentation velocity of chains with and without reacted —SH groups

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>$t_{1/2,wp} \times 10^4$ sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin A</td>
<td>4.50</td>
</tr>
<tr>
<td>Hemoglobin H</td>
<td>4.51</td>
</tr>
<tr>
<td>Unreacted, prepared $\beta$ chains</td>
<td>4.39 ± 0.04</td>
</tr>
<tr>
<td>$\beta$ chains carboxymethylated at —SH 112</td>
<td>1.88 ± 0.12</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In contrast to their state when combined with $\alpha$ chains, isolated $\beta$ chains have two reactive —SH groups (2). These groups react at different rates with both iodoacetate and EM. The partition of radioactive label between the two reactive sites shows that the slowly reacting —SH group is at position 93 and that the rapidly reacting —SH group is at position 112. The half-time for the reaction of —SH 93 with EM is the same as that of the CO hemoglobin A control. The rate of reaction of cysteinyl residue 93 with iodoacetate can be roughly estimated from the extent of its reaction after 50 and 60 min. The estimated half-time of 8 hours is in the same range as the half-time from the extent of its reaction after 50 and 60 min. The estimated half-time of 8 hours is in the same range as the half-time of 112, 1.88 ± 0.12

The reaction kinetics of —SH 93$^p$ have been shown in normal hemoglobin to depend upon the conformation of the chains and to vary markedly depending on the presence or absence of ligand on the chains (20). The fact that the reaction of —SH 93$^p$ in hemoglobin $\beta_4$ goes on at the same rate as it does in liganded normal hemoglobin suggests that the conformation of the individual chains in hemoglobin $\beta_4$ is like that of liganded hemoglobin A chains. However, the arrangement of the chains in crystals of liganded hemoglobin H is the same as that of deoxyhemoglobin. Thus, the arrangement of the chains does not necessarily show the conformation of the chains themselves. Our findings agree with those of Benesch et al. (21), who found that in the reaction with carbon monoxide, hemoglobin $\beta_4$ behaved like rapidly reacting hemoglobin, that is, like hemoglobin A having the conformation of liganded hemoglobin.

The —SH group at position $\beta 112$ is not reactive in native human hemoglobin. In horse hemoglobin, on which the x-ray crystallography was done, this position is occupied by a leucyl residue which lies in the $\alpha$-$\beta$ contact and has van der Waals interactions with 5 residues on the $\alpha$ chain (22). Presumably, in human hemoglobin, the cysteinyl residue is similarly situated. In the absence of the $\alpha$ chain, the normal $\alpha$-$\beta$ interactions are not possible and the $\beta 112$ cysteinyl residue reacts rapidly with the reagents tested. Although the rate of reaction of EM at this site is 3-fold faster than the rate with —SH 93, it is still 300 times slower than the velocity of reaction of EM with a model compound such as glutathione (23). The environment of the —SH group must, therefore, be acting to decrease the rate. In this respect, it is analogous to —SH 93 and a comparison of the kinetics of reaction of this group in liganded and unliganded chains might reveal changes in the conformation of the individual chains which are too small to be apparent in the over-all crystal structure.

Because the $\beta$ chain itself is asymmetrical, it has two different surfaces in contact with $\alpha$ chains in the normal tetramer. From the nature and number of interatomic contacts at the $\alpha$-$\beta$ interface, Perutz et al. (22) concluded that in concentrated salt solutions horse hemoglobin must split into $\alpha\beta_2$ dimers. The corresponding surface on CO hemoglobin $\beta_1$ is much less sensitive to salt concentration than it is on CO hemoglobin A, less sensitive even than in deoxyhemoglobin A. Benesch et al. (5) report a molecular weight of 55,700 at 20° in 2 M NaCl for oxyhemoglobin H, whereas deoxyhemoglobin A under the same conditions has a molecular weight of 45,000.

The large, salt-resistant contact is, according to Perutz et al. (22), the $\alpha$-$\beta$ interface. The fact that the —SH 112 group which is normally buried in this contact region becomes available when the $\alpha$ chain is replaced by a $\beta$ indicates that the region which corresponds to the $\alpha$-$\beta$ interface is different in hemoglobin $\beta_4$ from hemoglobin A. Nonetheless, it is important in maintaining the integrity of the tetramer because reaction of —SH 112 with iodoacetate prevents polymerization of monomeric chains. In 0.2 M NaCl, modified $\beta$ chains have a value for $t_{1/2,wp}$ of 1.77 s, a value similar to that for myoglobin (19).

From these experiments, one cannot distinguish between two possible explanations of the data. On one hand, the introduction of a negative charge at position 112 may lead to an over-all change in the conformation of the protein which makes aggregation impossible by affecting all the contact surfaces. On the other hand, if the chains are assembled in the following way

\[
\begin{align*}
\beta &- 1123 \\
\beta &- 112 \\
\beta &- 112 - \beta
\end{align*}
\]

the carboxymethyl group may directly prevent combination.

In addition to the inferences which may be drawn regarding the structure of hemoglobin $\beta_4$, the possibility of making a derivative of the $\beta$ chain which is modified only at cysteinyl residue 112 gives us a tool with which to investigate the role of this —SH group in the formation of $\alpha$-$\beta$ subunits. The physical and functional properties of the molecule which results when $\beta$ chains modified in this way are combined with $\alpha$ chains are reported in the following paper (16).

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**REFERENCES**

The Reactions of the Sulphhydryl Groups of Human Hemoglobin $\beta_4$
Eva J. Neer