Chemical Modification of Human Hemoglobin by Antisickling Concentrations of Nitrogen Mustard

By Eugene F. Roth, Jr., Arthur Arnone, Robert M. Bookchin, and Ronald L. Nagel

We have examined the alkylation of human hemoglobins A, S, and H by nitrogen mustard (HN2). Two types of adducts are formed: Alkali labile adducts, which are mostly esterified carboxyl groups easily removed by dialysis against weakly alkaline solutions, and stable alkali-resistant adducts. The stable adducts, which are responsible for the inhibition of polymerization of deoxy-HbS, do not alter the pl of hemoglobin. Higher pHs enhance the binding of HN2 to hemoglobin as does the deoxy conformation. Separation of alpha and beta globin chains reveals that more than 90% of 14C-HN2 is bound in a stable manner to beta chains; however, peptide mapping did not yield a ninhydrin-positive, radioactive peptide because of elution of the label during this procedure. There was also evidence that about 25% of the rapidly titratable -SH groups of HbA and HbS were reacted with HN2. X-ray crystallographic study of HbA crystals revealed that beta histidines 97, 117, and 143 were reacted with HN2. In addition, the beta chain N terminus (the location of the beta 6 Val substitution in HbS) was displaced but not alkylated. By means of gelation studies with deoxy-HbS, it was found that alkylation of beta S chains inhibited gelation, whereas alkylation of alpha chains was without effect. Moreover, raising the pH during the HN2 reaction (but not during the gelation study) enhanced the inhibitory effect of HN2 on gelation. A plot of the minimum gelation concentration as a function of the pH during alkylation produced a curve that closely resembled the titration of a group with a pK near 7. This is consistent with alkylation of a histidine imidazole group. It is concluded that the stable adducts of HN2 and hemoglobin consist mainly of histidine adducts with some involvement of -SH groups from the beta 93 cysteine. In addition, the antisickling properties of HN2 are likely due to the alkylation of the beta 2 and beta 117 histidine residues that reside in the intertetrameric contact areas of the deoxy-HbS polymer.

In 1972, we reported that nitrogen mustard (bis beta chloroethyl) methylamine hydrochloride (HN2) reacts with HbS and inhibits polymerization of the deoxy form. This inhibition was achieved without significant changes in oxygen affinity or cooperativity; in addition, stability of the hemoglobin was not affected. In collaboration with Fung and Ho, it was subsequently shown by means of high resolution 1H-NMR studies, that the alkali stable reaction products of HbS and HN2 consisted largely of modified histidine side chains. Because hemoglobin is a histidine-rich protein, it was not possible to assign all of the hyperfine resonances to specific residues; nevertheless, by comparing the hyperfine 1H-NMR spectrum of the mutant Hb Deer Lodge (beta 2 His → Arg), to the normal 1H-NMR spectrum, it was possible to demonstrate that the beta 2 His or the region close to it was altered by HN2 alkylation. This finding was deemed a sufficient explanation for the mechanism whereby HN2 inhibits polymerization, because the beta 2 His has been shown to participate in the intertetrameric contact region of the deoxy-HbS polymer. However, it was recognized that the HN2 alkylation was not specific for any one histidine residue and that other surface histidines were likely to be involved, especially at higher concentrations of HN2. This report is concerned with some of the other residues reacted with HN2 that we have examined by different techniques. The aim of this study is to learn more about the mechanism whereby nitrogen mustard inhibits sickling, not to describe all the possible sites of alkylation by nitrogen mustard.
ALKYLATION OF HbS BY NITROGEN MUSTARD

MATERIALS AND METHODS

Hemoglobin Preparation

Heparinized blood samples were obtained from both normal volunteers and untransfused patients with hemoglobinopathies. Hemoglobins used in these studies were A, S, and H; hemoglobin H was separated by starch block electrophoresis.6 Lysates of all samples were made according to Drabkin16 and examined by electrophoresis on starch gel at pH 6.8 and on agar gel at pH 6.2. Alkali-resistant hemoglobin was quantified by the method of Singer.17 Hemoglobins S and A were purified on DE52 columns using 0.05M Tris-HCl buffer, pH 8.15. A Chromaflex Kontes-25 glass column in two separable portions was used. After elution of the HbA2 fraction and after migration of the HbS, the top portion of the column was removed, and both components were rapidly eluted with 0.5M NaCl.

Alkylation of Hemoglobin by Nitrogen Mustard

Hemoglobin samples were first equilibrated with 0.15M potassium phosphate buffer, pH 7.35. HN2 was then added in an equal volume of the same buffer, and the mixture was allowed to stand at room temperature for 30 min. The final concentration of hemoglobin in this mixture was 2.5-3.0 mM in heme. To study the effect of pH on HN2 binding to hemoglobin, aliquots were first equilibrated with 0.15M potassium phosphate buffers at the indicated pH levels, and after the reaction with 14C-HN2, the samples were dialyzed for 24-48 hr against the potassium phosphate buffer, pH 7.35 (the standard conditions of our gelling experiments). It had previously been determined that the Hb samples reach constant specific activity after 24 hr of dialysis at 4°C. Hemoglobin concentrations were determined by the cyanmethemoglobin method, and aliquots were prepared for scintillation counting by bleaching in plastic scintillation vials in a mixture of isopropanol and Protosol (1:1) containing 30% H2O2, and were counted in Aquasol using a Packard Tri-Carb Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Ill.). Quench correction was performed with an internal standard.

In order to examine the reactions of HN2 with hemoglobin in the oxy and deoxy states at pH 7.35, hemoglobin samples at 4 g/100 ml were equilibrated in tonometers with air or with 100% nitrogen. HN2 (1 mole/mole heme) was dissolved in 0.15M potassium phosphate buffer, pH 7.35, and introduced into the tonometer. Hemoglobin samples were first equilibrated with 0.15M potassium phosphate buffer at pH 7.35. The precipitated material was dissolved in water and lyophilized, the buffer before addition of HN2. However, prior to each gelling experiment, the hemoglobin solution was reequilibrated with the phosphate buffer at pH 7.35.

Hybrid tetramers were made by isolating $\alpha$ and $\beta$ chains of alkylated HbS by the method of Parkhurst et al.18 and recombining these chains with unalkylated $\alpha$ and $\beta$ partners, respectively.

X-Ray Studies

Crystals of two different samples of nitrogen mustard treated hemoglobin A were grown in the deoxy form from phosphate-buffered (pH 6.5) solutions of ammonium sulfate according to the method of Perutz.19 In one sample, hemoglobin A was treated with a fivefold excess (per heme) of nitrogen mustard and then dialyzed to eliminate labile esters. This sample failed to yield well formed crystals. However, we found that crystals suitable for x-ray study could be grown by mixing the reacted sample with an equivalent amount of untreated hemoglobin A.

The second sample was prepared by treatment of hemoglobin A with an equimolar amount (per heme) of nitrogen mustard. This sample crystallized normally without the addition of untreated hemoglobin A.

Single crystals of native or modified deoxyhemoglobin were mounted in quartz capillary tubes in an atmosphere of high purity nitrogen. The diffraction data were then collected to a resolution of 3.5Å on an Enraf-Nonius CAD4 diffractometer using the omega scan mode. Degradation due to radiation damage never exceeded 10% as determined by repeated measurements of four standard reflections. An empirical correction for radiation damage was incorporated into the crystal scaling calculations, as described previously. A correction for absorption was applied according to the method of North et al.20

Difference electron density maps were calculated using the known phases of deoxyhemoglobin A21 and the difference amplitudes (IFobserved - IFcalc). If the difference map showed the twofold symmetry characteristic of the hemoglobin tetramer, it was then symmetry averaged about the twofold rotation axis that related equivalent $\alpha\beta$ dimers22 in order to enhance the signal-to-noise ratio.

Materials

HbS was obtained from the Sigma Co., St. Louis, Mo. and the K and K Labs, Plainview, N. Y. 14C-labeled nitrogen mustard (labeled in the chloroethyl group) was obtained from the Mallinckrodt Co., St. Louis, Mo., specific activity 0.1 mCi/2.62 mg. Trypsin treated with TPCk was obtained from the Worthington Biochemical Co., Freehold, N. J.

RESULTS

General Characteristics of the Reaction Between HN2 and Hb

Immediately following reaction with HN2 for 30 min, isoelectric focusing revealed marked heterogeneity of the hemoglobin, indicating a spectrum of molecules with a broad range of isoelectric points, mostly higher than the pI of the untreated Hb (Fig. 1). Following dialysis against mildly alkaline buffers, however, the pI of more than 95% of the HN2-treated Hb was returned to that of the original protein. This probably reflects the disruption of alkali labile esters between HN2 and protein carboxyl groups, as had been observed in other proteins.23
Fig. 1. Electrofocusing of hemoglobin S on acrylamide gels with LKB ampholine pH 6-8. (1) Control: from left to right bands are: HbA2, unidentified minor component, HbS (main band), HbF1 and FIl. (2) Nitrogen mustard:heme molar ratio 5:1 (undialyzed). (3) Number 2 after dialysis. (4) Nitrogen mustard:heme 20:1 molar ratio (undialyzed). (5) Number 4 after dialysis.

An electrophilic reagent such as HN2 would be expected to react primarily with unprotonated functional groups.24 The effect of varying the pH of the reaction mixture during the reaction of HN2 with Hb, shown in Fig. 2, is consistent with the expected behavior. As the pH of the reaction is raised from 6 to 9, there was a progressive rise in the binding of HN2. It should be noted that the binding shown in Fig. 2 refers to stable reaction products (after labile esters have been removed by dialysis).

The results of increasing the ratio of HN2:Hb in the reaction mixtures at pH 7.35 are shown in Table 1. As the molar ratio of these reactants was increased within the range studied, there was a proportionate increase in the amount of $^{14}$C-HN2 that was stably bound to the Hb. For each mole of HN2 per mole of heme in the reaction mixture, between 0.06 and 0.08 moles HN2 remain bound (per heme) after dialysis of the hemoglobin. Thus, even with reaction at a 10:1 ratio, less than 1 mole HN2/mole heme is stably bound. It should be noted that preliminary studies had already demonstrated that this level of alkylation of HbS is sufficient to prevent gelation and sickling upon deoxygenation.7

The effect of the ligand state of the hemoglobin on its reactivity with HN2 is shown in Table 2. In these experiments, $^{14}$C–HN2 (1 mole/mole heme) was added to tonometers containing oxy or deoxy-Hb and, after 30 min, the samples were dialyzed. It is apparent that the deoxy forms of both HbA and HbS bound 20%–30% more HN2 in a stable manner than did the oxy forms.

Localization of Binding Sites for HN2 by Chemical Methods

In order to localize the areas of the Hb molecule that form relatively stable reaction products with HN2, the subunit globin chains of $^{14}$C-HN2-reacted Hb were isolated, and subsequently, the tryptic peptides were studied. The relationship of radioactivity to the chromatographically separated protein chains of $^{14}$C-HN2-treated HbS is shown in Fig. 3A. Most of the HN2 appeared to be bound to the $\beta$ chains whose mobility on the column was indistinguishable from that of native (untreated) $\beta$ chains. Therefore, the net charge of these chains is not significantly altered by HN2.

A second radioactive peak occurred between the protein peaks of the $\alpha$ and $\beta$ chains, suggesting the

Table 1. Binding of $^{14}$C-HN2 at pH 7.35 in 0.15M KPO4 buffer at 25°C at Different Ratios of Reactants

<table>
<thead>
<tr>
<th>Ratio of Reactants in Moles (HN2:Heme)</th>
<th>Moles HN2 bound/Mole Heme (After 24 hr Dialysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:1</td>
<td>0.16</td>
</tr>
<tr>
<td>6:1</td>
<td>0.37</td>
</tr>
<tr>
<td>10:1</td>
<td>0.69</td>
</tr>
</tbody>
</table>
Table 2. Binding of ^14C-HN2 by Hemoglobins A and S at pH 7.35 in 0.15M KPO4 Buffer at 25°C; Effect of Conformational State on HN2 Binding

<table>
<thead>
<tr>
<th></th>
<th>mM HN2 mM heme</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxy A</td>
<td>0.099</td>
<td>100</td>
</tr>
<tr>
<td>Deoxy A</td>
<td>0.138</td>
<td>138</td>
</tr>
<tr>
<td>Oxy S</td>
<td>0.112</td>
<td>100</td>
</tr>
<tr>
<td>Deoxy S</td>
<td>0.139</td>
<td>123</td>
</tr>
</tbody>
</table>

The ratio of reactants was 1:1 (moles HN2/mole heme).

The presence of a small amount of protein with a high specific activity. Peptide maps of the protein under this second radioactive peak showed a mixture of α and β chains. However, when ^14C-HN2-treated HbH (a tetramer comprised only of β chains) was chromatographed under the same conditions (Fig. 3B), a similar second peak was found, indicating that α chains are not required to produce at least a part of this component. In order to study further the origin of this peak, a hemolysate containing both HbA and HbN Baltimore (β95 Lys → Glu) was alkylated and chromatographed (not shown). The βN Baltimore chains, which are more negatively charged than βA chains and are eluted well ahead of the βA peak, were found to have their own second radioactive peak (between βA and βA protein peaks); but, in addition, the α chain contributed appreciably to the interchain radioactive peak between the βA and α protein peaks. The separate contribution of the α chains to this interchain peak was further confirmed by similar experiments using HbF and with a hemolysate of horse erythrocyte that contains two Hb components that differ only in their α chains. The source of this high specific activity material was not investigated further, but since the amount of protein involved accounts for a very small portion of the total protein, it probably plays little role in the altered properties of HN2-treated HbS.

Peptide mapping of a tryptic digest of ^14C-HN2-reacted beta chains revealed no deleted or abnormal ninhydrin-positive peptides, but elution and loss of the label during this procedure did not permit interpretation of the binding sites in an unambiguous manner.

Nitrogen mustard is capable of reacting with -SH groups on the surface of proteins. To explore this possibility, oxy-HbA, S, and H were titrated with hydroxymercuribenzoate. In addition, HbA reacted with HN2 in the deoxy state was also titrated to study the influence of ligand state on the reactivity of the -SH group.

The results, depicted in Table 3, show the expected two reactive -SH residues per tetramer in the control HbA and HbS and the eight free residues in HbH. The alkylated oxy-HbA and oxy-HbS showed the loss of HN2 in the deoxy state was also titrated to study the influence of ligand state on the reactivity of the -SH group.

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Table 3. Parahydroxymercuribenzoate Titration of HbA, S, and H

<table>
<thead>
<tr>
<th>Ratio of Reactants (HN2/Heme)</th>
<th>Oxy-HbA (SH/Tetramer)</th>
<th>*Deoxy-HbA (SH/Tetramer)</th>
<th>Oxy-HbS (SH/Tetramer)</th>
<th>Oxy-HbH (SH/Tetramer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.98</td>
<td>1.98</td>
<td>2.04</td>
<td>7.61</td>
</tr>
<tr>
<td>2:1</td>
<td>1.65</td>
<td>1.34</td>
<td>1.69</td>
<td>7.32</td>
</tr>
<tr>
<td>5:1</td>
<td>1.58</td>
<td>1.56</td>
<td>1.50</td>
<td>7.16</td>
</tr>
<tr>
<td>10:1</td>
<td></td>
<td></td>
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</tbody>
</table>

*Reacted with HN2 in the deoxy state, but titration was performed in the oxy state.
of about 0.45-SH residues/tetramer. No difference was observed between hemoglobin A alkylated in the oxy or deoxy state. Only 0.45-SH residues/tetramer of HbH were lost in spite of the fact that eight residues were available for titration. This suggests that the β chain -SH groups in the β̂ tetramer are not all equally reactive with nitrogen mustard.

Localization of the Reaction Sites of HN2 by Difference Electron Density Analysis

Portions of the difference electron density map of deoxyhemoglobin A modified by treatment with a fivefold excess of nitrogen mustard are shown in Figs. 4B and 5B. As mentioned above, the reacted sample was mixed with an equal amount of untreated hemoglobin A to facilitate crystallization. Figure 4A shows the native electron density of deoxyhemoglobin A in the region of the N-terminus of the β chains, and in Fig. 4B, we have superimposed the difference map. The positive peak (labeled A) bridges between His 143 and Lys 82 in each β chain. A reasonable interpretation of this feature is that nitrogen mustard alkylates His 143 but does not alkylate the ε-amino group of Lys 82; the latter conclusion is suggested by the observation that isoelectric focusing shows no fraction with a pI lower than that of the native Hb. Negative peak c shows that the inorganic anion (labeled XO₄ in Fig. 4A), normally bound between Lys 32 and the N-terminal amino groups of each β chain, is displaced in the modified hemoglobin, which could be the consequence of steric hindrance from the adduct approaching Lys 82 or by changes in the N-terminal segment. The loss of XO₄ apparently results in a small shift in position of the β chain N-terminal dipeptide. This is shown in Fig. 4B by the negative and positive pair of peaks, (labeled b and B), which straddle the native electron density of the backbone of the first two residues.

Figure 5A shows several sections of the native electron density map at the level of the β heme group. The difference electron density section shown in Fig. 5B contains a large positive peak (and its symmetry related counterpart), which is located between His 97 and Cys 93 on the same β chain. This indicates that nitrogen mustard has reacted with His 97 and possibly also with Cys 93 to form a cross-link in the hemoglobin sample treated with a fivefold excess of nitrogen mustard.

In the case of the hemoglobin crystal grown from the sample that was treated with an equimolar amount of nitrogen mustard, no significant difference electron density is observed in the region of the β chain N-termini. However, in the region of the β heme groups, a weak positive peak (labeled C in Fig. 5C) is observed between His 97 and Cys 93, indicating that alkylation again occurs at this site, but at a lower level than in the hemoglobin sample treated with a fivefold excess of nitrogen mustard.

In addition, a very intense positive peak (labeled A
in Fig. 5C) occurs next to the imidazole side chain of His 117 of only one β chain in the difference map of the “1:1” treated sample, but not in the difference map of the “5:1” treated sample. A negative peak (labeled b in Fig. 5C) is adjacent to peak A and occurs at a site that is associated with His 117 (Arnone et al., unpublished work). It appears, therefore, that the crystal selected for study from the “1:1” treated sample consists of hybrids, with His 117 alkylated on one β chain but not the other, and the alkylation at this site displaces a bound anion (probably sulfate or phosphate). Symmetric tetramers with both β chains alkylated at position 117 must not crystallize because of steric factors involved in crystal packing. In this connection, Wishner et al.2 have pointed out that all crystal forms of human deoxyhemoglobin are composed of very similar linear strands of tetramers and the β His 117 is at the tetramer-tetramer interface in these strands.

It can be concluded from the data presented that β His 117 and β His 97 form adducts with nitrogen mustard, with possible intrasubunit cross-links between His 97 and Cys 93. However, these adducts are not necessarily present in all molecules of the solutions examined. In addition, the difference electron density maps show that alkylation at β His 143 and the approach of the new side chain to the Lys 82 site result in the movement of the β chain N-terminal dipeptide, but no evidence was found for the alkylation of β His 2. The discrepancy between the x-ray studies and the NMR studies of Fung et al.6 (which demonstrated a significant decrease in the resonance assigned to β His 2) may be due to the mobility of the side chain of β His 2, which is on the surface of the β chain, or to differences between HbA and HbS relating to the pK of this residue. This kind of disorder can reduce the intensity of a difference electron density peak to the level of background noise, especially if the extent of reaction at this site is low.

In the interpretation of the crystallographic data, we must consider the possibility that some modified forms may not have crystallized, so that x-ray crystal-
lography of a heterogeneous mixture of modified proteins has value in assigning residues reacted, but not in excluding them.

The Effect on Gelation of the Alkylation of HbS by Nitrogen Mustard

We have previously demonstrated that HN2 inhibits the polymerization of HbS\textsuperscript{7} as judged by the increase in the minimum gelling concentration. We will now attempt to define more precisely the characteristics of this inhibition.

The first series of experiments were designed to answer the question as to whether HN2-treated HbA would affect the gelation of HbS in binary mixtures. Previous data from our laboratory\textsuperscript{26} demonstrated that HbA in mixtures with HbS facilitates the gelation of the latter, either by the formation of hybrids or by simple non-ideality contributions. As shown in Table 4, the effect of addition of HN2-treated HbA on gelation of HbS was no different from the effect of addition of native HbA. This finding could result from the "sparing" effect of HN2-HbA on gelation due to its contribution to non-ideality, without incorporation into the polymer.

The next series of experiments, shown in Table 5, attempt to answer the question of the chain participation in the inhibitory effect. We have already shown that little radioactivity is incorporated in the $\alpha$ chains and most of the HN2 reacts with the $\beta$ chains. Is the inhibition of polymerization also based entirely on the $\beta$ chain? To answer this question, we have reconstituted hybrids with the $\alpha^*$ or the $\beta^*$ chains from Hb reacted with HN2 (the complementary chain is native). As we can see from the results, the MGC of the fully alkylated HbS tetramers was 35 g/dl and 34.6 g/dl for the hybrid containing only the alkylated $\beta$ chain. We can conclude that the inhibitory effect of HN2 on HbS is entirely based on the modification of the $\beta$ chains.

The effect of the alklylation pH on the polymerization of HN2-treated HbS is of great interest (Fig. 6). In these experiments, the pH is altered during exposure to mustard and then restored to the standard pH 7.35 for the gelling experiment. Since alkylation occurs only at unprotonated functional groups, it was expected that manipulation of the pH during alkylation would reveal the pK of any functional group(s) involved in the alkylation reaction that inhibits gelling. It can be seen that at an alkylation pH below 6.5, there is little effect of HN2 on the MGC. Above this pH, there is rapid rise in the MGC. This rise appears to follow the titration of a functional group with a pK near 7, with the pK of histidine imidazole groups and $\alpha$ amino groups. This result is consistent with the involvement of histidine groups as the structural basis for the inhibitory effect.

**DISCUSSION**

From the data presented here, we can conclude that HN2 forms stable adducts with several residues in the surface of the hemoglobin molecule. The reaction seems to be restricted to the $\beta$ chains, and the crystallographic evidence suggests that adducts are formed

### Table 5. Minimum Gelling Concentrations (MGC) of Hybrids Containing Native Chains ($\alpha$ or $\beta$) or HN2-Reacted Chains ($\alpha^*$ or $\beta^*$)

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>1 MGC (g/100 ml)</th>
<th>2 MGC (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_2\beta_2^*$ (control)</td>
<td>23.5</td>
<td>24.0</td>
</tr>
<tr>
<td>$\alpha_2\beta_2^*$ (unseparated)</td>
<td>35.0</td>
<td>31.1</td>
</tr>
<tr>
<td>$\alpha_2\beta_2$</td>
<td>34.6</td>
<td>31.0</td>
</tr>
<tr>
<td>$\alpha_2\beta_2$</td>
<td>24.0</td>
<td>24.4</td>
</tr>
</tbody>
</table>

1: Hemoglobin S was reacted with HN2 in a ratio of 4.5/heme.
2: H Hemoglobin S was reacted with HN2 in a ratio of 3.5/heme.
Involving primarily \( \beta \) His 117, \( \beta \) His 143, and \( \beta \) His 97, with the possible additional involvement of \( \beta \) Cys 93. Previous NMR data have also implicated \( \beta \) His 2. It should be understood that under the conditions of the reaction, not all molecules have all these residues modified. In addition, other modified forms might not have crystallized under the conditions studied. Hence, this analysis does not exclude the possibility that other residues could be modified and be significant in terms of the antisickling effect.

The gelation data presented here clearly show that the inhibition of the HbS polymerization by HN2 is based exclusively on the modifications of the \( \beta \) chains and particularly of one or more side chains characterized by a pK of about 7.0. These results are in complete agreement with the above-mentioned structural data.

Finally, it is easy to rationalize the mechanism of the inhibition of polymerization with the data available. The HbS polymer seems to have a supramolecular structure characterized by 14 strands arranged in a complex helical array. There is abundant mutant evidence that the double-strand crystal contacts are retained in the fiber. Modification of \( \beta \) Cys 93 can be excluded as a source of the inhibitory effect because this residue does not appear in the contact sites of the Wishner-Love double strands and, in addition, its modification by para hydroxymercuribenzoate and iodoacetamide has no effect on the polymerization of deoxy-HbS. Nitrogen mustard seems to modify two residues involved in the areas of contact of the HbS crystal double strand. One of them is \( \beta \) His 2, for which there is NMR evidence of alteration by HN2. This residue participates in the side-to-side interactions. The other very important residue is \( \beta \) His 117; we have presented here crystallographic evidence of its modification in HN2-treated hemoglobin. This residue participates in the up-down strand contacts of the HbS crystal between \( \beta 1 \) and \( \beta 2 \). Interestingly, it appears in both sides of the contact area, hence, its modification would tend to alter drastically the stability of the polymer. Hemoglobin S molecules with one or two of these sites modified will have very low affinity for the polymer.

ACKNOWLEDGMENT

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