CHEMICAL MODIFICATION OF HEMOGLOBINS: A STUDY OF
CONFORMATION RESTRAINT BY INTERNAL BRIDGING*

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The equilibrium between mammalian hemoglobins and oxygen has long been
recognized to be a reflection of functional interactions within individual α2β2
tetramers. The physical basis for such interactions has been the subject of many theo-
retical analyses, most of which postulate the existence of at least two forms of the
protein with different reactivities toward the binding of oxygen.1–10 Any hypothe-
sis attempting to relate structure and function must also account for such diverse
phenomena as the pK of certain amino acid side chains, the rate of digestion of the
protein with carboxypeptidase A, the dissociation of the tetramer into subunits,
and the particular crystalline form of the protein.11–14 These properties seem to be
intimately associated with the binding of oxygen to hemoglobin, so that they behave
as "linked functions."15, 16

We have approached the study of the cooperative interactions which give rise
to the characteristic oxygen equilibrium of hemoglobin, and to those properties to
which it is linked, by attempting to impose a constraint on the conformation of the
protein. We hoped to achieve this by introducing covalent bridges in the hemo-
globin molecule with a bifunctional reagent. Studies on the reaction of substituted
maleimides with native human hemoglobin have demonstrated that the sulfhydryl
group at position 93 in the β chain reacts faster than any other residue in the mole-
cule.17–19 The second most reactive site is the N-terminal α-amino group of the
α chain.17 Still other residues may react with maleimides, although no other prod-
ucts have been characterized as yet.18, 20 Considerations of the selectivity exhibited
by N-ethylmaleimide (NEM) in its reaction with hemoglobin, as well as the poten-
tial reactivity of a bifunctional maleimide derivative, led to the choice of bis(N-
maleimidomethyl)ether (BME) as a reagent to introduce covalent bridges into human
and horse hemoglobin. We will present evidence that the conformational transition
normally accompanying ligand exchange has been eliminated in these
modified hemoglobins, and that BME hemoglobin assumes a deoxyhemoglobin-like
conformation, even with the ligand attached. The physical and functional prop-
erties of these derivatives are consistent with the hypothesis that the normal con-
formation transition is intimately linked with cooperative interactions.

Experimental.—Bis(N-maleimidomethyl)ether (methyl-C14) was synthesized according to
the method of Tawney et al.,11 by reacting maleimide with C14-formaldehyde to form the methylo-
derivative, and, by elimination of water, condensing two molecules of N-methylolmaleimide to
yield the final product. BME was reacted for 24 hr at 4°C with a 10% human or horse carbon-
monoxyhemoglobin (COhemoglobin) solution, prepared from washed pooled erythrocytes by the
method of Drabkin,21 the BME:hemoglobin tetramer ratio was 2:1. Separation of products
was achieved with the column chromatographic system of Clegg and Schroeder,22 using a 4 ×
50-cm column of BioRex 70 and a discontinuous sodium phosphate gradient from 0.05 M to 0.2 M
in NaCl, at pH 6.80. Peaks were analyzed for radioactive content by counting aliquots on
planchets. Ultracentrifugation studies were performed in double sector cells in the Spinco model
E, using schlieren optics. Hemoglobin solutions (0.5%) were run at 40,000 rpm, and data were
evaluated according to the methods described by Kirshner and Tanford,41 and by Kawahara

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et al. Oxygen equilibria and Bohr effect measurements were made by a modified spectrophotometric procedure similar to those described by Rossi-Fanelli and Antonini and by Benesch et al. Digestion with carboxypeptidase A (Worthington) was carried out according to the procedure of Antonini et al. Crystals of normal and BME-reacted horse hemoglobin were grown according to the method of Boyes-Watson et al. Deoxygenation of hemoglobin solutions and crystal suspensions was achieved by addition of small quantities of solid chromous chloride or chromous fluoride. Completion of deoxygenation was verified by spectral analysis in a Cary 11 spectrophotometer.

Results.—Separation and characterization of the derivatives: Analysis of the hemoglobin reaction mixture, as seen in Figure 1, reveals the presence of two products, plus some unreacted material. The major product, in the second peak, contains two molecules of BME per $a_2b_2$ tetramer of hemoglobin, as indicated by radioactive content (cf. Table 1). The first peak contains a minor product in which three molecules of BME appear to have been incorporated into the hemoglobin tetramer. Some unreacted normal hemoglobin is eluted in the third peak. No material containing one molecule of BME per tetramer has ever been detected.

We have concentrated our studies on the material containing two molecules of BME per hemoglobin tetramer, hereafter referred to as BME-hemoglobin. After hydrolysis of this product in $6\ N\ HCl$ for 24–72 hr and amino acid analysis according to the procedure of Spackman et al., the only modified amino acid detectable is S-succinyl cysteine, which is present to the extent of two residues per $a_2b_2$ tetramer (cf. Table 1). This derivative, which results from the hydrolysis of an N-substituted S-succinimidocysteiny1 residue in the protein, can account for the reaction of only two of the four maleimide rings incorporated into the BME-hemoglobin tetramer. That the other two rings have not reacted, however, seems unlikely, since no additional succinyl cysteine can be detected if BME-hemoglobin is denatured with sodium lauryl sulfate and then treated with free cysteine in several hundredfold excess before hydrolysis. Thus, it appears that the other two rings react with the protein to form either an acid labile adduct, or some product which has escaped detection by conventional amino acid analysis.

Studies to locate the sites of reaction of BME with hemoglobin are still in progress.

![Figure 1](chromatographic_pattern.png)

**Fig. 1.**—Chromatographic pattern as explained in text.
TABLE 1

COMPOSITION OF HEMOGLOBIN DERIVATIVES

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Radioactive content per 66,000 mol wt</th>
<th>Succinyl cysteine content 56,000 mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioReX 70 Peak 1</td>
<td>2.97-3.14 moles/mole</td>
<td>2 moles/mole</td>
</tr>
<tr>
<td>BioReX 70 Peak 2</td>
<td>1.96-2.03 moles/mole</td>
<td>2 moles/mole</td>
</tr>
<tr>
<td>BioReX 70 Peak 3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CM-cellulose* Peak 1</td>
<td>3.47 moles/mole†</td>
<td>3.34 moles/mole†</td>
</tr>
<tr>
<td>PCMB-treated protein Peak 1</td>
<td>0†</td>
<td>0†</td>
</tr>
<tr>
<td>PCMB-treated protein Peak 2</td>
<td>0†</td>
<td>0†</td>
</tr>
</tbody>
</table>

* According to Bucci and Fronticelli.†
† Protein contains 80% β chains by amino acid analysis.
‡ Protein obtained in poor yield with extensive loss of heme.

However, the presence of one residue of succinyl cysteine per αβ subunit after hydrolysis leads us to believe that one of the sites substituted is cysteine β93, assuming analogy with the reaction of NEM with hemoglobin. It is unlikely that reaction of hemoglobin with BME yields inter-β chain cross-links, involving these reactive cysteines, because 2.5 M guanidinium hydrochloride solutions dissociate the derivative completely into dimers. By analogy with normal hemoglobins, these dimers are most likely αβ subunits. A tetramer with β-β cross-links would not be expected to dissociate into αβ dimers at all. It is also unlikely that α and β chains are cross-linked by the BME, since separation of chains can be accomplished at neutral pH by the method of Bucci and Fronticelli. Radioactivity determinations and amino acid analyses, shown in Table 1, indicate that the BME is incorporated solely into the β chains of human hemoglobin, apparently forming one intra-β chain bridge per αβ dimer.

Dissociation into subunits: Studies on the behavior of normal and BME-reacted human hemoglobin in the presence of various dissociating agents are summarized in Table 2. The data may be considered in the light of the findings by Kawahara et al. that, under the conditions listed, S20,w values are a good reflection of molecular weight for human hemoglobin. The values obtained by Kawahara et al. for the

TABLE 2

DISSOCIATION OF HEMOGLOBIN

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Normal Hemoglobin</th>
<th>BME-Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxy-Hydroxy</td>
<td>Deoxy-Hydroxy</td>
</tr>
<tr>
<td>0.02 M NaCl, pH 6.8</td>
<td>4.50*</td>
<td>—</td>
</tr>
<tr>
<td>2 M NaCl, pH 6.8</td>
<td>3.5*</td>
<td>4-4.5*</td>
</tr>
<tr>
<td>0.25 M HClO4, pH 4.7</td>
<td>2.9*</td>
<td>—</td>
</tr>
<tr>
<td>0.125 M HClO4, pH 4.7</td>
<td>3.0*</td>
<td>4*</td>
</tr>
<tr>
<td>0.25 M MgCl2, pH 6.7</td>
<td>3.63</td>
<td>4.35</td>
</tr>
<tr>
<td>0.5 M MgCl2, pH 6.7</td>
<td>3.10</td>
<td>4.3</td>
</tr>
<tr>
<td>2.5 M GuHCl, pH 6.8</td>
<td>2.9*</td>
<td>—</td>
</tr>
</tbody>
</table>

* Data of Kawahara et al.
† Data of Kirshner.
‡ Calculated from sedimentation equilibrium data of Benesch et al. and Briehl.
§ Data of Gilbert and Chiancone.
| Data of Deoxygenation of acetic acid solutions with Cr+++ is rendered difficult by the formation of insoluble red ppt. of Cr(OAc)3.
\[\text{Approximated from the data of Briehl.}\]
theoretical $S_{20,w}$ of undissociated hemoglobin tetramers and fully dissociated $\alpha\beta$ dimers are $4.60 \pm 0.1$ and $2.90 \pm 0.06$, respectively. Under conditions which cause normal oxyhemoglobin to dissociate, oxy-BME-hemoglobin is also dissociated, but to a much smaller extent. Moreover, the striking difference between the dissociation behavior of normal oxy- and deoxyhemoglobin seems to be absent in the BME-protein. The fact that guanidinium hydrochloride apparently does separate the BME-hemoglobin completely into dimers strongly argues against a partial contamination with an interchain cross-linked derivative which could not dissociate at all. Finally, the similarity of both oxy- and deoxy-BME-hemoglobin to normal deoxyhemoglobin with respect to dissociation behavior suggests that the derivative might possess some fundamental deoxyhemoglobin-like properties, regardless of the presence or absence of the ligand. If this is indeed the case, then other properties which may be reflections of conformational differences between oxy- and deoxyhemoglobin would be expected to resemble those of normal deoxyhemoglobin.

At present, seven parameters have been used as a measure of conformational differences: molecular weight under conditions of partial dissociation; digestion rate with carboxypeptidase $A$; solubility; crystal structure; the binding of bromthymol blue; the rates of alkylation of the reactive sulphydryl group with iodoacetamide; and the binding to haptoglobin.

**Digestion with carboxypeptidase $A$:** The rates of digestion of normal and BME-reacted human oxyhemoglobin with carboxypeptidase $A$ are shown in Figure 2 along with the data of Zito et al. for normal deoxyhemoglobin. The rate of digestion of oxy-BME-hemoglobin is much slower than that of normal oxyhemoglobin, and is much more comparable to that of normal deoxyhemoglobin. Since only the $\beta$ chains are subject to digestion by carboxypeptidase $A$, the slow rate of digestion of BME-hemoglobin could conceivably be due to a slow decomposition of a covalent bond formed between the imidazole group of the C-terminal histidine and BME. This possibility is ruled out, however, by the fact that the product of digestion of BME-hemoglobin still contains two molecules of fully reacted BME per tetramer. Furthermore, identical products are obtained if hemoglobin is first digested with car-
boxypeptidase A and then treated with BME, or vice versa. Thus, it appears that the slow digestion rate of oxy-BME-hemoglobin is due to some conformational property, perhaps similar to that which results in the slow rate of digestion of normal deoxyhemoglobin by carboxypeptidase A.

**Oxygen equilibria:** The equilibria between oxygen and both normal and BME-reacted human hemoglobin at pH 6.8 are presented in Figure 3. It is apparent that BME-hemoglobin, which is predominantly in the form of tetramers under these conditions, nevertheless possesses an oxygen equilibrium virtually identical with that of the monomeric hemoprotein, myoglobin. The value of $n$ is equal to 1, and the Bohr effect is drastically reduced. No change in $n$ was observed when the equilibrium of BME-hemoglobin was measured at different pH values. These observations have led us to conclude that cooperative interactions in BME-hemoglobin have largely been eliminated. The only naturally occurring multimeric hemoglobin with a hyperbolic oxygen binding curve is hemoglobin H, which, as will be discussed later, also appears to undergo no conformational changes with binding or release of ligands.

**Crystal deoxygenation:** Horse hemoglobin, upon treatment with BME and chromatographic separation on BioRex 70 in a manner identical with that used for human hemoglobin, also yields a BME-derivative containing two molecules of reagent per hemoglobin tetramer. Horse oxy-BME-hemoglobin can be crystallized in a fashion similar to that used for crystallization of normal horse oxyhemoglobin. However, somewhat higher concentrations of (NH$_4$)$_2$SO$_4$ must be used, indicating that the BME-derivative has a solubility between those of normal oxy- and deoxy-hemoglobin.

Oxyhemoglobin crystals of both normal and BME-reacted proteins were suspended in their respective mother liquors on microscope slides. After microscopic examination, a few grains of chromous chloride were added and the slides were sealed with petroleum jelly-ringed cover slips. Deoxygenation of the crystals was apparent from the dramatic color change from orange to purple. Within 30 min, the crystals of deoxygenated normal hemoglobin began to undergo extensive crazing. Disintegration ensued shortly thereafter, and was facilitated by gentle agitation of the slide. Crystals of BME-reacted horse hemoglobin appear to
deoxyanxygenate completely under these conditions, and yet, after several weeks, the crystals have remained perfectly stable in the deoxygenated state, with no signs of disintegration or deterioration whatsoever.

Discussion.—Of the many theoretical discussions of the mechanism whereby cooperative interactions are achieved during the oxygenation of hemoglobin, those which postulate two protein conformations with two different reactivities to oxygen have received considerable attention. The elegant studies of Perutz and co-workers on the structures of oxy- and deoxy- human and horse hemoglobin have demonstrated the existence of two possible conformations in the solid phase, but this finding alone does not necessarily link conformational changes to cooperative interactions. Hemoglobin H, the β chain tetramer, lacks cooperative interactions, as evidenced by an insignificant Bohr effect and a value of n equal to 1. Moreover, it appears to have the same conformation in the oxygenated and deoxygenated crystals. This molecule, however, does not contain α chains; thus, theories which explain cooperative interactions by differential reactivities of α and β chains, without involving conformational changes, would not be invalidated by such findings. Similarly, it can be argued that conformational differences in crystals of normal oxy- and deoxyhemoglobin might no longer be present when the crystals are dissolved. However, observations of differential reactivities of oxy- and deoxyhemoglobin solutions to the action of the carboxypeptidases to alklation by iodosacetamide, and to the binding of bromthymol blue and haptoglobin, all suggest that conformational differences do indeed exist in solution as well.

The classic experiment of Haurowitz, in which crystals of deoxyhemoglobin were observed to disintegrate upon oxygenation, is an indication that the conformational changes are not merely events concomitant with ligand binding in normal hemoglobin; rather, the two events seem to be necessarily interdependent. The results presented here offer support for this hypothesis. BME-hemoglobin, by all the criteria we have used, shows little or no cooperative interactions. Moreover, simple crystallographic evidence suggests that BME-hemoglobin also undergoes no conformational changes associated with ligand binding. Since this derivative is made from a normal hemoglobin, which possesses both cooperative interactions and conformational flexibility, it would appear that these two phenomena are inseparably interrelated.

Measurements of "linked functions" in human BME-hemoglobin indicate that the conformational state into which the protein is apparently constrained might resemble that of normal human deoxyhemoglobin quite closely. The rate of digestion of BME-hemoglobin with carboxypeptidase A is certainly much more comparable with that of normal deoxyhemoglobin than with that of the normal oxygenated protein. The possibility that dissociation of the αβ tetramer into αβ dimers is a linked function, in the strictest sense, analogous to the Bohr effect, has been open to question. Our findings indicate that dissociation is indeed a true linked function. Furthermore, the dissociation properties of BME-hemoglobin substantiate its deoxyhemoglobin-like behavior, in the presence or absence of ligand. BME-hemoglobin, like hemoglobin H, has a high affinity for oxygen in spite of its apparent deoxyhemoglobin-like conformation. It has been suggested that such high oxygen affinity would be a property more typical of the oxyhemoglobin-like conforma-
tion. These apparently contradictory observations will require further investigation.

A principal feature of several models for the mechanism whereby cooperative interactions are achieved in hemoglobin is the postulated existence of an equilibrium between two conformationally distinct states of the protein. The presence or absence of ligands would not eliminate the equilibrium; rather, the position of the equilibrium would be shifted to one of these conformational states. Evidence presented here for the existence of a chemically modified oxygenated hemoglobin with a deoxyhemoglobin-like conformation suggests that oxy- and deoxy-like conformational states might indeed be in equilibrium in normal human hemoglobin solutions, even when all the molecules are in the fully oxygenated or deoxygenated form.

Note added in proof: Dr. Max F. Perutz has determined that crystals of horse CO-BME-hemoglobin are in fact isomorphous with those of normal horse oxyhemoglobin. The authors express their appreciation to him for his interest in this work. Further crystallographic studies are now in progress.

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