Titratable Sulfhydryl Groups of Horse, Sheep, Dog, and Cow Hemoglobins at 0° and 38°*

Makio Murayama†

From the Department of Biochemistry, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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In a previous paper (1) it was reported that normal hemoglobin (Hb-A) and sickle cell hemoglobin (Hb-S) have eight argentometrically titratable sulfhydryl groups per molecule at 38°. The mercurimetric-amperometric titration data suggested that there are two \( S^-\text{Hg}^-\text{S}^- \) linkages possible in the Hb-A molecule, whereas three such bridges are possible in the Hb-S molecule. A subsequent report (2) indicated that there are eight argentometrically titratable sulfhydryl groups in the hemoglobin C (Hb-C) molecule; since 8 mercury atoms are also bound per molecule, it was suggested that these are "lone" \( S^-\text{Hg}^-\text{S}^- \) groups. It was also reported that fetal hemoglobin (Hb-F) contains six titratable sulfhydryl groups per molecule which occur as three close, neighboring pairs so that three \( S^-\text{Hg}^-\text{S}^- \) linkages are possible in this molecule.

Ingram (3) has reviewed the literature on sulfhydryl groups of hemoglobins and made a comprehensive study of the \( S^-\) groups of hemoglobins from man, horse, ox, and sheep. He reported that native horse and ox hemoglobins have four sulfhydryl groups in two pairs of closely neighboring groups. Native sheep hemoglobin has eight \( S^-\) groups in four such pairs. According to Ingram, horse hemoglobin denatured with dodecyl sodium sulfate has six \( S^-\) groups probably situated symmetrically with respect to a diad axis in two clusters of three. Benesch et al. (4) also reported that there are eight argentometrically titratable sulfhydryl groups per molecule of horse hemoglobin. They reported ten sulfhydryl groups per molecule of dog hemoglobin. These investigators also noted that the sulfhydryl groups of sheep hemoglobin are fully reactive while most of those of canine and human hemoglobins are highly inaccessible.

The present study is an extension of the previous reports (1, 2, 5) on the conformational differences of sulfhydryl groups in hemoglobins of horse, sheep, dog, and cow. The maximal number of titratable \( S^-\) groups in horse and cow hemoglobins is the same; there are six sulfhydryl groups per molecule in both instances. However, the cow hemoglobin has a negative temperature coefficient of binding of heavy metals. Sheep and dog hemoglobins have eight and ten titratable \( S^-\) groups per molecule, respectively. From the analysis of the data reported here, a discussion on the nature of mercapto-mercapto (or sulfhydryl-sulfhydryl) interactions in hemoglobins is presented.

**EXPERIMENTAL**

The dialyzed hemoglobin solutions were prepared as previously described (2). The last trace of electrolytes was removed by electrodialysis.

The hemoglobin concentration was determined spectrophoto metrically. An aliquot is converted to cyanohe moglobin and its optical density determined in a 1 cm. Corex Beckman cell at 540 m\( \mu \) in the Beckman model DU spectrophotometer. A molecular extinction coefficient of 11.5 \( \times 10^3 \) at 540 m\( \mu \) and a molecular weight of 16,700 per heme were adopted for the calculations (6).

The apparatus\(^1\) and technique used for the amperometric titrations were essentially the same as previously described (2). The argentometric-amperometric titration method of Benesch et al. (4) was utilized. The titrations were performed in 30 ml. of tris(hydroxymethyl)aminomethane buffer,\(^2\) pH 7.4. The temperature of the solution was controlled in a thermostatic cell, as previously described (2).

The apparatus was deoxygenated,\(^3\) the hemoglobin solution was then added from a self-adjusting micropipette (9). The titrating agents were added from a syringe microburette as previously described (2). In all titrations, 20 \( \mu \)l. of 5 \( \times 10^{-4} \) M titrating agent were added at 1 minute intervals. A total of 400 \( \mu \)l. of the titrating agent were used in each titration.

**RESULTS**

**Horse Hemoglobin**—At 0° the steric hindrance effect on titratable mercapto groups appears to be slightly increased so that the maximal number of titratable \( S^-\) groups are not available to the titrating agents; on the average, 5 silver and about 2

\(^1\) An automatic amperometric titration apparatus is available although it was not used in this investigation. It is described by M. Murayama (7).

\(^2\) Potassium nitrate was used in place of KCl.

\(^3\) To minimize protein denaturation the buffer was deoxygenated before the hemoglobin solution was added into the titrating vessel. Pure nitrogen was washed through a solution of the same composition as that used in the beaker. Ddeoxygenated hemoglobin was being titrated in all instances.
mercury atoms are bound per molecule. At 38°, however, 6 silver and 3 mercury atoms are bound per molecule (Table I). The data suggest that sulfhydryl groups are so arranged that three —S—Hg—S— linkages are possible at the equivalence point of the mercurimetric-amperometric titration. In —S—Hg—S— the centers of the sulfur atoms are separated by twice the covalent Hg—S bond distance, giving S—Hg—S = 5.60 Å.

**Sheep Hemoglobin**—The argentometric-amperometric titration data indicate that there are eight titratable sulfhydryl groups per molecule of sheep hemoglobin at 0° as well as at 38° (Table I). Since 4 Hg atoms are bound per molecule, the data suggest that four —S—Hg—S— linkages are possible for this hemoglobin molecule at 0° as well as at 38°.

**Dog Hemoglobin**—About 10 silver atoms and about 5 mercury atoms are bound per molecule of dog hemoglobin at 0°. At 38° the values found are about the same within the 1st hour of incubation of the hemoglobin solution. However, as the hemoglobin solution is incubated longer than an hour, the number of mercury atoms bound per molecule increases asymptotically up to about 9 per molecule, i.e. during the 1st hour of incubation, about 5 mercury atoms are bound per molecule, during the second hour 6, and so on. The number, however, does not rise above 9 at 38°. This suggests that the molecule undergoes a slow architectural alteration which brings about conformational changes of sulfhydryl groups so that more mercury atoms are bound per molecule.

**Cow Hemoglobin**—At 0° about 6 silver atoms are bound per molecule of cow hemoglobin, and about 3 mercury atoms are bound per molecule (Table I). The data suggest that about three —S—Hg—S— linkages are possible at 0° in the molecule. But in contrast to the other hemoglobins investigated thus far, the cow hemoglobin is different in that it has a negative temperature coefficient of binding of heavy metals. It appears that the molecule undergoes an architectural alteration when the temperature of the solution is changed from 0° to 38° in such a manner that some of the —SH groups become more sterically hindered by the protein part of the molecule. On the average, about 4 silver atoms and 2 mercury atoms are bound per molecule (Table I) at 38°.

**Discussion**

The present study of the titratable sulfhydryl groups of horse, sheep, dog, and cow hemoglobin gives additional support to the views previously presented (1, 2, 5) that there is a close relationship between the genetics of an individual organism and the kind of protein it produces; and, further, that the heterogeneity of hemoglobins may, in part, be due to the conformational differences of the peptide chains which are reflected in the conformational differences of —SH groups. These particular differences are made apparent by the number of —S—Hg—S— linkages which are possible in a mercurimetric-amperometric titration.

The data reported here agree well with those reported by Ingram (3) on —SH groups of hemoglobins of horse, sheep, and ox, as well as those reported by Benesch et al. (4) on sheep and canine hemoglobin.

In the discussion on the steric hindrance theory of heme-heme interaction, St. George and Pauling (10) showed that the affinity of hemoglobin for the ligand depended upon the size of the alkyl groups; the larger the alkyl groups, the lower the affinity. The situation described here involves a converse relation. Since the ligand remains the same, the affinity may be inferred to depend on the difference in the “looseness” of the polypeptide chains with respect to —SH groups; i.e. the more tightly the chains are coiled or folded in the region of the sulfhydryl group, the lower the affinity for the heavy metal ions.

In a previous paper (1) it was reported that at 0° four —SH

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**Table I**

**Titratable—SH groups of horse, sheep, dog, and cow hemoglobins at 0° and 38°**

<table>
<thead>
<tr>
<th>Kind of hemoglobin</th>
<th>Silver atoms bound per 4 of Fe</th>
<th>Mercury atoms bound per 4 of Fe</th>
<th>Silver atoms bound per 4 of Fe</th>
<th>Mercury atoms bound per 4 of Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 degrees</td>
<td>38 degrees</td>
<td>0 degrees</td>
<td>38 degrees</td>
</tr>
<tr>
<td>Horse</td>
<td>4.90 ± 0.70</td>
<td>6.04 ± 0.43</td>
<td>6.04 ± 0.11</td>
<td>3.02 ± 0.11</td>
</tr>
<tr>
<td>Sheep</td>
<td>8.12 ± 0.32</td>
<td>5.09 ± 0.39</td>
<td>5.09 ± 0.11</td>
<td>5.09 ± 0.11</td>
</tr>
<tr>
<td>Dog</td>
<td>10.05 ± 0.48</td>
<td>11.33 ± 0.41</td>
<td>11.33 ± 0.12</td>
<td>8.66 ± 0.56</td>
</tr>
<tr>
<td>Cow</td>
<td>6.25 ± 0.51</td>
<td>4.45 ± 0.23</td>
<td>4.45 ± 0.23</td>
<td>2.35 ± 0.38</td>
</tr>
</tbody>
</table>

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**Figure 1**

![Plot of log [y/(1 - y)] against log [Hg++] free at 25° and pH 7.3, in solutions of native and denatured dog hemoglobin. The slope of the curve indicates the degree of interaction between the mercapto groups. The native dog hemoglobin curve (O——O) with a slope of 2.9 indicates a strong interaction. Upon the addition of dodecyl sodium sulfate, the slope of the curve (O——O) becomes 1, and it appears that the interaction vanishes.**
groups of hemoglobins A and B are sterically hindered, but that
they become accessible to the titrating agents at 38°. In hemo-
globin C the four groups which become accessible at 38° have a
lower affinity for the titrating agents (2) than the -SH groups
denoted as 1, 2, 3, and 4. These designations are used for conve-
ience to denote those -SH groups at the periphery of the
diagrams in reference (2), whereas the -SH groups which are
titratable at 38° are designated by the numbers 5, 6, 7, and 8.
Sulfhydryl groups numbers 5, 6, 7, and 8 appear to behave
between them. The uptake of the mercuric ion by -SH
group number 5 appears to make it easier for the next to come onto
the denatured molecule. The mercapto-mercurato interaction
of the heavy metal ion for mercapto-mercury complex formation
requires that the energy barrier due to steric hindrance be
about 0.9 kilocalorie mole⁻¹ greater for the native than for
the denatured molecule. The mercapto-mercaptop interaction
of hemoglobins appears to arise from steric hindrance.

\[ y = \frac{K_m y}{(1 + K_m y)} \]  

where \( y \) is the fractional saturation of \(-\text{SH}\) groups numbers 5, 6, 7, and 8 with respect to the mercu-
ric ion; \( m \) is the molar concentration of unbound mercuric ion; \( K \) is the association constant; and \( n \) is the interaction constant (where \( n = 1 \), this corresponds to no mercapto-mercaptop interaction). By re-
arrangement

\[ \frac{y}{1 - y} = K_m^n \]  

or on taking logarithms of both sides of equation (2):

\[ \log \left( \frac{y}{1 - y} \right) = n \log m + \log K \]

A plot of \( \log \left( \frac{y}{1 - y} \right) \) against \( \log m \) gives a straight line with
a slope \( n \) of about 3 for those sulfhydryl groups which become
accessible at 38°. However, upon the addition of dodecyl sodium
sulfate (400 molecules per 4 of Fe), the interaction constant \( n \)
becomes about 1, and the interaction then vanishes. Similar
observations are made with dog hemoglobin and will be elabo-
rated below.

The mercurimetric-amperometric titration curves for dog
hemoglobin are essentially the same as those shown for hemo-
globin C (2). Plots of \( \log \left( \frac{y}{1 - y} \right)^n \) against the log of concentration
of unbound mercurometric ions is shown in Fig. 1 where it can
be seen that the slope is about 2.9 for the native molecule and
about 1 for the denatured molecule. Furthermore, the affinity
for the heavy metal ion is increased on denaturation, and an
increase of about 4-fold in the affinity constant is observed
when the dog hemoglobin is denatured with dodecyl sodium
sulfate (400 molecules to 4 of Fe). This difference in the com-
bining power of mercuric ion for mercapto-mercury complex
formation requires that the energy barrier due to steric hindrance
be about 0.9 kilocalorie mole⁻¹ greater for the native than for
the denatured molecule. The mercapto-mercaptop interaction
of hemoglobins appears to arise from steric hindrance.

**SUMMARY**

1. Amperometrically titratable sulfhydryl groups of electro-
dialyzed hemoglobins of horse, sheep, dog, and cow have been
investigated with argentometric and mercurimetric methods at
0° and 38°.
2. Horse hemoglobin binds about 5 silver and about 2 mercury
atoms per molecule at 0°; at 38°, 6 silver and 3 mercury atoms
are bound per molecule.
3. Sheep hemoglobin binds either 8 silver atoms or 4 mercury
atoms per molecule at the ice point, at room temperature, and
at body temperature.
4. Dog hemoglobin binds either 10 silver atoms per molecule
at 0° or about 5 mercury atoms per molecule. However, at
38° the number of mercury atoms bound per molecule gradu-
ally increases as a function of incubation time, up to about 9. The
number of silver atoms bound per molecule remains essentially
unchanged.
5. Cow hemoglobin resembles horse hemoglobin with respect
to the maximal number of sulfhydryl groups titratable; how-
ever, it has a negative temperature coefficient of binding of
heavy metals. At 0° it binds 6 silver atoms per molecule and 3
mercury atoms per molecule. At 38° only about 4 silver atoms
are bound per molecule and about 2 mercury atoms.
6. A discussion on the nature of mercapto-mercaptop (or
sulfhydryl-sulfhydryl) interaction is presented.

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