Observations on the Mechanical Precipitation of Oxy Hb S and Other Mutants

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Oxyhemoglobin S exhibits greater mechanical instability than oxyhemoglobin A. The rate of precipitation of Hb S when agitated by vortexing depends upon the geometry of the tube, the volume of the hemoglobin solution, and the concentration of hemoglobin. The rate of precipitation is inversely related to concentration. Precipitation is inhibited by temperatures near 4°C and alkylureas whose protective capacity is approximately proportional to the carbon chain length of the alkyl group. Blocking the β93-SH group with para-hydroxymercuribenzoate has only a small enhancing effect on the precipitation rate. Other mutants such as Hb Gun Hill, Leiden, (both heat unstable), and C_Hb are also unstable. In the case of C_Hb, the precipitation rate is greater than that for Hb S. The heat-unstable mutants are not as well protected by cold temperatures or alkyl ureas. D2O has only a minor stabilizing effect on hemoglobin S, but NaCl and related salts markedly enhance precipitation at concentrations of 0.5 M. It is concluded that mechanical instability of oxyhemoglobins is a multifactorial process involving surface denaturation, pH, ionic strength, hydrophobic interactions, protein conformation, and primary protein structure. This phenomenon will require more extensive investigation.

OXYHEMOGLOBINS S and A exhibit very different rates of mechanical precipitation. The finding of the greater mechanical instability of oxy Hb S has identified for the first time a measurable conformational difference between oxyhemoglobins S and A. Until this discovery, it had been generally thought that the only conformational differences between these two hemoglobins (apart from net charge) were limited to the deoxy state.

Since the rate of mechanical precipitation depends in some way on the primary structure, we have extended the studies of Asakura et al. to other structural mutants in order to determine which mutations destabilize or stabilize oxyhemoglobin in solution. We have also tried to gain some insight into the nature of the precipitation process by varying both the physical and chemical environment of the experiment. Finally, we have concerned ourselves with the specificity of the precipitation test as a means of identifying hemoglobin S, because this method is being offered as a valid but untried means of identifying hemoglobin S in both the homozygous and the heterozygous state.

*Mechanical precipitation of dilute solutions of hemoglobin can be easily demonstrated by manually shaking a small volume of solution in a standard test tube for 1 min. After this period of agitation, solutions of Hb S appear turbid, while solutions of Hb A remain almost totally clear.

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MATERIALS AND METHODS

Hemoglobin solutions were prepared according to the method of Drabkin. Total hemolysates which were to be used without further fractionation were dialyzed against 0.15 M KPO₄ buffer, pH 7.35, at 4°C. Hemoglobins C₇₂ Harlem and S were separated from Hb A on a DES2 Sephadex column in 0.05 M Tris buffer, pH 8.10. After visible separation had occurred on the column, the top portion of the column was removed, and the abnormal component was then rapidly eluted by the addition of 0.5 M NaCl to the buffer. After isolation, the abnormal hemoglobin was equilibrated against the 0.15 M KPO₄ buffer, pH 7.35. In those experiments in which the pH was varied, the KPO₄ buffer was adjusted to the indicated pH. For mechanical precipitation studies, the hemoglobin concentration was adjusted to 200 mg/dl. Four milliliters of this solution were placed in 30 ml Corex centrifuge tubes approximately 15 x 150 mm. The samples were vortexed on a Scientific Industries Vortexer Model K550G with an intensity setting of 4 (scale, 1-10). The tube was held at about a 60° angle and enough contact was made to elevate the column of fluid two-thirds up the tube. After 2 min of vortexing, the samples were centrifuged in a Sorvall refrigerated centrifuge Model RC-2 at 10,000 rpm for 5 min. The supernatants were carefully placed in fresh tubes with Pasteur pipets, and 0.2-ml aliquots were removed and placed in 3 ml of Drabkin’s reagent. These were later read at 419 nm on a Gilford Model 240 spectrophotometer. All samples were allowed to come to room temperature before repeating the vortexing. It was determined that during the 2-min period of vortexing, a temperature rise of no more than 3°C occurred.

In order to assess the mechanical stability of the hybrids (α₂β²β⁺; α₂β⁻β⁺C₇₂ Harlem), solutions of Hb SA, C₇₂ Harlem-A, and A alone were vortexed with radioactive Hb A labeled with ¹⁴C-HN₂ (nitrogen mustard). Hb concentration and temperature were the same as described above. To prepare the labeled hemoglobin, approximately 100 mg of Hb A were dialyzed against a 0.15 M KPO₄ buffer at 4°C. Fifty microcuries of ¹⁴C-HN₂ were added, and the sample was allowed to react at room temperature for 1 hr. The sample was then dialyzed against three changes of the same buffer at 4°C for 48 hr. The final specific activity of the hemoglobin was 36000 dpm/mg Hb. It had previously been found both by us and by Asakura that mustard-treated Hb A or S has the same mechanical stability as the native proteins. We have also determined that HN₂ is largely bound to the β-chain of hemoglobin, so that changes in radioactivity of a vortexed sample would reflect the precipitation of the β-chain only, irrespective of any other coprecipitated chain. After the usual vortexing and centrifuging, 0.1-ml aliquots were placed in plastic scintillation vials, bleached with 30% H₂O₂ in Protosol. One milliliter of methyl cellosolve was added together with 10 ml Omnifluor toluene (6 g/liter), and the samples were counted in a Packard Tri-carb Scintillation Spectrometer. Efficiency was generally 85% for ¹⁴C, and quenching was monitored with an internal standard.

To test the effect of blocking the β₉₉ sulphydryl group on mechanical precipitation, hemoglobins S and A were reacted with parahydroximercuribenzoic acid (PMB) at room temperature in 0.15 M KPO₄, pH 7.35. After 5 min, the samples were passed through a Sephadex G-25C column equilibrated with 0.15 M KPO₄ buffer, pH 7.35. The ratio of PMB to heme was 5:1. Controls were handled similarly, with the exception of exposure to PMB. After passage through Sephadex, the samples were diluted to 0.2 g/dl and vortexed in the usual manner. Aliquots of these samples were equilibrated against a 0.15 M KPO₄ buffer, pH 8.35, and 24 hr later, the same experiment was performed. To insure that all rapidly titratable S-H groups were blocked, samples were titrated with PMB by the method of Boyer.

The effect of D₂O on mechanical precipitation was examined by equilibrating hemoglobin samples with D₂O in an Amicon Model 8MC Micro-UF system equipped with a type UM-10 membrane (lot No. 397, Amicon Corp., Lexington, Mass.).

In order to examine the α-helix content of the hemoglobin remaining in the supernatant after vortexing, measurements of optical rotatory dispersion (ORD) were made on a Cary Model 60 recording spectropolarimeter and were calculated according to the following equation:

\[ [m]_{233} = \frac{\alpha M_p}{C L} \left( \frac{3}{n^2 + 2} \right) \]

where \( \alpha \) is the observed rotation at wavelength 233 nm, \( C \) is the concentration of protein in g/dl, \( n \) is the refractive index of the solvent at this wavelength, and \( L \) is the path length in decimeters. The mean residue molecular weight \( M_p \) of 112.5 was used.
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MATERIALS

The unstable mutant hemoglobins Gun Hill (β92-96 deleted) and Leiden (β6 or 7 Glu → 0) were provided as red cells from heterozygous patients by Dr. Ronald Rieder of Downstate University, Brooklyn, N.Y. These unstable hemoglobins were dialyzed as total lysates against a 0.15 M KPO₄ buffer, pH 7.35, but purification of the unstable mutant was not attempted for fear of partially denaturing it in advance. Hemoglobin Deer Lodge (β2His → Arg) was provided as whole blood from a heterozygous patient by Dr. François Vella of Canada. Hb F was prepared from pooled cord blood samples and was separated on a Biorex 70 column using Developer No. 4. After separation, the purified Hb F was equilibrated with a 0.15 M KPO₄ buffer, pH 7.35. D₂O was obtained from the New England Nuclear Co., and ¹⁴C-HN₂ (nitrogen mustard) was obtained from the Mallinkrodt Co., St. Louis, Mo.; specific activity, 100 μCi/2.62 mg). Alkylureas, methyl, ethyl, propyl, and butyl ureas were obtained in the purest quality commercially available and recrystallized twice from hot ethanol and dried in a vacuum oven.

RESULTS

We were able to confirm Asakura’s observation of an increased mechanical instability of hemoglobin S as compared to A. We also confirmed that raising the pH in the vicinity of 8 increased the instability of both Hb S and A. In addition, we have found that the geometry of the tube and the volume of hemoglobin solution placed in it played important roles in determining the amount of precipitation. For example, after 3 min of shaking 3 ml of Hb S (200 mg/dl) in a 30-ml Cortex tube, there was a 20% loss of hemoglobin. After shaking 5 ml in the same tube for 3 min, there was only a 10% loss. If a 15-ml tube was used instead of a 30 ml one, the difference was even greater. In this case, a 2-ml volume resulted in a 20% loss, while a 3-ml volume produced only a 7% loss under the same conditions. It appears that the amount of surface exposure (both glass and air interface) may exert major controls over the per cent denatured. It was also found that different amounts were precipitated when five 2-min intervals of vortexing were used (with centrifuging in between vortexing) on the one hand, and simply vortexing for 10 min continuously. Since the amount of precipitation was greater in the latter case, it was assumed that both the greater production of heat and the greater turbulence produced by the accumulation of precipitated material contributed to the enhancement observed by a single long continuous period of vortexing.

When the tube size and the volume of the hemoglobin solutions are kept constant, it can be seen that the concentration of the hemoglobin influences the per cent precipitated by vortexing. Figure 1 shows the result of vortexing solutions of oxyhemoglobin S in the concentration range of 0.1–1.6 g/dl (0.13–1.0 × 10⁻¹ M heme). There is quite clearly a different slope for each concentration in the range studied. This is in contrast to the findings of Asakura, who found no relation of precipitation to concentration.

Because hemoglobin S is the result of a single substitution at the β6 position (β6Glu → Val), it was of interest to determine the mechanical stability of other hemoglobins with substitutions near this site. It was also of interest to investigate the doubly substituted hemoglobin C_Harlem (β6 Glu → Val and β73Asp → Asn). Figure 2 shows the precipitation rates of hemolysates from patients who are heterozygous for Hb Deer Lodge or C_Harlem. It can be seen that the substitution of Arginine at β2, only four amino acids away from the β6 site, does not affect mechanical
precipitation of Hb Deer Lodge-A. The data for S and SA are similar to the results of Asakura and co-workers. C_{Harlem} precipitates more rapidly than SA. Both these samples contain 60% A and 40% S or C_{Harlem}. The purified samples confirm that it is in fact the C_{Harlem} which precipitates faster than Hb S.

Because mechanical instability of oxyHb S has been proposed as a screening test for Hb S, it was also of interest to test other mutants which are known to be heat unstable. Figure 3 compares the precipitation rates of Hb Leiden-A, Hb Gun-Hill-A, and Hb SA at 25°C. It is clear that all these hemoglobins are mechanically unstable. It was also of interest to note that, while vortexing in the cold at 5°C protected the SA mixture (same slope as A alone), this was not
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Fig. 3. Mechanical precipitation of hemoglobins Gun Hill-A, Leiden-A, and SA at pH 7.35, 25°C. Coordinates are the same as in Figs. 1 and 2. Initial Hb concentration, 200 mg/100 ml.

the case with Gun-Hill-A and Leiden-A (data not shown). Although denaturation was reduced in the cold for these mutants, it was by no means absent. These differences may indicate that different processes are occurring in the case of the heat-unstable mutants on the one hand and Hb S on the other. A parallel difference is seen in response to alkylureas.

Figure 4 shows the effect of a series of alkylureas on the precipitation rate of Hb S at 25°C at pH 7.35. It can be seen that, at equimolar concentrations of each urea, there is increasing protection of the hemoglobin with increasing alkyl chain length. In the case of butylurea, 0.1 M is sufficient to inhibit virtually all precipitation during the observation period.

The alkylureas were not as successful in protecting the heat-unstable mu-

Fig. 4. The effect of urea and the alkylureas (methyl, ethyl, propyl, and butylurea) on the rate of mechanical precipitation of Hb S at pH 7.35, 25°C. Coordinates are the same as in Fig. 1. Filled circles, control; open circles, urea, 0.2 M; open triangles, methylurea, 0.2 M; closed triangles, ethylurea, 0.2 M; open squares, propylurea, 0.2 M; closed squares, butylurea, 0.1 M. Initial Hb concentration, 200 mg/100 ml.
Figure 5 shows the effect of these agents on the precipitation rates of Hb Gun-Hill-A and Leiden-A. In comparison with their effect on Hb S, it can be seen that 0.2 M concentrations of ethyl and propyl ureas are not as effective in protecting these mutants. Hb Leiden is particularly resistant even to 0.1 M butylurea which totally protected Hb S under the same conditions.

We turn now to the question of the stability of hybrids with Hb S. Oxyhemoglobins SA and C_Harlem would be expected to form the hybrids $\alpha_2\beta^S\beta^A$ or $\alpha_2\beta^A\beta^C_{Harlem}$. If these hybrids were unstable, then the removal of $\beta^A$ would be expected to be accelerated by the presence of Hb S or C_Harlem. Using the $^{14}$C-mustard-labeled Hb A, which contains almost all the label bound to the $\beta$-chain, it was possible to test the hypothesis that hybrids of $\beta^S$ and $\beta^A$ or $\beta^A$ and $\beta^C_{Harlem}$ are unstable. Figure 6A and B show the results of these experiments. Figure 6B shows that A, SA, and C_Harlem-A all precipitate at different rates as previously noted. This was measured by conventional spectrophotometry at the Soret absorption wave length of 419 nm. Figure 6A shows that radioactivity from Hb A-$^{14}$C-HN$_2$ disappears at a constant rate regardless of which other mutant is present. This rate is similar to the precipitation rate of Hb A as judged by optical methods. Hence, it is concluded that hybrids of the type described above are not particularly unstable, and only the tetramers $\alpha_2\beta^S$ or $\alpha_2\beta^C_{Harlem}$ or their dimer forms display instability.

Another possible factor in the precipitation of oxyhemoglobins from solutions is oxidation. Asakura originally concluded that denaturation appears to...
involve an oxidative process because the rate of precipitation was dependent on the oxygen concentration. Oxidative changes readily affect the -SH groups of proteins. It was therefore of interest to investigate the effect of blocking the \( \beta_93 \) -SH group with parachloromercuribenzoate. Figure 7 shows the result of vortexing native and PMB-treated samples of Hb A and S at pH 7.35 and 8.35. There is only a modest difference between the blocked and free -SH samples, and in the case of Hb S at pH 8.35, there is no apparent difference. Spectrophotometric titration of the \( \beta_93 \) -SH group with PMB at 250 nm confirmed that no free rapidly titratable -SH groups remained. It would appear from these data that the formation of mixed disulfides involving the \( \beta_93 \) -SH group are not important in the precipitation process. It is very likely that -SH groups in general are not involved in the mechanical precipitation process, since the presence of an excess of dithiothreitol does not affect the precipitation rate.

In addition to interacting with ligands and dissolved gases, hemoglobin is also in contact with the surrounding water and the small ions dissolved in it. We therefore varied these substances both qualitatively and quantitatively in order to discern any pattern of effects on mechanical stability. Table 1 summarizes the effect of inorganic salts and D_2O on the stability of oxyhemoglobin S. After 10 min of vortexing (five periods of 2 min each), there appears to be a small, reproducible stabilizing effect of D_2O on the precipitation of oxyHb S both in the presence and absence of KPO_4. The addition of 0.5 M NaCl, NaI, and MgCl_2 to the KPO_4 buffer appears to enhance the precipitation according to the increase in ionic strength which they produce.

Finally, an attempt was made to discover partially denatured but still soluble species of hemoglobin by serial optical rotatory dispersion (ORD) measurements after five successive 2-min periods of vortexing at 24°C at pH 7.35. Table 2 shows the mean residue rotation at 233 nm for each sample. There is no discernible decline in the \( \alpha \)-helix content of the material left in the supernatant.
Table 1. The Effect of \( D_2O \) and Salts on the Stability of Oxy Hb S at 25° C

<table>
<thead>
<tr>
<th>Hb Solution</th>
<th>Soluble Protein (%) After 10 min Vortexing</th>
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<tbody>
<tr>
<td></td>
<td>H( _2O )</td>
</tr>
<tr>
<td>KPO( _4 ) buffer, 0.15 M, pH 7.35</td>
<td>63%</td>
</tr>
<tr>
<td>Hemoglobin S (without buffer)</td>
<td>53%</td>
</tr>
<tr>
<td>0.5 M NaCl</td>
<td>36%</td>
</tr>
<tr>
<td>0.5 M NaI</td>
<td>42%</td>
</tr>
<tr>
<td>0.5 M MgCl( _2 )</td>
<td>31%</td>
</tr>
</tbody>
</table>

*The \( pD \) of these solutions is about 0.4 U higher than the pH in \( H_2O \). This difference would tend to destabilize Hb S slightly.

Table 2. Effect of Shaking on the Mean Residue Rotation of Oxy Hb S

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>(-[m]_{233})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8600 ± 300</td>
</tr>
<tr>
<td>2</td>
<td>8300</td>
</tr>
<tr>
<td>4</td>
<td>8600</td>
</tr>
<tr>
<td>6</td>
<td>8300</td>
</tr>
<tr>
<td>8</td>
<td>8500</td>
</tr>
<tr>
<td>10</td>
<td>8800</td>
</tr>
</tbody>
</table>

Fig. 7. The effect of parahydroxymercuribenzoate (PMB) on the mechanical precipitation of Hb S and A at pH 7.35 and 8.35, 25° C. Ordinate, per cent hemoglobin remaining in supernatant; abscissa, time in minutes.
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DISCUSSION

Precipitation by shaking of oxyhemoglobin appears to be caused by a complex array of factors. Our studies do suggest that a surface denaturation phenomenon is involved. The contribution of surface denaturation may be more significant for Hb S than for the other heat-unstable mutants studied here. Our data concerning the importance of the tube geometry and the volume of the solution certainly suggest that a relationship exists between the surface area (Hb versus glass and Hb versus air) and the extent of precipitation. The protection afforded by low temperature and alkylureas is not inconsistent with this view. The partial failure of low temperature and alkylureas to protect some of the other unstable mutants does seem to indicate that precipitation can occur by more than one mechanism. Further studies will be needed to determine if alkylureas, D2O, ionic strength, ligand state, and protein primary structure alter precipitation rate because they affect the surface properties of the hemoglobin solution or because they affect the structure of the protein itself.

It was hoped that the kinetics of precipitation would yield some insight into the process or processes involved. It was therefore of interest to note that a relatively sharp transition from native to insoluble protein occurs, as judged by ORD studies. Asakura has observed that the rate of precipitation follows first-order kinetics, i.e., is independent of the initial concentration. In contrast, we find that precipitation is a function of concentration. This finding is consistent with surface denaturation, because when the surface area is kept constant, a progressively smaller proportion of the total will be denatured as the concentration of hemoglobin increases.

The precipitation of hybrids gives some insight into the conformational requirements for rapid precipitation. It is believed that gelation of Hb S in the deoxy state requires only one β6Val site per tetramer. Thus, as the hybrid α3βαβ8 is capable of participating in polymer formation, there is no absolute requirement for α3βαβ8 for gel formation. In contrast, mechanical precipitation of oxyhemoglobin S does require the tetramer α2β2, or rapid denaturation is not observed. This is another indication that a very different conformational effect is involved in precipitation as opposed to gelation. This disparity is also seen in the data for the precipitation of Hb C_Harlem. In the deoxy form, the loss of the β73 carboxyl group inhibits gelling, but in the oxy form, the same hemoglobin is even more unstable than Hb S. There is no obvious explanation for this difference.

Finally, Hb S mechanical instability has been proposed as a test for both the homozygous and the heterozygous forms of this condition. Our data clearly show that several other mutants also display instability. It is therefore incumbent on anyone using this method as a screening test for the presence of Hb S to confirm the diagnosis by conventional means.

In summary, the data presented here suggest that mechanical precipitation is probably related to surface denaturation. It also demonstrates that precipitation is dependent on concentration, the presence of alkylureas, and on other mutations at sites other than the β6 valine.

Further studies on the basis of the difference between Hb A and Hb S in the oxy state are clearly indicated.
ACKNOWLEDGMENT

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REFERENCES

Observations on the mechanical precipitation of oxy Hb S and other mutants

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