Stabilizing Effect of Various Organic Solvents on Protein*

(Received for publication, July 25, 1977)

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The rate of denaturation of hemoglobin and other proteins by mechanical shaking is strongly affected by organic solvents. A group of solvents, including alcohols and ketones, was found to stabilize proteins at low concentrations, although these same organic solvents denatured proteins at high concentrations. The stabilizing effect of alcohols increased with increasing chain lengths. The second group of solvents, including toluene and chloroform, showed only a destabilizing effect, while the third group of solvents such as formamide and pentane had no effect over a wide range of concentrations. Organic solvents may be used to protect or denature a specific protein in solutions containing various proteins.

Various compounds, among them organic solvents, are used to denature proteins in solution (1-3). Organic solvents alter the native structure of proteins by disrupting hydrophobic interactions between the nonpolar side chains of amino acids. Relatively high concentrations of these solvents are required to unfold the ordered structure of polypeptide chains (3).

During a study of the mechanism of denaturation of sickle hemoglobin (Hb S) by mechanical shaking, a method recently developed in our laboratories for the study of protein denaturation (4-6), we noticed that the rates of denaturation of hemoglobin were strongly affected by the addition of small amounts of organic solvents. Some solvents showed a strong stabilizing effect, while others showed either no effect or a strong destabilizing effect. Since the amounts of organic solvents used for these experiments were far less than those used for classical denaturation experiments, the mechanism of these effects appears to differ from that of the well known denaturation effect of organic solvents on proteins.

EXPERIMENTAL PROCEDURES

In experiments testing the effects of various amounts of organic solvents on the rate of denaturation of protein, a protein solution (2 ml) in a glass vial (10 x 50 mm) was shaken vigorously at a shaking frequency of 30 or 50 Hz with a high speed Technical Consulting Services shaker specifically designed for these experiments. After shaking for various time intervals, the vial was centrifuged at 5000 x g for 5 min and the amount of protein remaining in the supernatant was determined either spectrophotometrically or by measuring the biological activity of the enzyme before and after shaking. Extensive studies on shaking conditions have shown that the denaturation curves for various proteins follow essentially first order kinetics and are highly reproducible for each protein when shaking conditions are kept constant (6). The first order rate constant of denaturation curves can be used as a parameter of stability of proteins in solution. Denaturation of glycolytic enzymes was studied by measuring the enzyme activity before and after shaking (7). All enzyme activities were measured according to the method described by Beutler (8).

RESULTS AND DISCUSSION

Denaturation of proteins by mechanical shaking is thought to be due to surface denaturation in which protein molecules exposed to the air-liquid interface spread over the surface of the bubble, resulting in the unfolding of the molecule. It is well known that aeration or bubbling causes denaturation of proteins. The effect of ethanol and toluene on the rate of denaturation of sickle oxyhemoglobin is shown in Fig. 1. In the absence of an organic solvent, Hb S precipitates according to first order kinetics (6) and the rate of precipitation is approximately 10 times faster than that of Hb A (5). The addition of ethanol (2.5%, v/v) totally inhibits the denaturation of the Hb S, while the addition of toluene (0.25%, v/v) increases the rate of denaturation 4-fold. These effects are concentration-dependent, as are the effects of other organic solvents. The effect of organic solvents on Hb A was essentially similar to that of Hb S except that slightly higher concentrations of organic solvents were required to reveal this same effect.

The concentration dependency of two representative types of organic solvents on oxy-Hb S is shown in Fig. 2. The stabilizing effect of various alcohols, classified for our purposes as Group I solvents, increases with concentration. The C_{50x}, defined as the molar concentration (or the volume percentage of organic solvent in the medium) required to inhibit denaturation by 50%, decreases in the order of methanol, ethanol, propanol, and butanol. There is no spectral change in hemoglobin in the visible and UV regions. The concentration of organic solvents required to reveal this effect.

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Effect of Organic Solvents on Stability of Protein

The effect of various organic solvents on the denaturation of Hb S is summarized in Table I. Hexamethylphosphoramide has been the most effective stabilizing agent among various solvents employed. The addition of only 15 μl of hexamethylphosphoramide in 100 ml of Hb solution inhibits mechanical denaturation by 50%.

The mechanism of the stabilizing or destabilizing effects of low concentrations of organic solvents on hemoglobin is unknown. Klotz and Tam (10) reported that aqueous solutions containing 0.1 M ethanol markedly weaken the binding of uncharged small molecules by serum albumin. It should be pointed out that the rate of denaturation of hemoglobin is depressed when a concentrated hemoglobin solution is used (6, 9). This was explained by the relative decrease of bubble formation to the total number of protein molecules and insufficient mixing due to increased viscosity (6). Similar inhibitory effect was seen when a concentrated hemoglobin solution was shaken with Group II solvents. As shown in Fig. 3, much more organic solvent is required to reveal the same effect on concentrated hemoglobin solution.

The denaturation by mechanical shaking is not restricted to merely hemoglobin, but is applicable to other proteins, too (7). To study if the organic solvent used for hemoglobin has a similar effect on other proteins, the effect of organic solvents on the rate of denaturation of glycolytic enzymes of red blood cells was investigated. The enzymes tested were hexokinase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, pyruvate kinase, aldolase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, monophosphoglyceromutase, enolase, pyruvate kinase, and lactate dehydrogenase. Since the amount of these enzymes in hemolysate is extremely small, denaturation experiments were carried out by measuring enzyme activities before and after shaking. It was found that the addition of organic solvents belonging to Group I always stabilized these enzymes, while those of Group II destabilized them. One example of the stabilizing effect of ethanol in glucose-6-phosphate dehydrogenase is shown in Fig. 4. In this specific experiment, the hemolysate was passed through a Sephadex G-25 column to remove NADP and glucose 6-phosphate because these compounds were found to have a stabilizing effect on glucose-6-phosphate dehydrogenase. The C50 value for glucose-6-phosphate dehydrogenase by ethanol was 0.7%, a value similar to that for hemoglobin (Table I). The stabilizing effect of Group I organic solvents appears to be less sensitive to protein concentration than the destabilizing effect of Group II solvents. All the other enzymes described above showed similar results.

### Table I

<table>
<thead>
<tr>
<th>Compounds</th>
<th>C50 (mM)</th>
<th>% (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>303.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>130.0</td>
<td>0.75</td>
</tr>
<tr>
<td>Propanol</td>
<td>66.8</td>
<td>0.60</td>
</tr>
<tr>
<td>Butanol</td>
<td>54.7</td>
<td>0.50</td>
</tr>
<tr>
<td>Ether</td>
<td>32.0</td>
<td>0.15</td>
</tr>
<tr>
<td>Dioxane</td>
<td>42.1</td>
<td>0.36</td>
</tr>
<tr>
<td>Acetone</td>
<td>70.1</td>
<td>0.52</td>
</tr>
<tr>
<td>Methyl cetyl ketone</td>
<td>42.0</td>
<td>0.38</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>45.2</td>
<td>0.35</td>
</tr>
<tr>
<td>Hexamethylphosphoramide</td>
<td>0.83</td>
<td>0.015</td>
</tr>
<tr>
<td>2,2-Dimethylpropyl</td>
<td>12.3</td>
<td>0.15</td>
</tr>
<tr>
<td>n-Amyl Alcohol</td>
<td>75.9</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>28.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Chloroform</td>
<td>12.1</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentane</td>
<td></td>
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</table>

*a* Indicates midpoint concentration for stabilization in Group I solvents and for destabilization in Group II solvents.

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**Fig. 1.** Effect of ethanol and toluene on mechanical denaturation of sickle oxyhemoglobin. A solution (2 ml) of sickle oxyhemoglobin (10 μM) in 0.1 M potassium phosphate buffer, pH 8.0, was shaken in the presence or absence of organic solvents with a Technical Consulting Services shaker at a frequency of 30 Hz at 20°C. After centrifugation, the amount of remaining hemoglobin in the supernatant was determined spectrophotometrically (5).

**Fig. 2.** Effect of various organic solvents on mechanical denaturation of hemoglobin.

**Fig. 3.** Effect of hemoglobin concentration on the destabilization effect of chloroform. Washed and packed red blood cells (RBC) were hemolyzed with 5 mM phosphate containing 0.5 mM EDTA, pH 7.4, at the ratio indicated in the figure. Two milliliters of each hemolysate were shaken in the presence of three different amounts of chloroform for 30 s. After shaking, the fraction of remaining undenatured hemoglobin was determined spectrophotometrically as described elsewhere (5, 6).
Effect of Organic Solvents on Stability of Protein

It was interesting to note that there are slight differences in the denaturation effects of organic solvents on different proteins. As shown in Fig. 5, hexokinase denatures more rapidly than hemoglobin when a 1:20 hemolysate was shaken in the presence of 5% chloroform. In contrast, hemoglobin precipitated more rapidly than glucose phosphate isomerase when a 1:200 hemolysate was shaken in the presence of 0.5% chloroform.

Although the physiological significance of the effect of small amounts of organic solvents on proteins is unknown, there may be a number of applications of these properties of organic solvents in laboratories and industries. One major application is the use of organic solvents in enzyme purification procedures to remove unstable proteins or to stabilize unstable proteins. A classical application of this method is known as the Tsuchihashi method (11) in which chloroform is used to purify catalase from hemolysate. We have found that hexamethylphosphoramide can be used to stabilize unstable hemoglobins during preparation or during measurement of oxygen equilibrium curves of hemoglobin. Organic solvents, including toluene and chloroform, are used routinely in clinical laboratories to hemolyze red cells (12). Results in our laboratory clearly indicate that toluene and chloroform are not suitable for this purpose because these organic solvents belong to Group II and thereby accelerate the rate of denaturation of proteins. In fact, precipitation of hemoglobin, particularly unstable hemoglobins, was found to be significant when toluene or chloroform was used as the hemolyzing solution. In instances where an organic solvent is needed for efficient red cell hemolysis without much dilution of hemoglobin in aqueous solutions, ether, which belongs to Group I and has a stabilizing effect on proteins, appears to be the most appropriate choice. Some organic compounds are known to inhibit sickling of red blood cells containing Hb S (6, 13, 14). Extensive screening of organic solvents may lead to the finding of an effective anti-sickling agent with low toxicity.

REFERENCES