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Stabilization of 20β-Hydroxysteroid Dehydrogenase by Glycerol

Tsuyoshi Tanimoto, Takao Hayakawa, and Jiro Kawamura

Division of Biological Chemistry and Reference Standards, National Institute of Hygienic Sciences[1)]

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20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* is partially inactivated in sodium phosphate buffer within a short time. Inactivation of the enzyme was accelerated at acidic pH, and the enzyme was also inactivated by incubation with rose bengal. The inactivation due to acidic pH and rose bengal was prevented by glycerol; 20% glycerol completely restored the activity lost at pH 5.5. The mechanism of stabilization of the enzyme by glycerol is not yet clear.

Keywords——20β-hydroxysteroid dehydrogenase; stabilization; glycerol; stabilization of enzyme; pH stability; rose bengal; reversible inactivation; stability

The present paper is concerned with the stability of 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans*,[2)] which transfers the hydrogen from NADH to 20-oxo steroids, giving 20β-hydroxy steroids.[3)] The enzyme is known to be stable in crystalline form, and has been widely used for enzymatic analyses of some 20-oxo steroids,[4)] and also as a model protein for studies of the interaction between steroids and proteins.[5)] We have undertaken studies to examine thoroughly the active site of the enzyme and the interactions of the enzyme with steroids and the coenzyme. During these investigations, it was found that the enzyme was partially inactivated within 30—60 min when the enzyme was dissolved in sodium phosphate buffer and Tris buffer or when rose bengal was added in the enzyme solution in the dark. This resulted in experimental difficulties, so our initial studies were aimed at stabilization of the enzyme.

Experimental

Materials——20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* was obtained in crystalline form from Boehringer Mannheim Co. Polyacrylamide gel electrophoresis of the enzyme gave a single band, so the enzyme was used without further purification. Cortisone was obtained from E. Merck AG. NADH was purchased from Sigma Chemicals Co. and Oriental Yeast Co.

Enzyme Assay——20β-Hydroxysteroid dehydrogenase activity was determined by following NADH oxidation at 340 nm with a Union High-Sens SM-401 spectrophotometer equipped with a National X–Y recorder at 25°. The assay mixture contained 100 mM sodium phosphate buffer (pH 6.4), 1 mM EDTA, 0.46 mM cortisone, 0.15 mM NADH and an appropriate amount of the enzyme in a final volume of 3.0 ml. The reaction was initiated by adding the enzyme, and the decrease of absorption at 340 nm was followed for 3 min. Cortisone was dissolved in methanol, and the final concentration of this solvent was 3.3% in all assays. NADH solution was made up immediately before use by dissolving the compound in 10 mM Tris buffer at pH 8.2.

1) Location: *1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan.*
2) H.J. Hübener, F.H. Sahrholz, J. Schmidt-Thome, G. Nesemann, and R. Junk, *Biochim. Biophys. Acta*, **35**, 270 (1959).
3) K.R. Hanson, *J. Am. Chem. Soc.*, **88**, 2731 (1966); W. Gibb and J. Jeffery, *Eur. J. Biochem.*, **34**, 395 (1973).
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Enzyme Concentration——The concentration of the enzyme was determined from the absorbances at 280 and 260 nm,[6] and was estimated by the specific activity which was 18.65 μmol/min per mg enzyme when assayed in 0.1 M sodium phosphate buffer (pH 6.4) containing 1 mM EDTA, 0.15 mM NADH and 0.46 mM cortisone at 25°.

Results

Stabilization of 20β-Hydroxysteroid Dehydrogenase by Glycerol and Various Glycols

When 20β-hydroxysteroid dehydrogenase was incubated at 25° in 0.1 M sodium phosphate buffer at pH 6.5, 30% of the enzyme activity was lost within 1—3 hr. However, as shown in Fig. 1, this inactivation was completely prevented by adding 20% glycerol. Various glycols and sucrose also showed a protective effect. Exceptionally, propyleneglycol accelerated the inactivation of the enzyme, and the loss of activity was more than 90% after incubation for 2 hr. Glycerol concentrations of more than 10% stabilized the enzyme completely (Table I).

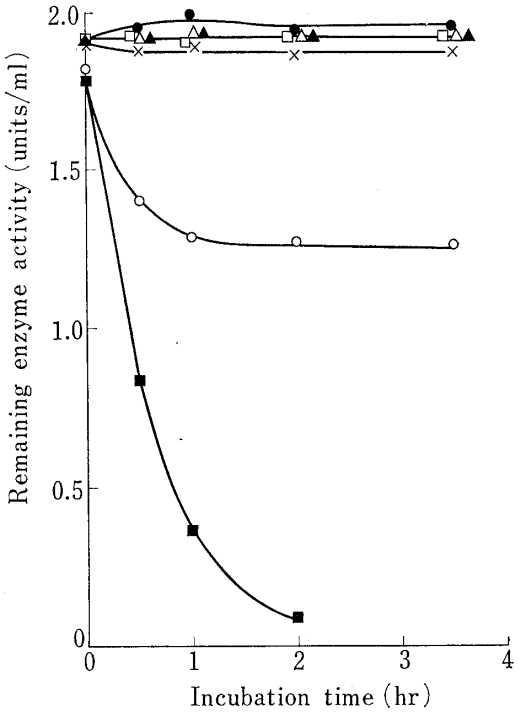

Fig. 1. Stabilization of 20β-Hydroxysteroid Dehydrogenase by Glycerol, Sucrose and Various Glycols

The enzyme (0.1 mg/ml) was dissolved in 0.1 M sodium phosphate buffer at pH 6.5 containing 20% glycerol, sucrose or a glycol. The enzyme solutions were incubated at 25° and assayed after various times of incubation for enzyme activity.
—○—, none; —●—, glycerol; —△—, diethyleneglycol; —▲—, triethyleneglycol; —□—, tetraethyleneglycol; —■—, propyleneglycol; —×—, sucrose.

Effect of pH on the Enzyme Stability

Figure 2A shows the effect of pH on the stability of 20β-hydroxysteroid dehydrogenase during incubation in 0.1 M sodium phosphate buffer at 25°. The enzyme appeared to be more stable at neutral pH than at acidic pH. At pH 7.5, only about 20% of the original activity was lost on incubation for 3 hr, while about 50 and 70% of the original activity was lost in 0.5 and 3 hr, respectively, at pH 5.5. However, 20% glycerol was able to protect the enzyme significantly against inactivation over a wide pH range (pH 6.0—8.0) (Fig. 2B). Inactivation at pH 5.5 was also partially prevented by addition of glycerol; only about 25% of the original activity was lost after incubation for 3 hr.

Effect of Glycerol on the Inactivation by Rose Bengal

During the course of photochemical modification of 20β-hydroxysteroid dehydrogenase, it was observed that the enzyme activity decreased in the dark in the presence of rose bengal.[7] Although about 30 and 55% of the original activity were lost on incubation with 10^{-6} and 10^{-5} M rose bengal, respectively, for 2.5 hr at 25°, the loss of activity was almost completely prevented in the presence of 20% glycerol. Only about 10% of the original activity was lost in 2.5 hr at 10^{-5} M rose bengal in the presence of glycerol, and no decrease of activity was observed at 10^{-6} M rose bengal (Fig. 3).

Inactivation at pH 5.5 and Recovery of the Activity with Glycerol

20β-Hydroxysteroid dehydrogenase was inactivated by treatment at pH 5.5, but the activity could be restored rapidly by the addition of 20% glycerol (Fig. 4). The reactivation

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by glycerol was complete in the case of loss of about 40% of the original activity after incubation for 1 hr, whereas enzyme that had lost about 60% of the original activity after incubation for 2.5 hr was reactivated only slightly by glycerol. The lost enzyme activity was not recovered when the pH of the incubation medium was changed from pH 5.5 to pH 7.0 in the absence of glycerol.

Table I. Effect of Glycerol Concentration on the Stability of 20β-Hydroxysteroid Dehydrogenase

Concentration of glycerol (%)	Remaining enzyme activity (units/ml) 0 hr	1 hr	4 hr
	%	%	%
0	1.81 (100)	1.45 (80.1)	1.24 (68.5)
5	1.89 (100)	1.72 (91.0)	1.60 (84.7)
10	1.91 (100)	1.92 (100.5)	1.87 (97.9)
20	1.87 (100)	1.89 (101.1)	1.86 (99.5)

The enzyme (0.1 mg/ml) was dissolved in 0.1 M sodium phosphate buffer at pH 6.5 containing various concentrations of glycerol. The enzyme solutions were incubated at 25° and assayed after 1 and 4 hr for enzyme activity.

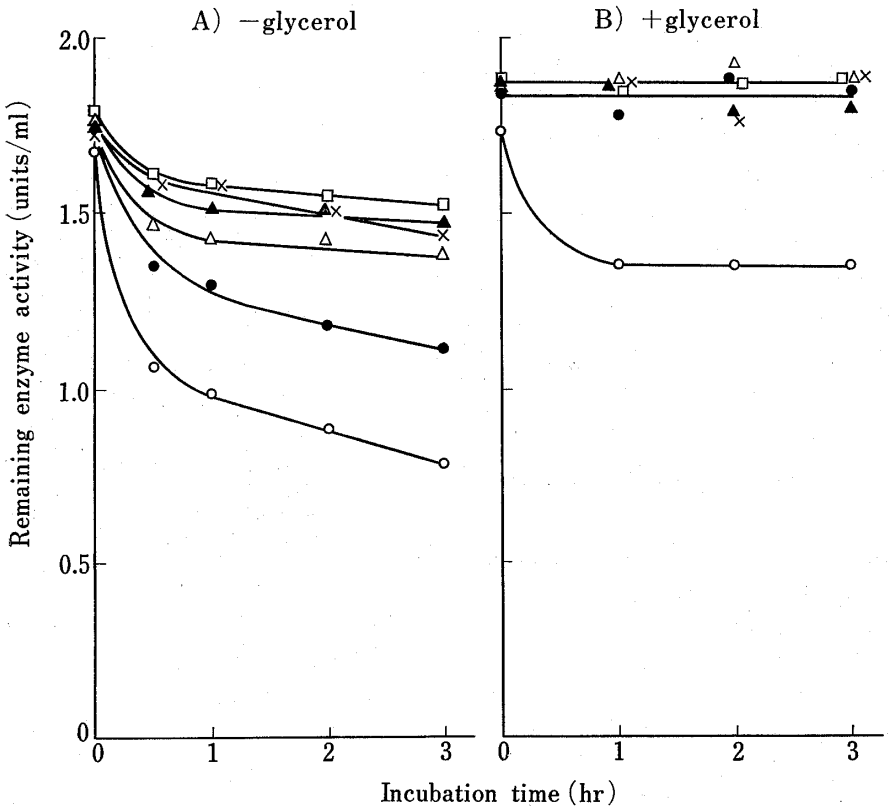

Fig. 2. Effect of pH on the Stability of 20 β-Hydroxysteroid Dehydrogenase

The enzyme (0.1 mg/ml) was dissolved in 0.1 M sodium phosphate buffer at various pH values (A) or in the same buffer containing 20% glycerol (B). The enzyme solutions were incubated at 25° and assayed after various times of incubation for enzyme activity.

—○—, pH 5.5; —●—, pH 6.0; —△—, pH 6.5; —▲—, pH 7.0; —□—, pH 7.5; —×—, pH 8.0.

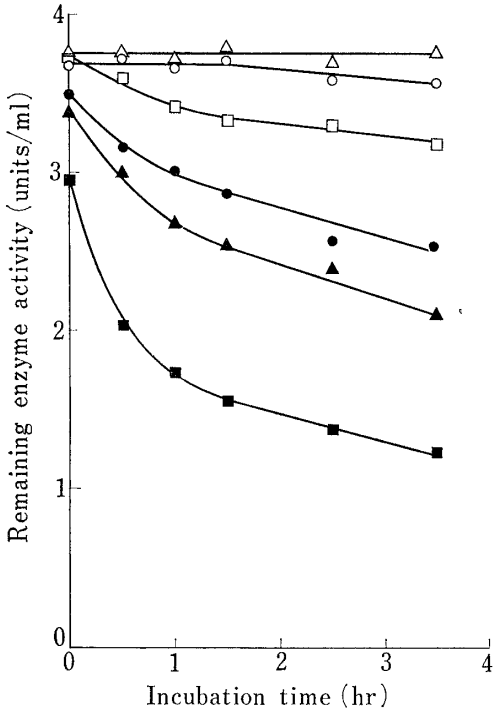

Fig. 3. Effect of Glycerol on the Inactivation of the Enzyme by Rose Bengal in the Dark

The enzyme (0.2 mg/ml) was dissolved in 0.1 M sodium phosphate buffer at pH 7.5 containing 0 (○, ●), 10^{-6} M (△, ▲), or 10^{-5} M (□, ■) rose bengal. Two aliquots were taken; 20% glycerol was added to one (open symbols), and no glycerol was added to the other (closed symbols). Each sample was incubated at 25° and assayed after various times of incubation for enzyme activity.

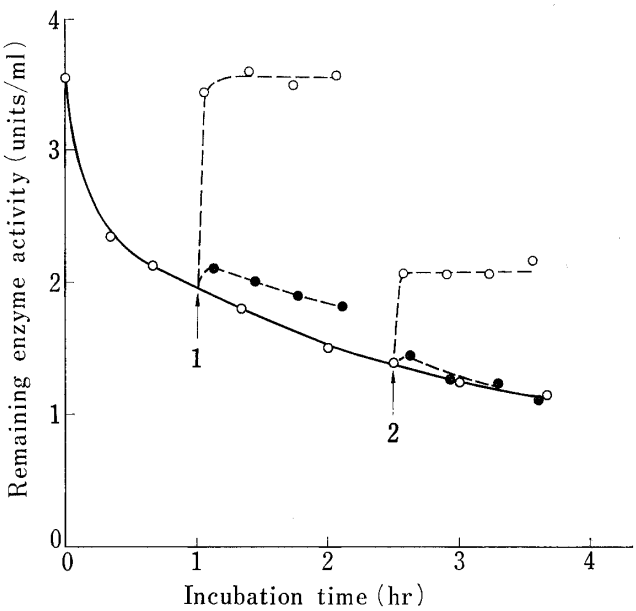

Fig. 4. Inactivation of 20β-Hydroxysteroid Dehydrogenase at pH 5.5 and Reactivation by Glycerol

The enzyme (0.2 mg/ml) was dissolved in 0.1 M sodium phosphate buffer at pH 5.5 and incubated at 25°. The enzyme solution was assayed after various times of incubation for enzyme activity. ——○——, incubated at pH 5.5; ——○——, incubated at pH 7.0 in the presence of 20% glycerol after preincubation at pH 5.5 for 1 hr (arrow 1) or 2.5 hr (arrow 2); ——●——, incubated at pH 7.0 in the absence of glycerol after preincubation at pH 5.5 for 1 hr (arrow 1) or 2.5 hr (arrow 2).

Discussion

The stability of 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* is affected by pH, but not by salt concentration (not shown). The enzyme was susceptible to acidic pH. In addition, inactivation of the enzyme was enhanced by rose bengal. However, these inactivations were completely prevented by the addition of glycerol. The inactivation due to acidic pH was reversible. The enzyme activity loss after incubation for 1 hr in pH 5.5 was restored by the addition of glycerol, though prolonged incubaton caused a reduction in the extent of reactivation. It is possible that the crystalline enzyme may undergo a rapid, reversible conformational change when dissolved in buffer solution, and that this becomes irreversible at acidic pH. The molecular weight of the enzyme was about 110000, and the enzyme was composed of four identical subunits having a molecular weight of about 27000.[8] When native enzyme and enzyme partially inactivated at pH 5.5 (about 50% inactivation) were applied to a Sephadex G-200 column, the enzymes showed similar elution patterns and were eluted at elution volumes corresponding to an approximate molecular weight of 100000. The CD spectra of the enzymes were also similar. Thus, the inactivation of the enzyme is presumably not due to a major conformational change such as dissociation–association, but to a relatively small local conformational change.

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Propyleneglycol accelerated the inactivation of the enzyme. The reason for this is not clear, though Contaxis and Reithel[9] suggested that propyleneglycol affects the degree of hydration near hydrophobic areas.

Glycerol has been empirically used in many enzymological studies to stabilize proteins. For instance, tryptophan synthetase from *Bacillus subtilis*,[10] placental 17β-hydroxysteroid dehydrogenase,[11] anthranilate synthetase from *Acinetobacter calcoaceticus*,[12] and carbamylphosphate synthetase from frog liver[13] were completely protected against loss of activity by addition of glycerol. However, the mechanism of the stabilizing effect of glycerol is not known. It was surmised that the effect may involve a nonspecific strengthening of intersubunit or intramolecular hydrophobic interactions. In addition, it has also been suggested that glycerol may have the ability to stabilize networks of structured water molecules[14] which are essential for the maintenance of the correct spatial configuration of the protein in the native state. McDuffie *et al.*[15] indicated the existence of "packages" of glycerol and water of different composition in glycerol–water mixtures, so the formation of water–glycerol structures around the protein molecules seems quite possible, and may account for the stabilization of the protein. However, the confirmation of such proposals based on stabilization of the hydrated structure of proteins must await a far better understanding of the structure of water surrounding biological macromolecules.

At any rate, the finding that glycerol was able to protect 20β-hydroxysteroid dehydrogenase against inactivation resulting from dilution with buffer solution made possible reliable kinetic analysis using enzyme solution diluted to appropriate concentrations in one set of experiments. Some kinetic studies previously carried out using the enzyme solution diluted with 128 mM phosphate buffer at pH 7.0[16] or 5 mM Tris buffer at pH 8.2[17] should be reexamined in view of the possibility that the activity of the enzyme diluted with buffer might have been partially lost during the course of the experiments. The protective effect of glycerol against inactivation of the enzyme in the presence of rose bengal in the dark may make possible a photochemical approach to elucidation of the active site of this enzyme. Such studies, based on the present work, will be described in a subsequent paper.

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