Role of Cobalt in Stabilizing the Molecular Structure of Glucose Isomerase from *Streptomyces griseofuscus* S-41

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The role of cobalt in the stabilization of the molecular structure of glucose isomerase from *Streptomyces griseofuscus* S-41 was investigated using various denaturants. The enzymatic activity and molar ellipticity at 220 nm were significantly reduced in 8 M urea solution, but restored to the original values on removing urea. Therefore, the enzyme was thought not to suffer a drastic conformational change with urea. On the other hand, the destruction of ordered structure involving a complete loss of activity was observed from circular dichroism and fluorescence spectra in 6 M guanidine hydrochloride solution. The enzyme showed somewhat peculiar behavior in organic solvents; in linear-chained solvents the activity correspondingly decreased with solvent concentrations, whereas it increased slightly in side-chained solvents and acetone. The change in activity observed here were not reflected in the circular dichroism and fluorescence spectra. Three of four cobalt ions originally contained in the enzyme were eliminated by treatment with EDTA or 8 M urea without significant loss of activity. However, a cobalt-free enzyme was quite difficult to obtain in stable form. For elimination of all cobalts, drastic treatment was required, such as with 6 M guanidine hydrochloride, acid–8 M urea or EDTA–8 M urea, indicating considerable dissociation into subunits. The cobalt addition showed a protective effect on the enzyme from denaturation in such drastic conditions, whereas it did not in less drastic conditions, with 8 M urea or organic solvents. It was, therefore, considered that one of the four cobalts was tightly bound to the enzyme and had an essential role in holding the ordered conformation, especially the quaternary structure of the enzyme, while the other three were bound loosely and might be less important in stabilizing the structure.

In the previous paper,1) the authors reported that glucose isomerase (α-xylose ketol-isomerase EC 5.3.1.5) from *Streptomyces griseofuscus* S-412) is a metal activated enzyme and the activation mechanism with divalent metal ions, magnesium and cobalt, is represented by a rapid equilibrium rate expression, in which the combination order of metal and substrate with an enzyme is random, analogous to the case of a two-substrate reaction involving random addition of substrates to an enzyme. The dissociation constants of cobalt from the enzyme–cobalt complex was found to be two orders of magnitude smaller than that of magnesium by kinetic measurements, indicating very much higher affinity for the enzyme. While, magnesium was two-fold effective over cobalt as an activator at the saturating concentration. The affinity of the enzyme for glucose, which was only weak in the absence of a metal, was enhanced by combination of metal and the enzyme, and vice versa. Magnesium and cobalt were shown to be competitive activators in the isomerase reaction, they combine with the same or very close sites of the enzyme, though the competitive profile alternated depending on the relative concentrations of them. It was considered to be due to the difference in affinities of the enzyme for the two metals that the Co²⁺-activated enzyme has higher activity than the Mg²⁺-activated enzyme at a low concentration of the metal ion and that the former is more tolerant to the inhibitory effect of other metals, especially heavy ones. However, it is still unexplainable only by the
difference in affinity why the Co\(^{2+}\) -activated enzyme is stable to heat or acid treatment and the other is not.

In order to get more detailed information on the role of cobalt in stabilization of the molecular structure of glucose isomerase from *Streptomyces griseofuscus* S-41, the effects of denaturants on activity and stability of the enzyme were investigated, and the results are presented in this paper.

**MATERIALS AND METHODS**

**Chemicals.** Guanidine hydrochloride and urea were purchased from Nakarai Co. (Japan), and both were recrystallized from hot methanol before use. Organic solvents for fluorescence spectroscopy were purchased from Merck.

**Enzyme preparation.** Glucose isomerase prepared in a crystalline form from cells of *Streptomyces griseofuscus* S-41 was used throughout the experiments. Cultivation of the strain, and purification and crystallization of the enzyme were carried out according to the procedures described in the previous paper.\(^3\) The crystallized enzyme was dialyzed against 0.01 M MOPS* buffer (pH 7.0) at 4°C for 24 hr. The final preparation was found to be homogeneous on polyacrylamide gel electrophoresis and ultracentrifugal analysis, indicating it to be sufficiently purified. Enzyme activity was assayed, unless otherwise stated, in a reaction mixture containing 0.8 M glucose, 0.1 M Mg\(^{2+}\), 0.05 M MOPS buffer (pH 8.0) and the enzyme in a total volume of 2.0 ml. After incubation at 60°C for 30 min, a 0.5 ml aliquot of the reaction mixture was poured into 4.5 ml of 0.5 n perchloric acid to stop the reaction. Fructose formed was determined by the cysteine carbazole method.\(^4\)

**Protein determination.** Protein content was determined spectrophotometrically with an extinction coefficient (\(E_{1\text{cm}}^{1%}\)) of 11.4.\(^5\) or determined by the method of Lowry et al.\(^6\) using bovine serum albumin as a standard.

**Polyacrylamide gel electrophoresis.** Disc gel electrophoresis was carried out according to the method of Davis\(^7\) in the presence of 8 M urea. The sample was loaded on top of a gel (7.5%, 5 x 70 mm) containing 8 M urea and a constant current of 3 mA was passed through each gel for 40 min. After that, gels were stained with 1% Amide Black 10B for 1 hr and then, diffusonally destained in 7.5% acetic acid.

**Circular dichroism.** CD was measured with J-500 spectropolarimeter (JASCO). The data obtained were expressed in terms of mean residue ellipticity, using a mean residue weight for the enzyme of 110.6.\(^5\)

**Fluorescence spectrometry.** Fluorescence measurement was performed with a Shimadzu 510 spectrofluorophotometer. When excited by irradiation at 280 nm, the enzyme showed a maximum emission at 335 nm. Therefore, fluorescence intensity was measured at 335 nm.

**Assay of cobalt.** Determination of cobalt was carried out with an atomic absorption spectrophotometer, Nippon Jarrel Ash 781, using a carbon rod atomizer at 240.7 nm.

**RESULTS**

**Effect of urea**

Glucose isomerase was incubated at 40°C for 1 hr in the presence of various concentrations of urea and then, remaining activity, CD spectrum and fluorescence intensity were assayed. As shown in Fig. 1(A), the activity was reduced corresponding with urea concentration; in 8 M urea it went down to 60% of the initial activity, however, it recovered almost completely from the inactivation on dilution of the urea. A similar result was obtained with respect to the CD spectrum as shown in Fig. 1(B). The molar ellipticity at 220 nm also decreased corresponding to urea concentration, but returned to the initial value after dilution of urea. Metal addition had no effect on denaturation of the enzyme. Fluorescence intensity increased significantly in the presence of urea, but little changed on addition of cobalt, as shown in Fig. 2. Denaturation by urea was very dependent on pH of the incubation mixture. In acidic urea solution, the denaturation was considerable and enzyme activity was not recovered after removal of urea. In this case, however, addition of cobalt was effective for protection of the enzyme from denaturation. Even in the presence of 8 M urea, the enzyme showed conspicuous dissociation at neutral pH, whereas it showed considerable dissociation into subunits at acidic pH, especially below pH 5.0 (Fig. 3).

\(^*\) MOPS, 3-(N-morpholino)propanesulfonic acid.
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Fig. 1. Effect of Urea.

(A) Activity: The enzyme (9.17 × 10⁻⁵ M) was incubated with various concentrations of urea (in 0.05 M MOPS, pH 7.0) at 40°C for 1 hr, then a 0.1 ml aliquot of each mixture was poured into 2.9 ml of a reaction mixture (0.8 M glucose, 0.1 M Mg²⁺ and 0.05 M MOPS, pH 8.0) containing the same concentrations of urea as in the incubation mixture, or into a reaction mixture containing no urea. Fructose content was determined after 30 min reaction at 60°C.

○—○, treated with urea and assayed in the presence of it; ○—○, treated with urea and assayed in the absence of it; ○—○, treated with urea and 10⁻³ M Co²⁺, and assayed in the presence of them.

(B) CD profile: After incubation with various concentrations of urea at 40°C for 1 hr, the CD spectrum of the enzyme was measured. The data are expressed in terms of mean residue ellipticity at 220 nm.

○—○, measured in the presence of urea; ○—○, measured in the absence of urea (20-fold dilution); ○—○, treated with urea and 10⁻³ M Co²⁺, and measured in the presence of urea.

Effect of guanidine hydrochloride

The enzyme was incubated at 40°C for 1 hr in the presence of various concentrations of guanidine hydrochloride, and then, remaining activity, CD and fluorescence spectra were determined. The results are illustrated in Fig. 4(A), (B) and Fig. 5, respectively. The activity was lost completely in the presence of 6 M guanidine hydrochloride and was never recovered on dilution of guanidine hydrochloride, in contrast with the case of urea. The molar ellipticity decreased with the concentration of denaturant and was little restored to the initial value after dilution of guanidine hydrochloride, indicating an unfolding of the molecular structure. Cobalt showed a protective effect against enzyme denaturation, while magnesium did not. As shown in Fig. 5, the fluorescence profile was significantly changed in the presence of guanidine hydro-
Fig. 4. Effect of Guanidine Hydrochloride.

(A) Activity: The enzyme \((9.17 \times 10^{-3} \text{ m})\) was incubated with various concentrations of Gu-HCl (in 0.05 M MOPS buffer, pH 7.0) at 40°C for 1 hr, then a 0.1 ml aliquot of each mixture poured into 2.9 ml of a reaction mixture (0.8 m glucose, 0.1 m Mg^{2+} and 0.05 m MOPS buffer, pH 8.0) containing the same concentrations of Gu-HCl as in the incubation mixture, or into a reaction mixture containing no Gu-HCl. Fructose content was determined after 30 min reaction at 60°C.

- O--O, treated with Gu-HCl and assayed in the presence of it;
- O--O, treated with Gu-HCl and assayed in the absence of it;
- ●--●, treated with Gu-HCl and \(10^{-3} \text{ m} \text{Co}^{2+}\), and assayed in the absence of them;
- O--O, treated with Gu-HCl and \(10^{-2} \text{ m} \text{Mg}^{2+}\), and assayed in the absence of them.

(B) CD profile: After incubation with various concentrations of Gu-HCl at 40°C for 1 hr, the CD spectrum of the enzyme was measured. The data are expressed in terms of mean residue ellipticity at 220 nm.

- O--O, measured in the presence of Gu-HCl;
- O--O, measured in the absence of Gu-HCl (20-fold dilution);
- ●--●, treated with Gu-HCl and \(10^{-3} \text{ m} \text{Co}^{2+}\), and measured in the presence of Gu-HCl.

drochloride. In addition to the decrease in intensity, a shift of peak position from 335 nm to 350 nm was observed at 6 m guanidine hydrochloride, suggesting a collapse of the hydrophobic region of glucose isomerase. The intensity was little influenced by cobalt addition, while, on the other hand, the shift of peak position was slightly restrained by it.

Fig. 5. Profile of Fluorescence Spectra of the Gu-HCl-treated Enzyme.

Conditions of Gu-HCl treatment were the same as those described in Fig. 4(B). The data are expressed as relative intensity. Concentrations of Gu-HCl are shown in the figure.

Fig. 6. Changes in Activity and Cobalt Content of the EDTA-treated Enzyme.

The enzyme \((6.36 \times 10^{-5} \text{ m})\) was incubated with \(10^{-2} \text{ m} \text{EDTA}\) at pH 8.0 at room temperature, and remaining activity and cobalt content were determined at the times indicated. The reaction mixture was composed of 0.8 m glucose, 0.1 m Mg^{2+}, 0.05 m MOPS buffer (pH 8.0) and 143 \(\mu\text{g/ml}\) enzyme. The sample was dialyzed against 0.01 m MOPS buffer before the assay.

- O--O, activity;
- ●--●, cobalt content.

Effect of EDTA

The effects of EDTA on the activity and dislocation of cobalt are shown in Fig. 6. The enzyme was dialyzed against 0.01 m MOPS buffer (pH 8.0) containing \(10^{-2} \text{ m} \text{EDTA}\) at room temperature, then, remaining activity and the amount of cobalt in the enzyme were assayed at the times indicated. After four days, the cobalt content was reduced to 1 mol per
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Fig. 7. pH Dependence of Inactivation with EDTA.
After incubation with $10^{-2}$ M EDTA at 60°C for 1 hr at pH 8.0, remaining activity was assayed in the reaction mixture composed of 0.8 M glucose, 0.1 M Mg$^{2+}$, 0.05 M MOPS buffer (pH 8.0) and 143 µg/ml enzyme. ○—○, control; ●—●, incubated with EDTA; ○—○, incubated with $10^{-3}$ M Co$^{2+}$.

Fig. 8. Time Course of Inactivation with Mercaptoethanol.
The enzyme (6.36 x $10^{-5}$ M) was incubated with 0.2 M 2-mercaptoethanol at pH 7.0 in the presence or absence of 8 M urea, and remaining activity was assayed at time intervals in the reaction mixture composed of 0.8 M glucose, 0.1 M Mg$^{2+}$, 0.05 M MOPS (pH 8.0) and 143 µg/ml enzyme. ○—○, incubated without addition of urea (40°C); ○—○, incubated with addition of urea (40°C); ●—●, incubated without addition of urea (60°C); ●—●, incubated with addition of urea (60°C).

mol of enzyme, however, that remaining was never eliminated by EDTA under the conditions described. The enzymatic activity was not influenced so much by dislocation of cobalt in this case. On the contrary, when incubated with EDTA in the presence of 8 M urea, considerable loss of activity was observed depending on incubation temperature. The effect of pH on inactivation of the enzyme with EDTA is shown in Fig. 7. EDTA and cobalt behaved conversely toward the enzyme activity; the former caused significant inhibition of the enzyme at low pH, whereas, the latter showed a protective effect at low pH. These results suggested contribution of cobalt to the stability of the enzyme.

Effect of mercaptoethanol
Figure 8 shows the effect of 2-mercaptoethanol on the activity of glucose isomerase. The loss of activity was 50% after incubation at 60°C, while it was almost complete in the presence 8 M urea, suggesting disulfide linkages in the native enzyme were hard to attack with 2-mercaptoethanol. Figure 9 represents the visible region spectra of the enzyme in the presence and absence of mercaptoethanol. The spectra of the native enzyme did not have any characteristic peak in that region (the enzyme solution is a light brownish yellow) except a few indistinct peaks, and showed no change on addition of 8 M urea. However, when incubated with mercaptoethanol in the presence of 8 M urea at 60°C, two clear peaks appeared at
Effect of organic solvents

Figure 10 represents the effects of various organic solvents on the activity of glucose isomerase. The results were different for the two groups, linear-chained and side-chained compounds. In the former (n-type), enzyme activity simply decreased corresponding with both increases of solvent concentration and length of carbon chain, that is, hydrophobicity [Fig. 10(A)]. In this case, cobalt showed no protective effect. On the contrary, in the latter group (iso-type) and acetone, the enzyme was activated at a low concentration of solvent, significantly by acetone and slightly by iso-propanol and iso-butanol [Fig. 10(B)]. These activations, however, effaced by cobalt addition. In fluorescence measurement, a strong quenching was observed in the presence of acetone, and a little increase in intensity in the presence of methanol as shown in Fig. 11. The CD spectrum little changed in the presence of solvents of both types even after treatment at 60°C. Thus, profile of the effects of organic solvents on the enzyme were different from those of other denaturants.
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The role of cobalt in stabilization of the molecular structure of glucose isomerase isolated from cells of *Streptomyces griseofuscus* S-41 was studied through inspection of the effect of the metal on inactivation of the enzyme with various denaturants.

The profiles of changes in activity and in molar ellipticity at 220 nm of heat treated enzyme were well correlated with each other, so both were used as criteria for the degree of denaturation of the enzyme. When excited by ultraviolet irradiation at 280 nm, the enzyme showed a symmetrical fluorescence spectrum having a peak at 335 nm, suggesting typical emission from tryptophan residues. So, the fluorescence spectrum was also adopted for inspection of conformational change of the enzyme.

The activity and molar ellipticity of the enzyme were significantly decreased corresponding with urea concentration, however, both were restored to the original values by removing urea. Different from the case of D-xylose isomerase reported by Yamanaka,8) reversible dissociation was not observed. Therefore, glucose isomerase from *Streptomyces griseofuscus* S-41 seemed to retain an ordered conformation even in 8 M urea solution, similar to that from *Bacillus coagulans.*9) It has been known that in high con-

**DISCUSSION**

**Number of cobalts in denatured enzyme**

The relation between activity of the enzyme treated with a denaturant and number of cobalts contained in that enzyme is shown in Fig. 12. After incubation with various reagents at 60°C for 2 hr (pH 8.0), remaining activity and cobalt content of the enzyme were determined. Samples were dialyzed against 0.01 M MOPS buffer for 48 hr before the assay. The reaction mixture was composed of 0.8 M glucose, 0.1 M Mg^{2+}, 0.05 M MOPS buffer (pH 8.0) and enzyme. ①, native enzyme; ②, urea (40°C, 2 hr); ③, 0.5 M mercaptoethanol; ④, 8 M urea; ⑥, 10^{-2} M EDTA; ⑤, 6 M Gu HCl +0.5 M mercaptoethanol; ⑦, 8 M urea +0.5 M mercaptoethanol; ⑧, 8 M urea (pH 4.0); ⑨, 6 M Gu HCl; ⑩, 8 M urea +10^{-3} M EDTA.

Fig. 12. Relation between Activity and Cobalt Content of the Enzyme Treated with Various Denaturants.

After incubation with various reagents at 60°C for 2 hr (pH 8.0), remaining activity and cobalt content of the enzyme were determined. Samples were dialyzed against 0.01 M MOPS buffer for 48 hr before the assay. The reaction mixture was composed of 0.8 M glucose, 0.1 M Mg^{2+}, 0.05 M MOPS buffer (pH 8.0) and enzyme. ①, native enzyme; ②, urea (40°C, 2 hr); ③, 0.5 M mercaptoethanol; ④, 8 M urea; ⑥, 10^{-2} M EDTA; ⑤, 6 M Gu HCl +0.5 M mercaptoethanol; ⑦, 8 M urea +0.5 M mercaptoethanol; ⑧, 8 M urea (pH 4.0); ⑨, 6 M Gu HCl; ⑩, 8 M urea +10^{-3} M EDTA.

The unfolded enzyme was thus found to contain very little cobalt. It was therefore considered that cobalt, especially one of four originally contained in the enzyme, is closely related to retention of the ordered structure of the protein molecule. When incubated with 6 M guanidine hydrochloride and urea each with 2-mercaptoethanol, the samples still had 2.5 and 2.6 mol of cobalt, respectively, regardless of complete dissociation. In these cases, cobalt ions were thought to combine with other sites of the enzyme in a form of a complex with 2-mercaptoethanol as previously described, although most of them were removed from their original binding sites.

The role of cobalt in stabilization of the molecular structure of glucose isomerase isolated from cells of *Streptomyces griseofuscus* S-41 was studied through inspection of the effect of the metal on inactivation of the enzyme with various denaturants.

The profiles of changes in activity and in molar ellipticity at 220 nm of heat treated enzyme were well correlated with each other, so both were used as criteria for the degree of denaturation of the enzyme. When excited by ultraviolet irradiation at 280 nm, the enzyme showed a symmetrical fluorescence spectrum having a peak at 335 nm, suggesting typical emission from tryptophan residues. So, the fluorescence spectrum was also adopted for inspection of conformational change of the enzyme.

The activity and molar ellipticity of the enzyme were significantly decreased corresponding with urea concentration, however, both were restored to the original values by removing urea. Different from the case of D-xylose isomerase reported by Yamanaka,8) reversible dissociation was not observed. Therefore, glucose isomerase from *Streptomyces griseofuscus* S-41 seemed to retain an ordered conformation even in 8 M urea solution, similar to that from *Bacillus coagulans.*9) It has been known that in high con-
centrations of urea, cyanate ions derived from urea at neutral and alkaline pH often react with amino or sulphydryl groups of proteins and result in a decrease of activity. Glucose isomerase was, however, rather activated by addition of cyanate, and did not show any change in electrophoretic profile. Furthermore, no competition of urea with the enzyme substrate, D-glucose, was observed on kinetic measurement. Therefore, the decrease in activity at high concentrations of urea might arise from a “reversible” conformational change in the microenvironment near the active site of the enzyme, though it was indistinguishable whether a cause or an effect. While, the enzyme was rapidly inactivated and dissociated by incubation with urea at low pH.

Decreases in enzymatic activity and molar ellipticity were also induced by treatment with guanidine hydrochloride. In this case, however, they did not return to the original values after removal of guanidine hydrochloride, and concurrent formation of a precipitate of inactive protein was observed. The red shift and decline of intensity in the fluorescence spectrum of tryptophan indicate an increase of polarity in its surroundings. From the results of fluorescence measurements it was considered that the conformation of the hydrophobic region of glucose isomerase was transformed and tryptophan residues buried were, in consequence, exposed to the surface of the enzyme protein in 6 M guanidine hydrochloride. The inactivation profile with guanidine hydrochloride was somewhat different from the case of the enzyme from *Bacillus coagulans*, in which the effect of that denaturant was reported to be completely reversible at a concentration less than 4.0 M and irreversible at 5.8 M. The ordered structure of glucose isomerase thus seemed to be severely disrupted with guanidine hydrochloride.

The enzyme was stable to organic solvents and showed somewhat particular behavior in solvents. The enzyme was activated by solvents of the side-chain type and acetone to some extent. Different from denaturation with urea and guanidine hydrochloride, the CD spectrum was little changed in solvents regardless of activity change. Therefore, the inhibition by solvents might seem to be due to a minor conformational change of the hydrophobic region which is undesirable for enzyme action, though satisfactory data on this were not obtained by fluorescence analysis.

It is well known that the thermostable enzymes sometimes owe their stability to metals bound to them. Glucose isomerase from *Streptomyces griseofuscus* S-41 was found to have four cobalt atoms per molecule and these are of no significance for initiation of activity. Furthermore, cobalt addition is effective for protection of the enzyme from denaturation by heat, acid or SDS. Three of the four cobalts could be removed from the enzyme by treatment with EDTA or 8 M urea without significant loss of activity, whereas the other one could not. The cobalt free enzyme was difficult or might be impossible to obtain in a stable form, since for elimination of all cobalts, drastic treatment was required such as with 6 M guanidine hydrochloride, acid-8 M urea or EDTA-8 M urea, inducing considerable dissociation. The addition of cobalt was effective for protection from denaturation in such drastic conditions, while ineffective in less drastic conditions, 8 M urea or organic solvents, on the other hand. From these results, it was considered that one of four cobalts contained in glucose isomerase from *Streptomyces griseofuscus* S-41 is tightly bound to the enzyme and is probably essential for holding the ordered structure, especially the quaternary structure, whereas the other three are loosely bound and might be less significant in stabilizing the structure. The one cobalt atom may be a sort of reinforcement of polypeptide chains, similar to zinc in α-amylase from *Bacillus subtilis*, or zinscs in nuclease α from *Penicillium citrinum*. The role of the other three is, however, still unclear. The protective effect of added cobalt may be due to repression of cobalt dislocation which leads to dissociation into subunits.
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