0273-2289/99/77–79/0325/$12.75

Subunit Analyses of a Novel Thermostable Glucose Dehydrogenase Showing Different Temperature Properties According to Its Quaternary Structure

Tomohiko Yamazaki, Wakako Tsugawa, and Koji Sode*

Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei-shi, Tokyo 184-8588, Japan, E-mail: sode@cc.tuat.ac.jp

Abstract

We previously reported a novel glucose dehydrogenase (GDH) showing two peaks in the optimum temperature for the reaction at around 45°C and at around 75°C. Each peak derived from hetero-oligomeric enzyme, constructed from two distinct peptides with an α-subunit (MWs 67,000) and β-subunit (MWs 43,000), and a single peptide enzyme containing an α-subunit alone. The function of the two subunits in the thermostable co-factor binding GDH was investigated. The results of spectroscopic analyses indicated that the α-subunit contained an unknown co-factor showing specific fluorescence spectra like pyrroloquinoline quinone (PQQ), and the β-subunit was cytochrome *c*. Moreover, the results of a urea denaturation and reconstitution experiment suggested that the dissociation of the hetero-oligomeric complex to a single peptide was reversible. The kinetic parameter analyses for glucose and the electron mediator also suggested that the β-subunit was responsible for electron transfer from the catalytic center of the α-subunit to the electron mediator.

Index Entries: Glucose dehydrogenase; thermal stability; catalytic subunit; electron transfer subunit; quaternary structures.

Introduction

Various microbial enzymes capable of oxidizing glucose are known, such as glucose oxygen-oxidoreductases (EC 1.1.3.4), glucose dehydrogenases (GDHs) ($NAD(P)^+$) (EC 1.1.1.47), and glucose dehydrogenases (pyrroloquinoline quinone; PQQ) (EC 1.1.99.17). Among these glucose oxidoreductases, co-factor binding types such as PQQGDHs are consid-

*Author to whom all correspondence and reprint requests should be addressed.

ered to be favorable constituents for mediator type glucose sensors, because of their negligible effect on the presence of oxygen in the reaction mixture *(1–5)*.

We recently reported a novel co-factor binding type GDH isolated from a moderate thermophilic bacterium, SM4 *(6)*. SM4 was isolated from soil near a hot spring. This strain was Gram negative, long-rod, oxidase negative, catalase-positive, and showed motility. Because this enzyme neither required an additional co-factor to show dye-mediated GDH activity, nor utilized oxygen as an electron acceptor, its further application in biosensor components is expected. In addition to this practical potential, this enzyme showed different temperature properties according to its quaternary structure. This enzyme was a hetero-oligomeric complex composed of two distinct subunits, α (MW 67,000) and β (MW 43,000). This enzyme showed the optimum temperature for reaction at about 45°C. Curiously, it shows another peak at around 75°C. After 30 min of incubation at 70°C, the β-subunit precipitated and consequently this oligomeric complex became a single peptide enzyme composed of the α-subunit. The single peptide enzyme showed the optimum temperature for reaction at 75°C and also showed higher thermal stability than the oligomeric complex enzyme. Therefore, this enzyme changes its temperature properties by altering its quaternary structures. However the correlation between oligomeric enzyme dissociation and the alteration in the temperature properties has not yet been elucidated.

In this study, we investigated the function of two subunits in GDH and their role in altering temperature properties. We carried out spectroscopic analyses and an investigation of kinetic parameters in order to see the role of each subunit in dye-mediated GDH activity. We also found that this change in temperature properties was reversible under urea denaturation conditions, which also triggered oligomeric enzyme dissociation.

Materials and Methods

Chemical

All chemicals and reagents used in this work were reagent grade. Nitro blue tetrazolium (NBT), 2,6-dichlorphenolindophenol (DCIP) and phenazine methosulfate (PMS) were purchased from Kanto Chem (Tokyo, Japan).

Bacterial Culture Condition and Enzyme Preparation

A bacterial strain producing a thermostable GDH, SM4, was cultured as described previously *(6)*. The enzyme was purified by sequential chromatographies as in our previous report *(6)* and an electrophoretically homogeneous sample was prepared and stored at 4°C until use. The heat-treated GDH was prepared as follows. Purified GDH was incubated at 70°C for 30 min and centrifuged at 8000*g* for 5 min to remove precipitates.

Determination of Kinetic Parameters of GDH

A GDH assay was carried out spectrophotometrically by measuring the reduction in electron acceptors *(6)*. Parameters for the mediator, PMS, were obtained by varying PMS concentration within the range of 0.59 and 3.9 m*M*. Five microliter of enzyme sample was added to 19 µL of reaction mixture (0.38 m*M* DCIP and various concentration PMS in 10 m*M* potassium phosphate buffer, pH 6.0, containing 0.2% (w/v) Triton X-100 and 1 µL of 2 *M* glucose, and the decrease in absorption of DCIP owing to glucose oxidation was measured at 40°C. The final concentrations of glucose and DCIP were 40 and 0.26 m*M*, respectively. One unit of enzyme was defined as the oxidation of 1 µmol glucose/min under the standard assay conditions.

The parameters for glucose were obtained by varying glucose concentration within the range of 0.6 and 20 m*M*. It was determined that these were basically the same as for the PMS method. The final concentrations of PMS and DCIP were 16 and 0.26 m*M* respectively. The concentration of GDH was determined by the Lowry method.

Spectroscopic Analyses

For the spectroscopic analyses of GDH, an UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a micro cell (120 µL) was used. For the fluorescence analyses of GDH, an FL5500 fluorescence spectrophotometer (Shimadzu) equipped with a micro cell (400 µL) was used. Enzyme samples were dissolved in 10 m*M* potassium phosphate buffer, pH 6.0, containing 0.2% (w/v) Triton X-100.

Urea Denaturation Experiments

Purified enzyme samples with an enzyme concentration of 0.12 mg/mL before and after heat treatment were incubated for 30 min in the presence of various concentrations of urea (0–8.3 *M*) at 25°C. Residual enzyme activity was determined as follows: 40 µL of urea-treated samples were added to 560 µL of reaction mixture containing PMS, DCIP, and glucose in 10 m*M* potassium phosphate buffer, pH 6.0, containing 0.2% (w/v) Triton X-100, and the decrease in absorption of DCIP owing to glucose oxidation was measured at room temperature. The final concentrations of PMS, DCIP and glucose in the reaction mixture were 16, 0.26, and 20 m*M*, respectively.

The effect of temperature on GDH activity in the urea-denatured sample was determined to be basically the same as described in our previous study *(6)*, but using 0.1 mg/mL sample denatured in the presence of 5.8 *M* urea for 30 min. Reconstitution of the oligomeric complex after urea denaturation was carried out by dialyzing the urea-denatured sample in a dialyzing tube (Viskase Sales Corp., Chicago, IL) in 10 m*M* potassium phosphate buffer, pH 6.0, containing 0.2% (w/v) Triton X-100 at 4°C for 2 d. Native polyacrylamide gel electrophoresis (PAGE) and active staining of gels were carried out as in our previous report *(6)*.

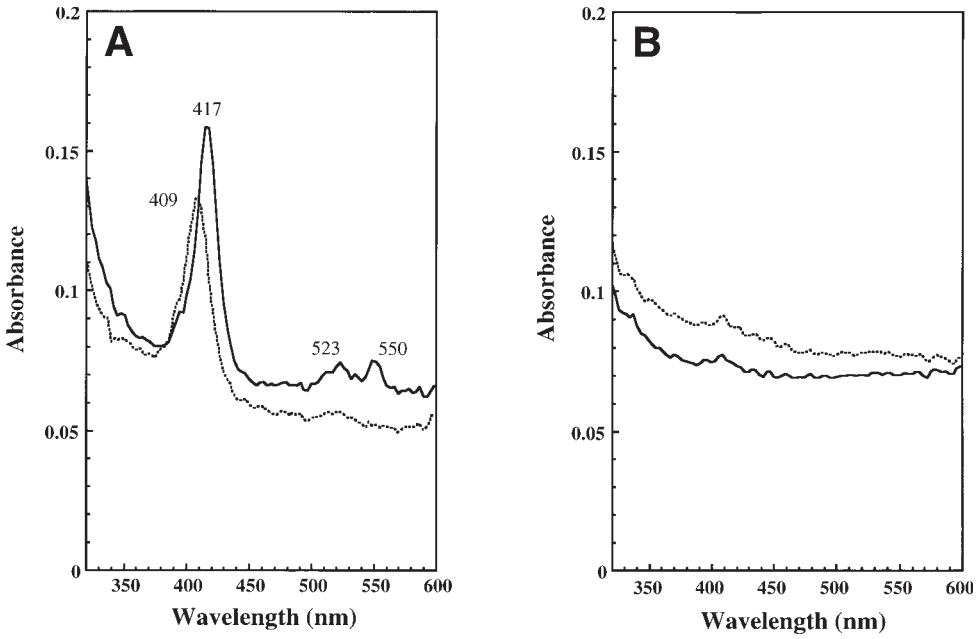

Fig. 1. Absorption spectra of purified GDH. **(A)** Native GDH and **(B)** heat-treated GDH. Dotted line is oxidized form. Solid line is reduced form obtained by adding glucose. Purified GDH was used at protein concentration of (A) 294 µg/mL and (B) 148 µg/mL in 10 m*M* potassium phosphate buffer, pH 6.0, containing 0.2% (w/v) Triton X-100.

Results

Spectroscopic Analyses of GDH Before and After Heat Treatment

In order to investigate the role and co-factor of each subunit, we carried out spectroscopic analyses of GDH before and after heat treatment. Figure 1A,B shows absorption of GDH in the oxidative and reduced (in the presence of glucose) forms, before and after heat treatment. The spectra of oxidized GDH before heat treatment, a native GDH, showed a characteristic peak at 409 nm, although it shifted to 417 nm in the presence of glucose, and two additional peaks were observed at 523 and 550 nm (Fig. 1A). In contrast, after the heat treatment, the characteristic peak at 409 nm disappeared (Fig. 1B) and no significant difference was observed between the oxidized and reduced forms. The absorption spectra of oxidized GDH before heat treatment, a native GDH, was similar to the absorption spectra of alcohol dehydrogenase and aldehyde dehydrogenase composed of dehydrogenase-cytochrome complex *Gluconobactor* sp. or *Acetobactor* sp. *(7–11)*. These results indicated that this oligomeric complex of SM4 GDH possibly contained cytochrome *c* as the co-factor. Therefore, the observed cytochrome *c*-like spectrum was derived from the β-subunit, which was lost during the heat treatment.

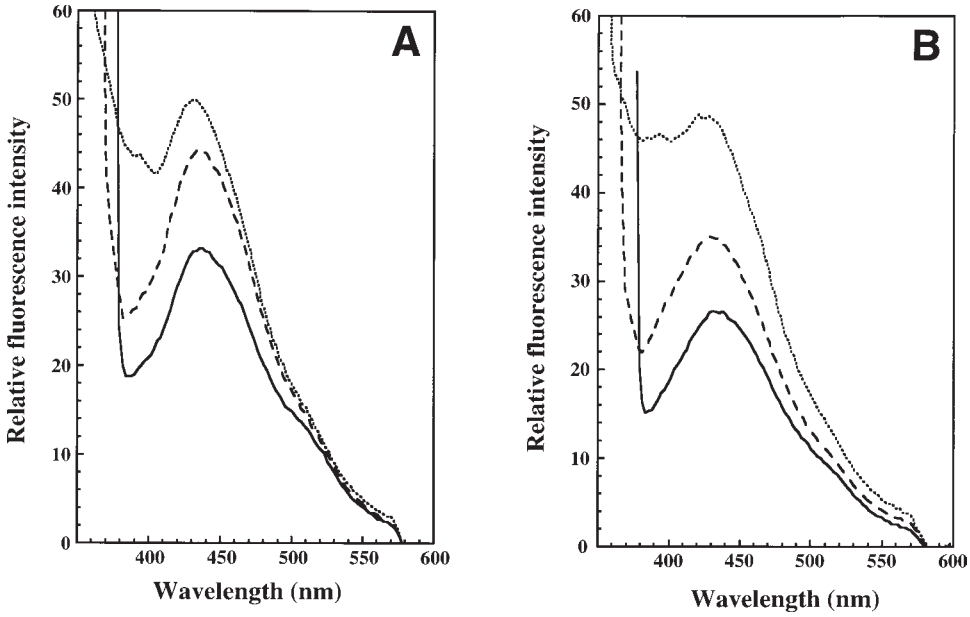

Fig. 2. Fluorescence spectra of purified GDH. **(A)** Native GDH and **(B)** heat-treated GDH. Emission spectra were measured with excitation at 340 nm (—), 350 nm (– – –), and 360 nm (·····). Purified GDH was used at protein concentration of (A) 327 µg/mL and (B) 164 µg/mL in 10 m*M* potassium phosphate buffer, pH 6.0, containing 0.2% (w/v) Triton X-100.

Figure 2A,B shows the fluorescence spectra of GDH before and after heat treatment. With the excitation wavelength at 340–360 nm, a characteristic emission peak was observed at 440 nm in both stages, before and after heat treatment. Because the co-factor showing this fluorescence was not lost during the heat treatment, this fluorescence was derived from the α-subunit. These fluorescence characteristics were similar to PQQ *(12)*.

Spectroscopic analyses indicated that the α-subunit contained an unknown co-factor showing a characteristic fluorescence like PQQ, and the β-subunit was a cytochrome *c*-like molecule.

Kinetic Parameters of GDH Before and After Heat Treatment

Kinetic parameters for both glucose and the electron mediator PMS were investigated (Table 1). K_m values for both glucose and PMS were slightly different before and after heat treatment. The V_{max} value for glucose in the heat-treated, single peptide GDH was about 20% of that of the oligomeric native GDH. However, a very significant difference was observed in the V_{max} values for PMS before and after heat treatment. Heat-treated GDH showed less than 1% of the activity of the oligomeric native GDH. From the results of native PAGE for the active staining, after heat treatment GDH showed a mobility of around only 85 kDa, which was composed of a single polypeptide with a MW 67 kDa by SDS-PAGE *(6)*. On the basis of these

Table 1
Kinetic Parameters of Native and Heat-Treated GDH[a]

	PMS		Glucose	
	K_m (m*M*)	V_{max}[b]	K_m (m*M*)	V_{max}[b]
Native GDH	1.4	470	2.5	290
Heat-treated GDH[c]	2.7	4.3	1.3	66

[a]Enzymatic reaction was carried out at 40°C.
[b]V_{max} is expressed in U/mg protein.
[c]Heat treatment: 70°C, 30 min.

results, we had previously concluded that heat-treated GDH was composed of only an α-subunit *(6)*. Therefore, these results indicated that single peptide GDH had a significantly reduced ability to transfer electrons to an artificial electron mediator, PMS, rather than catalytic activity toward glucose, compared with the parental oligomeric complex GDH.

Urea-Denaturation of GDH

Denaturation curves for GDH before and after heat treatment are shown in Fig. 3A. With the increase in urea concentration, the heat-treated GDH activity gradually decreased, although it retained more than 60% of its activity even in the presence of 8 *M* urea. In contrast, with the increase in the urea concentration up to 5 *M*, GDH activity in the oligomeric complex drastically decreased from the initial activity. However at a higher urea concentration, the denaturation curve was similar to that of the heat-treated GDH, and was not drastically further inactivated. Samples were taken from each stage and analyzed by native PAGE and active staining (Fig. 3B). With the increase in urea concentration, the oligomeric complex obviously dissociated (lanes 1–2). At a urea concentration of 8 *M*, GDH showed a mobility of around 85 kDa, which was similar to that of heat-treated GDH (lanes 4 and 5). From the previous results of native PAGE and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) *(6)*, after heat treatment this GDH showed a mobility of around 85 kDa, which was composed of an α-subunit with a MW of 67 kDa. Thus, we predicted that urea denaturated GDH comprised only one α-subunit. Figure 4 shows the effect of temperature on GDH activity in the presence of 5.8 *M* urea. The maximum activity was observed at 70°C, which was a similar temperature property to heat-treated GDH *(6)*. We concluded that the higher optimum temperature is owing to the α-subunit. Therefore, by urea denaturation, the oligomeric complex also dissociates and generates single peptide GDH, showing similar temperature properties to those observed with heat-treated GDH

We also found that this phenomenon was reversible. Figure 5 shows the active staining of native PAGE of the samples after urea denaturation. In the presence of 5.8 *M* urea, only one active band was observed in a low

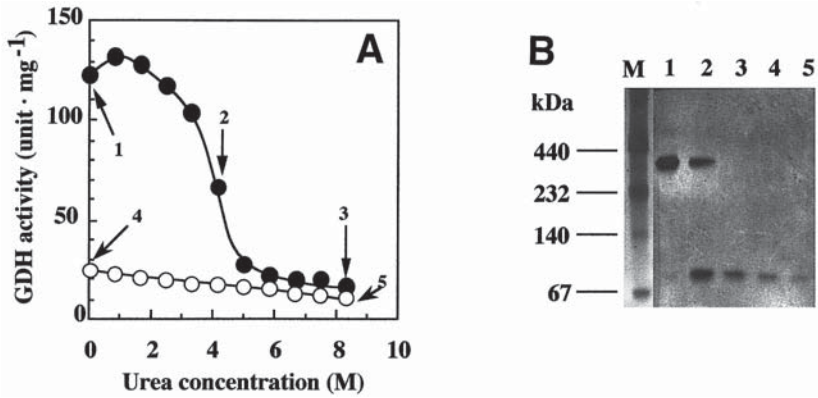

Fig. 3. Denaturation curve and native PAGE. **(A)** Denaturation curve of purified GDH (●) and heat-treated GDH (○). **(B)** Native PAGE for active staining. Lanes represent: M, molecular weight marker; 1–5, corresponding to numbers of denaturation curve. Enzyme samples were incubated in the presence of various concentrations of urea at 25°C for 30 min.

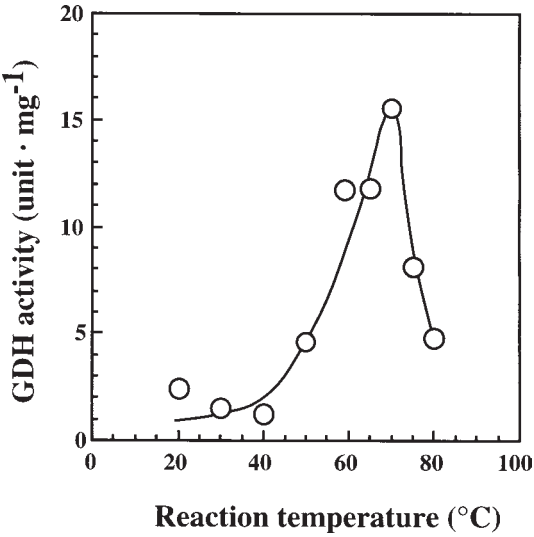

Fig. 4. The temperature profiles of urea-treated GDH. Purified GDH was incubated in 5.8 *M* urea solution for 30 min at 25°C. GDH activity was determined in the same way as in our previous study *(6)*.

molecular-weight migrating position (lane 2). However, after dialysis for 2 d, an active band was also observed in a high mol wt migrating position (lane 3), which was the same position observed in the parental GDH (lane 1). Moreover, two active bands were observed in a migrating position corresponding to mol wts around 150 and 105 kDa. These molecular weights might correspond to an α–α and α–β complex, respectively, which might be formed during the reconstitution of the oligomeric complex.

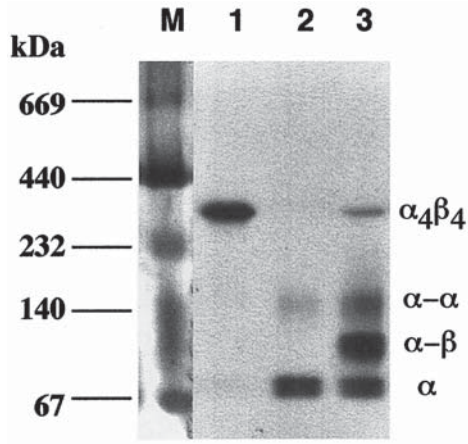

Fig. 5. Native PAGE of urea treated and refolding GDH. Lanes represent: M, Molecular weight marker; 1, purified GDH; 2, urea-treated GDH; 3, refolding GDH. Active staining of the native PAGE was performed by incubating the gel at 37°C by PMS and NBT in the presence of 100 m*M* glucose. The reconstitution experiment was carried out by dialysis of the denatured enzyme sample in 10 m*M* potassium phosphate buffer, pH 6.0, containing 0.2% (w/v) Triton X-100 without urea for 2 d at 4°C.

Since the urea denaturation was found to be reversible, the alteration of temperature properties caused by the dissociation of subunits might also be reversible.

Discussion

In our previous study, we found a novel GDH that showed different temperature properties according to its quaternary structure. The results obtained from this study enabled us to explain this curious phenomenon.

An α-subunit, which alone showed catalytic activity with an optimum reaction temperature of 70°C, contained an unknown co-factor showing characteristic fluorescence. A β-subunit that was lost during thermal treatment was a cytochrome *c*-like molecule. Considering the kinetic parameters of Table 1, it was obvious that the β-subunit had a significant role in electron transfer to an electron mediator, PMS, during dye-mediated glucose oxidation, because the α-subunit alone showed an extremely low V_{max} value toward PMS compared with that of an oligomeric complex containing the β-subunit. On the basis of these findings, we arrived at the following hypothesis to explain this phenomenon, as shown in Fig. 6. At the oligomeric stage, consisting of α- and β-subunits, this enzyme showed dye-mediated glucose dehydrogenase activity by electron transfer from the catalytic center of the α-subunit via the cytochrome *c*-like β-subunit to PMS, which showed an optimum reaction temperature of 45°C. Incubation at 70°C resulted in the dissociation of these subunits. Considering the drastic decrease in the V_{max} value after heat treatment toward PMS, the α-sub-

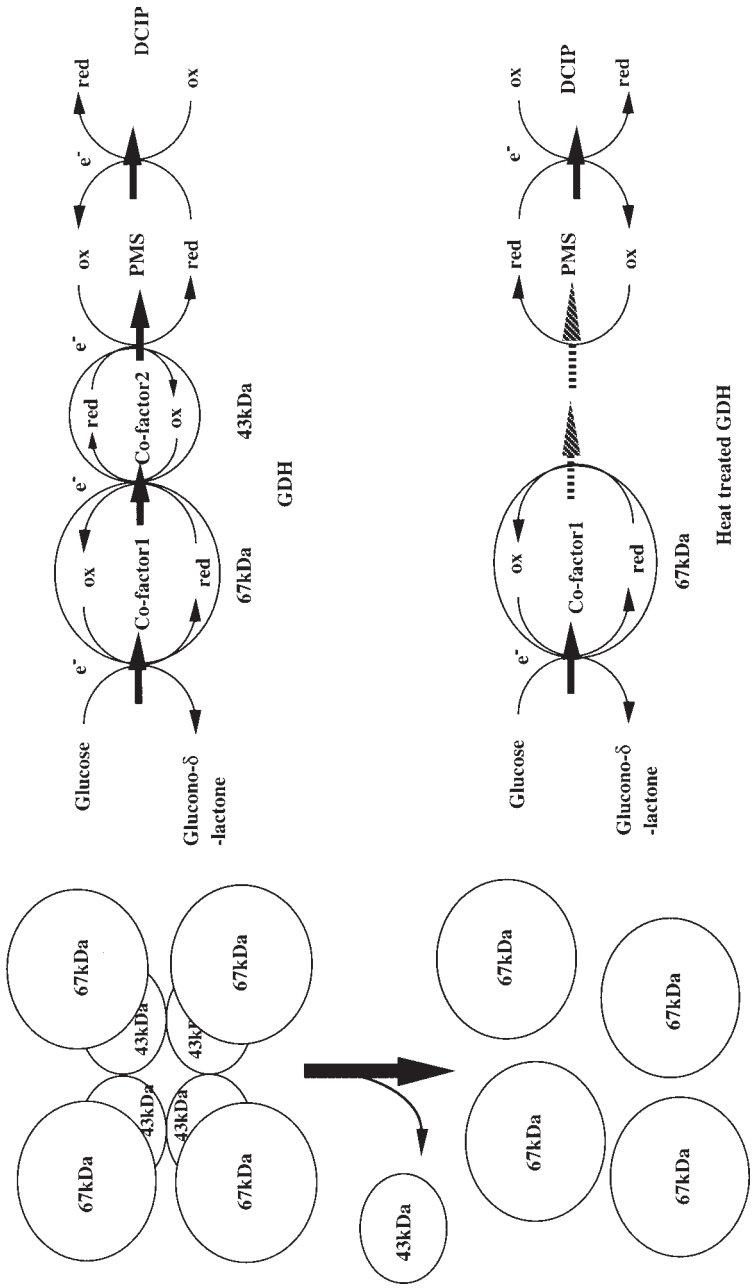

Fig. 6. Predicted change in quaternary structure of GDH induced by heat or urea treatment. α is α-subunit (MWs 67,000) which had a role in glucose oxidation catalysis. β is β-subunit (MWs 43,000), which transferred electrons from the catalytic center of the α-subunit to PMS.

unit alone might scarcely transfer electrons to PMS at a temperature of around 45°C in the absence of the β-subunit. However with the increase in temperature, the rate of electron transfer directly from the catalytic center to PMS might also increase, and at 70°C, the α-subunit showed its maximum activity. This was owing to the high thermal stability of the α-subunit, possessing the catalytic site of this GDH.

The slight change in K_m values toward both PMS and glucose might be owing to the following assumption. Native GDH composed of α- and β-subunits showed enzyme activity by electron transfer from the catalytic center of the α-subunit via the β-subunit to PMS. The K_m value toward PMS represented the affinity of the β-subunit for PMS. On the other hand, heat-treated GDH is composed of only an α-subunit, and showed direct electron transfer from the α-subunit to PMS. Therefore, the K_m value toward PMS of heat-treated GDH represents the affinity of the α-subunit for PMS, which is a lower affinity than that for the β-subunit. Consequently, heat-treated GDH showed a high K_m value compared with that of native GDH. On the other hand, heat-treated GDH showed extremely low V_{max} values toward PMS. Therefore, the electron transfer from the α-subunit to PMS is rate-limiting step as compared to the reaction of glucose oxidation, consequently GDH activity saturated at low glucose concentration. For this reason, heat-treated GDH showed a low K_m value compared with that of native GDH.

Conclusion

A novel GDH from thermophilic bacterium was constructed from two distinct peptides, α- and β-subunits. The roles of each subunit are glucose oxidation catalysis and electron transfer, from the catalytic center of the α-subunit to an electron mediator, respectively. The reason for altering temperature properties was the dissociation of the electron-transfer subunit. Therefore the electron-transfer rate from the α-subunit to PMS was limited, and consequently GDH activity was decreased. However, the α-subunit possessed high thermal stability, so the optimum reaction temperature shifted to a high temperature.

References

1. Turner, A. P. F., D'Costa, E. J., and Higgins, I. J. (1989), *Ann. NY Acad. Sci.* **501,** 283–287.
2. Sode, K., Nakasono. S., Tanaka, M., and Matsunaga, T. (1993), *Biotechnol. Bioeng.* **42,** 251–254.
3. Ye, L., Hammerle, M., Oisthoorn, A. J. J., Schuhmann, W., Schmidt, H.-L., Duine, J. A., and Heller, A. (1993), *Anal. Chem.* **65,** 238–241.
4. Yokoyama, K., Sode, K., Tamiya, E., and Karube, I. (1989), *Anal. Chim. Acta.* **218,** 137–142.
5. Sode, K., Witarto, A. B., Watanabe, K., Noda, K., Ito, S., and Tsugawa, W. (1994), *Biotechnol. Lett.* **16,** 1265–1268.
6. Sode, K., Tsugawa, W., Yamazaki, T., Watanabe, M., Ogasawara, N., and Tanaka, M. (1996), *Enzyme Microb. Technol.* **19,** 82–85.
7. Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K., and Ameyama, M. (1978), *Agr. Biol. Chem.* **42,** 2045–2056.

8. Adachi, O., Miyagawa, E., Shinagawa, E., Matsushita, K., and Ameyama, M. (1978), *Agr. Biol. Chem.* **42,** 2331–2340.
9. Ameyama, M. and Adachi, O., (1982), *Methods Enzymol.* **89,** 450–457.
10. Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K., and Ameyama, M. (1980), *Agr. Biol. Chem.* **44,** 503–515.
11. Ameyama, M. and Adachi, O., (1982), *Methods Enzymol.* **89,** 491–497.
12. Ameyama, M., Hayashi, M., Matsushita, K., Shinagawa, E., and Adachi, O. (1984), *Agr. Biol. Chem.* **48,** 561–565.