ALTERATION OF THERMAL STABILITY OF GLUCOSE OXIDASE ASSOCIATED WITH THE REDOX STATES

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SUMMARY: By the method of differential scanning calorimetry, it was found that thermal stability of glucose oxidase was dependent on its redox states. The oxidized form showed an apparent denaturation temperature at 76°C and the denaturation enthalpy was approximately 865 kcal/mol. On reduction of the enzyme, the denaturation temperature increased by about 10°, but no significant change was seen in the denaturation enthalpy. The activation energies of the denaturation of the oxidized and the reduced enzymes were about 89 and 103 kcal/mol, respectively. These results may imply conformational changes in the catalytic turnover of this enzyme.

DSC[1] has recently been used for the investigation of thermal behaviors of biological materials (1). By this method, several proteins have been found to increase thermal stability when bound to inhibitors, metal ions, cofactors or second proteins, or when they are immobilized (2-6). A new type of stabilization of a protein will be reported in the present paper. Glucose oxidase (EC 1.1.3.4), an FAD-containing enzyme, which is known to turnover between the oxidized and the fully reduced forms during catalysis (7-9), was found to acquire a higher thermal stability on reduction with the substrate glucose. The details will be described.

MATERIALS AND METHODS

Aspergillus niger glucose oxidase purchased from Kyowa Hakko Kogyo Co., Tokyo (Lot No. 93005), was used in the present study. The purification

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[1]Abbreviation used: DSC, differential scanning calorimetry.

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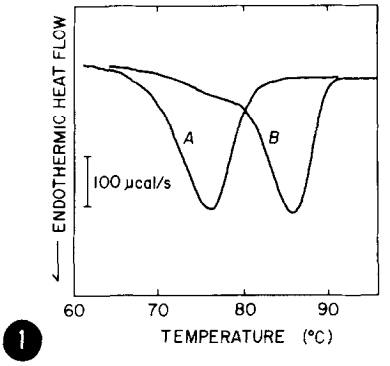

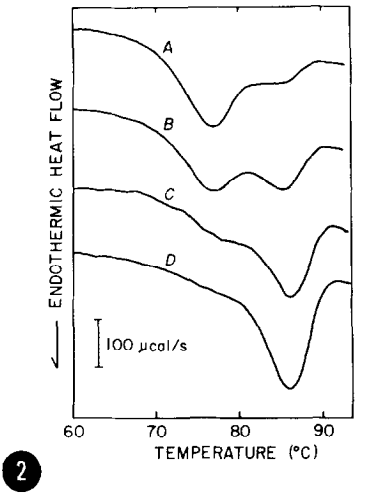

Fig. 1 DSC thermograms of the oxidized (A) and the reduced (B) forms of glucose oxidase.
The sample pans contained 25 µl of 1.28 mM glucose oxidase in the absence (A) and presence (B) of 170 mM glucose.
The DSC heating rate was 5°/min.

Fig. 2 Thermograms of partially reduced glucose oxidase samples.
Each of the sample pans contained 20 µl of 1.28 mM enzyme solution.
Glucose concentrations initially added were: A, 1.3 mM; B, 2.1 mM; C, 4.2 mM; and D, 33.3 mM.

procedure was essentially the same as described before (10). Purified sample was homogeneous on ultracentrifugation and on a disc gel electrophoresis at pH 8.9. The concentration of the enzyme was determined spectrophotometrically by the use of the extinction coefficient: ε(452 nm) = 21.6 $mM^{-1}cm^{-1}$ for the oxidized form (10). Fully and partially reduced enzyme samples were prepared simply by adding various amounts of glucose into the enzyme solutions contained in the DSC pans. After consuming oxygen in the sample solution, enzyme became reduced to various degrees depending on the substrate added. After addition of glucose, DSC pans were sealed to avoid the diffusion of oxygen from the air phase. The DSC measurements were started at 5 min after sealing.

DSC measurements were made with a Rigaku Denki DSC-meter. Determinations of the thermal denaturation parameters were essentially after Donovan and Ross (2). A DSC-heating rate of 5°/min was adopted in the present study.

The buffer solution used was 0.05 M acetate, pH 5.5.

RESULTS

The DSC thermograms of the oxidized and the reduced glucose oxidase samples are shown in Fig. 1 (A and B). The oxidized enzyme showed a peak temperature of the thermogram at 75.8 ± 0.6°C, and the endothermic enthalpy change associated with the thermal denaturation was estiamted to be 865 ± 45 kcal/mol. The

reduction of the enzyme by the substrate glucose shifted the peak to a higher temperature by about 10° (peak temperature: 86.6 ± 0.6°C), but the denaturation enthalpy remained practically unchanged (857 ± 17 kcal/mol). The transition of thermogram from the low-peak-temperature type to the high-peak-temperature type was well documented by the experiments with the enzyme samples reduced partially to various degrees (Fig. 2, A to D). This is a strong evidence that the reduced form of the enzyme is thermally more stable than the oxidized form. That the marked alteration in the denaturation temperature really resulted from the reduction of the enzyme was further supported by the following observations: 1) the inorganic reducing agent $Na_2S_2O_4$ also shifted the peak temperature to about 89°C; 2) the reaction product, δ-gluconolactone, when added to the oxidized enzyme solution did not show any stabilizing effect at a concentration of 33 mM; and 3) addition of N-acetylglucosamine, which does not serve as a substrate nor as an inhibitor, did not affect the thermogram.

Since the change in the thermal stability was thus ascribable to the reduction of the enzyme itself, a conformational change of the protein moiety associated with the reduction is suggested.

The rate constant of the denaturation reaction, k_d, was then determined according to the method of Donovan and Ross (2), and the results are shown in Fig. 3 in the forms of the Arrhenius plots. The activation energy estimated from this figure was 89.1 ± 6.4 kcal/mol for the oxidized enzyme and 103.0 ± 7.4 kcal per mol for the reduced enzyme, respectively. The difference in the activation energy between the enzyme forms of different redox states also indicates that a conformational alteration will accompany with the oxidation-reduction of the enzyme.

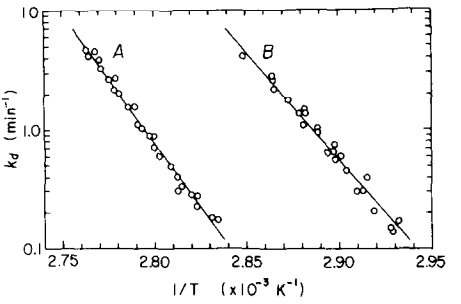

Fig. 3 Arrhenius plots for the denaturation reaction of glucose oxidase. A, reduced enzyme; B, oxidized enzyme.

DISCUSSION

By utilizing DSC, changes in the thermal stability have been demonstrated with several proteins. The stability increase was usually observed when proteins were bound to specific stabilizing substances such as metal ions or inhibitors. The present paper may be the first to describe that the flavin-containing oxidoreductase, glucose oxidase, increases thermal stability on reduction with the substrate or inorganic reducing agent. This is probably because of its conformational alteration of the protein moiety associated with the reduction. Since free flavin compounds are known to change the steric configurations with the change of the redox states (11), the prosthetic FAD in the present enzyme will also take a different configuration on reduction, which may in turn facillitate the conformational change of the protein moiety.

Conformational changes of proteins on binding with specific ligands are widely known with various enzymes (12). However, few proteins have been reported to cause structural alterations associated with their redox states. Among them, cytochrome *c* is an established example which changes its conformations depend-

ing on the redox states of the heme-iron (13). Glucose oxidase may be an additional example which alters conformations accompanying with the turnover of the enzyme between the oxidized and the reduced forms. However, these changes would not be large enough to be detected by the usual physicochemical measurements such as optical rotatory dispersion or circular dichroism. In this context, promising application of DSC in the investigation shall be emphasized.

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