A ROLE OF THE CARBOHYDRATE MOIETY OF GLUCOSE OXIDASE: KINETIC EVIDENCE FOR PROTECTION OF THE ENZYME FROM THERMAL INACTIVATION IN THE PRESENCE OF SODIUM DODECYL SULFATE

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1. Introduction

Glucose oxidase of fungal sources is known to be a glycoprotein [1—4]. Quantitative analyses have shown that the enzyme contains 11 to 16% of carbohydrate depending on the sources [2—4]. As is shown with other glycoenzymes [5], glucose oxidase is very stable on storage and fairly resistant to proteolysis [2], but no detailed study on the role of the carbohydrate moiety has been published so far.

In the present paper, a comparative study on the native and periodate-oxidized glucose is reported. It was revealed that no significant alteration was caused by the oxidation in catalytic properties, protein conformations, and stability against heating in the absence of denaturing agents. The only detectable change brought forth by the periodate oxidation was a reduction of the stability against heating in the presence of SDS*. This phenomenon was quantitatively shown by a marked decrease in the entropy of activation as compared with that of the native enzyme. These results indicate that the periodate-oxidized glucose oxidase is much more susceptible to the detergent than the native enzyme. It is, then, conceivable that the carbohydrate moiety contributes to increase the stability of the enzyme although it does not affect the gross structure of the protein moiety.

* Abbreviations used are: SDS, sodium dodecyl sulfate; CD, circular dichroism.

2. Materials and methods

Crude samples of *Aspergillus niger* glucose oxidase were obtained from Kyowa Hakko Kogyo Co., Ltd., Tokyo and purified as described elsewhere [4]. The enzyme activity was measured by the use of a Clark oxygen electrode at 25°C in a 0.05 M acetate buffer, pH 5.5. The enzyme concentration was determined spectrophotometrically in terms of the enzyme-bound FAD by using its molar extinction coefficient, 10.8 $mM^{-1} \cdot cm^{-1}$ [4]. The glucose concentration was conveniently expressed by the total amount added although the enzyme is known to be active on its β-form only [6]. Total carbohydrate content was estimated by the phenol—sulfuric acid method [7], and the qualitative identification of component sugars was carried out by the use of a Shimadzu Gas-chromatograph, GC-4BM. CD spectra were measured at room temperature with a Jasco recording spectropolarimeter, Model J-20. Periodate oxidation was carried out in a 0.1 M sodium metaperiodate solution at 0° in the dark as described by Yasuda et al. [8].

3. Results

3.1. *Carbohydrate content of the native enzyme*

It has been reported that glucose oxidase from *Asp. niger* contains some 16% carbohydrate by weight, whose main component is mannose with minor sugar components: galactose and glucosamine [2]; or glucose and hexosamine [4]. The present sample

showed a 10.5 ± 1% carbohydrate content by the phenol–sulfuric acid method. Gas-chromatographic analyses revealed that mannose (75 to 76% of the sugar components), galactose (16 to 17%), glucose (0.6%), galactosamine (5 to 6%) and glucosamine (2%) were contained. No sialic acid was detected.

3.2. *Periodate oxidation of the enzyme*

Upon incubation of the enzyme with 0.1 M sodium metaperiodate, it was found that the amount of carbohydrate was reduced to approximately half of the original value in two hours with little change in the overall catalytic activity, but no further decrease in the carbohydrate was seen even after 16 hr-incubation (fig. 1). Gas-chromatographic analyses

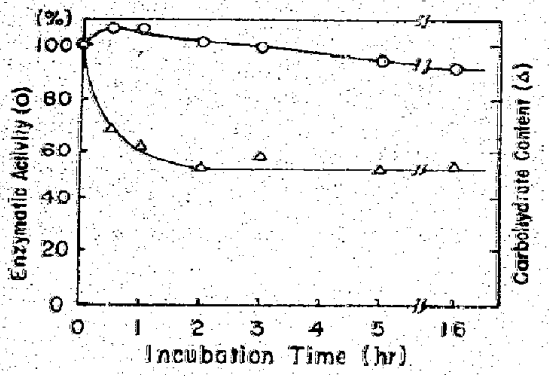

Fig. 1. Effect of periodate oxidation on carbohydrate content and overall enzymatic activity of glucose oxidase. Carbohydrate contents were determined by the phenol–sulfuric acid method with mannose as a standard. Enzymatic activity was measured by the oxygen uptake at 25°C, pH 5.5 in the presence of 57 mM D-glucose.

showed that the main carbohydrate component which had been decreased was mannose during the oxidation. No significant alteration in the catalytic properties was caused by the oxidation since the apparent Michaelis constants for glucose and for molecular oxygen of the 5 hr-oxidized enzyme sample were 28 mM and 0.18 mM, while those of the native enzyme were 26 mM and 0.20 mM, respectively.

3.3. *Conformations of the enzyme protein*

CD spectra of the native and the 5 hr-oxidized enzyme samples are shown in fig. 2. Of interest is

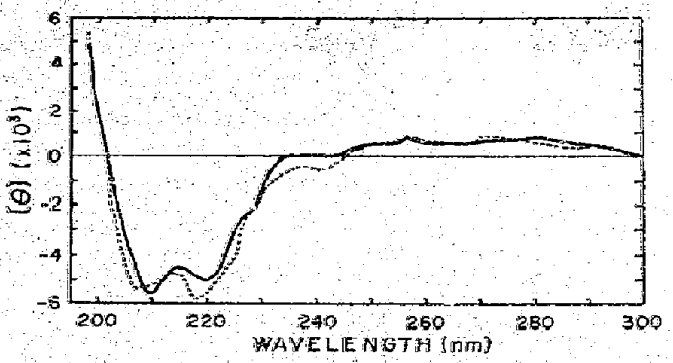

Fig. 2. Circular dichroism spectra of glucose oxidase. CD spectra were measured with a 1 mm cell at room temperature, pH 5.5. The mean residue weight of amino acids was taken as 110 in the calculation of molar ellipticity (θ). (——) Native enzyme; (– – –) 5 hr oxidized enzyme.

the finding that this enzyme protein has a very small amount of regular structure, such as α-helix and/or β-structure. Assuming that the CD behavior is mainly due to the α-helix conformation, the helix contents of the native and the 5 hr-oxidized enzyme samples are calculated as 11 and 11.5%, respectively [9,10]. It is thus evident that the periodate oxidation did not cause a significant alteration in the protein structure.

3.4. *Stability of the enzyme*

Glucose oxidase was found to be very stable against denaturing agents, such as SDS and urea. It retained a full activity after 30 hr-incubation in a 1% SDS solution at 30°C, pH 5.5. The activity was also fully restored after 1 hr-incubation with 4 M urea. As shown in fig. 3 (Curve *a*), the enzyme was found to be fairly stable at an elevated temperature. The stability of the enzyme, however, was reduced to a significant degree in the presence of 1% SDS (Curve *c*). It is noteworthy that the 5 hr-oxidized enzyme sample (Curve *b*) showed a similar or even higher stability against heating in comparison with the native enzyme when no SDS was added. On the other hand, the 5 hr-oxidized enzyme was much less stable than the native enzyme in the presence of SDS (Curve *d*). Kinetic analyses showed that the heat inactivation process was expressed by a first order rate constant, k_d, and the temperature dependence of the rate constant was shown in the form of the

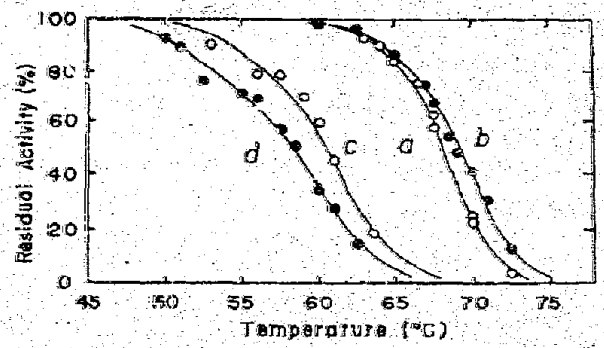

Fig. 3. Heat stability of glucose oxidase with and without 1% SDS. The enzyme was incubated for 5 min at each temperature, and an aliquot was taken for the assay of the enzymatic activity. Curves *a* (−SDS) and *c* (+SDS) are for the native enzyme, and curves *b* (−SDS) and *d* (+SDS) are for the 5 hr oxidized enzyme, respectively. Assay conditions were: glucose oxidase, 8×10^{-9} M (as FAD); D-glucose, 57 mM; temperature, 25°C; pH 5.5.

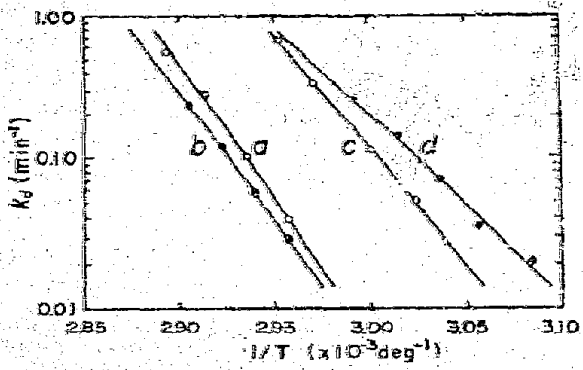

Fig. 4. Arrhenius plot for thermal inactivation of glucose oxidase with and without 1% SDS. Curves *a* (−SDS) and *c* (+SDS) are for the native enzyme, and curves *b* (−SDS) and *d* (+SDS) are for the 5 hr oxidized enzyme, respectively. Assay conditions were the same as in fig. 3.

Arrhenius plot (fig. 4). Activation parameters for the heat inactivation kinetics were calculated by the use of the activation energy obtained from this figure, and are listed in table 1. An important observation is a dramatic change in the activation enthalpy ($\Delta H^{\neq}$) of the 5 hr-oxidized enzyme when 1% SDS was added. Since the free energy of activation ($\Delta F^{\neq}$) was nearly constant in all cases, the entropy of activation ($\Delta S^{\neq}$) was varied in accordance with the change in the enthalpy.

Table 1
Properties of the native and periodate-oxidized glucose oxidase

	Native enzyme	5 hr oxidized enzyme
Total carbohydrate content (%)	10.5	5.5
Overall activity (moles/min/mole FAD)	6.6×10^3	6.2×10^3
K'_m for glucose (mM)	26	28
K'_m for oxygen (mM)	0.20	0.18
α-Helix content (%)	11	11.5
Enthalpy of activation[a] ($\Delta H^{\neq}$) (kcal/mole)	+87.3 (−SDS) +74.4 (+SDS)	+81.0 (−SDS) +53.2 (+SDS)
Free energy of activation ($\Delta F^{\neq}$) (kcal/mole)	+24.1 (−SDS) +23.1 (+SDS)	+24.5 (−SDS) +23.7 (+SDS)
Entropy of activation ($\Delta S^{\neq}$) (cal/deg/mole)	+184.8 (−SDS) +153.0 (+SDS)	+165.6 (−SDS) + 89.5 (+SDS)

[a] Activation parameters were calculated from $\Delta H^{\neq} = E^* - RT$, where E^* is the experimental activation energy; $\Delta F^{\neq} = -RT \cdot \ln[(h/kT)k_d]$, where k_d is the experimental rate constant for the heat inactivation, R, h and k are the gas constant, Planck's constant and Boltzmann's constant, respectively; and $\Delta S^{\neq} = (\Delta H^{\neq} - \Delta F^{\neq})/T$. Values listed here are the average of four to five calculations on the basis of the experimental data in fig. 4.

4. Discussion

Possible roles of the carbohydrate moiety of a glycoenzyme are: 1) to participate in the catalytic activities of the enzyme; 2) to maintain the tridimensional structure of the enzyme protein; 3) to protect the enzyme from proteolysis; and 4) to facilitate the transport of the enzyme through cellular membranes [5].

For the investigation of these possibilities, one of approaches is to compare the properties of the native enzyme with those of the enzyme from which the carbohydrate moiety is removed. Glucose oxidase was treated by metaperiodate and a 50%-carbohydrate enzyme was obtained, suggesting that the residual carbohydrate moiety might be deeply buried in a core of the polypeptide chain. Since no significant alterations were found in kinetic parameters and in the far-ultraviolet CD spectrum with the 5 hr-oxidized enzyme, it is able to conclude that the removed sugar component plays a primary role neither in the catalytic activities nor in maintaining tridimensional structure of the enzyme protein. However, the periodate-oxidized enzyme showed a much less thermal stability in the presence of SDS than the native enzyme, although it was as much stable as the native enzyme when no SDS was present. Therefore, a protective effect of the carbohydrate moiety is to be present, which does increase the stability of the protein moiety against heating. The protective effect is quantitatively reflected as the values of the entropy of activation (table 1). Since a decrease in this value, in general, might as well be ascribed to a less-ordered state of the protein structure as compared with a standard state, the large decrease observed with the 5 hr-oxidized enzyme in the presence of SDS implies that the protein is forced to take a somewhat disordered conformation by the detergent. Hence, the oxidized enzyme is much more susceptible to the heat treatment than the native enzyme in the presence of SDS.

Further studies are in progress.

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References

[1] Pazur, J. H., Kleppe, K., and Ball, E. M. (1963) Arch. Biochem. Biophys. 103, 515–518.
[2] Pazur, J. H., Kleppe, K. and Cepure, A. (1965) Arch. Biochem. Biophys. 111, 351–357.
[3] Swoboda, B. E. P. and Massey, V. (1965) J. Biol. Chem. 240, 2209–2215.
[4] Nakamura, S. and Fujiki, S. (1968) J. Biochem. (Tokyo) 63, 51–58.
[5] Pazur, J. H. and Aronson, Jr., N. N. (1972) in: Advances in Carbohydrate Chemistry and Biochemistry (Tipson, R. S. and Horton, D. eds.), Vol. 27, pp. 301–341, Academic Press, New York.
[6] Bentley, R. and Neuberger, A. (1949) Biochem. J. 45, 584–590.
[7] Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[8] Yasuda, Y., Takahashi, N. and Murachi, T. (1971) Biochemistry, 10, 2624–2630.
[9] Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D. and Davidson, B. (1966) Biochem. Biophys. Res. Commun. 23, 163–169.
[10] Cassim, J. Y. and Yang, J. T. (1967) Biochem. Biophys. Res. Commun. 26, 58–64.