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EFFECT OF PERIODATE OXIDATION ON THE STRUCTURE AND PROPERTIES OF GLUCOSE OXIDASE

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Summary

In order to elucidate the molecular structure of glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) and the roles of its carbohydrate moiety, chemical, physiochemical and immunological experiments were performed with enzyme samples before and after periodate oxidation. Hydrodynamic parameters indicated that the native enzyme was a globular protein with values of 1.21 for the frictional ratio and 43 Å for the Stokes radius. The enzyme contained about 12% carbohydrate by weight, of which the main component was mannose. The periodate treatment decreased the carbohydrate content to about 40% of its original value. Slight modifications were detected in the absorbance spectrum and the content of arginyl residue. However, no significant alteration was brought about by this treatment in the catalytic parameters, immunological reactivities or the gross structure, nor in the secondary and quaternary structures of the protein moiety. Thermal denaturation temperature (about 72.5° C) and the enthalpy of denaturation (about 450 kcal/mol) were common to the native and the periodate-oxidized enzymes. The native enzyme was found to be quite resistant to sodium dodecyl sulfate and fairly stable to urea and heating. The periodate-oxidized enzyme was also stable to heat treatment, but it showed a diminished stability when denaturing agents were present. Kinetic analyses of the thermal inactivation processes showed that the entropy of activation was greatly decreased by the denaturing agents, especially in the case of the periodate-oxidized enzyme.

It is concluded that the carbohydrate moiety of the enzyme plays a role in increasing the stability of the protein moiety, but does not directly participate in the catalytic activity, in the immunological reactivity, or in maintaining the conformation of the enzyme protein.

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

Introduction

Glucose oxidases (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) from fungal sources are flavoproteins with molecular weights of around 150 000 [1—6]. It has been shown that these enzymes contain 11—16% carbohydrate by weight depending on the sources [5,7—10]. Like other glycoenzymes, glucose oxidases are known to be very stable on storage and fairly resistant to proteolysis, but no intensive study on the roles of the carbohydrate moiety of these enzymes has been reported.

We reported recently [10,11] that a glucose oxidase isolated from *Aspergillus niger* (Kyowa Hakko Kogyo Co.) contains some 13% of carbohydrate, and that this enzyme has a relatively small proportion of "ordered structures" such as α-helical and β-pleated sheet conformations, suggesting that the major part of the polypeptide chains might be of an "unordered structure".

These findings prompted us to investigate in detail the gross structure and to elucidate the roles of the carbohydrate moiety of this glycoprotein. For the former purpose, hydrodynamic parameters as well as the secondary and quaternary structures of this enzyme have been studied; for the latter, the molecular properties of the native and the periodate-oxidized enzymes have been investigated comparatively.

Portions of this study have been presented [11,12].

Materials and Methods

Glucose oxidase. Asp. niger glucose oxidase purchased from Kyowa Hakko Kogyo Co., Tokyo (Lot No. 93 005), was used throughout the present study. The purification procedure was essentially the same as described before [5]. Purified sample was homogeneous on ultracentrifugation and on the disc gel electrophoresis at pH 8.9. The concentration of the enzyme was determined spectrophotometrically by the use of the extinction coefficient: $\epsilon_{452} = 21.6$ $mM^{-1} \cdot cm^{-1}$ [5].

Glycosidases. Endo-β-*N*-acetyl glucosaminidase H from *Streptomyces griseus* [13], endo-β-*N*-acetyl glucosaminidase D from *Diplococcus pneumoniae* [14], β-galactosidases from jackbean, from *Diplococcus pneumoniae* and from *Escherichia coli*, and α-mannosidase from jackbean were gifts of Dr. T. Muramatsu, Kobe University School of Medicine.

Preparation of periodate-oxidized glucose oxidase. Periodate oxidation of glucose oxidase was performed as described by Yasuda et al. [15]. The enzyme sample was incubated with 0.1 M sodium metaperiodate in the dark at 0°C for the required time. The oxidation was stopped by the addition of 50% ethylene glycol and the mixture was allowed to stand for 30 min at room temperature. The oxidized protein was recovered by gel-filtration on a Sephadex G-25 column, 0.05 M acetate buffer, pH 5.5, followed by dialysis against water overnight at 4°C. No turbidity was observed during the treatment. Since the carbohydrate content of the enzyme was not appreciably further changed by an incubation of more than 5 h (Table III), the enzyme sample treated with the periodate for 5 h was used as the "periodate-oxidized" glucose oxidase throughout the present study.

Carbohydrate analyses. Total carbohydrate content of the enzyme was estimated with mannose as standard by the phenol-sulfuric acid method [16], orcinol-sulfuric acid method [17] and Park-Johnson method [18]. Quantitative and qualitative analyses of neutral sugar components were carried out with a Shimadzu Gas-Chromatograph, GC-4BM. Amino sugar components were determined by the Elson-Morgan method [19] and by the use of an amino acid analyzer, Hitachi 034.

Amino acid analyses. Amino acid analyses were done with the samples after 24-, 48-, and 72-h hydrolysis using a Hitachi amino acid analyzer 034. Cysteine (and/or cystine) was determined after performic acid oxidation. Tryptophan was determined spectrophotometrically by the use of *p*-dimethylaminobenzaldehyde with free tryptophan as standard [20].

Catalytic activity. The overall catalytic activity of glucose oxidase was measured by oxygen uptake by the use of an oxygen electrode from Yellow Springs Instruments Co., Ohio, at 25° C in a 0.05 M acetate buffer, pH 5.5.

Immunological experiments. Antiserum against the native glucose oxidase was prepared as described previously [10]. Immunodouble diffusion tests were carried out by the method of Ouchterlony [21]. Quantitative immunoprecipitin reaction was performed under the conditions reported by McDuffie and Kabat [22].

Thermal inactivation kinetics. Kinetic measurements of the inactivation processes were as described before [10].

Spectrophotometric measurements. The spectrophotometer used was a Hitachi double-beam spectrophotometer, Model 124.

CD measurements. CD spectra were measured with a Jasco spectropolarimeter, J-20, at room temperature with a 1-mm cell.

Ultracentrifugation. Ultracentrifuge analyses were performed by the use of a Beckman Model E ultracentrifuge at 20° C.

Hydrodynamic parameters. Diffusion coefficient was measured by a free boundary method using a Tiselius-type electrophoretic apparatus, Tukasa HTD-1, at 20° C. Ostwald-type capillary viscometers were used to measure relative viscosity at 25° C. Partial specific volume was measured pycnometrically. The intrinsic viscosity and the partial specific volume were calculated on the basis of dry weight of the enzyme, which was determined with the samples dried on P_2O_5 in vacuo at 105° C for 12 h.

The Stokes radius, r, and the frictional ratio, f/f_0, were calculated by the following equations:

$$r = \frac{RT}{6\pi\eta ND},$$

and

$$f/f_0 = r \cdot \left(\frac{4\pi N}{3\nu M}\right)^{1/3},$$

where R is the gas constant, N is the Avogadro's number and M_r is the molecular weight of the enzyme.

Differential scanning calorimetry. The measurements were made with a Rigaku Denki Differential Scanning Calorimeter. Determinations of the thermal

denaturation temperature and the enthalpy of denaturation were essentially as done by Donovan and Beardslee [23]. A sample of 15—20 μl sealed in an aluminum pan was placed in the calorimeter, and the denaturation thermogram was obtained with a buffer-sealed pan as reference. The temperature at the peak of a thermogram was taken as the apparent denaturation temperature (T_d). Since the peak temperature was found to be slightly dependent on the heating rate of the calorimeter, the denaturation temperature (T_d^0) was estimated by an extrapolation to a heating rate of 0°C/min. The enthalpy of denaturation (ΔH_d) was determined by integrating the area between the denaturation curve and a baseline drawn under the peak. This value was virtually independent of the heating rate within experimental error.

Results

Carbohydrate analyses. The total carbohydrate content and the component sugars were analyzed by various methods. The results are listed in Table I, together with the methods used for analyses. The total carbohydrate content was approximately 12%, which agreed well with our previous results [11], but the value was slightly lower than those reported by several groups who had studied the *Aspergillus* enzymes [5, 7—9]. However, our observations on the enzyme samples from different manufacturers and from different fungal origins have indicated that the carbohydrate content of glucose oxidase depends on the production lots or the manufacturers [10]. For this reason, it is required that a single lot of sample from a single company should be used when carrying out quantitative carbohydrate analyses. Hence, the present investigations were done with a sample from Lot No. 93 005 from Kyowa Hakko Kogyo Co. throughout the study. In accordance with other reports [5,7,9], the enzyme contained mannose as the main carbohydrate component with galactose and glucose as the minor components. Glucosamine was the only hexosamine identified, and no sialic acid was detected. In our previous communication [11], the presence of a small amount of galactosamine was described, but this was not detected in the present study by the gas chromatography, by the amino acid analyzer, or by paper chromatography (pyridine/ethanol/acetic acid/water, 5 : 5 : 1 : 3); so that the previous result was not reproducible. However, when the analysis was carried out with the amino acid analyzer, an unidentified peak at a position very close to that expected for

TABLE I

CARBOHYDRATE COMPOSITION OF GLUCOSE OXIDASE

Method	Total content (%)	Component carbohydrate (residues/mol)				
		Man	Gal	Glc	Hexosamine	(GlcN)
Phenol-sulfuric acid	12.3					
Orcinol-sulfuric acid	11.6					
Park-Johnson	13.2					
Gas chromatography		108.8	8.8	2.0		
Elson-Morgan					19.2	
Amino acid analyzer						(22.2)
Average	12.4	109	9	2	(20)	

galactosamine was usually observed. Further study will be required for this problem.

Effect of glycosidases. In attempting to prepare a carbohydrate-deleted glucose oxidase sample, application of various glycosidases was examined. The purified glucose oxidase was incubated with a single glycosidase or with a combination of various glycosidases at 30°C for 10 h. The reaction was stopped by adding trichloroacetic acid, and the amount of the carbohydrate in the supernatant was measured by the phenol-sulfuric acid method. The results are listed in Table II. As this table shows, the carbohydrate moiety of the glucose oxidase was not effectively removed by the glycosidases; only about 25% at most, of the original content could be released. Since the action of glycosidases was thus revealed to be ineffective, the effect of periodate oxidation was investigated.

Effect of periodate treatment on the carbohydrate moiety. Upon incubation of the native glucose oxidase with sodium metaperiodate, the amount of the carbohydrate was found to be decreased to approximately 40% of the original content in about 3 h, but no further change was seen even after a 20-h incubation (Table III). Quantitative analyses revealed that the main carbohydrate component, mannose, underwent the greatest decrease during the treatment. It is noteworthy that the content of glucosamine was not significantly changed by this treatment, suggesting that this component is probably linked directly to the polypeptide chains, so that it is deeply buried in the framework of the protein moiety.

Amino acid composition. The glucose oxidase samples before and after the periodate treatment were subjected to amino acid analysis in order to know how the periodate treatment affected the amino acid residues of the protein. As shown in Table IV, the amino acid composition of the periodate-oxidized

TABLE II

EFFECT OF GLYCOSIDASES ON THE CARBOHYDRATE MOIETY OF GLUCOSE OXIDASE

Glycosidase(s)	pH of incubation mixture	Per cent decrease in carbohydrate content *
Endo-β-*N*-acetyl glucosaminidase H	5.5	12
Endo-β-*N*-acetyl glucosaminidase D	6.2	14
β-Galactosidase **	6.2	19
β-Galactosidase ***	4.0	26
α-Mannosidase ***	4.0	20
Endo-β-*N*-acetyl glucosaminidase H and α-mannosidase ***	5.5	14
Endo-β-*N*-acetyl glucosaminidase H, α-mannosidase *** and β-galactosidase **	5.5	18
Endo-β-*N*-acetyl glucosaminidase D and β-galactosidase **	6.2	18
Endo-β-*N*-acetyl glucosaminidase H, α-mannosidase ** and β-galactosidase †	4.0	25

* Carbohydrate removed by glycosidase treatment was determined by the phenol-sulfuric acid method on the supernatant of trichloroacetic acid-treated reaction mixture.

** *Diplococcus pneumoniae.*

*** Jackbean

† *E. coli*

TABLE III

EFFECT OF PERIODATE TREATMENT ON THE CARBOHYDRATE CONTENT OF GLUCOSE OXIDASE

Incubation time (h)	Residues/mol				
	Man *	Gal *	Glc *	Hexosamine **	(GlcN) ***
0	103	8.8	1.8	19.2	(22.2)
0.5	49	3.0	trace	18.2	(22.8)
1.0	47.4	trace	trace	17.6	(21.8)
2.0	ND	ND	ND	16.4	ND
3.0	32.8	trace	trace	15.6	ND
5.0	37.0	trace	trace	16.0	(19.0)
20.0	33.2	trace	trace	14.8	ND

* Gas-chromatography.
** Elson-Morgan method.
*** Amino acid analyzer method. ND: not determined.

enzyme was in general the same as that of the native enzyme. It is interesting that the threonyl, seryl, methionyl, cysteinyl and tyrosyl residues, which might be expected to be destroyed by periodate, were found intact within errors of analysis. The only significant change was found in the content of the arginyl residue, which was decreased by about 30% of the original content. No further investigation was performed yet to identify the product or products of this reaction.

Absorbance spectrum. It was found that the periodate treatment caused insignificant alteration in the absorption spectrum of glucose oxidase. This indicates that the FAD moiety as well as most of the protein moiety remain un-

TABLE IV

AMINO ACID COMPOSITIONS OF THE NATIVE AND THE PERIODATE-OXIDIZED ENZYME SAMPLES

The numbers of the amino acid residues were calculated by assuming the molecular weight of the protein moiety to be 134 000 on the basis of the molecular weight and the total carbohydrate content of the native enzyme; All values in residues per mol.

Amino acid	Native enzyme	Periodate-oxidized enzyme
Lys	34.8	33.3
His	30.7	33.4
Arg	47.0	33.3
Asx	159.5	162.1
Thr	90.0	89.4
Ser	75.7	76.9
Glx	108.4	114.3
Pro	55.2	54.0
Gly	122.7	122.6
Ala	126.8	130.9
Cys	6.1	6.2
Val	85.9	91.4
Met	22.5	20.8
Ile	53.2	56.1
Leu	110.4	114.3
Tyr	55.2	54.0
Phe	34.8	35.5
Trp	20.5	18.7

attacked by the reagent. A slight increase in the absorbance, however, was observed in the near-ultraviolet region, 300—350 nm. There seemed to be a possibility that the reaction product(s) of the arginyl residues might cause this absorbance change. But this possibility was eliminated, since no such absorbance increase was seen when arginine-rich proteins, histone and protamine, were treated by periodate under identical conditions.

CD spectrum. CD spectra of the native and periodate-oxidized enzyme samples are shown in Fig. 1. Since the spectrum of the periodate-oxidized enzyme was essentially identical with that of the native enzyme, it is concluded that the secondary structure of the glucose oxidase was practically unaltered by the periodate oxidation. The helical contents of the native and the periodate-oxidized enzyme samples calculated from their respective spectra were both around 15% [24,25]. The major part, about 85%, of the polypeptide chains could possibly be of a randomly-extended or "unordered structure".

Ultracentrifugation. The native and the periodate-oxidized enzymes were subjected to ultracentrifugation. Both were known to be homogeneous, and the sedimentation coefficients, $s^0_{20,w}$, of the native and the periodate-oxidized enzymes were 8.06 and 8.37 S, respectively. It is thus evident that the periodate treatment caused neither aggregation nor dissociation of this enzyme molecule; in other words, the quaternary structures of the native enzyme and the periodate-oxidized enzyme are virtually identical. Interestingly, it was observed that the periodate-oxidized enzyme had a slightly higher sedimentation coefficient than the native enzyme. This implies that the periodate-oxidized enzyme molecule is somewhat more compact with a higher density than the native enzyme molecule. The molecular weights determined by the meniscus-depletion method [26] using the values for the partial specific volumes obtained in the present study (see below), were 153 000 for the native enzyme and 150 000 for the periodate-oxidized enzyme. These values are in good agreement with those reported before [3—6].

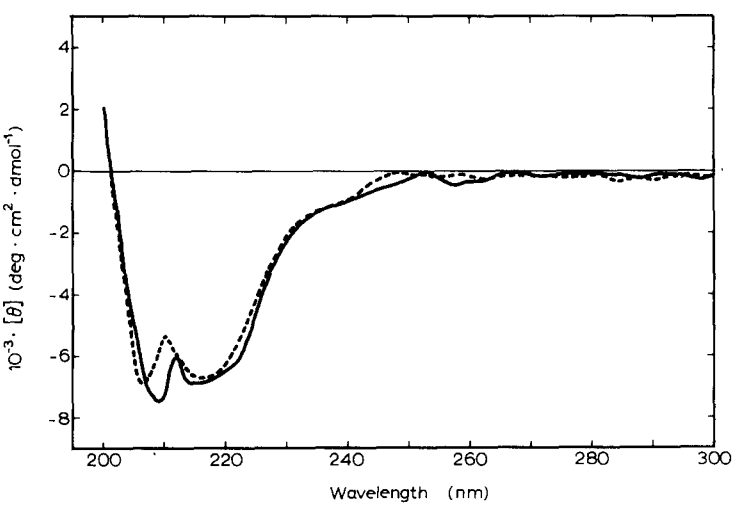


Fig. 1. CD spectra of the native (———) and the periodate-oxidized (- - - - -) enzymes. The spectra were measured with a 1-mm cell at room temperature. The mean residue weight of amino acids was taken as 109 in the calculation of molar ellipticity, $[\theta]$, [5,10].

Partial specific volume. Partial specific volumes of the native and the periodate-oxidized enzymes were determined pycnometrically to be 0.727 and 0.721 ml/g, respectively. These values are slightly larger than those reported earlier for the *Aspergillus* enzymes [4,5], but distinctly smaller than those reported for the *Penicillium* enzymes in accordance with previous results [2,4, 5].

Diffusion. Diffusion coefficients, $D_{20,w}$, measured by the free boundary method using a Tiselius-type electrophoretic apparatus were $4.94 \cdot 10^{-7}$ cm^2/s for the native enzyme and $4.74 \cdot 10^{-7}$ cm^2/s for the periodate-oxidized enzyme, respectively. The value obtained under high ionic strength conditions (0.1 M acetate buffer with 0.1 M NaCl) with the native enzyme was also essentially identical ($4.98 \cdot 10^{-7}$ cm^2/s).

Viscosity. The intrinsic viscosity, $[\eta]$, of the native enzyme was 4.57 ml/g and that of the periodate-oxidized enzyme was 4.63 ml/g (Fig. 2). Both values were nearly identical, indicating that the periodate treatment did not affect the gross structure of the enzyme protein. These values are slightly larger than those reported for typical globular proteins, but much smaller than those for fibrous proteins or randomly-extended polypeptides [27]. The addition of high concentration of neutral salt (0.1—0.3 M NaCl) did not affect the viscosity of the native enzyme (Fig. 2a).

Catalytic properties. The overall catalyzed reaction was investigated in the presence of varying concentrations of glucose and oxygen, and the catalytic parameters were estimated by Lineweaver-Burk plots. The Michaelis constants

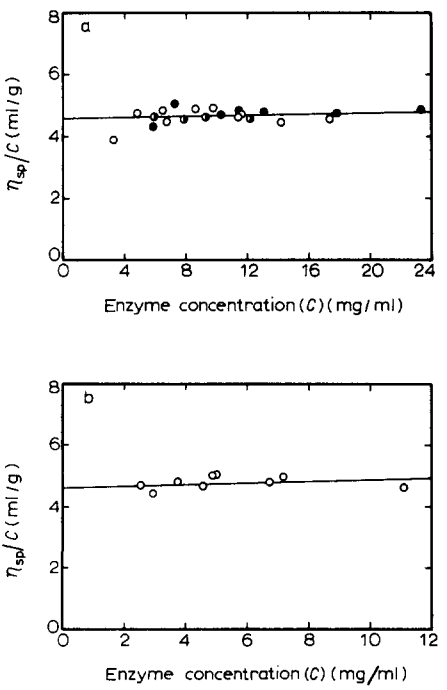


Fig. 2. Viscosities of the native enzyme (a) and the periodate-oxidized enzyme (b). Viscosities measured under low ionic strength conditions: (0.05 M acetate buffer) (○); and high ionic strength conditions: 0.1 M acetate buffer with 0.1 M NaCl (○), or with 0.3 M NaCl (●).

for glucose and for molecular oxygen of the periodate-oxidized enzyme were 28 and 0.18 mM, while those of the native enzyme were 26 and 0.20 mM, respectively. The values of turnover number, V/e_0, estimated by an extrapolation to infinite glucose concentration were 20 200 and 19 400 mol/min per mol of enzyme for the native and the periodate-oxidized enzymes, respectively.

Immunological studies. Both the native and periodate-oxidized enzymes were examined by the immunodouble diffusion technique against the antiserum to the native enzyme. As shown in Fig. 3, the antiserum cross-reacted with the periodate-oxidized enzyme as with the native enzyme. The precipitin line by the periodate-oxidized enzyme fused well with the line by the native enzyme.

Quantitative immunoprecipitation was also investigated with the antiserum, and the results are shown in Fig. 4. It is evident from this figure that the cross-reactivity of the periodate-oxidized enzyme with the antibody is quantitatively identical with that of the native enzyme itself. Immunological association constants estimated by a double reciprocal plot were $6.6 \cdot 10^6$ M^{-1} and $7.4 \cdot 10^6$ M^{-1} for the native enzyme and for the periodate-oxidized enzyme, respectively.

We may therefore conclude that the periodate oxidation did not modify the immunological properties of the glucose oxidase, and that the antigenic deter-

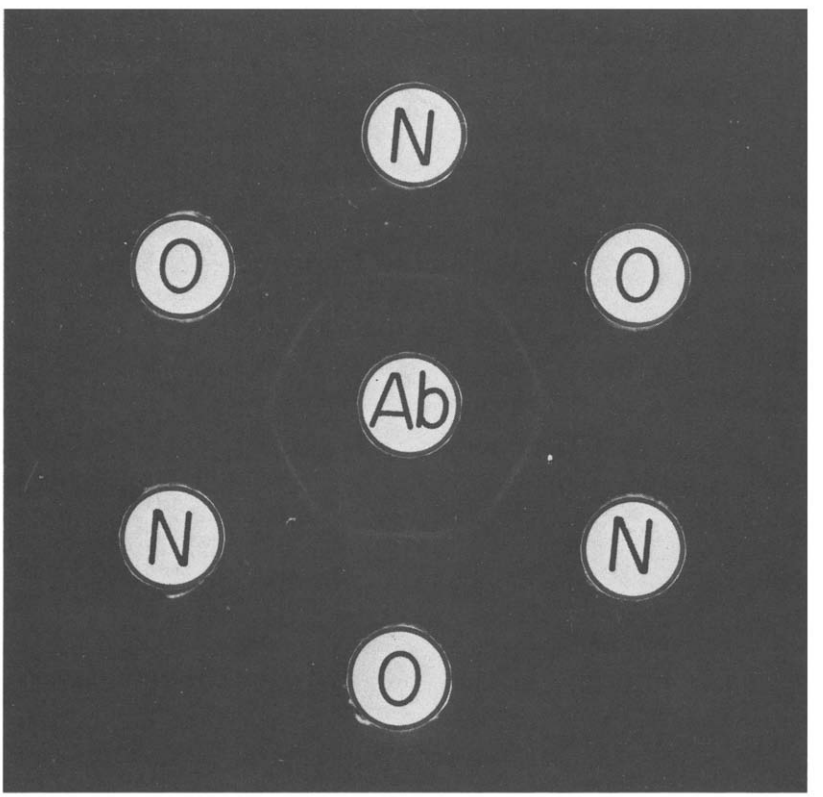


Fig. 3. Agar immunodouble diffusion experiments with the antiserum against native glucose oxidase. Ab: antiserum against the native enzyme, 0.75 mg; N: the native enzyme, 4.3 μg; and O: the periodate-oxidized enzyme, 4.2 μg.

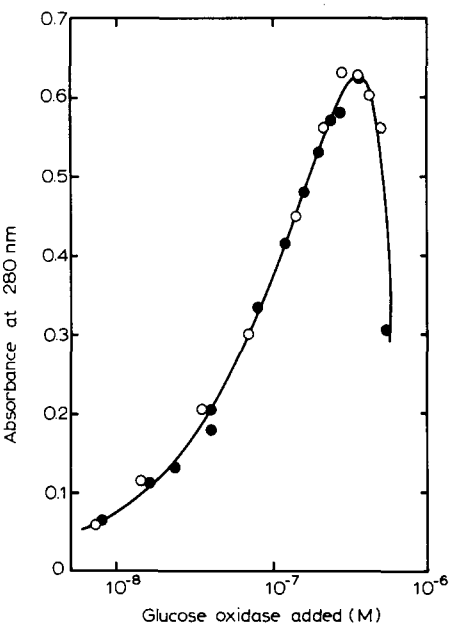


Fig. 4. Quantitative immunoprecipitin reactions with the antiserum against the native enzyme. Reactivities of the native enzyme (○), and the periodate-oxidized enzyme (●). 7.5 mg antiserum added.

minant of this enzyme is located on the protein moiety rather than on the carbohydrate moiety.

Kinetics of thermal inactivation. As reported previously [12], glucose oxidase was very stable against denaturing agents such as SDS and urea. It retained full activity after a 30-h incubation in a 1% SDS solution at 30° C, pH 5.5. The activity was fully restored after a 5-min contact with 7 M urea.

The enzyme was found fairly stable at an elevated temperature. The stability of the enzyme, however, was reduced to an appreciable degree in the presence

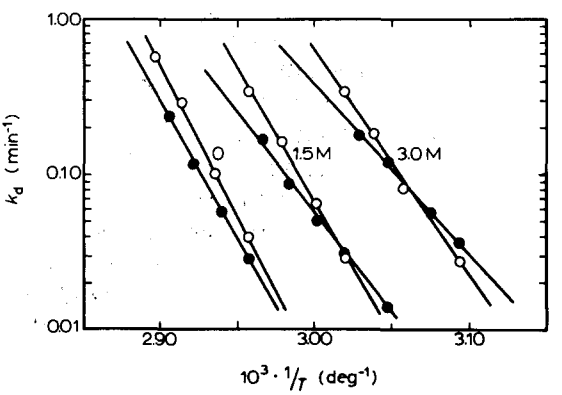


Fig. 5. Arrhenius plots for thermal inactivation of the native and the periodate-oxidized enzymes in the presence and the absence of urea. The native enzyme: ○; the periodate-oxidized enzyme: ●. Urea concentrations were as indicated in the figure.

TABLE V

ACTIVATION PARAMETERS FOR THERMAL INACTIVATION OF GLUCOSE OXIDASE IN THE PRESENCE AND ABSENCE OF DENATURING AGENTS

Activation parameters were calculated from the following equations: $\Delta H^{\ddagger} = E^* - RT$, $\Delta F^{\ddagger} = -RT \cdot \ln [(h/kT) \cdot k_d]$, and $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta F^{\ddagger})/T$, where E^* is the experimental activation energy; $\Delta H^{\ddagger}$, $\Delta F^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy, free energy and entropy of activation; R, h and k are the gas constant, the Planck's constant and the Boltzmann's constant, respectively. Values listed here are the average of four to five calculations on the basis of the experimental data.

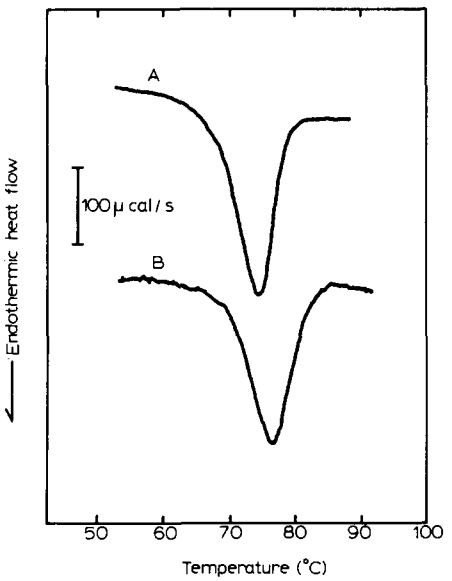
	Conditions	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta F^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/deg per mol)
Native enzyme	Control	87.3	24.1	184.8
	+1% SDS	74.8	23.1	153.0
	+1.5 M urea	77.8	23.1	161.2
	+3.0 M urea	66.1	23.3	130.5
Periodate-oxidized enzyme	Control	81.0	24.5	165.6
	+1% SDS	53.2	23.7	165.6
	+1.5 M urea	59.8	24.3	106.8
	+3.0 M urea	50.0	23.5	81.2

of SDS or urea. It is noteworthy that the periodate-oxidized enzyme was as stable as the native enzyme when no denaturing agent was added (Fig. 5).

Kinetic analyses of thermal inactivation processes were done with both the native and periodate-oxidized enzymes in the absence and the presence of the denaturing agents. The inactivation was known to follow first-order reaction kinetics through about 70% of the total process. The rate constant, k_d, of the process was determined at various temperatures, and the results are shown in Fig. 5 in the forms of the Arrhenius plots. Activation parameters for the thermal inactivation processes calculated are listed in Table V. It should be noted that the enthalpy of activation decreased markedly when the denaturing agents were added, especially in the cases of the periodate-oxidized enzyme. The free energy of activation, in contrast, was found to be nearly constant in all cases. As a result, the entropy of activation was varied in accordance with the changes of the enthalpy.

Differential scanning calorimetry. Fig. 6 shows typical thermograms for thermal denaturation of the native and the periodate-oxidized enzymes. The thermograms were nearly symmetrical with no detectable shoulders or minor peaks, indicating that both samples were thermally homogeneous. In agreement with the results of thermal stability tests and the inactivation kinetics studies (Fig. 5), the periodate-oxidized enzyme was as stable as or even more stable than the native enzyme, since the peak temperature of the thermogram, T_d, of the periodate-oxidized enzyme was slightly higher than that of the native enzyme (Figs. 6 and 7). The denaturation temperature T_d^0, was estimated to be 72.4°C for the native enzyme and that of the periodate-oxidized enzyme 72.8°C (Fig. 7). The enthalpy of denaturation, ΔH_d, for the periodate-oxidized enzyme (459 kcal/mol) was virtually identical with that of the native enzyme (445 kcal/mol).

The molecular properties of the native and the periodate-oxidized enzymes obtained in the present paper are summarized in Table VI.



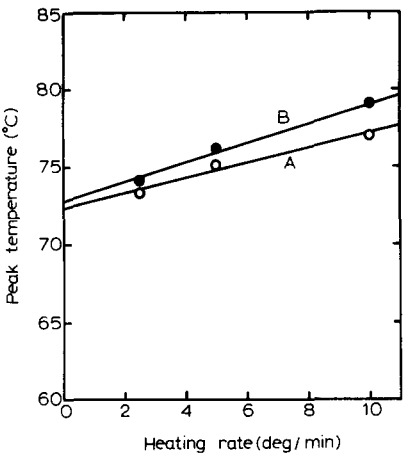


Fig. 6. Differential scanning calorimetric thermograms for thermal denaturation of the native and the periodate-oxidized enzymes. Curve A: the native enzyme, 7.1 mg; curve B: the periodate-oxidized enzyme, 7.2 mg. Heating rate: 5°C per min.

Fig. 7. The dependence of the peak temperature of the thermogram on the heating rate. The native enzyme: ○; the periodate-oxidized enzyme: ●.

TABLE VI

MOLECULAR PROPERTIES OF THE NATIVE AND THE PERIODATE-OXIDIZED GLUCOSE OXIDASE SAMPLES

	Native enzyme	Periodate-oxidized enzyme
Total carbohydrate content (%)	12.4	ca. 5
Molecular weight	153 000	150 000
V/e_0 (mol/min per mol) *	20 200	19 400
K_m for glucose (mM)	26	28
K_m for oxygen (mM)	0.20	0.18
$s_{20,w}$ (S)	8.06	8.37
$D_{20,w}$ (10^{-7} cm^2/s)	4.94	4.74
$\bar{v}$ (ml/g)	0.727	0.721
$[\eta]$ (ml/g)	4.57	4.63
Stokes radius (Å)	42.7	44.5
f/f_0	1.21	1.20
Axial ratio (a/b) **	2.50	2.40
Activation energy for thermal inactivation (kcal/mol)	88.0	81.7
Denaturation temperature (°C)	72.4	72.8
Enthalpy of denaturation (kcal/mol)	445	459

* e_0: total enzyme concentration.

** 0.3 g of bound water per g of dry protein was assumed.

Discussion

The purposes of the present study were: (1) to investigate the gross structure of the glucose oxidase molecule, since it seemingly had a randomly extended conformation in the major part of the polypeptide chains (Fig. 1); (2) to elucidate the possible roles of the carbohydrate moiety of the glycoenzyme by comparing various properties of the periodate-oxidized enzyme with those of the native enzyme; and (3) to examine the effects of the periodate treatment on the properties of the enzyme, since this treatment is usually used to delete the carbohydrate moiety of glycoproteins without necessary consideration of possible alterations in the stucture and properties of the protein moiety.

On the bais of the hydrodynamic parameters obtained in the present study (Table VI), the size and shape of the glucose oxidase molecule can be depicted. The molecule has a value of about 43 Å for the Stokes radius with a frictional ratio of 1.21, which may correspond to an axial ratio (a/b, $a \geqslant b$) of 2.5/1 assuming a prolate ellipsoidal form and 0.3 g of bound water per g of dry protein. These results may enable us to conclude that glucose oxidase is a slightly elongated globular protein with a rigid structure, but not a protein of a disordered configuration. In favor of this interpretation, no alteration was observed in the hydrodynamic parameters, such as diffusion coefficient and viscosity, upon addition of neutral salt (0.1—0.3 M NaCl). This would give rise to such an alteration by weakening the intrapeptide ionic interactions in the case of a disordered polypeptide.

The size and shape of the periodate-oxidized enzyme were revealed to be essentially the same as those of the native enzyme (Table VI). The catalytic properties, immunological reactivities, and the secondary and quaternary structures of the enzyme were also not significantly altered by the periodate treatment. Furthermore, when denaturing agents were absent, the thermal stability, the denaturation temperature and the enthalpy of denaturation of the periodate-oxidized enzyme were much the same as those of the native enzyme. Accordingly, the protein moiety of the enzyme remained practically intact throughout the periodate treatment, although minor modifications were detectable in the amino acid composition (Table IV) and in the absorption spectrum. At the same time, it is relevant to conclude that the carbohydrate moiety of this enzyme does not participate directly in the catalytic activity, in the immunological reactivity, or in maintaining the spatial configuration of the enzyme molecule.

The only remarkable alteration brought about by the periodate treatment was the reduced thermal stability of the enzyme in the presence of the denaturing agents, SDS and urea. This alteration was quantitatively reflected in the values of the activation parameters, especially in those of the entropy of activation (Table V). A decrease of this value might in general be ascribed to a less-ordered state of the protein structure as compared with a standard state. Therefore, the notable decrease observed with the periodate-oxidized enzyme implies that the protein moiety of the periodate-oxidized enzyme may easily take a somewhat disordered conformation in the presence of the denaturing agents. Since the protein moiety of the periodate-oxidized enzyme was kept practically intact, the reduction of the thermal stability is ascribed to the removal of the

carbohydrate moiety. Accordingly, a protective effect of the carbohydrate moiety can be postulated, which contributes to an increase in the stability of the protein moiety when the denaturing agents are present.

Although glucose oxidase, as elucidated in the present study, is a globular protein, the enthalpy of denaturation, ΔH_d, was found to be unusually low compared with those of other globular proteins. Specific enthalpies of denaturation have been reported to be 4.6 cal/g for avidin [28], 3.7—5.7 cal/g for chymotrypsinogen [29,30], 3.8—4.2 cal/g for conalbumin [31], 4.8—6.5 cal/g for ribonuclease [28], 8.2 cal/g for β-trypsin [23] and 8.8 cal/g for lysozyme [28]. In contrast, the value for glucose oxidase, calculated on the basis of a molecular weight of 153 000, was only 2.9 cal/g. This low value may possibly be attributed, at least in part, to the low content of the ordered secondary protein structure of this enzyme. Further studies are in progress.

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References

1 Cecil, R. and Ogston, A.G. (1948) Biochem. J. 42, 229
2 Kusai, K. (1960) Annu. Rep. Sci. Works, Osaka Univ. 8, 43—74
3 Pazur, J.H. and Kleppe, K. (1964) Biochemistry 3, 578—583
4 Bodmann, O. and Walter, M. (1965) Biochim. Biophys. Acta 110, 496—506
5 Nakamura, S. and Fujiki, S. (1968) J. Biochem. 63, 51—58
6 Abalikhina, T.A., Morozkin, A.D., Bogdanov, V.P. and Kaverzneva, E.D. (1971) Biokhimiya 36, 191—198
7 Pazur, J.H., Kleppe, K. and Cepure, A. (1965) Arch. Biochem. Biophys. 111, 351—357
8 Swoboda, B.E.P. and Massey, V. (1965) J. Biol. Chem. 240, 2209—2215
9 Bogdanov, V.P., Abalikhina, T.A., Chukhrova, A.I., Morozkin, A.D., Degtyar', R.G. and Kaverzneva, E.D. (1973) Biokhimiya 39, 771—777
10 Hayashi, S. and Nakamura, S. (1976) Biochim. Biophys. Acta 438, 37—48
11 Nakamura, S. and Hayashi, S. (1974) FEBS Lett. 41, 327—330
12 Nakamura, S., Hayashi, S. and Hasumi, H. (1976) in Flavins and Flavoproteins (Singer, T.P., ed.), pp. 691—701, Elsevier, Amsterdam
13 Tarentino, A.L. and Maley, F. (1974) J. Biol. Chem. 249, 811—817
14 Koide, N. and Muramatsu, T. (1974) J. Biol. Chem. 249, 4897—4904
15 Yasuda, Y., Takahashi, N. and Murachi, T. (1971) Biochemistry 10, 2624—2630
16 Dubois, M., Gillis, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350—356
17 Brückner, J. (1955) Biochem. J. 60, 200—205
18 Park, J.T. and Johnson, M.G. (1949) J. Biol. Chem. 181, 149—151
19 Rondle, C.J. and Morgan, W.T.J. (1955) Biochem. J. 61, 586—589
20 Spies, J.R. and Chambers, D.C. (1948) Anal. Chem. 20, 30—39
21 Ouchterlony, O. (1958) Prog. Allergy 5, 1—78
22 McDuffie, F.C. and Kabat, E.A. (1953) J. Immunol. 77, 193—197

23 Donovan, J.W. and Beardslee, R.A. (1975) J. Biol. Chem. 250, 1966—1971
24 Townend, T., Kumosinski, T.F., Timasheff, S.N., Fassman, G.D. and Davidson, B. (1966) Biochem. Biophys. Res. Commun. 23, 163—169
25 Cassim, J.Y. and Yang, J.T. (1967) Biochem. Biophys. Res. Commun. 26, 58—64
26 Yphantis, D.Y. (1964) Biochemistry 3, 297—317
27 Tanford, C. (1968) Adv. Protein Chem. 23, 121—282
28 Donovan, J.W. and Ross, K.D. (1973) Biochemistry 12, 512—517
29 Jackson, W.M. and Brandts, J.F. (1970) Biochemistry 9, 2294—2301
30 Biltonen, R., Schwartz, A.T. and Wadsö, I. (1971) Biochemistry 10, 3417—3423
31 Donovan, J.W. and Ross, K.D. (1975) J. Biol. Chem. 250 6022—6025