ON THE OLIGOMERISATION OF PERIODATE-OXIDIZED GLUCOSE OXIDASE*

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1. Introduction

Some effects of periodate oxidation of glucose oxidase (EC 1.1.3.4) have been described by Nakamura et al. [1]. In agreement with these authors we have found that periodate oxidation hardly affects the enzymatic activity though it causes glucose oxidase to lose 50% of its carbohydrate content. Our value of 8.06 S for the sedimentation coefficient of the native enzyme is the same as found by Nakamura et al. [1] while for the periodate-oxidized enzyme we find 8.47 S compared with 8.37 S in [1]. We have confirmed that periodate oxidation causes a 4% decrease in the enzyme's diffusion coefficient, corresponding to a small increase in the Stokes radius of the protein. From these results the Japanese workers concluded that periodate oxidation causes only small changes in the size and shape of the molecule and that the typical globular protein properties of glucose oxidase are retained.

As we have already reported [2], there are some discrepancies that require explanation. Periodate oxidation causes a reduction in mass of the molecule of at least 7%, in the present experiments 14% (see later), which has to be reconciled with the changes in sedimentation and diffusion coefficients and in Stokes radius mentioned above. We therefore investigated the effects of periodate oxidation using gel-permeation chromatography. The results are reported here. They indicate that the oxidized enzyme exists as a complex oligomerising system.

* Part of the work described in the dissertation of Maria Gerber, Mainz (1977).

2. Materials and methods

Powdered *Aspergillus niger* mycelium as DeeO-powder manufactured by Takamine Laboratories, Clifton, USA, was supplied by Deutsche Naarden GmbH, Hamburg, and was purified by ion exchange chromatography as described previously [3]. The purified and, according to sedimentation behaviour, homogeneous glucose oxidase contained 16% of carbohydrates, had a molecular weight, M = 144 000, and a enzymatic activity of $(17.6 \pm 0.7) \times 10^3$ mol glucose min^{-1} $(mol\ FAD)^{-1}$ as measured at 25°C, in standard acetate buffered solutions of 0.1 M glucose. Solutions of this native enzyme in 0.05 M acetate buffer, pH 4.66 were mixed in the dark at 0°C with a solution of sodium metaperiodate in the same buffer [2,4]. The enzyme concentration in the reacting solution was 6.54 g l^{-1}, and the ratio of periodate to enzyme was 1:800 mol/mol. The reaction was stopped by 3 mol erythritol/mol sodium metaperiodate. Samples were taken between 2 min and 72 h, and they were immediately dialysed until free of iodate. Unless otherwise noted, a reaction product is described here which is obtained after 5 h oxidation time. All the experiments were carried out in standard acetate buffer at 20°C, and the activity measurements at 25°C.

The gel chromatographic separation of the oligomers of the oxidised glucose oxidase was performed on Sephadex G-200 columns (100 × 2.6 cm). Molecular weights larger than 480 000 were separated on Sepharose gel C1-6B from Pharmacia, Uppsala. The gels were equilibrated with standard acetate buffer, and standardized with several globular proteins. A

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wavelength of 254 nm was used to monitor the eluates from the columns.

Sedimentation coefficients, s, and molecular weights, M, were measured using a Beckman Spinco Model E Ultracentrifuge with UV scanner, at a wavelength of 280 nm. The molecular weights were determined from sedimentation equilibrium measurements.

The diffusion coefficients, D, were determined by inelastic light scattering (homodyne method) [5,6]. Alternatively they were calculated from the sedimentation coefficients and the s/D ratio obtained from sedimentation equilibrium measurements.

Density measurements for the determination of partial specific volumes were performed using a Digitale Dichtemesseinrichtung DMA 02C from A. Paar KG, Graz, Austria.

Protein concentrations were estimated from the dry weight (110° in vacuum). The carbohydrate content was determined by the phenol-sulfuric acid method [7] using mannose as a standard. For the amino acid analysis the Beckman Model Unichrom was used. Enzymatic activity was measured in a Warburg apparatus.

3. Results and discussion

Periodate-oxidised glucose oxidase subjected to gel permeation chromatography shows molecular inhomogenities. For example the elution diagram in fig.1 exhibits several distinct components, the molecular weights of which are nearly twice, three times, and four times greater than that of the monomer unit, for which $M \approx 120\,000$. Compared with the molecular weight of the native enzyme that of the oxidised monomer is somewhat decreased. Components, $M = 7.6 \times 10^5$ and $M = 2 \times 10^6$, were resolved in eluates from a Sepharose column. All of these components show glucose oxidase activity. The activities of monomer, dimer, and trimer compared with that of the native enzyme are about 10% higher. The activities of the $M = 7.6 \times 10^5$ and $M = 2 \times 10^6$ components are reduced to 50% and 40% respectively. It must be remembered, however, that at the low concentrations in the Warburg flasks the oligomers may partly dissociate.

Not shown in fig.1 is a further split-off component of $M \approx 10\,000$, which absorbs at 250 nm without any activity. An iodate test [8] of this component is negative.

A similar elution diagram as in fig.1 is obtained if the separated monomer is stored at 8°C and 7–9 g l^{-1} for four weeks and then applied to the G-200 column. In addition, the same results are obtained after an oxidation time of 1 h or 50 h.

The absorption spectra of the monomer, dimer, and trimer are identical (fig.2) and differ from that of the native enzyme (dashed line) by a distinct shoulder at 320 nm and slight deviations at 250, 278, 410, 490,

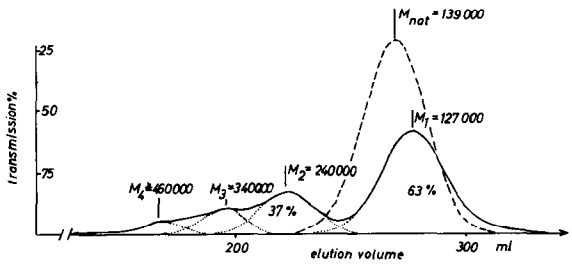

Fig.1. Elution-diagram of native glucose oxidase (- - -) and of a 5-h periodate-oxidized glucose oxidase (——) kept at 7.4 g l^{-1} for 4 weeks after oxidation (Sephadex G-200 column.) The dotted lines (. . .) indicate the different peaks of the oligomers.

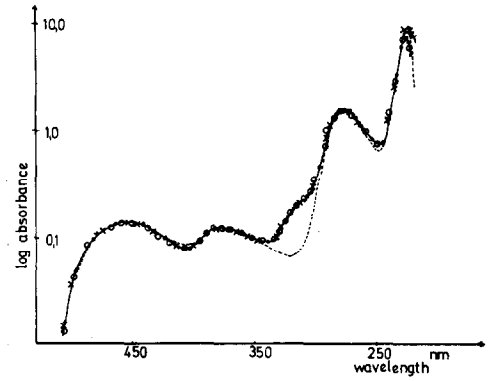

Fig.2. Absorption spectra of periodate-oxidized glucose oxidase, native enzyme (- - -), 5-h periodate-oxidized enzyme (——): monomer (•), dimer (×), trimer (○). The logarithm of the absorbance is plotted against the wavelength. Solutions of equal absorbance at 450 nm were used. The spectra in the UV range were measured at a 10-fold dilution of the concentration used in the visible range.

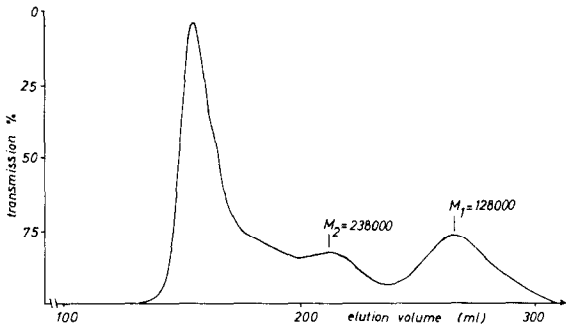

Fig.3. Elution diagram of a 5-h periodate-oxidized glucose oxidase kept at 24 g l^{-1} at 8°C for 16 days after oxidation on a Sephadex G-200 column.

and 520 nm, as stated already by Gerber [2] and by Nakamura et al. [1].

The oligomerisation rate of the periodate-oxidised samples and consequently their contents of oligomers (indicated in fig.1 and fig.4 as percentages) depend on the concentration of the native enzyme subjected to oxidation as well as on the concentrations at which the oxidised samples are kept.

As shown in fig.3, the high concentration of 24 g l^{-1} favours the formation of higher oligomers. The tetramer, hexamer, and polymer ($M = 2 \times 10^6$) appear within the exclusion volume of the Sephadex G-200 column, whereas the monomer and dimer are distinctly separated and the trimer appears as a shoulder.

Temperature and time also have a large influence on the polymerisation rate. pH does not have any effect between 4.6 and 6.8, but at 4.1 enhanced formation of higher oligomers appears.

Figure 4 shows that there is some evidence for reversible dissociation-association processes in the

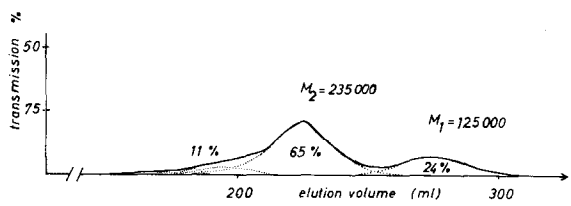

Fig.4. Elution diagram of the dimer of a 5-h periodate-oxidized glucose oxidase kept at 0.1 g l^{-1} and 8°C for 4 weeks after the first separation on a Sephadex G-200 column.

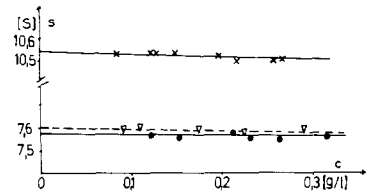

Fig.5. Dependence of the sedimentation coefficient on the concentration of the native enzyme (▽) and the 5-h periodate-oxidized enzyme: monomer (●), dimer (×).

case of the dimer component. A dimer fraction shows a redistribution of the oligomerisation components on a Sephadex G-200 column four weeks after the first separation.

The sedimentation behaviour of the monomers as well as that of the dimer were examined to determine the sedimentation coefficients and their dependence on the concentration, as characterized by k_s values (table 1). Measurements were performed in the range of 0.1–0.4 g l^{-1} by ultracentrifuge runs at 48 000 and 30 000 rev./min, respectively. In fig.5 the apparent sedimentation coefficients (measured in standard acetate buffered solutions at 20°C) are plotted against concentrations. The measurements were immediately performed after the components were separated on the G-200 column. The sedimentation coefficients extrapolated to zero concentration yielded 7.60 ± 0.06, 7.58 ± 0.06, and 10.54 ± 0.1 Svedberg units for the native enzyme, the monomer and dimer oxidised enzyme, respectively. In table 1 the values are reduced to standard conditions. The *molecular weights* stated in table 1 result from sedimentation equilibrium runs at 8000 and 6000 rev./min.

Diffusion measurements of monomer provided average values consisting of the diffusion coefficients of monomer and dimer, because the required concentration of 4–10 g l^{-1} accelerated oligomerisation. Therefore the value found at 4.36×10^{-7} cm^2 s^{-1} is lower than that of the native enzyme. The diffusion coefficients $D^{\circ}_{20,w}$ in table 1 of the monomer and dimer oxidised enzyme were evaluated by the Svedberg equation

$$M = \frac{RT s^{\circ}}{D^{\circ}(1-v_2 \varsigma)} \qquad (1)$$

Table 1
Molecular constants

		E_{nat}	$E_{ox\ mono}$	$E_{ox\ dim}$	$E_{ox\ mono + oligo}$
M (sed. equilibr.)		144 000 ± 4000	126 000 ± 3000	253 000 ± 6000	≈ 150 000
$s^{\circ}_{20,w} \cdot 10^{13}$	$[s^{-1}]$	8.06 ± 0.06	7.94 ± 0.06	11.17 ± 0.1	8.47 ± 0.1
$D^{\circ}_{20,w} \cdot 10^{7}$	$\left[\frac{cm^2}{s}\right]$	4.50 ± 0.06	5.0[a]	3.61[a]	4.36 ± 0.05
k_s	$\left[\frac{cm^3}{g}\right]$	11 ± 2	10 ± 2	13 ± 2	12 ± 2
$[\eta]$	$\left[\frac{cm^3}{g}\right]$	4.54 ± 0.06	4.44 ± 0.06		
Stokes Radius	[Å]	44	40	55	46
Enzym. activity $\cdot 10^{-3}$	$\left[\frac{mol\ gluc}{min\ mol\ FAD}\right]$	17.6 ± 0.7	19.6 ± 0.7	18.9 ± 0.7	≈ 20

[a] Calculated from eq. (1)

using both sedimentation velocity and sedimentation equilibrium experiments. From these values of $D^{\circ}_{20,w}$, Stokes radii were calculated. The partial specific volumes v_2 of the native and the periodate-oxidised enzymes are 0.691 and 0.694 $cm^3\ g^{-1}$, respectively.

The carbohydrate contents of the monomer and the dimer are identical, being 52 ± 3% of that of the native enzyme, and correspond to a loss of mass of 7.8%. The loss of nitrogen of the periodate-oxidised samples is 15% of the total nitrogen. As the native enzyme contained 16% nitrogen this indicates a loss of mass of 2.4%. An obvious reduction in the content of basic amino acids was found. In particular, 25% of the arginine was destroyed. The loss of mass after periodate-oxidation was determined by dry weight to be 14%. This is caused by the already mentioned reductions and the splitting off of the $M \approx 10\,000$ component. The data on intrinsic viscosities $[\eta]$ and k_s in the case of monomer hardly differ from those for the native enzyme, indicating, that the globular shape remains after periodate-oxidation. For the dimer it is supposed that the globular shape also remains, because the k_s value is essentially the same as that of the native enzyme.

Acknowledgment

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