The dissociation of glucose oxidase by sodium n-dodecyl sulphate

Malcolm N. JONES, Philip MANLEY and Alan WILKINSON

Department of Biochemistry, University of Manchester, Manchester M13 9PL, U.K.

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1. The enzymic activity of glucose oxidase was determined as a function of pH and sodium n-dodecyl sulphate (SDS) concentration. 2. Glucose oxidase is not deactivated by SDS at pH 6 even after prolonged incubation, but is deactivated at pH 4.3 and 3.65. 3. Sedimentation-rate analysis showed that glucose oxidase dissociates into its two subunits at pH 5 and below, and sedimentation-equilibrium experiments in the presence of SDS gave a subunit molecular weight of 73 500. 4. SDS binds to glucose oxidase in acid solutions; specific binding occurs at pH 3.65, but at pH 6 only co-operative binding was observed. 5. Glucose oxidases in which some of the carboxy groups were blocked with glycine methyl ester were deactivated by SDS at pH 6.0; the rate of deactivation increased with the extent of esterification. 6. Deactivation of esterified glucose oxidases correlated with thermal analysis of the initial SDS interaction, the exothermicity of the interaction increasing with the extent of esterification. 7. The results show that carboxy groups confer resistance to deactivation by SDS on glucose oxidase by screening the cationic residues and inhibiting specific interactions that facilitate dissociation into subunits.

Unlike most globular proteins that bind and are denatured by SDS, glucose oxidase (β -D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) has been reported to be resistant to SDS binding (Nelson, 1971) and to retain significant enzymic activity even after prolonged incubation with SDS (Swoboda & Massey, 1965; Tsuge *et al.*, 1975; Nakamura *et al.*, 1976; Solomon *et al.*, 1977). It is noteworthy that glucose oxidase did not feature in the early surveys of the application of SDS/polyacrylamide-gel electrophoresis to determination of protein molecular weights (Dunker & Rueckert, 1969; Weber & Osborn, 1969).

Most of the molecular-weight measurements for the enzyme from *Aspergillus niger* lie in the range 150 000–160 000 (Pazur & Kleppe, 1964; Swoboda, 1969a; Klärner *et al.*, 1969; O'Malley & Weaver, 1972), and the molecule contains two strongly bound FAD molecules (Swoboda, 1969a,b; Okuda *et al.*, 1979). Multiple forms of glucose oxidase exist with identical protein composition but different carbohydrate compositions (Hayashi & Nakamura, 1981). Swoboda (1969a) reported that the apoenzyme produced on removal of the FAD has a molecular weight of 153 000 and can exist in two conformational states, with sedimentation coefficients of 4.5S and 8.0S. However, Tsuge *et al.*

(1975) suggest that the apoenzyme has the molecular weight of the subunit ($79\,000 \pm 4000$). Although there is general agreement that the holoenzyme has two subunits, O'Malley & Weaver (1972) argue that they are covalently linked by disulphide bonds.

We report here an investigation of the interaction of SDS with glucose oxidase and glucose oxidases in which the aspartic acid and glutamic acid residues have been chemically modified. Our prime objective was to find the origin of the stability of glucose oxidase to deactivation and dissociation by SDS.

Experimental

Materials

Glucose oxidase (highly purified) from *Aspergillus niger* was obtained from Miles Laboratories, Stoke Poges, Slough, Bucks., U.K. As received, it gave a single symmetrical schlieren peak on ultracentrifugation, and the amino acid analysis was in good agreement with the literature values (Pazur *et al.*, 1965; Tsuge *et al.*, 1975; Nakamura *et al.*, 1976; Hayashi & Nakamura, 1981). SDS (especially pure grade) was used as supplied from BDH Chemicals, Poole, Dorset, U.K. All other reagents used were of analytical grade.

Abbreviation used: SDS, sodium dodecyl sulphate.

The buffers used were as follows: pH 3.65, 10.0055 (50 mM-glycine + HCl); pH 4.3, 10.0057 (10 mM-acetic acid + NaOH); pH 6.0, 10.0155 (1.23 mM- Na_2HPO_4 + 8.77 mM- NaH_2PO_4). The ionic strengths include a contribution of 0.00308 from the addition of NaN_3 . A series of acetate buffers (10 mM-acetic acid + NaOH), pH 4.5, 4.7, 5.0 and 5.5, was also used.

Three samples of glucose oxidase were prepared in which the carboxy groups were selectively coupled to glycine methyl ester by using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (Hoare & Koshland, 1967). To 15 cm³ of a solution of glucose oxidase in water (35.3 μM) was added 1.0 g of glycine methyl ester hydrochloride followed by 0.25 g of the carbodi-imide. The pH was adjusted to 4.75, and the reaction was allowed to proceed for 10 min. The reaction mixture was passed down a Sephadex G-50 column (2.6 cm diam. $\times$ 27 cm) equilibrated with 50 mM-glycine buffer, pH 3.5, to separate the reagents, and the glucose oxidase fractions were collected and dialysed twice against distilled water before dialysis against the required buffer. The procedure gave a sample in which 38% of the carboxy groups were blocked. Two further samples were prepared for which the concentrations of glycine methyl ester hydrochloride and carbodi-imide were decreased to give lower degrees of blocking.

Equilibrium dialysis

This was performed as previously described (Jones & Manley, 1979) with dialysis bags cut from Spectrapor membrane tubing with molecular-weight cut-off 6000–8000 [MSE (Fisons), Crawley, Sussex, U.K.]. The glucose oxidase concentration was 0.0828% (w/v), and the free surfactant concentration outside the bag in equilibrium with the complexes was assayed by the Rosaniline hydrochloride method of Karush & Sonnenberg (1950).

Ultracentrifugation

Sedimentation-velocity measurements were made in double-sector cells with 12 mm Epon centre-pieces with 4° sector angles in an AnD rotor in a Beckman model E analytical ultracentrifuge operating at 56 100 rev./min (229 100 g, r_{av} 6.51 cm) at 20°C. Schlieren optics were used and photographs were taken at 8 min intervals.

Sedimentation-equilibrium measurements to determine the molecular weight (M) of glucose oxidase subunits in SDS were made in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ solvent mixtures covering a range of density (ρ). The method is based on the equation of Reynolds & Tanford (1976):

$$M(1 - \phi' \rho) = M[(1 - \bar{v}_p \rho) + \delta_{\text{SDS}}(1 - \bar{v}_{\text{SDS}} \rho)] \quad (1)$$

where $\bar{v}_p$ and $\bar{v}_{\text{SDS}}$ are the partial specific volumes of

the protein and SDS respectively, and δ_{SDS} is the number of grams of SDS bound per gram of protein. ϕ' is the partial specific volume of protein in Donnan equilibrium with the solvent. Samples of glucose oxidase (0.3 mg/ml) were dialysed for 5 days in 10 mM-SDS (buffered at pH 4.3 with acetate) and made up in water, a 1:1 mixture (by weight) of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ and pure $^2\text{H}_2\text{O}$.

Sedimentation analysis was performed with interference optics to determine the concentration (c)–distance (r) profile, which was used to determine $M(1 - \phi' \rho)$, at an angular velocity ω (12 590 rev./min; $1.317 \times 10^3 \text{ rad} \cdot \text{s}^{-1}$), by using the procedure of Nazarian (1968) based on the equation:

$$M(1 - \phi' \rho) = \frac{2RT}{\omega^2} \cdot \frac{d \ln c}{dr^2} \quad (2)$$

M was obtained by extrapolation of the plots of $M(1 - \phi' \rho)$ versus ρ to a solvent density $\rho = (\bar{v}_{\text{SDS}})^{-1}$, at which point $M(1 - \phi' \rho)$ is independent of the bound SDS. The partial specific volume of glucose oxidase was taken to be 0.72 cm³/g (Swoboda, 1969a) and that of SDS to be 0.874 cm³/g (Dohnal *et al.*, 1980).

Microcalorimetry

Enthalpy measurements were made with an LKB 10700 batch microcalorimeter as previously described (Jones & Manley, 1980). The enthalpies of SDS dilution were balanced out in the reference cell, and the enthalpies of dilution of the glucose oxidases with diffusate buffers were negligible. The final protein concentration after mixing was 0.0828% (w/v).

Protein concentrations and enzymic activities

Protein concentrations were based on a specific absorption coefficient $A_{1\text{cm}}^{1\%}$ at 280 nm of 16.00 ± 0.32 determined from dry weights of protein samples after dialysis against distilled water. The specific absorption coefficient is in accord with literature values (Swoboda & Massey, 1965; Solomon *et al.*, 1977; Bentley, 1963).

Glucose oxidase activity was determined by the coupled peroxidase/o-dianisidine system (Keston, 1956) described in the *Worthington Enzyme Manual* (1972, p. 19).

Amino acid analysis

Samples were hydrolysed in 6 M-HCl for 24 h at 105°C, and the amino acid composition was determined with a JEOL 6AH analyser.

Results and discussion

Binding of SDS and dissociation of glucose oxidase subunits

Equilibrium-dialysis measurements gave the binding isotherms shown in Fig. 1. The shapes of the

Fig. 1. Binding of SDS to glucose oxidase ($\bar{v}$ = mol of SDS/mol of glucose oxidase) as a function of the logarithm of the free SDS concentration at 25°C

For full experimental details see the text. ○, Glycine buffer, pH 3.65 and I 0.0055; □, acetate buffer, pH 4.3 and I 0.0057; ◇, phosphate buffer, pH 6.0 and I 0.0155. Measurements were made by equilibrium dialysis.

Table 1. Sedimentation analysis of glucose oxidase and its derivatives

For full experimental details see the text.

Protein (concentration, mg/ml)	Medium	Sedimentation coefficient (S)
Glucose oxidase (4.4)	pH 4.3	7.78 ± 0.09
Glucose oxidase (3.3)	pH 4.3 + 25 mM-SDS*	2.92 ± 0.01
Glucose oxidase (3.3)	pH 5.0 + 25 mM-SDS*	3.76 ± 0.01 (peak 1)
		7.11 ± 0.08 (peak 2)
Glucose oxidase (4.2)	pH 6.0	7.57 ± 0.10
Glucose oxidase (3.2)	pH 6.0 + 25 mM-SDS*	7.13 ± 0.02
GO-M2 (1.7)	pH 6.0	8.09 ± 0.04
GO-M2 (1.9)	pH 6.0 + 10 mM-SDS*	3.50 ± 0.02
GO-M3 (1.6)	pH 6.0	7.96 ± 0.02
GO-M3 (1.9)	pH 6.0 + 10 mM-SDS*	3.57 ± 0.01

* Incubation period 24 h.

isotherms change over the pH range 3.65–6.0. At pH 3.65 the initial rise in the curve at low free SDS concentration (0.1–0.3 mM) is characteristic of specific interaction, most probably with the approximately 120 cationic amino acid residues in glucose oxidase. A Scatchard plot of this data gave approximately 130 specific binding sites. At this pH the complexes formed are insoluble. At high free

SDS concentration the rise in the curve is characteristic of co-operative binding.

The complexes formed at pH 4.3 and 6.0 are soluble and binding is predominantly co-operative. However, sedimentation analysis (Table 1 and Figs. 2a and 2c) shows that at pH 4.3 the sedimentation coefficient decreases from 7.8 S to 2.9 S on addition of SDS, owing to binding of SDS and dissociation

Fig. 2. Sedimentation analysis of glucose oxidases

Measurements were made in double-sector cells with 12 mm Epon centre-pieces with 4° sector angles in a Beckman model E analytical ultracentrifuge operating at 56 100 rev./min at 20°C . (a) Schlieren patterns of native glucose oxidase at pH 4.3 (lower pattern) and pH 6 (upper pattern). In the lower pattern the meniscus is displaced to the right-hand side owing to the lower solution volume in the sector. (b) Schlieren pattern of glucose oxidase, pH 5.0, plus 25 mM-SDS. The pattern shows peaks for the glucose oxidase subunit (3.76S) and the whole molecule (7.11S). (c) Schlieren patterns of glucose oxidase in 25 mM-SDS at pH 4.3 (lower pattern) and pH 6 (upper pattern). The rise in the upper pattern towards the meniscus is attributed to SDS micelles. (d) Schlieren patterns of modified glucose oxidase (GO-M2) at pH 6 (lower curve) and at pH 6 in 10 mM-SDS (upper curve). The photographs were taken after 32 min (a), 28 min (b), 36 min (c) and 24 min (d) from the time the centrifuge was up to speed.

into subunits. In contrast, at pH 6.0 addition of SDS does not dissociate the subunits (Fig. 2c), and only a small decrease in the sedimentation coefficient from 7.8S to 7.1S occurs on SDS binding. At the intermediate pH of 5.0 two Schlieren peaks were observed (7.1S and 3.8S), showing partial dissociation (Fig. 2b).

Fig. 3 shows the sedimentation-equilibrium measurements on the system at pH 4.3 (plus 10 mM-SDS). At a solvent density equal to $(\bar{v}_{\text{SDS}})^{-1}$, $M(1-\phi'\rho)$ has a value of 12951, which gives a subunit molecular weight of $73\,500 \pm 7200$, which, compared with 150 000–160 000 for the molecular weight of the native molecule, supports the assignment of the sedimentation coefficients of approx. 3S to the subunits. Dissociation of the subunits is accompanied by loss of 2 mol of FAD/mol of glucose oxidase. In the bound state the fluorescence of FAD at 530 nm is almost completely quenched (Swoboda, 1969a). At pH 4.3 above 2.5 mM, SDS fluorescence was detected at 530 nm after only 3 h incubation. At pH 3.65 and 4.3 total release of FAD occurred above an SDS concentration of approx. 3.3 mM after equilibrium dialysis.

Chemical modification of glucose oxidase

Since the mode of interaction with SDS was observed to change significantly in the pH range 4–6 over the ionization range of the carboxy groups, glucose oxidase was chemically modified by blocking the carboxy groups with glycine methyl ester. Some of the properties of these chemically modified glucose oxidases are shown in Table 2, in comparison with the unmodified enzyme.

The percentage of blocked groups has been estimated from the increase in the glycine content.

Fig. 3. Sedimentation equilibrium of glucose oxidase in the presence of SDS as a function of solvent density at 20°C

For full experimental details see the text. Glucose oxidase concentration was 0.3 mg/ml, SDS concentration was 10 mM and acetate buffer, pH 4.3 and 10.0057, was used. The arrow denotes the solvent density equal to the reciprocal partial specific volume of SDS.

Blocking of the carboxy groups decreases the enzymic activity relative to the native molecule, and in one case (GO-M3) results in loss of FAD as assessed from the absorbance ratio A_{456}/A_{280} (bound FAD in the presence of NaN_3 has an absorbance maximum at 456 nm, whereas the

Table 2. *Amino acid analysis and activity of native and chemically modified glucose oxidase*

For full experimental details see the text. Calculations were based on a molecular weight of 150 000 and a carbohydrate content of 14.3%. The literature values are in the following ranges: aspartic acid, 124–160; glutamic acid, 96–112; glycine, 111–136 (Tsuge *et al.*, 1975; Nakamura *et al.*, 1976; Pazur *et al.*, 1965; Hayashi & Nakamura, 1981). The percentage of blocked carboxy groups is defined as $100 \times \text{increase in the number of glycine residues}/(\text{numbers of aspartic acid} + \text{glutamic acid residues})$.

Protein	Relative activity (%)	A_{456} A_{280}	Cationic residues (Lys + His + Arg)	Residues/molecule			Blocked carboxy groups (%)
				Asp	Glu	Gly	
Native	100	0.102	119	140	119	150	0
GO-M1	75	0.115	113	138	115	184	13.4
GO-M2	65	0.095	119	141	117	198	18.6
GO-M3	31	0.053	102	141	117	247	37.6

maximum for free FAD is 450 nm). The modified samples gave single schlieren peaks with sedimentation coefficients close to those of the native enzyme, demonstrating that no dissociation into subunits occurs on chemical modification. However, in marked contrast with native glucose oxidase, sedimentation analysis (Table 1) shows that at pH 6 the modified samples are dissociated into subunits by SDS. Fig. 2(d) shows the change in the schlieren pattern for glucose oxidase GO-M2 at pH 6 on addition of 10 mM-SDS.

Enzymic activity in the presence of SDS

Fig. 4 shows the relative activity of native glucose oxidase and two modified samples (GO-M2 and GO-M3) as a function of SDS concentration. At pH 6.0 glucose oxidase retains 100% activity after 24 h incubation in SDS, but in more acid solution (pH 4.3 and 3.65) activity is decreased to zero in 2 mM-SDS. However, at pH 6.0 enzymic activity was retained, even after incubation for 5 days, when the dialysis measurements show that co-operative binding has occurred.

Blocking of the carboxy groups results in loss of activity at pH 6, and the loss of activity at a given SDS concentration increases with the extent of blocking.

The rate of change of activity at pH 6.0 and a fixed high SDS concentration (10 mM) is given in Fig. 5, which shows that the deactivation of modified glucose oxidase is a relatively slow process. These results illustrate that ionized carboxy groups are primarily responsible for inhibiting interaction between glucose oxidase and SDS, since activity is lost either by protonation at low pH or by chemically blocking the carboxy groups.

Thermochemical analysis

The enthalpy of the initial interaction between native glucose oxidase and SDS is shown in Fig. 6, as a function both of pH and SDS concentration. The measurements refer to the enthalpy change

Fig. 4. *Enzymic activity of glucose oxidases in SDS solution at 25°C*

For full experimental details see the text. Glucose oxidase concentration was 0.0828% (w/v). $\diamond$, Glucose oxidase, pH 6.0; $\circ$, glucose oxidase, pH 4.3; $\bullet$, glucose oxidase, pH 3.65; $\square$, modified glucose oxidase (GO-M2) with 19% of the carboxy groups blocked with glycine methyl ester, pH 6.0; $\blacklozenge$, modified glucose oxidase (GO-M3) with 38% of the carboxy groups blocked, pH 6.0. The glucose oxidases were incubated for 24 h with SDS before assay.

measured over a 20 min period after mixing. The greater part of this energy change probably occurs at the instant of mixing, and largely originates from specific ionic interactions between the SDS anion and cationic amino acid residues (Jones & Manley, 1980). At pH 6 no enthalpy change was detected, but at lower pH, where specific binding occurs, the enthalpy increases markedly.

The initial enthalpies of interaction of SDS with modified glucose oxidases at pH 6 are shown in Fig. 7. Progressive blocking of the carboxy groups leads to an increase in the initial enthalpy of interaction

Fig. 5. Relative activity of glucose oxidases in SDS as a function of time

For full experimental details see the text. Glucose oxidase concentration was 0.0828% (w/v), and SDS concentration was 10 mM. $\diamond$, Glucose oxidase, pH 6.0; $\blacksquare$, modified glucose oxidase (GO-M1) with 13% of the carboxy groups blocked with glycine methyl ester; $\square$, modified glucose oxidase (GO-M2) with 19% of the carboxy groups blocked; $\blacklozenge$, modified glucose oxidase (GO-M3) with 38% of the carboxy groups blocked.

Fig. 7. Initial enthalpies of interaction of glucose oxidases with SDS at 25°C

For full experimental details see the text. Glucose oxidase concentration was 0.0828% (w/v). $\blacklozenge$, Glucose oxidase, pH 6.0; $\diamond$, modified glucose oxidase (GO-M1) with 13% of the carboxy groups blocked with glycine methyl ester; $\square$, modified glucose oxidase (GO-M2) with 18% of the carboxy groups blocked; $\bullet$, modified glucose oxidase (GO-M3) with 38% of the carboxy groups blocked.

Fig. 6. Initial enthalpies of interaction of glucose oxidase with SDS as a function of pH and surfactant concentration at 25°C

For full experimental details see the text. Glucose oxidase concentration was 0.0828% (w/v). $\bullet$, Initial enthalpy on interaction with 5 mM-SDS. Inset: initial enthalpies at pH 6.0 ($\blacklozenge$), pH 4.3 ($\circ$) and pH 3.65 ($\square$).

with SDS. At a concentration of 5 mM-SDS the interaction enthalpy for glucose oxidase with 38% of the carboxy groups blocked at pH 6 is approx. -18 J/g , which is of the same order as the interaction enthalpy (-15 J/g) of unmodified glucose oxidase at pH 4.5.

Although there are numerous thermal contributions to the observed enthalpies of interaction arising from ion binding and subunit dissociation that prevent a rigorous interpretation, they clearly show that there is an almost instantaneous interaction between SDS and carboxy-group-blocked glucose oxidases at pH 6, in marked contrast with the athermal interaction with the unmodified enzyme.

Conclusions

The results show that the origin of the resistance of glucose oxidase to deactivation and subunit dissociation by SDS at and above pH 6 resides in the relatively high acidic amino acid content (the ratio of acidic to basic amino acids is 3.4). The ionized carboxy groups screen specific cationic binding sites from interaction with the anionic head group of the surfactant. Equilibrium dialysis shows that on

prolonged incubation with SDS only co-operative (hydrophobic) binding occurs with retention of activity, indicating that the bound SDS does not inhibit the active site. On protonation or chemical modification of the carboxy groups glucose oxidase becomes susceptible to deactivation and dissociates into its two subunits (molecular weight approx. 74000). The initial interaction of the modified enzyme with SDS occurs exothermically under conditions where interaction with the native enzyme is athermal. We find no evidence for any covalent linkage between the subunits as suggested by O'Malley & Weaver (1972).

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