Catalytic activity of *Aspergillus niger* glucose oxidase in water–polyol mixtures

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An experimental investigation was carried out on the kinetic and stability behaviour of *Aspergillus niger* glucose oxidase in water–polyol mixtures. Kinetic runs performed in systems containing up to 8 M polyhydric cosolvents (ethylene glycol, propylene glycol and glycerol) showed a strong dependence of the catalytic properties of the enzyme on the nature and composition of the reaction medium. Higher residual activities were measured in the presence of additives of lower polarity, according to the following order: propylene glycol > ethylene glycol > glycerol. Dilution and thermal unfolding experiments provided evidence that the activity reductions were not ascribable to solvent-induced structural modifications of the enzyme molecule but, rather, to the inhibitory action exerted by the polyols.

Keywords: glucose oxidase; catalytic activity; protein unfolding; water-miscible solvents

1. Introduction

Glucose oxidase catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone using oxygen as electron acceptor, according to the following reaction scheme:

$$\beta\text{-D-glucose} \rightarrow \text{D-glucono-}\delta\text{-lactone} + 2e + 2H^+$$

$$O_2 + 2e + 2H^+ \rightarrow H_2O_2$$

This enzyme is commercially produced from *Aspergillus niger* and *Penicillium glaucum* and is widely utilized in clinical analyzers for assaying glucose in whole blood, serum and plasma [1]. Furthermore it has been proposed as a potential candidate for the continuous monitoring of fermentation processes [2] and for the conversion of chemical into electrical energy in biochemical fuel cells [3].

The catalytic properties of glucose oxidase in pure buffer under optimal temperature and pH conditions have been extensively investigated [4–6]. The results obtained indicate that the enzyme activity is strictly related to the two flavin adenine dinucleotitide (FAD) cofactors which are present in the protein molecule as

prosthetic groups [7]. At physiologic conditions the cofactors are tightly bound to the apoprotein, but when the enzyme is subjected to stresses, such as heating or high concentrations of inactivating substances, the forces maintaining the integrity of the holoenzyme complex are first weakened and then broken, leading to the removal of the FAD components [8]. The molecular mechanisms involved in such process are only partly known, hampering the development of efficient stabilization strategies. This fact poses serious problems to the use of glucose oxidase at high temperature or in complex reaction media.

O'Malley and Ulmer studied the enzyme activity between 37 and 60°C in pure buffer and in the presence of various synthetic polymers [9]. These compounds were found to increase the enzyme half-life to a considerable extent and their protective action was shown to be strictly related to the polymer hydrophobicity. Stabilization, however, vanished at temperatures higher than 55°C, when a different inactivation mechanism was likely to prevail. Visible absorption spectra of the enzyme before and after thermal inactivation appeared to indicate that the observed activity loss could be ascribed to heat-induced flavin dissociation. Ye et al. analysed the effect of different additives (polyhydric alcohols, polyethylene glycol and salts) on the catalytic behaviour of glucose oxidase at 60°C [10]. The residual activity data showed that polyhydric additives and most of the polyethylene glycol used induced a sharp stabilization of the macromolecule. Furthermore, the protective action exerted by the monovalent ions was correlated to the lyotropic series of Hofmeister [11], suggesting an effect of solvent organization on the enzyme stability. In a recent work we investigated the influence of glycerol and sugars (fructose, sucrose and sorbitol) on heat-induced flavin dissociation, at temperatures ranging from 40 to 80°C [12]. Spectroscopic measurements performed on the heat-treated enzyme showed that at temperatures higher than 60°C an appreciable FAD dissociation occurred as a result of the extended conformational changes accompanying protein unfolding. Moreover, all of the polyhydric compounds examined were found to displace the dissociation equilibrium toward the biologically active holoenzyme form, probably by virtue of their ability to be preferentially excluded from the protein domain [13,14].

All these evidences clearly indicate that microenvironmental modifications attained by addition of appropriate organic compounds may affect positively the enzyme stability. However, the reason why some additives are effective at lower temperatures, as in the case of water–polymer systems, whereas some others manifest their beneficial action above 55–60°C, when the cofactor dissociation is the major pathway for the enzyme inactivation, is still obscure. Furthermore, very little is known about how properties such as activity, specificity and pH sensitivity can be influenced by the presence of an additive in the reaction medium.

In this contribution some experimental results are reported on the kinetic behaviour of *Aspergillus niger* glucose oxidase in water–polyhydric cosolvent mixtures. The main aim of the present investigation was to determine whether the catalytic properties of the enzyme were influenced by addition of a polyol and to evaluate the

effects of the reaction environment on the conformational stability of the macromolecule. Attention was focused on systems containing ethylene glycol, propylene glycol and glycerol. Both activity and heat-induced unfolding experiments were carried out. The results obtained were interpreted by a combined kinetic–thermodynamic approach to quantify the influence of the polyols on the intrinsic kinetic parameters and on the catalytically active enzyme fraction.

2. Experimental

Materials. Lyophilized glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* was obtained from Boheringer Mannheim (Germany). The claimed activity was 200 U/mg, where 1 U corresponds to the amount of enzyme that oxidizes one μmol of β-D-glucose per minute at 25°C and pH 5. Just before use the enzyme was dissolved in acetate buffer (0.1 M, pH 5.5). Crystalline β-D-glucose with an α-anomer content less than 2% was purchased from Sigma Co. (USA). Ethylene glycol, propylene glycol (1,2-propanediol) and glycerol, from Carlo Erba (Italy), were reagent grade with purities greater than 99.5 %.

Protein unfolding experiments. Glucose oxidase unfolding was investigated by UV difference spectroscopy. A detailed description of the procedure is reported elsewhere [15]. Briefly, a solution containing the enzyme and the additive (at a concentration ranging from 1 to 8 M) was prepared gravimetrically, passed through a 0.65 μm Millipore filter and transferred into the two cuvettes of a double-beam spectrophotometer (Perkin Elmer, Lambda 5). The reference cell was thermostated at 25°C, whereas the sample cell was continuously heated from 25 to 80°C by a digital temperature programmer (Haake, PG 41). Thermal unfolding curves were determined at constant wavelength (292 nm) by plotting the first derivative of the difference absorption spectrum against temperature. All runs were made at a final enzyme concentration of 4.2×10^{-6} M. Under the experimental conditions adopted, glucose oxidase unfolding was found to be completely reversible.

Kinetic experiments. Kinetic runs were carried out in a batch reactor consisting of a jacketed, magnetically stirred, pyrex vessel with an inner diameter of 2.5 cm and an overall length of 12 cm. The temperature was controlled within ±0.1°C by an external thermostat. About 40 ml of the buffer solution containing glucose and the additive (at a concentration ranging from 1 to 8 M) were poured into the reactor and allowed to mix. Then a stream of purified air was bubbled through the liquid at a flow rate of 30 Ncm^3/min. Reaction was started by adding 50 μl of the enzyme solution into the organic solvent–water mixture. Samples of the solution were periodically withdrawn, heated at 100°C for 5 min to stop the enzymatic reaction, and analysed for glucose content. This latter one was determined spectrophotometrically at 340 nm, by an enzymatic protocol making use of the enzymes hexokinase and glucose-6-phosphate dehydrogenase [16]. All runs were performed at 25°C. The glucose and the enzyme concentrations inside the reactor were 2.8×10^{-3} M

and 1.5×10^{-8} M, respectively. In order to detect possible activity changes resulting from irreversible solvent denaturation, some enzymatic solutions containing the additives at the highest concentrations used in the preceding runs (5 and 8 M) were prepared and incubated at 25°C for 3 h. Then samples of these solutions were withdrawn, diluted in buffer and poured into the batch reactor to perform kinetic measurements. After dilution, the additive concentration was less than 0.5 M.

3. Results

3.1. PROTEIN UNFOLDING EXPERIMENTS

The melting curves obtained from heat-induced unfolding experiments were very similar in shape, with a highly cooperative transition in a temperature range that was found to depend on the nature and concentration of the polyol. A typical example, relative to 5 M ethylene glycol, is shown in fig. 1a.

Glucose oxidase unfolding was assumed to follow the two-state model [17]: $F \rightleftharpoons U$, where F and U indicate the folded and unfolded protein, respectively. Spectroscopic measurements were interpreted as reported by Pace et al. [18] to determine the equilibrium constant for the above transition (K) and the melting temperature (T_m), i.e. the temperature at which the mole fractions of the species F and U are equal. The standard enthalpy change (ΔH_m^0) was calculated from the temperature dependence of the equilibrium constant, according to the van't Hoff equation (fig. 1b). The values of T_m and ΔH_m^0 were then used to evaluate the Gibbs free energy change at the various temperatures,

$$\Delta G^0(T) = \Delta H_m^0\left(1 - \frac{T}{T_m}\right) - \Delta c_P\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right], \qquad (1)$$

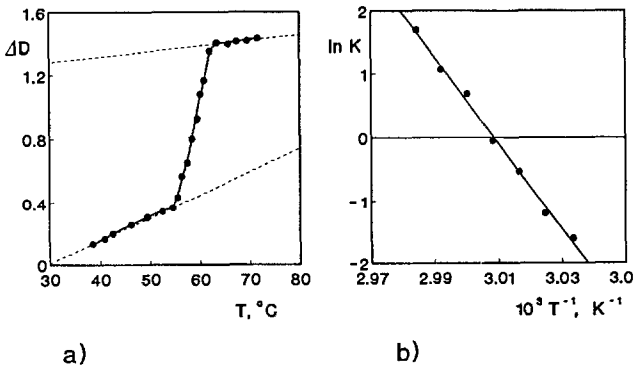

Fig. 1. Thermal transition curve (a) and van't Hoff plot (b) in 5 M ethylene glycol (ΔD is the first derivative of the difference spectrum at $\lambda = 292$ nm).

where Δc_P is the change in heat capacity between the folded and unfolded protein conformations. Once determined the function $\Delta G^0(T)$, the equilibrium constant upon unfolding can be computed, at each temperature, as

$$K(T) = \exp\left(-\frac{\Delta G^0(T)}{RT}\right). \tag{2}$$

From this equation, the folded enzyme concentration at 25°C, $[F]_{25}$, can easily be derived

$$[F]_{25} = \frac{[E_0]_{25}}{1 + K_{25}}, \tag{3}$$

where the quantity $[E_0]_{25}$ represents the total enzyme concentration at 25°C and K_{25} is the equilibrium constant upon unfolding at the same temperature. The results obtained in pure buffer and in water–polyol mixtures are listed in table 1.

3.2. KINETIC EXPERIMENTS

The influence of polyols on the kinetic behaviour of glucose oxidase was investigated by comparing the catalytic activity of the enzyme in the presence of the different organic compounds with that measured in their absence. Preliminary runs were made to analyse the enzyme kinetics in pure buffer. The results obtained showed that, up to 30 mM glucose concentration, the reaction rate was a linear function of substrate concentration. Therefore the first-order approximation was

Table 1
Thermodynamic parameters determined from thermal unfolding curves

Additive	Conc. (M)	T_m (°C)	ΔH^0_m (kcal/mol)	ΔS^0_m (cal/mol K)	ΔG^0_{25} (kcal/mol)	K_{25}
none		63.0 [a]	114 [a]	340 [a]	6.19	$2.89\,10^{-5}$
ethylene glycol	1	62.5	118	351	6.65	$1.33\,10^{-5}$
	3	60.7	133	398	8.27	$8.61\,10^{-7}$
	5	59.3	135	406	8.43	$6.56\,10^{-7}$
	8	56.9	140	424	8.45	$6.36\,10^{-7}$
propylene glycol	1	61.6	128	382	7.76	$2.04\,10^{-6}$
	3	58.7	147	443	9.61	$8.96\,10^{-8}$
	5	55.9	159	482	10.43	$2.24\,10^{-8}$
	8	51.6	168	517	10.39	$2.40\,10^{-8}$
glycerol	1	62.9	125	372	7.43	$3.55\,10^{-6}$
	3	62.0	133	397	8.31	$8.03\,10^{-7}$
	5	61.4	142	424	9.28	$1.57\,10^{-7}$
	8	60.1	158	473	10.89	$1.03\,10^{-8}$

[a] From ref. [28].

assumed for the Michaelis–Menten equation

$$(-r) = \frac{k_{cat}}{K_m}[E_0][S] = k^*[E_0][S], \tag{4}$$

where $(-r)$ is the reaction rate, k_{cat} and K_m are the Michaelis–Menten parameters, $[E_0]$ is the total enzyme concentration, [S] is the substrate concentration and k^* is the apparent first-order rate constant. The mass-balance equation for the substrate in the batch reactor yields the following relationship [19]:

$$\frac{1}{[E_0]}\ln\frac{[S]}{[S]_0} = k^* t, \tag{5}$$

where $[S]_0$ is the initial substrate concentration. A comparison between experimental and calculated results is shown in fig. 2. As apparent, the experimental kinetic data are well described by eq. (5), which can be used to estimate the first-order rate constant (k^*) from the glucose concentration decay inside the reactor. The results obtained for the different reaction media are summarized in table 2, along with the k^*-values determined after contact with 5 and 8 M cosolvent and subsequent dilution.

In order to better appreciate the influence of cosolvents on the catalytic properties of glucose oxidase, residual activities (a_R) were plotted against cosolvent concentration (fig. 3). The residual activity was defined as the ratio between the first-order rate constants in the presence and absence of additives.

4. Discussion

4.1. EFFECT OF ADDITIVES ON PROTEIN UNFOLDING

The results reported in table 1 show that, although all the additives cause a

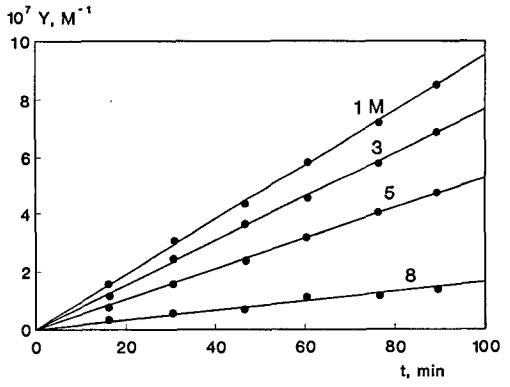

Fig. 2. Kinetic patterns in media containing 1, 3, 5 and 8 M glycerol ($Y = (1/[E_0])\ln[S]/[S]_0$).

Table 2
First-order rate constants (k^*, $10^5\ M^{-1}\ min^{-1}$) obtained from kinetic experiments. k_d^* are the same parameters determined after cosolvent contact and dilution

Additive	Conc. (M)	k^*	k_d^*
none		9.72	
ethylene glycol	1	9.23	–
	3	8.07	–
	5	6.12	9.75
	8	3.60	9.54
propylene glycol	1	9.62	–
	3	8.65	–
	5	6.99	9.64
	8	4.18	9.37
glycerol	1	9.52	–
	3	7.68	–
	5	5.25	9.55
	8	1.65	9.68

decrease in the melting temperature, ranging from 0.1°C (in 1 M glycerol) to 11.4°C (in 8 M propylene glycol), at 25°C glucose oxidase is more stable than in pure buffer. The displacement of the unfolding equilibrium toward the folded protein is reflected in the values of ΔG^0 at 25°C, which are higher than that in pure buffer: the greatest stabilization occurs in 8 M glycerol, where the free energy change increases from 6.19 to 10.89 kcal/mol.

The influence of cosolvents on glucose oxidase stability over the whole temperature range can be visualized by analysing the trend of the stability curves [20], which are defined by eq. (1). Fig. 4 shows a plot of the function $\Delta G^0(T)$ for media containing propylene glycol. Free energy changes were calculated using a Δc_P value of

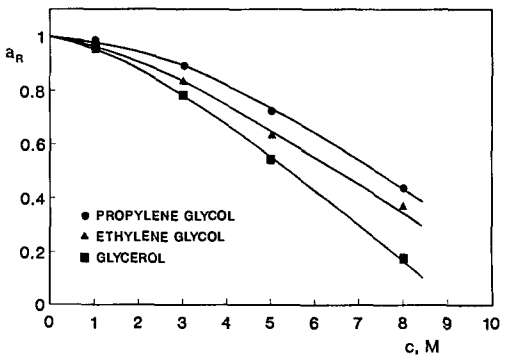

Fig. 3. Residual activity (a_R) versus cosolvent concentration (c) in the presence of polyhydric additives.

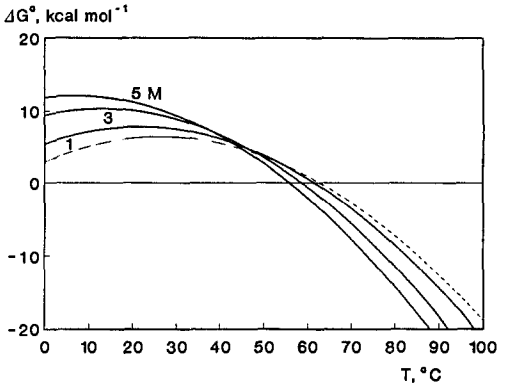

Fig. 4. Protein stability curves in media containing 1, 3 and 5 M propylene glycol. The dashed line is relative to pure buffer.

3 kcal/mol K [21]. Similar results were obtained in the presence of ethylene glycol and glycerol. As can be seen, additions of propylene glycol modify the stability curve, causing a shift of the maximum toward higher ΔG^0 values and lower temperatures. Moreover, below a given temperature (close to 40°C) glucose oxidase becomes more stable than in pure buffer.

It may be interesting to provide a physical interpretation of the observed behaviour. Turning attention to the enthalpy changes given in table 1, we note that glucose oxidase unfolding is an endothermic process. Furthermore, additions of polyols increase the ΔH^0_m value attained in pure buffer, making the F $\rightleftharpoons$ U transition even more endothermic and favouring the folded form as the temperature is decreased. The ability of polyhydric cosolvents to increase the enthalpy change upon unfolding also indicates that the enthalpy of transfer of the denatured protein from pure buffer to buffer–polyol mixtures is larger than that of the native protein. If we consider that the enthalpies of transfer of nonpolar residues from water to aqueous polyols are positive [22], it follows that the stabilization of glucose oxidase in the presence of polyols is probably due to the strengthening of the intramolecular hydrophobic interactions [23]. At high temperatures, however, the positive enthalpic contribution associated with protein unfolding is offset by a considerable entropy change (see the ΔS^0_m values listed in table 1). In other words, at higher temperatures (approximately > 40–45°C) the additives seem incapable of inhibiting the disorder of the system accompanying thermal denaturation, and this gives rise to a decreased melting temperature. At lower temperatures (approximately < 40–45°C) the situation is reversed, leading to glucose oxidase stabilization. Finally, at temperatures close to 40°C the increases in the enthalpy and entropy changes resulting from the addition of polyols give nearly comparable contributions to the free energy change: under these conditions the presence of additives has only a limited influence on glucose oxidase stability.

The last important point to be stressed is concerned with the close crossover tem-

peratures observed for the three polyols: such occurrence is probably ascribable to the structural homologies of these compounds, reflected in the similarity of their physico-chemical properties.

4.2. EFFECT OF ADDITIVES ON ENZYME ACTIVITY

The results summarized in table 2 and the residual activity plots depicted in fig. 3 clearly indicate that all the additives cause a reduction of glucose oxidase activity. This quantity decreases monotonously as the additive concentration is increased, and the detrimental action exerted by the different polyols varies according to the following order: propylene glycol < ethylene glycol < glycerol. At the lowest cosolvent concentration (1 M) the effects are very limited ($a_R > 95\%$). In media containing 3 M cosolvent, the residual activity varies between 79 and 89%. In the presence of 5 M cosolvent, these values lower to 54 and 72%, respectively. Finally, at the highest concentration investigated (8 M), the residual activity ranges between 17 and 43%. In table 2 the first-order rate constants determined after contact with 5 and 8 M cosolvent and subsequent dilution are also reported. As can be seen, no irreversible activity reduction occurs upon addition of polyols, suggesting that the observed activity loss is not ascribable to irreversible denaturation phenomena induced by the organic solvents. This is in agreement with the results obtained by other authors, and in particular by Ulbrich-Hofmann and Selisko [24], who investigated the influence of several water-miscible organic solvents on the activity of invertase and glucoamylase. These authors found a significant irreversible inactivation in the presence of methanol, ethanol and other additives, whereas no appreciable effect was observed when ethylene glycol and glycerol were added to the enzyme solution.

We must therefore assume that the observed reductions in activity are a consequence of modifications of the intrinsic catalytic parameters or of reversible changes in the active site configuration induced by the organic compounds. Of course these two phenomena could manifest themselves simultaneously. Referring to the second cause of inactivation, it should be noted that glucose oxidase denaturation by organic solvents is believed to originate from the solvent-mediated weakening of the non-covalent bonds that stabilize the holoenzyme complex [5,7]. Upon exposure to sufficiently adverse environmental conditions, this process may give rise to conformational alterations of the active site region and lead to a reversible activity loss. In a previous work we showed that additives hindering glucose oxidase unfolding also reduced the extent of flavin dissociation [12]. If we examine the stability behaviour of the enzyme at 25°C, we notice that in the presence of polyols the concentration of the folded protein is higher than in pure buffer (see table 1 and eq. (3)). Accordingly, we must admit that these compounds are also capable to protect the active site region or, at the most, that they do not cause any appreciable change in its configuration.

All these considerations appear to indicate that the catalytic behaviour of glu-

cose oxidase in water–polyol mixtures is purely a consequence of solvent inhibition. This result is in agreement with those found for other enzymes in similar reaction media [24–26]. Unfortunately, we cannot determine the effect of organic solvents on the individual Michaelis–Menten parameters, k_{cat} and K_m, since experiments were carried out at low substrate concentrations ([S] $\ll K_m$), where the only parameter attainable is k_{cat}/K_m. However, if we look at the polarity of the various cosolvents, expressed for instance by the dielectric constant or the partition coefficient between octanol and water (table 3), we notice that the residual activity decreases as the cosolvent polarity increases. This suggests that the observed reductions in activity could be, at least partially, due to electrostatic interactions between the additive molecules and some polar area in the active site region. These zones would be easily accessible to the low molecular weight cosolvents used, which could penetrate into the active site cavity without appreciable steric hindrances, thus inhibiting the enzyme activity. In the course of the reaction, the resulting competition between the substrate and the organic solvent would lead to an increase of K_m. This occurrence could, at least partially, explain the decrease in the first-order rate constant observed in the presence of polyhydric cosolvents.

5. Conclusions

The results emerging from the present investigation clearly attest that at low temperatures and up to 8 M concentrations, the polyhydric additives used do not decrease the conformational stability of glucose oxidase. Since under these conditions the integrity of the active site region is likely to be unimpaired, the observed activity losses seem to be consequent on variations of the intrinsic catalytic parameters due to cosolvent inhibition. This is particularly apparent in media containing glycerol, which was found to exert the most detrimental action on the enzyme activity. On the other hand, the stabilization of the macromolecule ensuing from addition of polyols supports the possibility to use these compounds for reinforcing the three-dimensional structure of glucose oxidase in the presence of non-thermal inactivating agents, as an alternative to strategies like immobilization or chemical modifications [27].

Table 3
Physico-chemical properties of pure compounds [a]

Compound	MW	ρ (mol/ℓ)	ε	log P
water	18	55.5	78.6	−1.38
ethylene glycol	62	17.9	37.7	−1.90
propylene glycol	76	13.6	32.0	−1.38
glycerol	92	13.7	42.5	−3.03

[a] MW: molecular weight; ρ: molar density at 25°C; ε: dielectric constant at 25°C; P: partition coefficient between octanol and water.

To conclude, the effects of the various additives on the enzyme activity and stability do not behave in a parallel way, suggesting a different sensitivity of the catalytic parameters and the active enzyme conformation to the reaction environment. At the present, the interdependence of these effects is not sufficiently understood, preventing the possibility to predict the enzyme behaviour in the absence of extensive experimental efforts.

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