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A kinetic study of soluble glucose oxidase using a rotating-disc electrode

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Abstract
In order to determine the kinetic parameters of glucose oxidation catalysed by the enzyme glucose oxidase (GO) the initial velocity of hydrogen peroxide formation was measured using a rotating disc electrode (RDE). The major advantage of this method is the possibility of continuous measurement of the increase in hydrogen peroxide concentration. This means that the real initial reaction rate \( v_0 \) can be determined, which is required for constructing a double-reciprocal plot. Several combinations of substrate concentrations (glucose and oxygen) were used. The method, in which a platinum black RDE was used, appeared to be very useful. Product inhibition experiments showed that the ping-pong mechanism is valid for GO. The three kinetic parameters of this mechanism were determined by initial velocity experiments.

1. Introduction
The enzyme glucose oxidase (GO) from *Aspergillus niger* catalyses the glucose oxidation in the presence of oxygen. A commonly accepted mechanism for this reaction is the ping-pong mechanism [1]:

\[
\beta-D-glucose + GO_{ox} \xrightarrow{k_1} GO_{red} + \text{gluconolactone}
\]

\[
GO_{red} + O_2 \xrightarrow{k_2} GO_{ox} \cdot H_2O_2
\]

\[
GO_{ox} \cdot H_2O_2 \xrightarrow{k_3} GO_{ox} + H_2O_2
\]

With the formation of hydrogen peroxide as the rate-determining step the following equation can be derived for steady state conditions [2].

\[

1 - \frac{1}{V_{max}} \cdot \frac{K_m(o)}{C_o} \cdot \frac{K_m(g)}{C_g}
\]

where \( v_0 \) M min\(^{-1}\) is the initial velocity of product formation, \( V_{max} \) M min\(^{-1}\) is the maximum initial velocity, \( K_m(o)/M \) and \( K_m(g)/M \) are the Michaelis constants for oxygen and glucose respectively, and \( C_o/M \) and \( C_g/M \) are the initial concentrations of these species. Furthermore,

\[
V_{max} = k_3 E_T
\]

\[
K_m(o) = \frac{k_3}{k_2}\]

where \( E_T/M \) is the total amount of enzyme present.

A primary plot for this mechanism can be obtained by plotting the reciprocal initial velocity versus the reciprocal initial oxygen concentration at a constant glucose concentration. A set of parallel lines is obtained, each corresponding to a fixed glucose concentration. A secondary plot can be deduced by plotting the intercepts of the primary plot as a function of the reciprocal glucose concentration. All three kinetic parameters, i.e. \( V_{max}, K_m(o) \) and \( K_m(g) \), are determined in this way. The major problem is measurement of the initial reaction rate \( v_0 \). Measurements should be performed under pseudo-steady-state conditions, i.e. the concentration of glucose and oxygen should remain almost constant. The electrochemical technique using a rotating-disc electrode (RDE) is suitable for this purpose.

2. Theory
The initial velocity can be determined by measuring the concentration of hydrogen peroxide as a function of time after addition of the enzyme GO. As hydrogen peroxide is an electrochemically active species an RDE is used. Levich [3] deduced a simple equation for the measured diffusion-controlled current \( I_d/A \) as a function of the concentration \( c_b/M \) of the electroactive
species in the bulk of the solution and the angular velocity \( \omega \)/rad s\(^{-1}\) of the RDE:

\[
I_d = 0.62 nF C_o D^{2/3} \nu^{-1/6} \omega^{1/2}
\]

(6)

where \( n \) is the number of electrons involved in the electrode reaction, \( F/C \) is the Faraday constant, i.e., the charge on 1 mole of electrons, \( A_e/m^2 \) is the geometric electrode area, \( D/m^2 s^{-1} \) is the diffusion coefficient of the electroactive species and \( \nu/m^2 s^{-1} \) is the kinematic viscosity of the solution.

Equation (6) is only valid for diffusion-controlled conditions, i.e. the applied potential is high enough to lower the concentration of the electroactive species at the electrode surface to virtually zero. If \( I_d \) is plotted versus \( \omega^{1/2} \) a straight line is obtained, from the slope of which \( D [4] \) or \( c_0 [5] \) can be calculated.

In the case of glucose oxidation both the concentration of the electroactive product hydrogen peroxide in the bulk solution and \( I_d \) will increase linearly with time at a fixed \( \omega \). The plot of \( I_d \) versus time (and hence hydrogen peroxide concentration versus time) is called a progress curve. The initial velocity \( v_0 \) can be calculated from the initial slope of the curve using the Levich equation. After some minutes the progress curve starts to show a clear deviation from linear behaviour, as a result of product inhibition, significant substrate consumption and/or enzyme instabilization. As this stage the pseudo-steady-state condition no longer exists. As the RDE technique is able to measure the product formation continuously, it is possible to calculate the initial pseudo-steady-state reaction rate.

3. Experimental

3.1. Reagents

Glucose oxidase from Aspergillus niger (E.C. 1.1.3.4, \( M = 150,000 \), lyophil, GO/catalse min 2000) was purchased from Serva. Phosphate-buffered saline (PBS) was prepared using NaH\(_2\)PO\(_4\) \( \cdot \) 2H\(_2\)O, Na\(_2\)HPO\(_4\) \( \cdot \) 12H\(_2\)O and NaCl purchased from Merck. \( d \)-glucose monohydrate was obtained from Jannsen Chimica. Product inhibition experiments were carried out using hydrogen peroxide (30% (w/w) aqueous solution) from Chempro Pack and gluconolactone from Sigma.

Platinum black electrodes were prepared using a solution of \( H_2PtCl_6 \cdot 6H_2O \) from H. Drijfhout and Sons and PbCl\(_2\) from Merck.

All solutions were prepared using demineralized distilled water.

3.2. Preparation of a platinum black electrode

A polished platinum RDE was scanned from -1500 mV to +1500 mV (vs. a saturated calomel electrode (SCE)) at a rate of 1 V s\(^{-1}\) in a 2 M H\(_2\)SO\(_4\) solution to remove all impurities from the electrode. The electrode was immersed in a 3% (w/w) H\(_2\)PtCl\(_6\) solution (with 0.02% (w/w) PbCl\(_2\)) and connected as the cathode with a platinum sheet as the anode. A current of about 5 mA was used to deposit a platinum black layer on the platinum RDE over a period of 10 min.

The platinum black electrode (platinized electrode) was then washed with running tap water for at least 30 minutes and with distilled demineralized water for a further 5 minutes.

3.3. Preparation of the enzyme stock solution

A 0.065 M acetic acid buffer (pH 4.4) was prepared by mixing 1.525 ml of a 4 M acetic acid solution and 0.53 g of CH\(_3\)COONa \( \cdot \) 3H\(_2\)O and diluting to 100 ml with distilled demineralized water.

Approximately 3 mg of the lyophilized enzyme was weighed exactly and dissolved in 10 ml of the acetate buffer. The solution was homogenized in a ultrasonic bath (Struers) for 5 minutes.

3.4. Preparation of the glucose solutions

Glucose-containing PBS was prepared by adding the appropriate amount of glucose to 9.22 g NaCl (0.16 mol), 17.8 g Na\(_2\)HPO\(_4\) \( \cdot \) 12H\(_2\)O (0.050 mol) and 8.00 g NaH\(_2\)PO\(_4\) \( \cdot \) 2H\(_2\)O (0.050 mol), and diluting to 1000 ml with distilled demineralized water. The pH was adjusted to either 7.0 or 7.4 with 4 M NaOH. The glucose solutions were allowed to mutarotate for at least 3 hours before use.

3.5. Measuring the variation of the hydrogen peroxide concentration with time

A platinum black RDE (\( A_e = 0.50 \times 10^{-4} m^2 \)) was used as the working electrode in all experiments. A platinum counter-electrode with a surface area of 5 \( \times \) 10\(^{-4} m^2 \) and an SCE with a Luggin capillary were placed in the single-compartment cell. A circulating water-bath (Colora NB-32981) was used to keep the temperature constant (25°C or 37°C). PBS (0.050 M NaH\(_2\)PO\(_4\) + 0.050 M Na\(_2\)HPO\(_4\) + 0.16 M NaCl, pH 7.0 or 7.4) was used as the supporting electrolyte. Glucose concentrations varied from 0 to 0.5 M. A Wenking POS 73 potentiostat, equipped with a digital multimeter (Fluke 8600 A) and a Motomatic E-550-M stirring motor, was used to carry out the RDE measurements. The rotation rate was chosen to be 2 rev s\(^{-1}\) (12.6 rad s\(^{-1}\)).

Before the enzyme was added and the variation of the hydrogen peroxide concentration with time was measured, the glucose solution was allowed to reach a constant temperature. The solution was saturated with argon. The diffusion-controlled background current of oxygen reduction was measured at -580 mV/SCE. Thereafter, the solution was saturated with oxygen or a nitrogen + oxygen mixture for at least 30 min while leaving the applied potential unchanged. In this way
the diffusion-controlled current for oxygen reduction could be determined and the oxygen concentration in the bulk solution calculated.

During experiments the appropriate gas was passed over the saturated solution, because bubbling through the solution would disturb the hydrodynamic profile created by the RDE.

After measurement of the oxygen concentration, the potential was changed to +700 mV/SCE and the diffusion-controlled background current of hydrogen peroxide oxidation was allowed to reach a steady value. An aliquot of the GO stock solution was pipetted into the solution (final concentration 1.12 mg l⁻¹) which was stirred vigorously for a few seconds, with the potential kept at +700 mV/SCE. Immediately after adding the enzyme the increase in hydrogen peroxide concentration was measured. The variation with time was recorded on a Kipp and Zonen x-t recorder (BD40) and followed until a clear deviation from linearity was observed.

For gluconolactone inhibition experiments the glucose solution also contained the required gluconolactone concentration. For hydrogen peroxide inhibition experiments an aliquot of a hydrogen peroxide stock solution was added after measuring the background current. After determination of this basal hydrogen peroxide level, GO was added and the variation with time was recorded.

4. Results and discussion

4.1. Measurements of progress curves

Before adding GO to a particular glucose solution the oxygen concentration was measured using the RDE. The solubility of oxygen appears to be affected by the glucose concentration [5]. The progress curve was then measured and it remained almost linear for several minutes. The exact duration of this pseudo-steady state varied slightly. At a fixed oxygen concentration, the pseudo-steady-state time is shorter at higher glucose concentrations (Fig. 1). Owing to a higher reaction rate at high glucose concentrations, significant consumption of oxygen occurs earlier. Furthermore, progress curves start to show deviation from linearity sooner when, at a fixed glucose concentration, the oxygen concentration is set at a low value.

The fact that the pseudo-steady state time is not reproducible indicates that kinetic experiments, performed by taking a sample after a fixed time interval, can be very risky. The chance of measuring a sample outside pseudo-steady-state conditions is considerably large. However, taking a sample within a very short time to ensure pseudo-steady-state conditions can cause difficulties with analysis as only a small amount of hydrogen peroxide has been formed.

The initial reaction rate for a fixed glucose and oxygen concentration was calculated from the initial slope of the progress curve using the Levich relation. Two electrons are transferred per mole of hydrogen peroxide (n = 2). The rotation speed was chosen to be 2 rev s⁻¹ (12.6 rad s⁻¹) and the electrode had a surface area of 0.50 × 10⁻⁴ m². Use of the Levich relation requires knowledge of the kinematic viscosity of the solution and the diffusion coefficient of the electroactive species. As the kinematic viscosity starts to increase significantly and the diffusion coefficient starts to decrease at high glucose concentrations (c₆ ≥ 100 mM), it was necessary to determine these parameters for solutions with a high glucose concentration [5]. Figure 1 can now serve as a calculation example. The initial slope of curve A (c₆ = 20 mM, c₈ = 1 mM) is equal to 0.0216 mA min⁻¹. For a kinematic viscosity of 0.95 × 10⁻⁶ m² s⁻¹ and a hydrogen peroxide diffusion...
TABLE 1
Primary plot and product inhibition behaviour for various BiBi mechanisms

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Primary plot</th>
<th>Product inhibition behaviour</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G vs. L</td>
<td>G vs. H</td>
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<tr>
<td>Random sequential</td>
<td>C</td>
<td>C</td>
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<tr>
<td>Ordered sequential</td>
<td>N</td>
<td>C</td>
</tr>
<tr>
<td>Ping-Pong</td>
<td>N</td>
<td>C</td>
</tr>
</tbody>
</table>

G, glucose; L, gluconolactone; O, oxygen; H, hydrogen peroxide; C, competitive inhibition; N, non-competitive inhibition.

Coefficient of $1.43 \times 10^{-9}$ m$^2$ s$^{-1}$, the initial increase $v_0$ in the hydrogen peroxide concentration with time is calculated to be 0.078 mM min$^{-1}$. Curve B is a progress curve for $c_g = 100$ mM and $c_o = 1$ mM. The initial slope of this curve is equal to 0.358 mA min$^{-1}$. For a kinematic viscosity of $0.98 \times 10^{-6}$ m$^2$ s$^{-1}$ and a hydrogen peroxide diffusion coefficient of $1.39 \times 10^{-9}$ m$^2$ s$^{-1}$, $v_0$ is calculated to be 0.135 mM min$^{-1}$.

Both initial velocities were used to construct the primary plot for $T = 25^\circ C$ and pH 7.0 (see below).

In this study a platinum black electrode was used to measure the progress curves. The advantages of this electrode are its high catalytic activity and its resistance to poisoning. A polished platinum electrode is easily poisoned by organic substances. However, the disadvantage is the long “lag” period before a steady background current is reached. This period can be as long as 1 h.

To optimize the RDE procedure, the choice of electrode material can be varied. Nowadays, research is carried out on new electrode materials for hydrogen peroxide detection. Carbon electrodes have the disadvantage of high overvoltages [6] and gold electrodes do not show hydrogen peroxide oxidation at pH $< 9$ [7]. Ti/IrO$_2$ electrodes seem to give reproducible results and show high stability [8].

4.2 Product inhibition behaviour

To verify the assumption of a ping-pong mechanism the product inhibition behaviour was studied. According to this mechanism hydrogen peroxide is a competi-

![Fig. 2. Product inhibition behaviour of gluconolactone versus glucose. The intersection of the double reciprocal lines on the negative abscissa indicates non-competitive inhibition. The experiments were performed at $T = 25^\circ C$ and pH 7.0. The initial oxygen concentration was 1.1 mM. The initial gluconolactone concentrations were 0 mM (+), 50 mM (○) and 100 mM (▲). All points indicated were calculated from an initial slope of a time dependence as shown in Fig. 1.](image)

![Fig. 3. Product inhibition behaviour of gluconolactone versus oxygen. The intersection of the double reciprocal lines on the ordinate indicates competitive inhibition. The experiments are performed at $T = 25^\circ C$ and pH 7.0. The initial glucose concentration used was 20 mM. The initial gluconolactone concentrations used were 0 mM (+), 10 mM (○) and 200 mM (▲).](image)
tive inhibitor for glucose and a non-competitive inhibitor for oxygen. Gluconolactone is a competitive inhibitor for oxygen and a non-competitive inhibitor for glucose [2]. Other possible mechanisms for the oxidation of glucose in the presence of GO are shown in Table 1 together with their product inhibition properties [2].

Only the ping-pong mechanism produces parallel lines when a double-reciprocal plot is constructed from initial velocity experiments. However, it is sometimes difficult to demonstrate parallelism as there is always the possibility of an intersection in the far third quadrant. Therefore it is worth examining the product inhibition behaviour. In this work, three inhibition cases were studied: gluconolactone versus glucose, gluconolactone versus oxygen and hydrogen peroxide versus oxygen (Figs. 2, 3 and 4). Competitive inhibition is shown by intersection of the double-reciprocal lines on the ordinate. Non-competitive inhibition is indicated by intersection on the negative abscissa. The product inhibition behaviour shown in Figs. 2–4, together with parallel lines in the primary double-reciprocal plots, indicates a ping-pong mechanism, or at least a very close approximation. Each point in Figs. 2–4 was calculated from a separate measurement of a progress curve.

4.3. The kinetic parameters of GO-catalysed glucose oxidation at \( T = 25^\circ C \) and pH 7.0

Hydrogen peroxide production was examined for several combinations of substrate concentrations at the standard conditions of \( T = 25^\circ C \) and pH 7.0. Figure 5 shows the results of all measurements. A set of parallel lines is obtained. However, at \( c_g \geq 100 \text{ mM} \), the well-known phenomenon of substrate inhibition [9] is observed, i.e. the reduced enzyme complexes with glucose which makes glucose a competitive inhibitor for oxygen. The mechanism allows gluconolactone to bind the reduced enzyme, but as glucose is incorporated into gluconolactone, it is possible that glucose binds the reduced enzyme at the gluconolactone site. Owing to substrate inhibition the lines for \( c_g = 100 \text{ mM} \) and \( c_g = 500 \text{ mM} \) exhibit a different slope to that obtained for lower glucose concentrations, but the intercepts i.e. [Fig. 5] can be used to construct the secondary plot, which is represented by the equation

\[
\mathrm{i.e. \ [fig. \ 5] = 2.38 + 167c_g^{-1}}
\]

which has a regression coefficient of 0.9993.
The intercept of the secondary plot (eqn. (7)) gives the reciprocal of the maximum velocity $V_{\text{max}}$. This is related to an infinite concentration of glucose as well as oxygen and has a value of 0.42 mM min$^{-1}$. As the GO concentration is chosen to be 1.12 mg l$^{-1}$, $k_3$ is calculated to be $3.74 \times 10^{-4}$ mol (mg enzyme)$^{-1}$ min$^{-1}$ or 935 s$^{-1}$ (turnover number). The Michaelis constants $K_m(o)$ and $K_m(g)$ are 0.80 mM and 70 mM respectively. These values indicate that the glucose concentration determines the reaction rate almost completely.

4.4. The kinetic parameters of GO-catalysed glucose oxidation at $T = 37^\circ C$ and pH 7.4

In the same way as for $T = 25^\circ C$ and pH 7.0, primary and secondary plots can be constructed from measurements of the rate of hydrogen peroxide production at $T = 37^\circ C$ and pH 7.4 (Fig. 6). These physiological conditions are chosen because GO is often used for medical purposes. Again, the ping-pong mechanism seems to be obeyed, as a set of parallel lines is obtained. Equation (8) represents the secondary plot derived from the intercepts i.e. [fig. 6] of Fig. 6 and has a regression coefficient of 0.9974:

$$i.c \ [\text{fig. 6}] = 1.96 + 138c_o^{-1} \tag{8}$$

$V_{\text{max}}$ has a value of 0.51 mM min$^{-1}$ for infinite concentrations of glucose as well as oxygen. The catalytic constant $k_3$ is equal to $4.56 \times 10^{-4}$ mol (mg enzyme)$^{-1}$ min$^{-1}$ or 1140 mol s$^{-1}$ (mol enzyme)$^{-1}$. The Michaelis constants $K_m(o)$ and $K_m(g)$ in this case are determined to be 2.4 mM and 70 mM respectively.

Again, substrate inhibition is observed at a glucose concentration above 100 mM. The temperature change from 25°C to 37°C combined with the pH change from 7.0 to 7.4 seems to have no effect on $K_m(g)$, and $V_{\text{max}}$ only changes slightly. The only parameter that changes significantly is $K_m(o)$. Gibson et al. [1] observed a change in $V_{\text{max}}$ and $K_m(o)$ with temperature, but not in $K_m(g)$.

5. Conclusions

The RDE method appears to be very useful for determining the kinetic parameters of GO-catalysed oxidation of glucose. The continuous measurement of the variation of hydrogen peroxide concentration with time makes it possible to determine the initial velocity. The method is also applicable to other (enzymatic) reactions, provided that one of the products is electrochemically active. If one of the substrates is electroactive, its consumption can be recorded as a function of time. In either case, there should be no interference from any of the other compounds present.

To optimize the procedure in the case of hydrogen peroxide detection, another electrode material can be chosen.

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