Insolubilized Enzymes

KINETIC BEHAVIOUR OF GLUCOSE OXIDASE BOUND TO POROUS GLASS PARTICLES

By MICHAEL K. WEIBEL AND HAROLD J. BRIGHT

*Department of Biochemistry, School of Medicine, University of Pennsylvania,
 Philadelphia, Pa. 19104, U.S.A.*

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1. The spectrophotometric and steady-state kinetic properties of glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*) that is covalently linked to porous glass beads have been examined. These properties have been compared with those of soluble glucose oxidase, for which the kinetic mechanism at pH 5.5 and 25°C has been established previously by a combination of conventional and rapid-reaction techniques to be the following:

where E_o and E_r represent oxidized and reduced forms of the enzyme, respectively. 2. The ratio k_{+4}/k_{+2} is unchanged after insolubilization, and evidence is presented which suggests that the absolute magnitudes of k_{+4} and k_{+2} are unchanged. 3. The kinetic efficiency of the insolubilized enzyme is greatly enhanced because of a 14-fold increase in the apparent affinity of glucose for E_o . This effect is attributed either to the binding of glucose to the glass surface or to a change in enzyme structure imposed by the insolubilization process. 4. Only 6% of the insolubilized enzyme which can be reduced by glucose is catalytically active. It is shown by calculation and direct experimental evidence that this fraction of catalytically active enzyme is bound to the exterior bead surface. The remaining 94% of the enzyme is bound within the pore network and may be subject to severe substrate diffusion control.

During the past decade considerable information has accumulated concerning the association of soluble enzymes with solid matrices. The potential of such systems, both as models for membrane-bound enzymes and in numerous practical applications, is considerable. A major difficulty that has hindered progress in the understanding of the properties of insolubilized enzymes has been the lack of precise information concerning the number of types, and the concentrations, of catalytically active species of the insolubilized molecule. Further, in addition to the complications of diffusion and other problems inherent to heterogeneous catalysis, the determination of changes in the physical and chemical properties of the enzyme after insolubilization is complicated by the fact that the insoluble system is often inhomogeneous on a macroscopic level. Because of these factors, we considered it worthwhile to study in insolubilized form an enzyme which is well characterized mechanistically in homogenous solution, and for which an optical determination of bound active enzyme could be devised.

Glucose oxidase (EC 1.1.3.4) catalyses the oxidation of β -D-glucose and other aldoses by O_2 to the corresponding lactone according to eqn. (1):

The kinetic mechanism of the glucose oxidase reaction has been thoroughly studied by a combination of rapid-reaction and conventional techniques (Gibson, Swoboda & Massey, 1964; Bright & Gibson, 1967; Nakamura & Ogura, 1967; Duke, Weibel, Page, Bulgrin & Luthy, 1969; Bright & Appleby, 1969; Weibel & Bright, 1971) and the important kinetic steps contributing to turnover with glucose as a substrate at pH 5.5 and 25°C are given in eqns. (2) and (3).

The enzyme has a molecular weight of 180 000 and contains two non-dissociable FAD molecules

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which undergo large spectral changes in the visible region during catalysis. Although there is a variety of established methods for insolubilizing glucose oxidase (Updike & Hicks, 1966; Pennington, Brown, Patel & Chattopadhyay, 1968; Weliky & Weetall, 1965; Selegny, Avrameas, Broun & Thomas, 1968), we have chosen to study the system in which the enzyme is covalently linked to porous glass beads.

We present kinetic and spectrophotometric methods for determining the effective concentration of catalytically active and substrate-reducible glucose oxidase that is covalently bound to porous glass beads. By using the evidence from spectrophotometric measurements and turnover kinetics, we find that 94% of the enzyme that can be reduced by substrate does not contribute significantly to turnover. The minor component of the bound enzyme, which can be reduced by substrate and which comprises 6% of the total, appears kinetically normal with the exception that the apparent binding constant for glucose is greatly enhanced.

MATERIALS

Glucose oxidase, covalently linked to silane-treated 40–60-mesh glass beads containing pores approx. 550 Å in diameter, was obtained from the Corning Glass Works, Corning, N.Y., U.S.A. These beads have a surface area of approx. 100 m²/g. The insolubilization procedure has been reported previously (Weetall, 1969; Weetall & Baum, 1970). The enzyme preparation (analytical grade, from *Aspergillus niger*) used for insolubilization and for studies of the soluble enzyme was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Anhydrous D-glucose was obtained from Pfanstiehl Laboratories, Waukegan, Ill., U.S.A. Glucose solutions were allowed to mutarotate to equilibrium for 24 h at 25°C and concentrations are expressed in terms of the β -D anomer.

All solutions were prepared with deionized glass-distilled water. The buffer solution used in the kinetic and spectral assays was 0.10 M-potassium acetate, pH 5.5, containing 0.5 mM-EDTA. Slurries of insolubilized enzyme were also prepared in this buffer. Since the enzyme preparation contained trace amounts of catalase, 0.1 mM-KCN was added to the oxygen-electrode-chamber solution for kinetic studies.

METHODS

Spectrophotometric determination of enzyme concentration. Enzyme concentration is expressed as the molarity of glucose-reducible E-FAD. The value of $\Delta\epsilon_{450}$ for oxidized-minus-reduced enzyme was taken to be 13.1 mM⁻¹·cm⁻¹ (Duke *et al.* 1969) and the procedure for determining the concentration of soluble glucose oxidase has been described (Weibel & Bright, 1971).

Spectrophotometric measurements of the concentration of insolubilized enzyme in packed bead beds were carried out with the Aminco-Chance dual-wavelength spectro-

photometer. A slurry of the insolubilized enzyme was gravity-packed to approx. 90% of the capacity of a 2 mm-path-length quartz cuvette. The beads were then further packed to constant volume by gently tapping the sides of the cuvette. As expected from the relative size uniformity of the beads, this packing procedure was reproducible. The quartz cell was heavily masked with black tape to decrease light scattering and presented an optical cross section of 0.5 cm² to the incident light beam. After packing, the cell was filled to the top with buffer and positioned in the spectrophotometer. The cuvette was thermostatically controlled at 25°C and after a stable reference signal was obtained, 10 μ l of a 2.0 M-glucose solution was carefully layered on the solution at the top of the cell. This addition produced a convex meniscus which extended slightly above the top of the cell. A small piece of parafilm was then used to seal the cell with no entrapped air bubbles. This procedure effectively prevented O₂ diffusion into the cuvette. As the glucose layer slowly settled and diffused throughout the bead bed, all of the O₂ was consumed. The extinction change resulting from enzyme becoming reduced in the light-path was followed on a strip chart recorder until a stable final reading was obtained. By using the dual-wavelength spectrophotometer, high incident light-intensities could be achieved. Even though much of the light was scattered by the bead bed, favourable signal/noise ratios could be achieved. In addition, this instrument will compensate for any gross refractive-index changes occurring in the light-path due to a highly concentrated glucose solution entering the bead bed. The reference wavelength used for this purpose was 510 nm, where neither reduced nor oxidized FAD absorbs, and the measuring wavelength was 450 nm. The slit width in all experiments was maintained at 1 mm. It was assumed that no significant spectral perturbations of enzyme-bound FAD occur on attaching the protein to glass.

After the spectrophotometric determination on the insolubilized enzyme had been made, the volume of the packed bead bed was measured. The beads were then quantitatively washed into a volumetrically calibrated glass cylinder and rinsed several times with buffer to remove both substrate and products. It was found that the glass beads could be uniformly suspended by rapid stirring in 10–15 ml of buffer with a small magnetic stir bar. Small known samples of this suspension were then transferred to the oxygen-electrode cell chamber for kinetic measurement. This was achieved by removing the capillary tip of a standard 1 ml graduated pipette and by using a small rubber bulb to rapidly fill a 0.4–0.6 ml portion of the pipette. The interior diameter of this pipette was at least 10 times the diameter of the beads. The beads were then washed into the cell chamber. This transfer procedure was calibrated by suspending a gravity-packed volume of 1.66 ml of glass beads in a total volume of 10.0 ml. The calculated volume of the beads transferred should be 0.166 ml/ml of sample. An average of six transfers yielded a measured gravity-packed bead volume of 0.166 ml/ml of sample with a standard deviation of 6.5%. With care, this technique allows quantitative sample transfer and dilution of the insolubilized enzyme.

To correct for absorption of scattered light in the cuvette packed with beads, a spectrophotometric deter-

mination of soluble glucose oxidase in the presence of porous glass beads was made. Light-scattering is a direct result of the inhomogeneous refractive medium comprising the particle bed. Scattering due to the enzyme in and around the particle bed at moderate protein concentrations is insignificant relative to that of the porous glass particles themselves. The bead bed was packed as before except that the glass beads contained no bound glucose oxidase and were packed in a solution of 0.1 mM-soluble glucose oxidase. Sufficient free liquid volume was left in the top portion of the cuvette so that the addition of 10 μ l of 2.0 M-glucose would not significantly perturb the concentration of soluble glucose oxidase in the cell. After the reaction was complete, the supernatant glucose oxidase solution above the bead bed was removed and the remaining volume of the bead bed plus soluble glucose oxidase was measured. The soluble glucose oxidase was then rinsed with the beads into a measured volume that was large compared with the initial volume contribution of the beads to the combined bed volume. Samples of this solution were examined for glucose oxidase enzyme activity and were compared with the standard activity of a soluble glucose oxidase preparation of known concentration. Entrapment or physical adsorption of soluble glucose oxidase by the porous glass beads, which would lower the concentration of free glucose oxidase available for the kinetic assay, was minimized by using high enzyme concentrations. This adsorption phenomenon has been previously reported for proteins on non-silane-treated porous glass (Messing, Weisz & Baum, 1969). In our experiments, the activity of washed beads that had been exposed to a high concentration of soluble glucose oxidase was negligible relative to that of the supernatant and no significant decrease in soluble enzyme concentration was noted.

The concentration of soluble glucose oxidase in the packed bead bed as determined spectrophotometrically was about 10% more than the concentration determined kinetically. This discrepancy was attributed to an increase in the effective path length of the cuvette. Significant absorption of scattered light by the chromophore FAD would account for the increase in path length. The results of two separate experiments yielded an effective path length for the 2 mm cuvette of 2.20 and 2.17 mm. Because of the high extinction of the glass beads containing insolubilized enzyme ($\Delta E_{450} > 3$ for a 2 mm path length) relative to that of untreated glass beads, it was not possible to obtain estimates of the total amount of insolubilized enzyme. This is because the diazotized arylamine used as a coupling agent has considerable absorption in the 300–500 nm region. Without this information we are unable to determine what fraction, if any, of the enzyme is inactivated by the insolubilization procedure. However, the consideration of any totally inactive enzyme will not be pertinent to our discussion.

It should be noted that on a macroscopic level the glass beads contain highly localized concentrations of bound enzyme. This is because the enzyme is covalently linked both to the external and pore network surfaces of an individual bead with the solid interiors of the bead comprising an excluded volume. Further, in a mechanically static system such as the packed bead bed, the interstitial volume between beads would also be considered an excluded volume. Nevertheless, if a large number of

beads is involved such as in a static spectrophotometric measurement, the localized enzyme concentrations may be averaged on a macroscopic level to yield an effective concentration. The same considerations apply to a stirred suspension with the exception that the external solvent is no longer an excluded volume for surface-bound enzyme.

Kinetic measurements. O₂-monitored turnover experiments were performed with the conventional membrane-covered Clark electrode. Both the electrode assembly and the power supply (model 53) were obtained from Yellow Springs Instrument Co. The thermostatically controlled electrode chamber is similar in design to the Yellow Springs Instrument Co. model 5301 bath assembly. The principal difference is that provision was made to achieve the faster stirring necessary to uniformly suspend the glass beads. The beads could be uniformly suspended (judged by visual inspection) at a stirring speed of approx. 150 rev./min and it was established that the reaction kinetics were unaffected when this speed was doubled. O₂ was recorded as a function of time with a Coleman model 165 recorder. The usual procedure of adding enzyme to an incubated substrate solution was reversed and substrate was added to the incubated suspension of insolubilized enzyme. This arrangement is preferred, as techniques for quantitatively introducing small volumes of the insolubilized enzyme into the equilibrated cell chamber and positioned electrode are not possible with the conventional electrode chamber configuration used. Typically 0.4–0.6 ml of an insolubilized enzyme suspension was delivered into several ml of buffer in the electrode chamber. The delivery pipette was rinsed with 0.5–1.0 ml of buffer into the electrode chamber and all glass beads were allowed to settle to the bottom. The volume of the cell was then decreased to 5 ml by aspirating the excess of supernatant buffer to a calibrated mark. Additions of concentrated substrate (1.0 or 2.0 M total glucose) ranged from 25 to 200 μ l. For a given set of related experiments, fresh beads from the same slurry were used for each experiment. This was found to be necessary, as rapid stirring for extended periods of time appeared to break a small percentage of the beads into small particles. These small particles did not settle rapidly enough to make routine rewashing and aspiration within the cell chamber feasible without some loss of enzyme. No destruction of active enzyme was observed on fracturing a small percentage of the beads during an experiment.

Slopes of the integral traces of percentage of O₂ remaining versus time were taken by hand and converted into reaction velocities at known O₂ concentrations. This is possible because $[S]_0 \gg [O_2]$ throughout the reaction and because there is no significant product inhibition (Gibson *et al.* 1964). The technique of obtaining the derivative of the integral data trace is based on approximating a small portion of the trace containing the point at which the derivative is to be taken as a small-angle arc on the circumference of a circle with a very large radius of curvature. Equal curvilinear distances along the data trace are marked off on each side of the point with a compass. A chord passing through the compass-drawn intersections with the curve represents a tangent to a point at the centre of the arc. This technique yielded tangents numerically equal to an average of those obtained by reflecting a portion of the curve back on itself with a

conventional tangentimeter. In addition, the technique proved to have less inherent error as judged by both reproducibility and negligible scatter of the data in double-reciprocal form. The value of $K_{m,app}$ for O_2 at each glucose concentration shown in Fig. 2 was the average of the abscissa intercepts from at least three independent experiments of the type shown in Fig. 1. It was demonstrated that for a given glucose concentration the value of $K_{m,app}$ for O_2 was independent of the number of glass beads (and hence the enzyme concentration) in the oxygen electrode chamber.

The O_2 concentration in 0.10M-acetate buffer, pH5.5, saturated with air at 25°C was estimated to be 0.235 mM (Robinson & Cooper, 1970).

RESULTS

The steady-state equation resulting from eqns. (2) and (3) is eqn. (4):

$$E_T/v = 1/(k_{red}[S]) + 1/(k_{ox}[O_2]) + 1/k_{cat}. \quad (4)$$

The parameter k_{red} is an apparent bimolecular rate constant for the interaction of substrate and the oxidized form of enzyme and has the form $k_{+1}k_{+2}/(k_{-1} + k_{+2})$, or $k_{+1}k_{+2}/k_{-1}$ if the rapid equilibrium assumption is used. The parameters k_{ox} and k_{cat} represent k_{+4} and k_{+2} , respectively.

Since E_T is not initially known, conventional O_2 -monitored turnover data will yield only the ratios k_{ox}/k_{red} and k_{ox}/k_{cat} . This is easily seen from eqn. (4) when the appropriate double-reciprocal plot of v versus $[O_2]$ is made (Fig. 1). The abscissa intercepts of this plot are represented by eqn. (5):

$$-1/K_{m,app} = k_{ox}(1/k_{red}[S] + 1/k_{cat}) \quad (5)$$

Typically three or more separate experiments at the same glucose concentration but with different enzyme concentrations were employed to determine the abscissa intercept. The enzyme concentration in each experiment can be calculated precisely from the slope of the line (see below) or by applying the appropriate dilution factor on transferring a sample into the reaction chamber. After corrections for differences in enzyme concentration, the data points of this figure would accurately fit a family of parallel lines, as is the case with soluble enzyme (see, e.g., Bright & Gibson, 1967).

The values of $1/K_{m,app}$ are then plotted against $1/[S]$ to yield an ordinate intercept of k_{ox}/k_{cat} and slope k_{ox}/k_{red} (Fig. 2). Two problems contribute to the high errors in $1/K_{m,app}$. First, the extrapolation to the abscissa in the double-reciprocal plot is a long one. Secondly, the steady-state kinetics under these conditions reflect only small contributions from the glucose term in eqn. (4). Use of very low glucose concentrations with air-saturated solutions are prohibited if we wish to keep the simplifying condition $[S]_0 \gg [O_2]$. Table 1 lists the results of the

Fig. 1. Kinetic behaviour of insolubilized glucose oxidase represented by double-reciprocal plot of steady-state velocity versus O_2 concentration at various concentrations of β -D-glucose. The O_2 -monitored experiments were carried out at 25°C in the presence of 0.1M-potassium acetate, pH5.5, and 0.1 mM-KCN. For ease of graphical presentation, we have selected experiments in which the enzyme concentration increases progressively with increase in glucose concentration. Kinetically determined enzyme concentrations (see the Results section) for each of the four β -D-glucose concentrations are as follows: 6.0, 7.3, 8.6 and 10.5 mM, progressing from lowest to highest substrate concentration.

parameter ratio determinations for free and insolubilized glucose oxidase at pH5.5 and 25°C. It is evident that the k_{ox}/k_{cat} ratio is unchanged by insolubilization, whereas the k_{ox}/k_{red} ratio is decreased by 14-fold in the case of the insolubilized enzyme. The fact that k_{ox}/k_{cat} is unchanged after insolubilization is initially interpreted as evidence that k_{ox} (and k_{cat}) for insoluble and soluble enzyme are the same. Additional arguments supporting this interpretation are presented in the Discussion section.

If k_{ox} is the same for both the insoluble and soluble glucose oxidases at this pH, then the active enzyme concentration may be calculated from the kinetic behaviour of that experiment. Again referring to eqn. (4), a double-reciprocal plot of v against O_2 ($[S]_0 \gg [O_2]$) yields a straight line of slope $1/(k_{ox} \cdot E_T)$. The value of k_{ox} at pH5.5 and 25°C for soluble glucose oxidase was determined in this work to be $2.05 \mu M^{-1} \cdot s^{-1}$ by using spectrophotometrically standardized soluble enzyme. This value agrees well with a value of $1.95 \mu M^{-1} \cdot s^{-1}$ determined from studies of the pH-dependence of soluble glucose oxidase (Weibel & Bright, 1971) and with values determined by Gibson *et al.* (1964) and Bright & Appleby (1969). Therefore, from the O_2 -dependence of the steady-state kinetics for insoluble glucose oxidase, we can calculate the concentration of kinetically active enzyme and compare it with the $[E-FAD]$ spectrophotometrically determined

Fig. 2. Results of Fig. 1 plotted according to eqn. (5) in the text. The broken line represents the same plot for soluble enzyme, and is calculated from the values of the steady-state parameters given in Table 2. $[S]$ denotes the β -D-glucose anomer concentration.

after reduction of the total enzyme with glucose. Table 2 gives the results of the spectrophotometric determinations for two sets of experiments. These results indicate that kinetically active E-FAD comprises only about 6% of the total amount of glucose-reducible E-FAD attached to the porous glass beads.

DISCUSSION

It should be emphasized that the success of comparative studies of the behaviour of an enzyme in soluble and insoluble form depends initially on the precision with which the kinetic mechanism of the homogenous enzyme reaction is known. In the case of glucose oxidase, the kinetic mechanism is understood with great precision over a wide range of experimental conditions through a combination of steady-state and direct rapid-reaction analyses. The rapid-reaction studies are essential for an understanding of both the reaction mechanism and the significance of each term in the steady-state rate equation.

The kinetic determination of the concentration of E-FAD that is participating in catalysis is based on the assumption that the bimolecular reoxidation rate constant k_{ox} is the same for soluble and immobilized enzyme. The observation that the k_{ox}/k_{cat} ratio is not changed on insolubilization of glucose oxidase is in itself strong evidence for this assumption. The possibility that both k_{ox} and k_{cat} are affected in magnitude to the same extent by insolubilization, hence yielding the same k_{ox}/k_{cat}

Table 1. Comparison of steady-state kinetic parameters for soluble and insoluble glucose oxidase

The values for glucose oxidase were determined by Weibel & Bright (1971). The values of the steady-state parameters for soluble glucose oxidase at pH 5.5 and 25°C, by using D-glucose, are the following: $k_{red} = 1.40 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{ox} = 1.95 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{cat} = 1.00 \times 10^3 \cdot \text{s}^{-1}$.

	k_{ox}/k_{red}	$k_{ox}/k_{cat} \cdot (\text{M}^{-1})$
Soluble glucose oxidase	1.4×10^2	2.0×10^3
Insoluble glucose oxidase	0.10×10^2	2.0×10^3

ratio for free and immobilized enzyme, seems remote for the following reasons. First, the parameter k_{cat} represents the first-order process in the reductive half-reaction and is associated with the flavin reduction process, $k_{1,2}$, at pH 5.5 and 25°C. The magnitude of $k_{1,2}$ is highly sensitive to the particular aldose used as substrate, whereas k_{ox} is independent of substrate (Gibson *et al.* 1964; Nakamura & Ogura, 1967). Secondly, with respect to both pH- and halide-dependence, k_{cat} and k_{ox} display very large differences in behaviour (Weibel & Bright, 1971; Bright & Appleby, 1969). Thirdly, the rate of reoxidation of E-FADH₂ at pH 5.5 and 25°C is very similar to the bimolecular rate constant ($1.6 \mu\text{M}^{-1} \cdot \text{s}^{-1}$) for the reaction of free FMNH₂ with O₂ under similar experimental conditions (Gibson & Hastings, 1962). This comparison would indicate that the reoxidation of reduced flavin is not significantly catalysed by apoprotein and hence not sensitive to structural changes within the protein environment. We therefore conclude that the processes associated with k_{cat} and k_{ox} are never in any way coupled, and are therefore unlikely to be perturbed in exactly the same fashion by the insolubilization procedure. Consequently, k_{ox} is probably not changed after insolubilization.

Assuming an average diameter for the 40–60 mesh beads of 0.2 mm, the approximate surface area of a bead is 0.125 mm² with a perfect spherical model. The approximate diameter for a typical protein of molecular weight 180 000 would be about 10 nm and this molecule would possess a surface packing cross-section of about 10² nm². This calculation shows that about 10⁹ enzyme molecules can be packed on the external surface of a bead. We have ignored the fact that there are two active sites per enzyme molecule as it is not known whether one or both will be exposed after insolubilization. The average number of beads in the chamber of the O₂ monitor is of the order of 1000. This was calculated from the volume of gravity-packed beads transferred to the chamber, assuming cubic body-centred packing of hard spheres with known diameter.

Table 2. Comparison of the amounts of glucose-reducible and kinetically active E-FAD on porous glass beads

Each of the two sets of results for glucose-reducible E-FAD represents a single spectrophotometric determination of glucose-reducible E-FAD and has been corrected for the dilution involved in transfer of the beads to the oxygen-electrode vessel. Each of the two sets of results for kinetically active E-FAD represents the concentration of catalytically active E-FAD calculated from O₂-monitored steady-state experiments containing the concentrations of glucose-reducible E-FAD shown in the first column.

Glucose-reducible E-FAD (nm)	Kinetically active E-FAD (nm)	Glucose-reducible E-FAD Kinetically active E-FAD
91.4	6.30	14.5
157.1	9.05	17.4
117.8	6.80	17.3
126.2	8.50	14.8
126.2	9.75	15.2
125.6	8.63	14.5
		15.6
		$\sigma = 1.2$
135.2	8.81	15.5
119.2	8.28	14.4
152.2	10.00	15.2
136.2	8.81	15.5
141.2	9.13	15.5
		15.2
		$\sigma = 0.1$

The kinetic assay indicates that there were about 10^{13} active sites in the reaction chamber of the O₂ monitor, or 10^{10} molecules per bead. Therefore, by assuming a smooth spherical surface, the beads can hypothetically accommodate all kinetically active enzyme on their exterior surfaces. The order-of-magnitude difference between the calculated number of enzyme molecules on the surface of the beads and the kinetically determined number is not significant considering the model and methods of calculation. The estimation of 10^9 enzyme molecules per bead represents a lower limit, since the surface area of the beads is much greater than that assumed because of the porous structure at the external surface and microscopically observable irregularities.

In the light of the above calculation, an explanation for the difference in concentration of total substrate-reducible E-FAD and that participating in catalysis in the well-stirred reactor becomes apparent. The function of enzyme molecules that are covalently linked to the glass structure deep within the pore network might be expected to be subject to considerable substrate-diffusion limitations, and even in the rapidly stirred bead suspension the kinetic contribution of this enzyme would

be expected to be negligible. However, these enzyme molecules would ultimately become reduced in a spectrophotometric determination where sufficient time is allowed for complete glucose diffusion and O₂ depletion to occur throughout the packed bead bed. In contrast, enzyme immobilized either at the external surface of the bead or within the pore network near the surface should be readily available to glucose or O₂ in both the packed bead bed and in the well-stirred O₂-electrode chamber. These conclusions were substantiated by the finding that total enzyme activity increased 2.5-fold after the beads were ground with a pestle and mortar. This enhancement is less than the hypothetical value of 15-fold because of the limit to which the beads can be decreased in size and because of enzyme damage incurred in the grinding process. Thus substrate diffusion barriers within a matrix such as the porous glass bead used in these studies may be serious rate-limiting processes in kinetic applications. Because the initial concentration of glucose is between 20- and 200-fold greater than that of O₂, and because, even for enzyme molecules at the exterior surface, the interaction of O₂ with E_r is the principal rate-limiting process in turnover under our conditions, we believe that the interior pore network must be largely anaerobic and that the enzyme is trapped in the form E_r. Product diffusion appears not to be an important factor, since D-glucono- β -lactone, which is a very weak inhibitor (Gibson *et al.* 1964), had no effect on the kinetics when present initially at the same concentration as glucose.

The parameters $k_{red.}$, $k_{ox.}$ and $k_{cat.}$ are all highly pH-dependent (Bright & Appleby, 1969; Weibel & Bright, 1971) and the question arises as to whether the 14-fold decrease in the $k_{ox.}/k_{red.}$ ratio might be explained by a perturbation of this pH-dependence brought about by the insolubilization process. The perturbation might consist of a change in pH in the region of insolubilized enzyme molecules, a change in the pK values of ionizable groups on the enzyme which govern the steady-state parameters, or a combination of these effects. These two mechanisms would not be distinguished by our method of kinetic analysis, and we choose to discuss this question in terms of a hypothetical change in the pH experienced by the insolubilized enzyme. The parameters $k_{red.}$, $k_{ox.}$ and $k_{cat.}$ for the soluble enzyme are maximal at pH 5.5 (Bright & Appleby, 1969; Weibel & Bright, 1971). At pH values less than 5.5, $k_{red.}$ is markedly decreased, whereas $k_{cat.}$ and $k_{ox.}$ are unaffected. At pH values greater than 5.5, $k_{ox.}$ and $k_{cat.}$ are decreased, whereas $k_{red.}$ is relatively constant. Therefore, if the pH experienced by the bound enzyme were less than 5.5, the $k_{ox.}/k_{red.}$ ratio would increase, rather than decrease as is observed. On the other hand, $k_{ox.}/k_{red.}$ would be decreased if

the local pH were 3 or 4 units greater than 5.5. However, if this were the case then the k_{ox}/k_{cat} ratio for insolubilized enzyme would be greatly altered, since the apparent pK values associated with k_{ox} and k_{cat} are 7.5 and 9.1 respectively. We may conclude, therefore, that the increase in k_{red} does not result from local pH effects or from the perturbation of the ionization of groups in the enzyme.

There appear to be two possible explanations for the 14-fold increase in the value of k_{red} , which is attributed to those enzyme molecules located at the bead surface after insolubilization. First, any mechanism that increases the frequency of collision of E_o with glucose would, by our method of kinetic analysis, cause k_{red} to be greater than the value characteristic of soluble enzyme. This is because we use the homogenous solution concentration for glucose (which is not measurably affected by the porous glass beads) in the application of eqn. (4). Although there appears to be no information concerning the affinity of glucose for glass surfaces, there is strong evidence for hydrogen-bonding interactions and possible stacking of methanol and other substances at porous glass surfaces (Fiat, Reuben & Folman, 1967; Folman & Yates, 1958). Secondly, the process of insolubilization might increase k_{+1} or decrease k_{-1} , or have both effects. This would represent an enhanced affinity of E_o for glucose (through a decrease in k_{-1}/k_{+1}) which results from an intrinsic change in enzyme structure. Although this seems to us to be a reasonable explanation, we are unable to comment further on this possibility until further information is available concerning the insolubilized enzyme preparation. We should note that k_{+2} (which together with k_{+1} and k_{-1} makes up the parameter k_{red}) is unaffected by insolubilization and therefore has nothing to do with the increase in k_{red} .

At present, sufficient information does not exist to differentiate between the two processes that we believe could explain the apparent increase in catalytic efficiency of the small fraction of insolu-

bilized glucose oxidase that is responsible for catalysis.

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