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THE EFFECTS OF BUFFER DIFFUSION ON THE ACTIVITY OF AN IMMOBILIZED ESTERASE

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Summary

The pH-activity profile of an immobilized esterase is shown to depend on the pK and the concentration of the buffer in which the activity is measured. The effect of buffer diffusion on the observed results is discussed on the basis of a simplified model.

Introduction

The pH-activity profiles of immobilized enzymes often differ from those of the free enzymes in a marked manner [1—4]. Apart from any direct influences of the carrier or of chemical binding on the enzyme itself [5], these differences are due to the changed microenvironment of the protein. The concentration of hydrogen ions at the site of action may differ from that in the bulk of the solution either on account of their unequal distribution between the solution and the support, or on account of diffusion limitations, or as a result of both factors. Thus the electrostatic field of anion and cation exchangers may increase or decrease the concentration of H^+ in relation to the solution with corresponding shifts of the profiles relative to the curve for the free enzyme [2,6]. Reactions producing base or acid generate pH gradients across the Nernst diffusion layer and within the matrix. In this case the pH-activity profiles depend on many factors including the concentration of the buffer, its pK and the diffusion rates of its components.

In their general form, the mathematics of such systems are very complex and the treatments described in the literature deal only with certain special cases. Thus solutions which take into account the pH gradients within particles containing entrapped enzymes have been reported for unbuffered systems [4,7,8]. Recently Engasser and Horvath [9] have analyzed the effects of buffer diffusion on reactions catalyzed by surface bound proteins. Their model would also describe the kinetics of a microencapsulated enzyme, if the contents of the

capsule were well mixed. Furthermore it has general implications that are independent of any internal pH gradients and which therefore apply to all types of immobilized enzymes. We report here some experimental results which bear out these predictions.

Experimental

The crude esterase, first described by Nüesch et al. [10], was obtained from *B. subtilis* ATCC 6633. Two different preparations were used, one with a specific activity of 1.1 units/mg protein for the entrapment in fibres and for determining the profiles of the free protein, and another with a specific activity of 13 units/mg for covalent binding to porous glass. Details of the isolation of the pure enzyme, which has a specific activity of 140 units/mg, will be described elsewhere.

The assay was carried out by the pH-stat method at pH 8.0 and 25° C in a solution containing 100 mM NaCl, 10 mM sodium phosphate and 85 mM (2-methoxyethyl)acetate as substrate. The units are expressed in μmol/min.

The enzyme was entrapped as a finely dispersed solution in cellulose triacetate fibres [11] by the method of Dinelli [12], the quantity immobilized being 107 units/g dry carrier. In calculating the catalytic efficiency relative to the free esterase it was assumed, in line with related observations [13], that no appreciable loss or inactivation occurred in the immobilization. The covalent binding to alkylamine/zirconia clad controlled pore glass CPG-550 (product of the Corning Glass Co., having a mean pore diameter of 550 Å and particle diameter of 177—840 μm) was carried out with glutaraldehyde in 0.1 M acetate pH 5.5 according to the standard methods, described in the Pierce Co. Bulletin. The catalyst had an activity of 82 units/g dry carrier.

The activity of the immobilized enzymes was measured at high flow rates in the recirculation reactor described by Ford and coworkers [14]. Its proper operation as a differential reactor, namely the insensitivity of the reaction rate per g catalyst to the flow rate and quantity of catalyst, was verified. The cellulose triacetate fibres, cut up into 5-mm pieces, were compressed in the reactor by a thick layer of glass beads. Their packing density corresponded to 0.18 g dry cellulose triacetate/ml.

The rates given are corrected for the non-enzymatic, base-catalyzed hydrolysis of the substrate, which may be represented by the equation

$$\log k(\mathrm{min}^{-1}) = -12.78 + \mathrm{pH}$$

in the region pH $\geqslant$ 8. All measurements were carried out at 25° C in the presence of 100 mM sodium chloride.

Results

The activity of the fibres as a function of sodium phosphate concentration at pH 7 and pH 8 is shown in Fig. 1, and the pH activity profiles of this catalyst and of the free enzyme in solutions containing 100 mM sodium chloride and 10 mM buffers, namely phosphate (pK 7.2), Tris (pK 8.1) and borate (pK 9.2), in

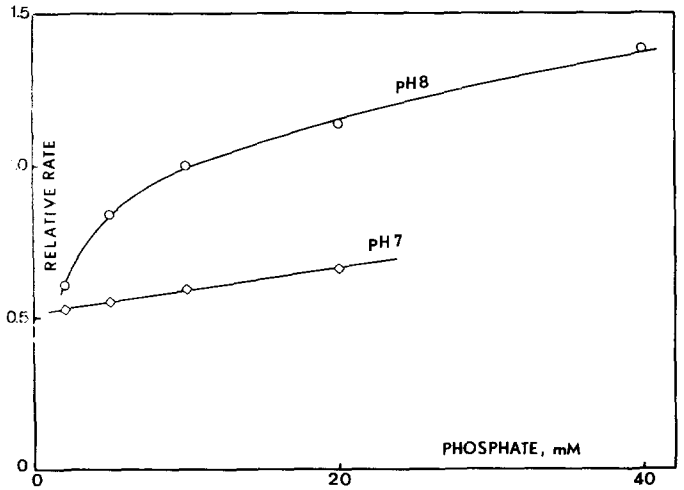

Fig. 1. The effect of phosphate buffer concentration on the activity of the fibres.

Fig. 2. The corresponding results for the enzyme covalently bound to porous glass are shown in Fig. 3.

The Michaelis-Menten constants for the reaction of the free and fibre entrapped enzyme in a solution containing 100 mM sodium chloride and 10 mM phosphate were K_m = 4.5 mM and K_m (app.) = 3.2 mM at pH 8.0. The plot of the reaction rate v against v/S (S = substrate concentration), which is more reliable than the conventional $1/v$ versus $1/S$ plot [15], is shown in Fig. 4.

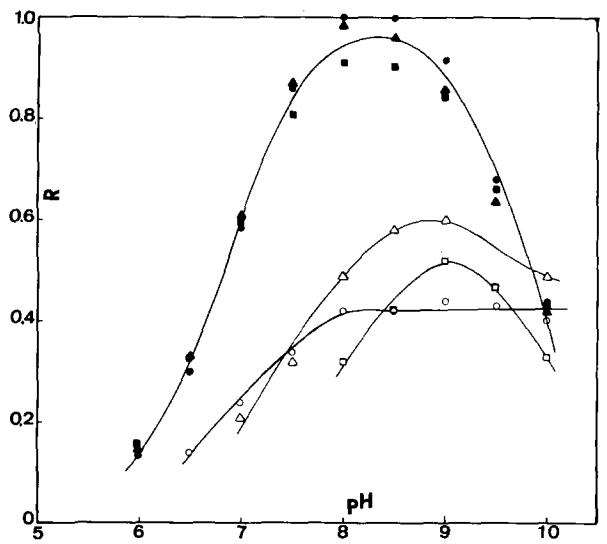

Fig. 2. The pH-activity profiles in 100 mM NaCl/10 mM buffers: Free enzyme in phosphate (●), Tris (▲) and borate (■); entrapped enzyme in phosphate (○), Tris (△) and borate (□). The rates R, expressed in μmol/min., are given for 1 unit of enzyme.

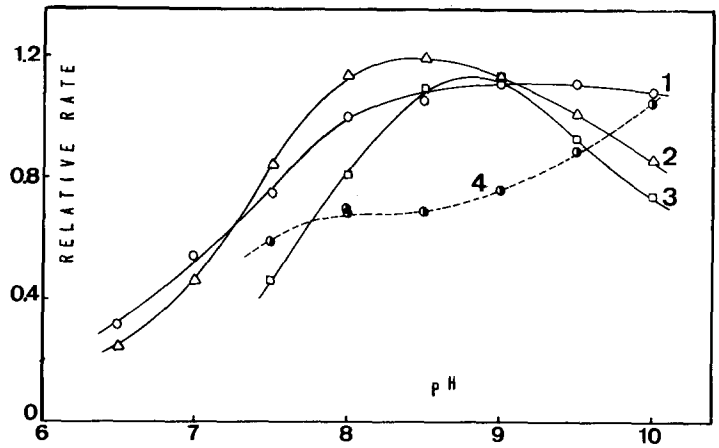

Fig. 3. The activity of the glass-bound enzyme as a function of pH in 10 mM phosphate (1), Tris (2) and borate (3) and in 2 mM phosphate (4).

Discussion

The observed results may be summarized as follows:

1. As shown in Fig. 1 the activities of the immobilized enzyme depend on buffer concentration, and the ratio of the two activities is also concentration dependent, especially in dilute buffer solutions.

2. At a given buffer concentration the pH-activity profile of the immobilized enzyme, unlike that of the free protein, depends markedly on the pK of the buffer. The rate maxima, when they exist, are at a higher pH than pH_m, the pH optimum of the free catalyst (pH_m = 8.3).

3. At equal concentrations the effectiveness of the buffers in repressing the rate in the region pH $\approx$ pH_m + 2 correlates with their buffer capacity, i.e. it increases as the ratio of the buffer components [AH]/[A^-] approaches unity.

4. The curves for 10 mM phosphate in Figs 2 and 3 have no maxima, the

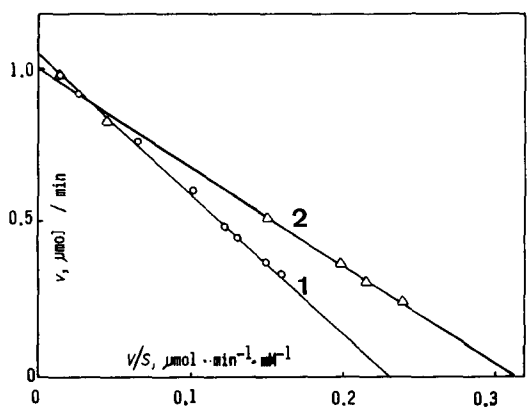

Fig. 4. Determination of the Michaelis-Menten constants for the free (1) and the fibre-entrapped (2) enzyme. Phosphate 10 mM, pH 8.0.

rates being practically independent of external pH in the region pH > 8.

5. The reaction rate in 2 mM phosphate buffer (curve 4 in Fig. 3) first levels off as external pH increases, but then increases again in more strongly alkaline solutions.

All these results follow from the equations of Engasser and Horvath [9] and can be deduced quite simply by considering which external variables are most effective in determining the internal pH (or the internal pH gradient) under the given conditions.

For a given buffer and given catalyst particle there are only four external variables, namely the concentrations of the buffer components AH and A^-, and of H^+ and OH^-, when the concentration of the substrate [S] $\gg K_m$ (app.). Their relative effectiveness in determining the internal pH depends on the relative values of the products k_x (X) where k_x is the diffusion coefficient of the species X. The two basic components, A^- and OH^-, raise the internal pH by neutralizing all or a part of the acid produced by the reaction. Assuming that the acid produced is much stronger than AH and that its reaction with A^- is very fast, we may distinguish the following situations:

1. The external concentrations of AH and A^- are much higher than those of H^+ and OH^-. In that case only the buffer components are effective external variables. The steady state equations of Engasser and Horvath then lead to the expression

$$[H^+]_s = K_{AH} \frac{[AH]_s}{[A^-]_s} = K_{AH} \frac{[AH]_b + v/k_{AH}}{[A^-]_b - v/k_{A^-}} \qquad (1)$$

(The subscripts s and b distinguish between the internal and external values of the variables, K_{AH} is the ionization constant of AH; the reaction rate v assumes a maximum value V when $pH_s = pH_m$*). This equation shows that $[H^+]_s$ is higher than

$$(H^+)_b = K_{AH} \frac{(AH)_b}{(A^-)_b} \qquad (2)$$

and approaches $[H^+]_b$ at sufficiently high values of $[AH]_b$ and $[A^-]_b$. Furthermore, for equal concentrations of different buffers and for comparable values of k_{AH} and k_{A^-} the difference $[H^+]_s - [H^+]_b$ has a minimum value in the region $[H^+]_b = K_{AH}$, where the concentrations of $[AH]_b$ and $[A^-]_b$ are comparable. The effectiveness of the three buffers in repressing the internal pH at $pH_b \approx pH_m + 2$ reflects this influence, as shown in Figs 2 and 3.

2. When, under the same conditions, the external pH $\gg pK_{AH}$, then the external concentration of AH is too small to have any effect on the internal pH, unless the buffer concentration is very high; practically all AH within the catalyst particle derives from the internal reaction of A^- with acid generated by the enzyme. Under these conditions there is only one effective external variable, $[A^-]_b$, and this has a practically constant value at a constant buffer

* The model predicts that the value of V will be the same for the free and the immobilized enzyme. This is a consequence of the assumption that there are no pH gradients within the particle.

concentration. The rate is therefore independent of external pH, as shown by the curves for 10 mM phosphate in Figs 2 and 3.

3. When the conditions under 2 apply and when the concentration of A^- is low, the effect of the diffusion of OH^- becomes appreciable at high external pH (see curve 4 in Fig. 3).

Finally, the foregoing considerations have also a bearing on the physical interpretation of Michaelis-Menten constants. In a series of experiments involving a constant external environment and increasing substrate concentrations, the internal pH is not constant but decreases with the increasing reaction rate (cf. Eqn 1). This means that the observed change of the reaction rate reflects not only substrate concentration but also this variable. The magnitude of these internal pH-changes will in general depend on the external pH, the concentration of the buffer and its pK. Their effect will be to decrease or increase the observed K_m (app.) relative to the "correct" value (corresponding to constant pH_s), depending on whether the values of pH_s are lower or higher than pH_m under the conditions of the measurements. For the rates increase with increasing pH_s on one side of pH_m, and decrease on the other. The lower value of K_m (app.) as compared with K_m is attributed to this factor.

Finally, such changes of internal pH, like the diffusion limited transport of substrate [16], may lead to apparent deviations from Michaelis-Menten kinetics.

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