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COMPARISON OF INTRINSIC STABILITIES OF FREE AND BOUND ENZYMES BY GRAPHICAL REMOVAL OF DIFFUSIONAL EFFECTS

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Summary

The enhanced stability usually exhibited by enzymes after immobilization may be attributed either to a stabilization effect of the solid matrix on the bound enzyme molecule or to the influences of diffusional limitations on the observed activity.

To allow the comparison of the intrinsic statilities of free and bound enzymes a simple graphical procedure for the removal of external diffusional effects on stability curves is described. It is based on the determination of substrate concentration differences between the enzyme micro- and macroenvironment.

Application of the method to aspartate aminotransferase bound to collagen membranes indicates that diffusional limitations for oxaloacetate are partly responsible for the observed stability enhancement. Comparison of the graphically obtained intrinsic profile with the stability curve of the soluble enzyme further demonstrate that the binding itself greatly increases the stability of aspartate aminotransferase.

Introduction

The immobilization of an enzyme on a solid support or inside a membrane often affects the stability of the enzyme. This phenomenon is not only of great practical interest in view of the numerous applications of bound enzymes, but may also give some fundamental insight in the phenomenon of enzymatic deactivation.

Enzymes generally exhibit an enhanced stability after immobilization. The question then arises whether such an observation can be interpreted as an actual stabilisation of the protein due to the vicinity of a solid matrix or

whether it may only be an apparent effect caused by diffusional limitations for the substrates or the products of the reaction. It has been recently demonstrated that concentration gradients between the enzymatic micro- and macro-environment result in an apparently slower decrease of the enzymatic activity with time [1,2].

Thus, before drawing any conclusions about the binding effect on the intrinsic enzyme stability it is necessary to first account for the possible diffusional limitations. In a previous study we have outlined a graphical method that allows the removal of external diffusional effects on curves of enzymatic activity versus substrate concentration [3]. We now propose an extension of this method to the removal of diffusional effects on stability profiles, and illustrate the simplicity and rapidity of the graphical procedure with stability data recently obtained with collagen-bound aspartate aminotransferase.

Theory

Intrinsic and apparent kinetic behavior

The graphical removal of the effect of external diffusional limitations is described in the case of an enzyme bound to an impervious solid surface. The rate of the enzymatic reaction is assumed unaffected by the product concentration so that only diffusional resistances for the substrate have to be taken into account.

When the transport of substrate by molecular and convective diffusion is a relatively slow process with respect to the substrate consumption at the active enzymatic surface the substrate concentration at the surface can be significantly lower than its concentration in the bulk solution [4]. As the bound enzyme activity is determined by the substrate concentration at the surface, the observed dependence of the enzymatic activity on the bulk substrate concentration does not represent the actual dependence of the enzymatic activity on the substrate concentration. When analyzing the kinetic behavior of bound enzymes it is thus necessary to distinguish between the intrinsic dependence of the activity on the substrate concentration seen by the enzyme, $v^*(s)$, and the apparent dependence of the enzyme activity on the substrate concentration experimentally measured in the bulk solution, $v(s)$.

In view of the above discussion the precise assessment of the effect of binding on the enzymatic molecule clearly requires the comparison of the kinetic behavior of the free enzyme with the intrinsic behavior of the bound enzyme. At a given time the intrinsic dependence of the activity on the substrate concentration, $v^*(s)$, can be graphically determined from the apparent dependence, $v(s)$, as previously reported [3]. In this paper we present another graphical procedure to determine the intrinsic time dependence of the bound enzymatic activity at a given substrate concentration, $v^*(t)$, from the apparent time dependence, $v(t)$.

Applicability of the procedure

First the value of the transport coefficient, h, between the bulk solution and the enzymatic surface has to be known. Measurements can be performed with a well defined system (open tubes, bed of particles) for which the trans-

TABLE I

TYPICAL VALUES OF THE EXTERNAL TRANSPORT COEFFICIENT PER UNIT AREA OF ENZYMATICALLY ACTIVE SURFACE IN DIFFERENT IMMOBILIZED ENZYME SYSTEMS

Immobilized enzyme system	External transport coefficient (cm/s)
Open tubular reactor with laminar flow	$5 \cdot 10^{-4}$ [8]
Open tubular reactor with turbulent flow	$2 \cdot 10^{-3}$ [8]
Enzymatic membranes in stirred solution	$1 \cdot 10^{-3}$ [9]
Packed-bed reactor	$2 \cdot 10^{-2}$ [10]

port coefficient can be calculated from available correlations. Alternatively, the value of the transport coefficient is independently determined when the rate of the enzymatic reaction is under diffusional control, thus equal to hs_b, the product of the transport coefficient by the bulk substrate concentration, s_b [4]. Table I indicates some typical values of the transport coefficient in immobilized enzyme systems.

The stability profile of an enzyme is usually determined by measuring the decrease with time of the enzymatic activity at a given bulk concentration of substrate. Yet, in order to remove diffusional interferences on such a profile the intrinsic dependence of the activity on the substrate concentration at the start of the stability experiment, $v_0^*(s)$, has also to be known. It can be graphically obtained [3] by removing diffusional effects from the measured initial dependence $v_0(s)$.

Finally the analytical procedure is generally applicable to any intrinsic enzyme kinetics, $v^*(s,t)$, provided the dependence on the substrate concentration and the variation of activity with time can be separated as:

$$V^*(s, t) = V(t)\, f(s)$$

One thus assumes that the denaturation of the enzyme only affects the maximum activity, V, which is proportional to the amount of active enzyme, and does not change the other kinetic parameters.

Graphical determination of the substrate surface concentration

Diffusional limitations, i.e. concentration gradients between the bulk solution and the catalytically active surface, result from the relative slowness of the diffusion of substrate with respect to its consumption at this surface. Because of denaturation, the number of active enzyme molecules is progressively decreasing and diffusional limitations become less important with time. In other words, whereas the substrate concentration is maintained constant in the bulk solution, its surface concentration will gradually increase, as illustrated in Fig. 1.

When enzyme activities are measured under steady-state conditions the rate of substrate transport is equal to the rate of substrate conversion at the enzymatic surface. Thus the initial substrate concentration at the surface, s_{s0},

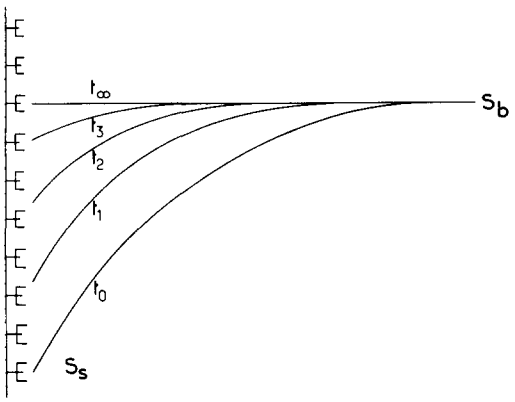

Fig. 1. Substrate concentration profiles in the vicinity of a surface-bound enzyme. As the amount of actieve enzyme decreases with time, diffusional limitations become less important and the substrate concentration at the surface gradually increases.

obeys the following equation:

$$v_0(s_b) = h(s_b - s_{S0}) = v_0^*(s_{S0}) = V_0 f(s_{S0}) \quad (1)$$

where $v_0(s_b)$ is the initial measured activity at bulk concentrations s_b, h the substrate transport coefficient, $v_0^*(s_{S0})$ the initial intrinsic activity at concentration s_{S0} and V_0 in the initial maximum activity.

The initial surface concentration, s_{S0}, can be graphically determined as shown in Fig. 2. One first draws the initial intrinsic dependence of the enzyme activity on the substrate concentration, $v_0^*(s)$, then the horizontal line at the

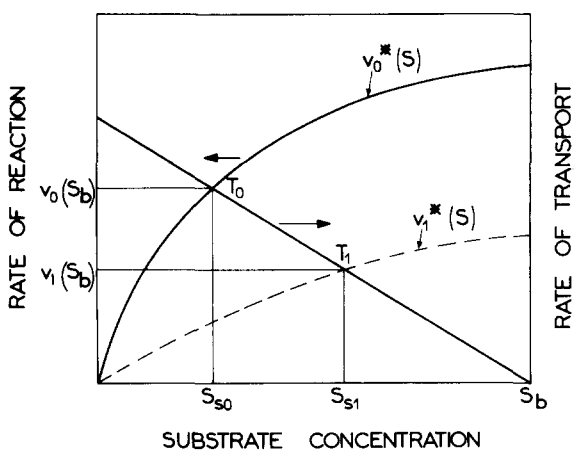

Fig. 2. Graphical determination of the substrate surface concentration, s_S, at a given bulk concentration, s_b, where the substrate transport coefficient, h is known. $v_0^*(s)$ represents the known initial intrinsic dependence of the bound enzyme activity on the substrate concentration, $v_1^*(s)$ the unknown intrinsic dependence at time t_1. One first draws the transport line of slope $-h$. The intersections T_0 and T_1 of this line with the horizontal lines drawn at the values of the measured activities, $v_0(s_b)$ and $v_1(s_b)$, yield on the abscissa the initial substrate concentration, s_{S0}, and the substrate concentration at time t_1, s_{S1}, at the enzymatically active surface.

value $v_0(s_b)$, the measured initial activity. Clearly the abscissa of the intersection T_0 of the two curves yields the initial surface concentration, s_{S0}.

According to Eqn. 1, T_0 is also the intersection of the curve $v_0^*(s)$ with the transport line which, at a given bulk concentration s_b, represents the rate of substrate transport to the surface versus the substrate concentration at the surface, i.e. $h(s_b - s_S)$. This line of slope $-h$ intercepts the abscissa axis at the value s_b and the ordinate axis at the values hs_b.

At a given time t_1, when because of denaturation the maximum activity has been reduced to V_1, the observed activity is $v_1(s_b)$ and the substrate concentration at the surface, s_{S1}, obeys the following relationship

$$v_1(s_b) = h(s_b - s_{S1}) = v_1^*(s_{S1}) = V_1 f(s_{S1}) \tag{2}$$

$v_1^*(s_{S1})$ being the new intrinsic activity at concentration s_{S1}. As indicated by Eqn. 2, the new surface concentration s_{S1} is graphically determined by the abscissa of the intersection T_1 of the previously introduced transport line and the horizontal line at the value $v_1(s_b)$.

According to Eqn. 2, T_1 is also the intersection of the transport line with $v_1^*(s)$, the intrinsic dependence of the enzymatic activity on the substrate concentration at time t_1, which, however, is yet unknown. We now outline the graphical method to determine $v_1^*(s_b)$, the intrinsic enzyme activity that would be measured at time t_1 in the absence of diffusional limitations.

Graphical determination of the enzymatic activity in the absence of diffusional limitations

With uniform substrate concentration s_b in the system the measured initial enzymatic activity, $v_0^*(s_b)$, would correspond to the ordinate of v_0^*, the intersection of the curve $v_0^*(s)$ with the vertical line at s_b, as shown in Fig. 3. At

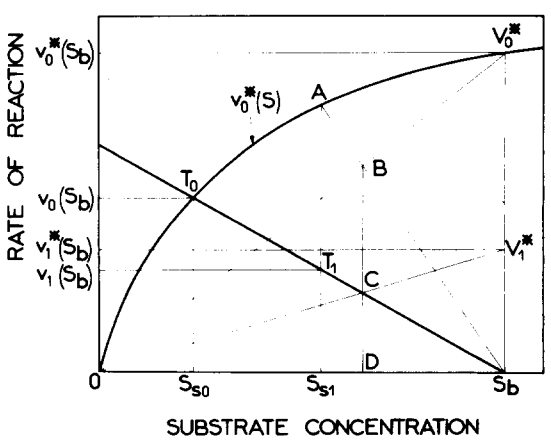

Fig. 3. Graphical determination of the enzymatic activity that would be measured at a given bulk substrate concentration, s_b, in the absence of external diffusional limitations. Initially, one would measure the activity $v_0^*(s_b)$, ordinate of the intersection V_0^* of the known intrinsic dependence $v_0^*(s)$ with the vertical line drawn at bulk concentration, s_b. At time t_1 the effect of diffusional limitations is removed as follows: the vertical line through T_1 intersects $v_0^*(s)$ at A; AS_b intersects OV_0^* at B; the vertical line through B intersects the transport line s_bT_0 at C; the ordinate, $v_1^*(s_b)$ of the intersection V_1^* of OC with $s_bV_0^*$ represents the activity that would be measured at t_1 without diffusional limitations.

time t_1 the intrinsic activity $v_1^*(s_b)$ can be determined by considering an alternative form of Eqn. 2, obtained by multiplying each member by V_0/V_1, i.e.

$$\frac{V_0}{V_1} v_1(s_b) = h \frac{V_0}{V_1} (s_b - s_{S1}) = v_0^*(s_{S1}) = V_0 f(s_{S1}) \tag{3}$$

Let A be the intersection of $v_0^*(s)$ with the vertical line through T_1. The abscissa of A is s_{S1} and its ordinate $v_0^*(s_{S1})$. According to Eqn. 3, A also represents the intersection of $v_0^*(s)$ with the line of slope $-hV_0/V_1$ which intersects the abscissa axis at s_b. Since the slopes of the two lines s_bT_1 and s_bA are $-h$ and $-hV_0/V_1$, respectively, the following equality is obeyed

$$\frac{s_{S1}T_1}{s_{S1}A} = \frac{V_1}{V_0} \tag{4}$$

The intrinsic activity $v_1^*(s_b)$ clearly represents the ordinate of a point v_1^* of $s_bv_0^*$ such as

$$\frac{s_bv_1^*}{s_bv_0^*} = \frac{V_1}{V_0} \tag{5}$$

This point v_1^* is obtained by the following graphical construction: let B be the intersection of Ov_0^* with s_bA. The vertical line through B intersects s_bT_0 at C and the abscissa axis at D. The intersection v_1^* of OC and $s_bv_0^*$ is such that

$$\frac{s_bv_1^*}{s_bv_0^*} = \frac{DC}{DB} = \frac{s_{S1}T_1}{s_{S1}A} \tag{6}$$

i.e. in view of Eqn. 4, satisfies Eqn. 5. Consequently the ordinate of v_1^* indeed represents the intrinsic bound enzyme activity at bulk concentration s_b and at time t_1.

By repeating this simple procedure at different times t_2, t_3, etc, it is thus possible to remove diffusional effects on the measured enzymatic activity and determine the intrinsic stability profile of the bound enzyme.

Example

To illustrate its convenience and rapidity this graphical method was used to determine the intrinsic stability of collagen-bound aspartate aminotransferase. A previous paper [5] described in details the preparation of aspartate aminotransferase collagen films and the assays of bound and soluble enzymatic activity. We examined the stability of the free and bound enzyme on storage in 0.05 M Tris · HCl buffer, pH 8.0, 4°C, by measuring from time to time the residual activity at 30°C in a reactive medium containing 0.05 M Tris · HCl buffer, 0.5 mM oxaloacetate and 50 mM glutamate.

The decrease of activity with time for the bound enzyme is shown in Fig. 4a. As seen, the activity remains constant for 5 months before decreasing. For comparison, under the same conditions the enzyme in dilute solution looses 25% of its initial activity in 15 days. In order to precisely assess the respective contributions of enzyme intrinsic stabilization and diffusional effects on this

apparent stability enhancement, in Fig. 4b we have determined the enzyme activities that would have been measured in the absence of diffusional limitations.

We have recently shown [7] that because of the great difference in concentration of oxaloacetate and glutamate diffusional limitations can only be important for one of the substrate, oxaloacetate; thus concentration gradients for glutamate are negligible under our experimental conditions. Furthermore the intrinsic kinetic parameters of the enzymatic reaction were found unaffected by the enzyme immobilization. Consequently, in Fig. 4b the initial intrinsic activity dependence on the oxaloacetate concentration at a glutamate concentration of 50 mM was calculated from the kinetic expression for the free enzyme as:

$$v_0^* = \frac{V_0[\mathrm{Ox}][\mathrm{Glu}]}{K_{\mathrm{Ox}}[\mathrm{Glu}] + K_{\mathrm{Glu}}[\mathrm{Ox}] + [\mathrm{Glu}][\mathrm{Ox}]} \tag{7}$$

where [Ox] and [Glu] are the oxaloacetate and glutamate concentrations, respectively, K_{Ox} the Michaelis constant for oxaloacetate and K_{Glu} the Michaelis constant for glutamate. The kinetic parameters were determined as V_0 = 270 nmol/min, K_{Ox} = 0.052 mM and K_{Glu} = 5 mM. In the previous study the value of transport coefficient h for oxaloacetate was also experimentally found to be equal to 0.45 cm^3/min for the whole film of 7.5 cm^2 active surface area, which allows the drawing of the transport line of slope $-h$.

Using the geometrical procedure outlined in this study we can see in Fig. 4b that diffusional resistances for oxaloacetate are significant in our system since the oxaloacetate concentration, which is equal to 0.5 mM in the bulk solution, varies between 0.1 mM and 0.3 mM at the surface. On the other hand, after removal of diffusional effects, one obtains a relatively stronger decrease of activity with time: 50% of the initial activity is lost between 5 and 10 months instead of 40% in the presence of diffusional effects. Diffusional limitations are thus partly responsible for the enhanced stability of the bound enzyme. Com-

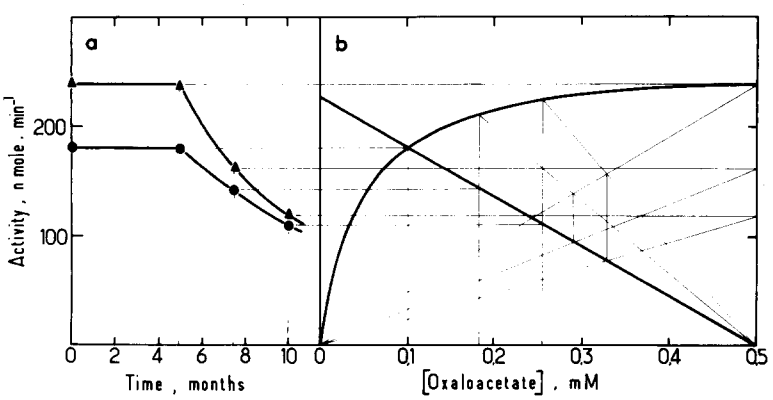

Fig. 4. Graphical removal of diffusional limitations on the stability profile of collagen-bound aspartate aminotransferase. In a, the bound enzyme activity (●———●) was measured [5] with a 7.5 cm^2 film in a 9 ml stirred solution containing 50 mM glutamate and 0.5 mM oxaloacetate. The corresponding activities in the absence of external diffusional limitations (▲———▲) are graphically determined in b knowing the substrate transport coefficient, h = 0.45 cm^3/min and the intrinsic kinetic parameters of the bound enzyme, V_0 = 270 nmol/min, K_{Ox} = 0.052 mM and K_{Gl} = 5 mM.

paring now the intrinsic stabilities of the soluble and bound enzyme indicates that the binding to the collagen membrane by itself also greatly increases the stability of aspartate aminotransferase, especially during the first 5 months.

Discussion

The present paper describes a simple graphical procedure that allows the removal of external diffusional interferences on stability curves obtained with bound enzymes. The method is generally applicable to any kinetic expression provided the following conditions are satisfied: (a) The enzymatic deactivation only affects the concentration of active bound enzymes. (b) Diffusional limitations are significant for a single substrate. This condition is of course satisfied for Michaelis-Menten type of kinetic expression, but may also prevail for many two-substrate enzymatic reactions. As in the case of aspartate aminotransferase, the concentration domain for the two substrates are often sufficiently different so that the diffusion of a single substrate is limiting. (c) The effect of product accumulation in the enzyme microenvironment is negligible. It has been shown that for reversible and product-inhibited enzymatic reactions diffusional limitations for the product can also significantly affect kinetic behaviors [6]. Such product interferences can be minimized by measuring the initial rate of reaction in the absence of products in the bulk solution.

The present graphical method is described for enzymes bound at the external surface of a solid support. In this case, the removal of diffusional effects between the bulk solution and the active surface yields the intrinsic kinetic behavior of the bound enzyme, thus allows a direct comparison between the kinetic and stability properties of free and bound enzymes. In many cases, however, enzymes are embedded inside membranes or porous media so that both external and internal diffusional resistances may be important [4]. Our procedure is still applicable but only allows the removal of external diffusional effects. Unfortunately, at the present time a simple method to further remove internal diffusional effects is not at hand. Yet the magnitude of internal diffusional limitations can be approximately evaluated by considering the relative importance of external and internal diffusional effects [4].

Finally the method is not restricted to stability curves. It can be more generally used to determine the contribution of diffusional effects on inhibition as well as on activation profiles.

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