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KINETIC BEHAVIOR OF IMMOBILIZED ALDOLASE IN A CONTINUOUSLY STIRRED TANK REACTOR

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Aldolase was bound covalently to CNBr-activated Sepharose. The immobilized enzyme was investigated in a continuously stirred tank reactor (CSTR). In spite of the complexity of the system, the stationary performance of the CSTR can be described by a very simple equation. There are essentially two control parameters, V_m and K_{eq}, that govern the stationary performance of the aldolase reactor.

INTRODUCTION

Considerable interest has recently been paid to immobilized enzymes, both from theoretical and practical points of view (1). A lot of work has been done on the kinetics of immobilized enzymes, but there is a need for kinetic studies on reactors working with immobilized enzymes that catalyze reversible reactions (2). In this paper we analyze the stationary kinetics of immobilized aldolase in a continuously stirred tank reactor.[1]

MATERIALS AND METHODS

Materials

Rabbit muscle aldolase (E.C. 4.1.2.13) was prepared according to Taylor et al. (3) and recrystallized three times. D-glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12) was prepared from pig muscle (4) and

[1]Abbreviations: CSTR, continuously stirred tank reactor; GAPD, D-glyceraldehyde-3-phosphate dehydrogenase; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxy-acetone-phosphate; NAD and NADH, oxidized and reduced nicotinamide-adenine-dinucleotide.

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recrystallized four times. Both enzyme preparations were free from triosephosphate isomerase and glycerophosphate dehydrogenase.

Sepharose 6B was purchased from Pharmacia. All other reagents were commercial preparations of analytical grade.

Methods

Sepharose 6B was activated with 20–60 mg CNBr/ml of settled gel, and aldolase was coupled to the CNBr-activated gel according to the standard procedure (5). Enzyme solutions (10–15 mg/ml) were made in 0.05 M phosphate buffer, pH 8.0.

Protein content of the immobilized aldolase preparations was determined in gravity-packed, 2-mm thick quartz cuvettes according to the method of Koelsch et al. (6), by measuring the extinction at 280 nm, assuming that the extinction coefficient of aldolase (7) $E_{280\,nm}^{1\,mg/ml} = 0.74$ had not changed upon immobilization.

Initial velocity measurements were made spectrophotometrically, with intermittent stirring in reaction mixtures containing 2×10^{-6} M GAPD, 1 mM Na-arsenate, 2 mM NAD, $1\text{–}7 \times 10^{-5}$ M FBP in 0.05 M pyrophosphate buffer, pH 7.5. A sufficient amount of aldolase was added to give an extinction change at 366 nm of about 0.15 unit/min. Typically, the volume ratio of gel to total reaction mixture in the cuvette was about $\frac{1}{60}$. Under these conditions the reaction is first order in aldolase. Maximal velocity, V_m, and apparent Michaelis constant, K_m, were estimated from Lineweaver–Burk plots. Substrate concentrations higher than the K_m were chosen to minimize the influence of diffusion limitations.

Substrate and product concentration determinations were made in the following way. FBP concentrations were determined in reaction mixtures containing 2 mM NAD, 2 mM Na-arsenate, about 2×10^{-7} M GAPD, about 2×10^{-7} M aldolase, and the FBP solution to be measured (adjusted to 0.01–0.1 mM), in 0.05 M pyrophosphate buffer, pH 7.5. The reaction mixture for the assay of GAP concentrations consisted of 2 mM NAD, 2 mM Na-arsenate, 2×10^{-7} M GAPD, with the GAP solution to be measured (adjusted to 0.03–0.08 mM) in 0.1 M glycine buffer, pH 8.5. In both cases the extinction differences at 366 nm were measured with an Eppendorf photometer before starting and completing the reaction, by using the extinction coefficient of NADH $E_{366\,nm} = 3330\ M^{-1}\ cm^{-1}$ (8).

Reactor experiments

Immobilized aldolase, usually 0.05 ml of settled gel, was allowed to act in the CSTR (described in Appendix I) at room temperature. The pH was

kept at 7.5, which is the optimal pH of immobilized aldolase, with 0.05 M pyrophosphate buffer. The feeding rate was kept constant, in most cases at 30 ml/h, and the stationary performance of the reactor was regarded as attained when in several subsequent outflow samples, taken at intervals of 10–20 min, the GAP concentration remained constant.

RESULTS AND DISCUSSION

Rabbit muscle aldolase was coupled to Sepharose 6B. The amount of protein fixed was high, 70–85 mg protein per g solid support, and immediately after coupling 20–50% of the added activity could be detected in the Sepharose-enzyme preparation. However, the activity yield decreased to 3–5% during some days of storage at 5°C, and then remained constant for at least some weeks. This phenomenon is probably due to changes in enzyme structure produced by immobilization, resulting in a heterogeneous enzyme population.

This immobilized aldolase with "constant activity" was used in the reactor experiments. With a given set of experimental conditions—flow rate, reactor volume, enzyme concentration, and temperature—one can establish a phenomenological relationship between inlet substrate and outlet product concentrations, if the reactor operates in the stationary state (Fig. 1).

In a first approximation, the simplest mechanism of the reactions will be considered, irrespective of the actual reaction mechanism of aldolase (9), which will be discussed later:

$$\xrightarrow{\text{inlet}} A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X \underset{k_X}{\overset{k_P}{\rightleftharpoons}} E + P + Q \xrightarrow{\text{outlet}} \qquad \text{(scheme I)}$$

together with the corresponding steady-state rate equation

$$v = \frac{dP}{dt} = V_m \frac{1 - PQ/AK_{eq}}{1 + K_m/A + k_X PQ/k_1 A} \left(K_{eq} = \frac{k_{-1}k_X}{k_1 k_P}; \quad K_m = \frac{k_P + k_{-1}}{k_1} \right) \tag{1}$$

The k_i's are the rate constants associated with the reactions, K_{eq} is the overall equilibrium constant, K_m is the apparent Michaelis constant for FBP, and V_m is the maximal velocity. For the sake of convenience, the chemical species and their concentrations are denoted by capital letters as follows: A = FBP, P = GAP, Q = DHAP, E = the free (uncomplexed) form of immobilized aldolase, and X = the intermediate complex.

In stationary CSTR experiments the reaction rate v is equal to $(u/V)P$, the so-called reactor productivity (10), where u is the flow rate (ml/sec), V is the reactor volume (ml), and P is the stationary concentration of the

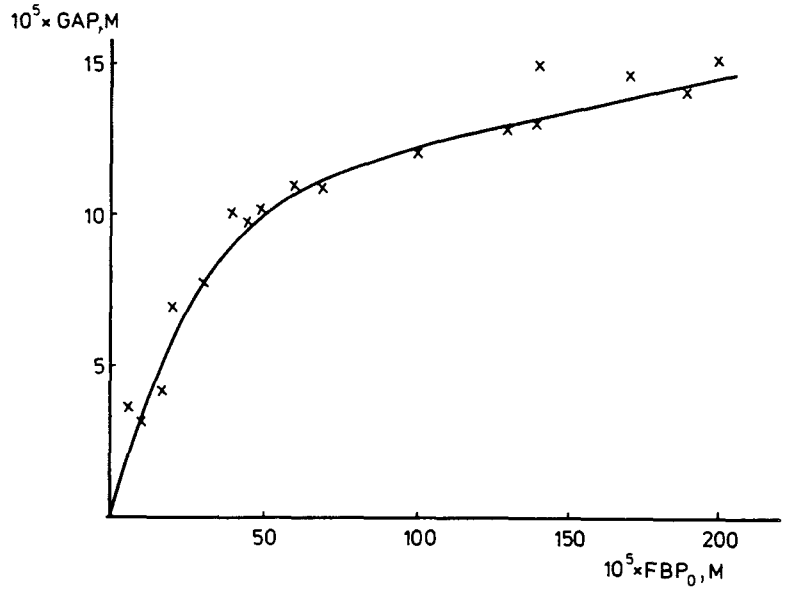

FIG. 1. Dependence of reactor outlet product (GAP) concentration on inlet substrate (FBP) concentration. GAP and FBP concentrations were determined as described under Methods. Reactor parameters: volume (V) = 10 ml, containing 0.05 ml settled gel (Sepharose-coupled aldolase); 0.05 M pyrophosphate, pH 7.5; flow rate (u) = 30 ml/h.

products GAP and DHAP in M. Under the conditions applied in these experiments the diffusion limitations are negligible; therefore, they are not considered here.

Since the value of K_m, determined as described above under Methods, is 2×10^{-5} M, and the inlet FBP concentration is $A_0 = A + P$, inspection of Fig. 1 shows that $A \gg K_m$. Moreover, if we assume that the intermediate X is formed mainly from FBP, i.e., $k_1 \times E \times A \gg k_X \times E \times P \times Q$, Eq. (1) reduces to a fairly simple formula relating P to A_0:

$$\frac{u}{V}P \approx V_m\left(1-\frac{P^2}{A_0-P}\frac{1}{K_{eq}}\right) \tag{2}$$

or, by rearrangement,

$$\frac{1}{P} \approx \frac{1}{(V/u)V_m} + \frac{1}{K_{eq}}\frac{P}{A_0-P} \tag{3}$$

In an independent experiment we determined K_{eq} to be 3.8×10^{-5} M. The value of $(V/u)V_m \approx 35\times10^{-5}$ M. A plot of experimental data according

to Eq. (3) is shown in Fig. 2, which supports the validity of Eq. (3): the ordinate intercept $= 10^5/41 \approx [(V/u)V_m]^{-1}$; the slope $10^5/3.5 \approx (K_{eq})^{-1}$. It should be pointed out that scheme I does not reflect the catalytic mechanism of soluble aldolase, which is as follows (9):

$$\xrightarrow{\text{inlet}} A+E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EQ+P\uparrow^{\text{outlet}} \qquad EQ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E+Q\uparrow^{\text{outlet}} \quad \text{(scheme II)}$$

where EA and EQ are enzyme complexes, and other symbols are as before.

A comparison of experimental results with theoretical predictions derived in Appendix II suggests that it is largely due to the relatively high value of the performance parameter $(V/u)V_m$ that scheme II is formally (kinetically) equivalent to scheme I.

In conclusion, the stationary process in the CSTR is described by a very simple equation [cf. Eq. (3)], which is in accord with other data as regards reaction mechanism. At a given value of residence time, V/u, there are essentially two control parameters, V_m and K_{eq}, that govern the stationary performance of the reactor. The substrate conversion of the CSTR can be

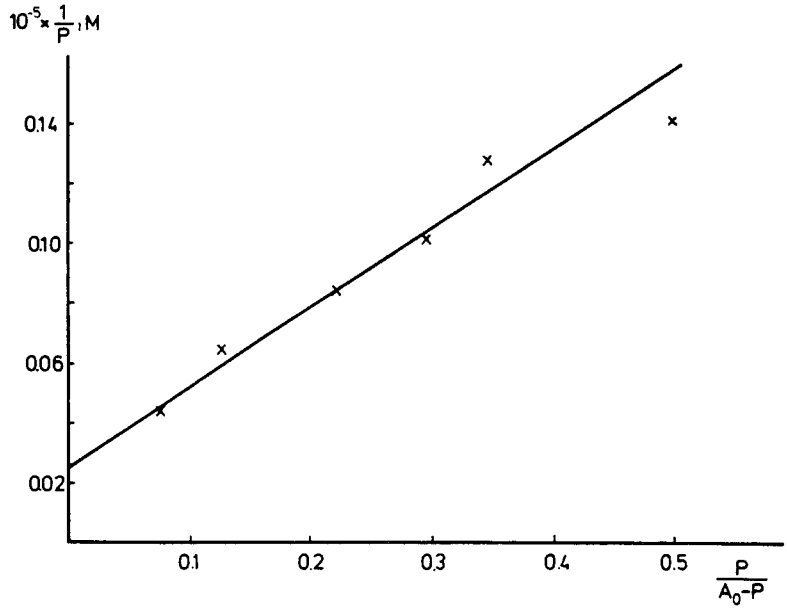

FIG. 2. Plot for characterizing the stationary performance of aldolase CSTR. Experimental conditions are the same as in Fig. 1. P = product concentration in the reactor outlet; A_0 = substrate concentration in reactor inlet solution. Data from one representative set of experiments.

calculated from the control parameters, or vice versa. We should like to point out that under different experimental conditions a nonsimplified kinetic investigation is required. This is possible by using the formulas given in Appendix II.

APPENDIX I: DESIGN OF THE CONTINUOUSLY STIRRED TANK REACTOR

The enzyme reactor employed in this work consisted of the reaction vessel with stirrer, the drive, and the twin peristaltic pump.

The reactor vessel is made of plexiglass and has an internal diameter of 25 mm. The bottom into which the inlet tubing is fixed is covered with a nylon net stabilized by a rigid support net. The vessel is mounted to the stirring equipment by means of flanges, and thus can be removed and cleaned easily. Two conical bores, inclined 20° against the horizontal, provide for the mounting of a calomel and a glass electrode (Fig. 3a and b, respectively). The vessel has an air-escape valve (Fig. 3c) and an outlet (Fig. 3d) with a stainless steel injection needle. A nylon net fixed by a tygon ring prevents the loss of material.

The stirrer (Fig. 3e), with a diameter of 23 mm, is made of polycarbonate and is shaped like a watch glass. Four slim fins on the side facing the bottom of the vessel distribute the entering substrate solution effectively. Four small bores between the fins at the center of the stirrer disk produce a

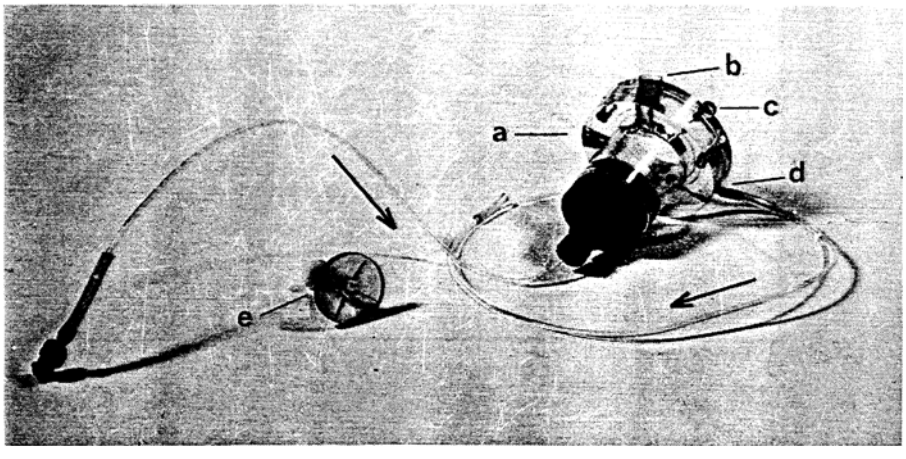

FIG. 3. Photograph of the continuously stirred tank reactor (CSTR) used in the experiments. For details see Appendix I.

vertical convection, which ensures ideal mixing without destroying the gel beads. The distance between the bottom and the lower edge of the stirrer is about 2 mm. The vessel, which can be operated with 10 ml of solution, is filled through the electrode bores.

The stirrer is driven by a dc motor from a pocket tape recorder by means of a rubber belt (1 × 1 mm). A variable precise voltage source and a flywheel guarantee a highly constant stirring speed. The rubber belt that drives the stirrer drives simultaneously a second pocket tape-recorder motor which works as generator so that the stirring speed can be read from a millivoltmeter. The instrument was calibrated by means of stroboscopic disks. A protective cover made of Teflon mounted on the upper part of the stirrer axis protects the stirrer bearings against the penetration of solution from the reaction vessel.

The solution is simultaneously pumped and sucked through the reactor vessel by means of a twin peristaltic pump. The best constancy of flow rate and reactor volume during operation, ±1%, was achieved by a pump with tubings of identical diameters.

APPENDIX II: THE KINETIC ANALYSIS

The steady-state rate equation of the Ordered Uni Bi mechanism (scheme II, which applies to aldolase) in terms of individual rate constants has been elaborated (11). For initial velocity conditions, at zero product concentration, it follows from this rate equation that

$$V_m = kE_0 \qquad 1/k = 1/k_2 + 1/k_3 \tag{4}$$

$$K_m = \frac{k_3}{k_2 + k_3} \cdot \frac{k_{-1} + k_2}{k_1} \tag{5}$$

where k is the specific activity and E_0 is the molar concentration of total active enzyme. In the general case the steady-state rate equation can be rearranged to yield the following relationship:

$$\frac{A_0 - P}{P} = \frac{A}{P} = \frac{K_m + \frac{P}{K_{eq}}\left(\frac{k}{k_{-1}}P + \frac{K_m}{K_3}K_{eq} + \frac{V}{u}V_m + \frac{k}{k_{-3}}\right)}{\frac{V}{u}V_m - P\left(1 + \frac{kP}{k_3K_2}\right)} \tag{6}$$

where

$$K_{eq} = \frac{K_2K_3}{K_1}, \qquad K_1 = \frac{k_{-1}}{k_1}, \qquad K_2 = \frac{k_2}{k_{-2}}, \qquad K_3 = \frac{k_3}{k_{-3}} \tag{7}$$

The denominator of the right-hand side of Eq. (6) tends to zero, as A_0 (and consequently A) tends to infinity:

$$\lim_{A_0 \to \infty} P = P_\infty \tag{8}$$

Or, in other words, P_∞ is the only positive root of the quadratic equation [cf. Eq. (6)]:

$$\frac{k}{k_3 K_2} P_\infty^2 + P_\infty - \frac{V}{u} V_m = 0 \tag{9}$$

By measuring P at fixed $(V/u)V_m$ and at different A_0 values, an estimation of the plateau of P vs. A_0 curve gives the value of P_∞. Because of Eq. (9),

$$\frac{k}{k_3 K_2} = \frac{1}{P_\infty}\left(\frac{1}{P_\infty}\frac{V}{u} V_m - 1\right) \tag{10}$$

Hence the denominator of the right-hand side of Eq. (6) can be directly converted to

$$\frac{V}{u} V_m - P\left[1 + \frac{P}{P_\infty}\left(\frac{1}{P_\infty}\frac{V}{u} V_m - 1\right)\right] = P\left(1 - \frac{P}{P_\infty}\right) \times \left[\frac{V}{u} V_m \left(\frac{1}{P} + \frac{1}{P_\infty}\right) - 1\right]$$

By using this relationship some elementary rearrangements were carried out on Eq. (6): multiplication by the denominator, division by P, etc. The resulting expression is linear with respect to an experimental variable y and also P:

$$y = mP + b \tag{11a}$$

where

$$y = \left(\frac{A_0}{P} - 1\right)\left(1 - \frac{P}{P_\infty}\right)\left[\frac{V}{u} V_m \left(\frac{1}{P_\infty} + \frac{1}{P}\right) - 1\right] - \frac{K_m}{P} \tag{11b}$$

The value of y is calculated from six measurable quantities: A_0, V_m, K_m, P_∞, P, and V/u; m and b represent slope and ordinate intercept, respectively, of the straight line y vs. P (A_0 is varied and the corresponding P measured):

$$m = \frac{k}{k_{-1} K_{eq}}$$
$$b = \frac{K_m}{K_3} + \frac{(V/u)V_m + (k/k_{-3})}{K_{eq}} \tag{12}$$

Note that if K_{eq} is known from independent measurements and the parameters m and b have been estimated, theoretically it is possible to calculate the rate constant ratios $k/k_{\pm i}$, where $i = 1, 2, 3$, by solving a set of six nonlinear algebraic equations (not expanded here).

In our experiments the following values were measured:

$$(V/u)V_m = 35\times10^{-5}\,\mathrm{M}, \qquad K_m = 2\times10^{-5}\,\mathrm{M}, \qquad P_\infty = 16\times10^{-5}\,\mathrm{M}$$

By plotting the data shown in Fig. 1 according to Eq. (11), a straight line was obtained, which ran approximately parallel with the P axis. This means $b \gg mP$, within experiment error. In an independent experiment we determined K_{eq} to be 3.8×10^{-5} M. These values allow one to estimate that $(V/u)V_m \gg K_{eq}K_m/P$ and $kP/k_3K_2 \lesssim 1$ [cf. Eq. (10)]. Consequently, Eq. (6) can be rearranged to give

$$\frac{1}{P} \approx \frac{1}{(V/u)V_m} + \frac{1+c}{K_{eq}}\frac{P}{A_0-P} \qquad c = \frac{1}{(V/u)V_m}\left(\frac{K_{eq}K_m}{K_3} + \frac{k}{k_{-3}}\right) \tag{13}$$

where $c \ll 1$ follows from the discussion under Eq. 3. Therefore, schemes I and II are indistinguishable in our case.

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