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Kinetics of Immobilized Pig Heart Fumarase

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Abstract

The kinetics of immobilized pig heart fumarase are described and compared with the properties of the enzyme free in solution.

1. An analogous pH dependence of initial activity is found for free and immobilized enzyme.
2. Immobilized and free fumarase deviate from classical Michaelis-Menten kinetics in the same way. The apparent K_m values are three to eight times higher for the immobilized (2 mg/g gel) enzyme.
3. The specific activity of immobilized fumarase is dependent on the final enzyme concentration on the gel; normal specific activities are observed when 50 μg fumarase is immobilized per gram of gel, whereas the specific activity decreases with increasing enzyme concentration.
4. The activation energies for free and immobilized fumarase (50 μg/g gel) were found to be identical between 22 and 32°C and with L-malate as substrate (E_a = 12,290 cal/mol at pH 7.9). Upon increasing the concentration of fumarase on the gel, the activation energy decreases.

Our results indicate that the true catalytic properties of fumarase are not affected by immobilization of this enzyme. The slight differences observed when fumarase is immobilized at concentrations higher than 50 μg/g gel must be attributed to diffusional limitation at the surface of the Sepharose matrix.

Index Entries: Kinetics, of immobilized pig heart fumarase; immobilized fumarase, kinetics of; fumarase, kinetics of immobilized; pig heart fumarase, kinetics of immobilized.

Introduction

In a recent publication (*1*), we demonstrated the existence of physical interactions between succeeding enzymes of the citric acid cycle and of the aspartate–malate shuttle. In our experiments, one of the enzymes was attached to a solid support,

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Sepharose-4B, and adsorption of the other enzymes on it was investigated. In these studies we immobilized either malate dehydrogenase (mitochondrial or cytoplasmic) or fumarase. Müller and Pfleiderer (*2*) have already reported studies on immobilized malate dehydrogenase and have claimed that diffusional limitation greatly affects the activity of this system. However, no important damage seems to be caused to this enzyme by the immobilization reactions. In order to confirm the "native" condition of immobilized fumarase, as it was used in our experiments (*1*), we report here a detailed study of its kinetic behavior in comparison with the free enzyme. Reference will also be made to an earlier publication of Erekin and Friedman (*3*) who described some earlier kinetic studies involving immobilized enzyme systems with fumarase and malate dehydrogenase.

Materials and Methods

Sepharose-4B was purchased from Pharmacia Fine Chemicals. L-Malic acid, fumaric acid, and Tris (Trizma base; Tris-(hydroxy-methyl)-aminomethane) were from Sigma Chem. Comp., as well as the electrode for pH measurements (N. E-4753), which was especially designed for use with Tris-buffered solutions. All other reagents were of highest purity available.

Optical density measurements were performed on a double-beam Shimadzu (210 UV, Bausch-Lomb) spectrophotometer.

Immobilization of Fumarase on Sepharose-4B

Pig heart fumarase was prepared as described previously (*4*) and stored as crystals in 0.55 saturated ammonium sulfate. An appropriate amount of this suspension, containing 20 mg of enzyme, was first dialyzed against 10 m*M* potassium phosphate buffer, pH 8.2, and afterwards against 0.1*M* sodium bicarbonate, pH 8.2, containing 0.02*M* L-malate. Substrate was added in order to protect the enzyme during the coupling step. The concentration of this enzyme solution was adjusted to 1 mg/mL. Ten grams, wet weight, of extensively washed (with bi-distilled water) Sepharose-4B was suspended in 20 mL bi-distilled water and activated with 2 g cyanogen bromide that was dissolved in a minimal volume of freshly distilled dimethylformamide. After an 8-min activation, during which the temperature was held at 25°C by the addition of crushed ice and the pH was kept between 11 and 11.5 by the addition of 4*M* NaOH (*5*), the gel was quickly washed with 1 L of cold (4°C) 0.1*M* $NaHCO_3$, pH 8.2, and suspended in the 20 mL fumarase solution. The enzyme was allowed to couple to the Sepharose by overnight shaking at 4°C. The gel was well washed with 1 L of 0.1*M* $NaHCO_3$ and the amount of immobilized fumarase was estimated from measurement of unbound enzyme, collected in the washing buffer. The immobilized fumarase was stored at 4°C in 0.1*M* $NaHCO_3$ buffer, pH 8.2, in the presence of 0.02% (w/v) sodium azide.

Two other immobilized fumarase gels were prepared by exactly the same procedure, but less concentrated enzyme solutions were used for coupling (respectively, 2.5 and 0.5 mg fumarase in 20 mL 0.01*M* sodium bicarbonate, pH 8.2, containing 0.02*M* L-malate).

The Sepharose (10 g wet weight) was, however, still activated with 2 g of cyanogen bromide.

Measurement of Enzyme Activity

The activity of free fumarase was determined spectrophotometrically with either L-malate or fumarate as substrate. When L-malate was the substrate, its concentration was 50 m*M* in 50 m*M* potassium phosphate buffer and an increase in absorbance was recorded at 250 nm ($\epsilon_{\text{fumarate, 250 nm}} = 1450\ M^{-1}\text{cm}^{-1}$) (*6*). The specific activity of free fumarase at pH 7.9 is 550 U/mg (one unit of activity is the amount of enzyme that catalyzes the formation of 1 μmol fumarate/min at 25°C). When fumarate was the substrate, its concentration was 10 m*M* in 50 m*M* potassium phosphate buffer and the decrease in absorbance was measured at 285 nm ($\epsilon_{\text{fumarate, 285 nm}} = 180\ M^{-1}\text{cm}^{-1}$) (*6*).

The activity of immobilized fumarase was determined spectrophotometrically under the same conditions as used for the free enzyme by suspending and carefully mixing a preweighed amount of the gel in 3 mL substrate solution.

Unless otherwise specified, all measurements were performed at 25°C.

Results

Studies with Higher Concentration Immobilized Fumarase (2 mg/g gel)

Amount of Immobilized Fumarase Subunits. Upon immobilization of pig fumarase, no more than 0.5% of the enzyme could be detected in the washing buffer, either by protein absorption [$E^{1\%}_{280\text{ nm, 1 cm}} = 5.1$ for pig heart fumarase (*4*)] or activity measurements. This gel thus contains 2 mg enzyme/g (wet weight) of Sepharose-4B.

It is known that fumarase easily dissociates in monomers by the action of urea (*7*). One gram of the Sepharose–fumarase gel and 1 g of untreated Sepharose-4B were washed with 1 m*M* potassium phosphate buffer, pH 7.3, and sucked dry (1 g of Sepharose corresponds roughly with 1 mL). One milliliter of 1 m*M* potassium phosphate buffer, pH 7.3, containing 9*M* urea, was added and both gels were shaken for 90 min at 25°C. The final concentration of urea was determined by optical rotation measurements of the supernatant (*8*) and was equal to 4.8*M*. From absorption measurements at 280 nm, it was calculated that no more than 0.1 mg fumarase was present in the supernatant; the supernatant of the untreated Sepharose was used as a blank. We may conclude that in our gel an average of only one subunit out of five fumarase tetramers is not directly attached to the Sepharose matrix by a covalent bond.

pH Dependence of Fumarase Activity. In 50 m*M* potassium phosphate buffer at pH 7.9 and with 50 m*M* L-malate as substrate, immobilized pig heart fumarase has a specific activity of 150 U/mg, which corresponds to 27% of the native enzyme activity. The dependence on pH of enzyme activity with L-malate as substrate is given in Fig. 1, for free and immobilized fumarase. They both display

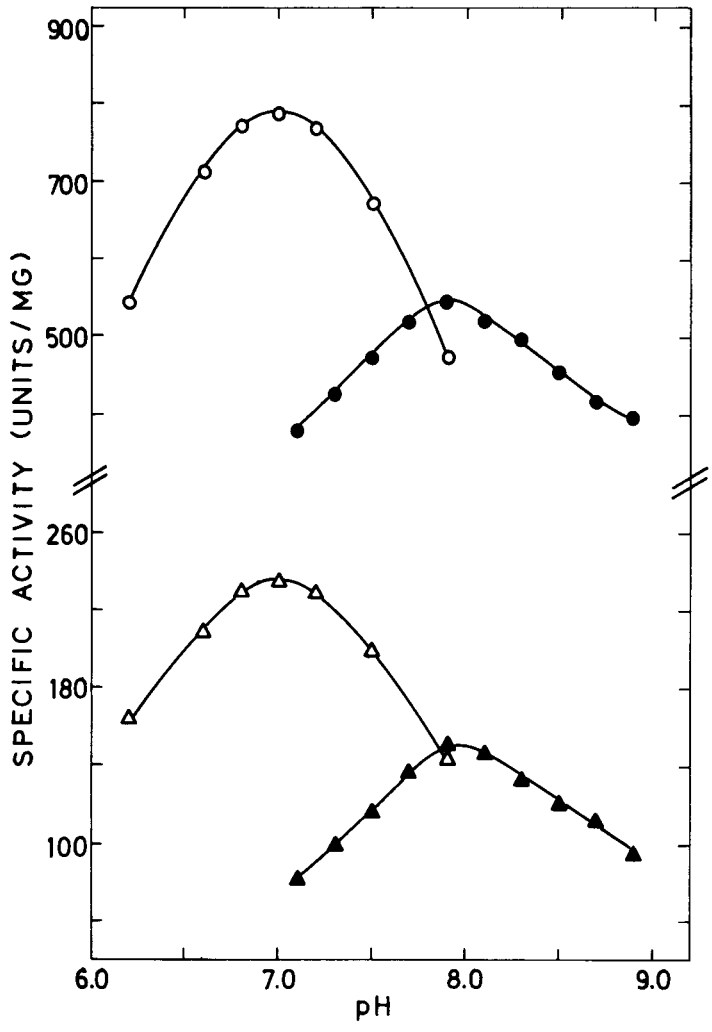

Fig. 1. Specific activities of free (circles) and immobilized (triangles) fumarase in function of pH. Activity was measured at 25°C in solutions containing 50 m*M* potassium phosphate buffer and either 50 m*M* L-malate (●, ▲) or 10 m*M* fumarate (○, △).

maximal activity at pH 7.9. It was calculated that the activity ratio between free and immobilized fumarase equals about 4 over the whole pH range studied.

In 50 m*M* potassium phosphate buffer at pH 7.0 and with 10 m*M* fumarate as substrate, immobilized fumarase has a specific activity of 235 U/mg, which corresponds to 30% of the native enzyme activity under the same conditions (785 U/mg). The activity towards fumarate of both free and immobilized fumarase in function of pH is shown in Fig. 1. Both free and immobilized enzyme show maximal activity at pH 7.0 and the activity ratio remains constant (about 3.4) over the whole pH range studied. The curves obtained for the free fumarase are in good agreement with literature data (*6*).

Determination of Michaelis Constants. It was stated that free pig heart fumarase does not follow classical Michaelis-Menten kinetics in the absence of phosphate buffers (*6, 9, 10*): a low K_m value is obtained in low substrate concentration ranges (0.01–0.5 m*M* L-malate), whereas the reaction displays much higher K_m values in high substrate concentration ranges (2.5–50 m*M* L-malate). Such behavior was ascribed to the existence of two different kinds of binding sites on the enzyme: one set of four active sites and another set of four (?) regulatory sites.

The kinetics of immobilized fumarase were compared at two different pH values with those of free enzyme in a wide range of substrate concentrations in the direction of fumarate formation. The results are shown in Figs. 2 and 3. Velocities are expressed with respect to $V = 100$ in conditions 50 m*M* potassium phosphate, 50 m*M* L-malate, pH 7.9 for free as well as immobilized fumarase. All solutions were buffered with 10 m*M* Tris-acetate. K_m values were determined from these Lineweaver-Burk plots and plots of activity versus log [*S*], and are given in Table 1. K_m^1 represents a true Michaelis constant and refers to the first (active) site. $K^2_{m,\ app.}$ is only an apparent Michaelis constant since it is determined from the intercept on the abscissa of the Lineweaver-Burk plot: it does not correspond with the definition of a Michaelis constant since it is not a representation of the substrate concentration at half maximal activity. In order to obtain a true Michaelis constant ($K_{0.5}$), the contribution of the first site has to be taken into account. Values for $K_{0.5}$ can be obtained from the plots activity versus log [*S*] by determination of the substrate concentration where the activity is half-way the two plateau values.

From Table 1 it can be concluded that the K_m values referring to the first site are increased upon immobilizing fumarase by a factor five to eight. The K_m values referring to the second site are increased by a factor three. The values of K_m^1 and $K_{0.5}$ for free fumarase are in good agreement with earlier determinations (*10, 11*).

Studies with Lower Concentration Immobilized Fumarase (250 and 50 μg/g gel)

Amount of Immobilized Subunits. Upon immobilizing fumarase at lower concentrations, no enzyme activity at all could be detected in the washing buffer, either by protein absorption or activity measurements. We assume that these gels respectively contain 250 and 50 μg fumarase/g (wet weight) Sepharose-4B.

The proportion of immobilized subunits was determined in the same way as mentioned above: no protein release could be measured after a 90 min incubation of the gels in the presence of 4.8*M* urea.

Specific Activities of Immobilized Fumarase. Activities of all immobilized fumarase preparations are given in Table 2 for both foreward and reverse reaction. Experimental conditions were as mentioned above. The apparent specific activities are obviously dependent on the amount of enzyme immobilized per gram gel. Low fumarase concentrations (50 μg/g gel) display normal specific activities.

Comparison of Activation Energies. The activation energies of all three immobilized fumarase preparations and native enzyme were compared at pH 7.9 in 50 m*M* potassium phosphate buffer and with 50 m*M* L-malate as substrate between 22 and 32°C. Results are shown in Fig. 4, together with the activation energies calcu-

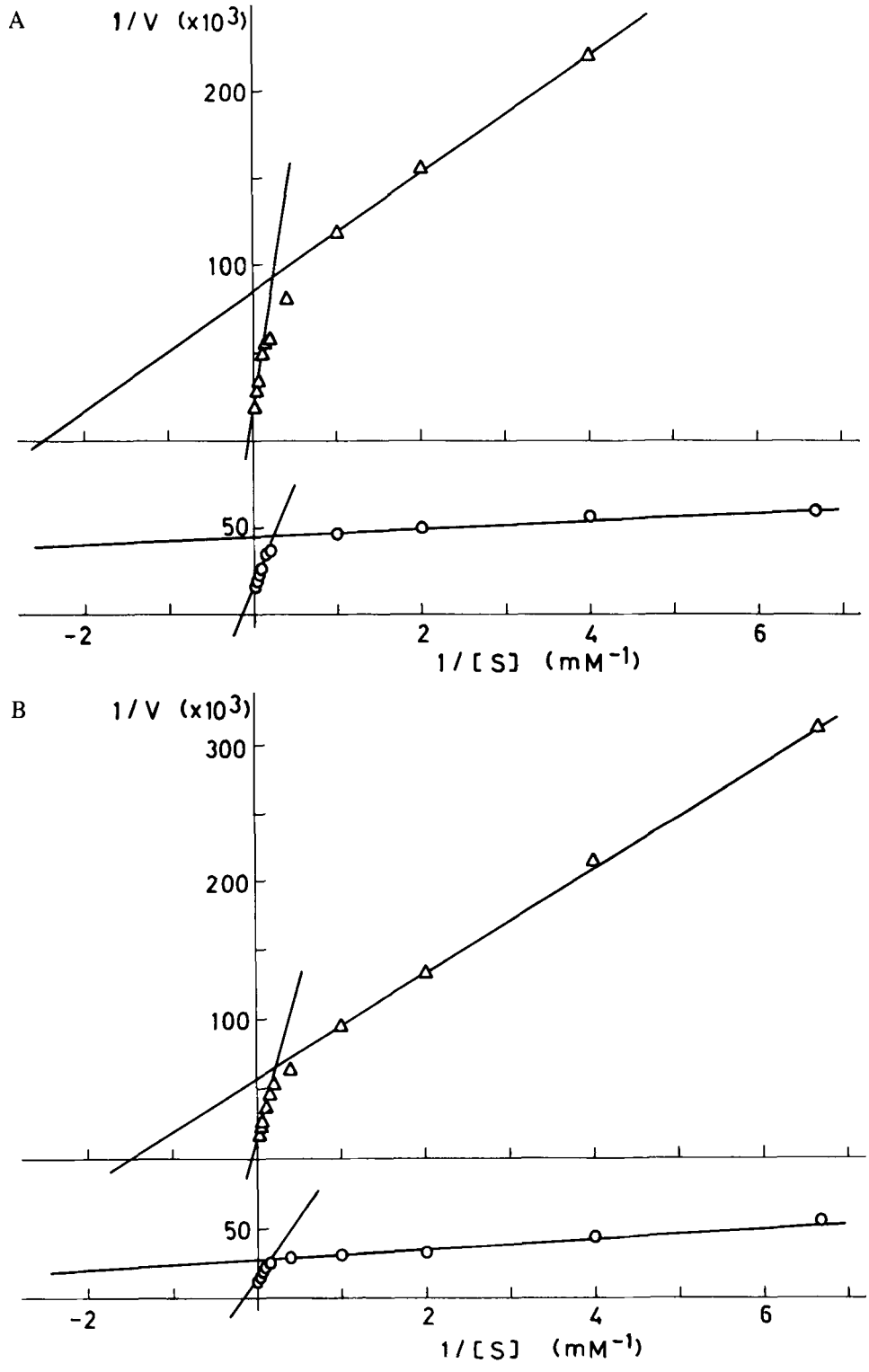

Fig. 2. Lineweaver-Burk plot of free (○) and immobilized (△) fumarase with L-malate as substrate at pH 7.5 (A) and at pH 8.5 (B).

lated from these Arrhenius plots. It may be noticed that the immobilized fumarase preparation, which has a normal specific activity (i.e., 50 μg enzyme/g gel), also has a normal activation energy in the temperature range studied. Our value corresponds well with earlier determinations performed by Massey (*12*). At higher concentrations of immobilized enzyme (250 μg/g and 2 mg/g gel), the activation energy lowers by a factor of 1.2–1.3.

Discussion

A detailed kinetic study of the immobilized pig fumarase preparation that we previously used for experiments of enzyme–enzyme interactions (*1*) (i.e., an enzyme concentration of 2 mg/g Sepharose-4B) reveals that no essential differences exist with respect to fumarase free in solution.

Plots of the pH dependence of initial activity for immobilized and free fumarase display the same features for either the forward or the reverse reaction. We may conclude that the pK values of the amino acid residues in the active site, which are essential for catalysis, are not affected by immobilizing the enzyme.

Both immobilized and free fumarase deviate from classical Michaelis-Menten kinetics in an analogous way. Two K_m values are observed for both systems, depending on the concentration of substrate present. However, the apparent K_m values are three to eight times higher for the immobilized enzyme. Similar increases in K_m have also been observed for other immobilized enzyme systems (*13–22*) and they have been explained in different ways. In some instances, the K_m value may be influenced by ionic charges present on the gel matrix. This possibility is improbable in our specific situation since Sepharose-4B will bear no appreciable amounts of charged groups if remaining active groups after the coupling step are hydrolyzed by standing at mildly alkaline pH (8.2) rather than blocked by primary amines (*23*). An alternative explanation is one of diffusion limitation, which is a consequence of the presence of an unstirred layer of solvent molecules on the surface of the insoluble support. Differences in activity may, however, also result from modifications brought about in the vicinity of the active site of the enzyme by the chemical reactions during immobilization, or alternatively by excessive conformational constraints on the enzyme as a consequence of a multipoint attachment to the matrix.

In order to distinguish between these possibilities, we also examined the properties of gels containing fumarase in lower concentrations that had, however, been activated to the same extent with cyanogen bromide. It is important to notice that the way in which fumarase is attached to the Sepharose matrix is the same for all preparations: they differ solely in the amount of enzyme bound per gram of gel. In the three gels constructed in this way, and containing respectively 2 mg, 250 μg, and 50 μg of fumarase per gram of Sepharose, it was shown that the tetramers are covalently attached to the matrix through practically all four the subunits. This imposes serious conformational restrictions on the enzyme that might eventually slow down its rate of catalysis. This possibility is, however, ruled out by the observation that fumarase, when immobilized at concentrations not exceeding 50 μg/g

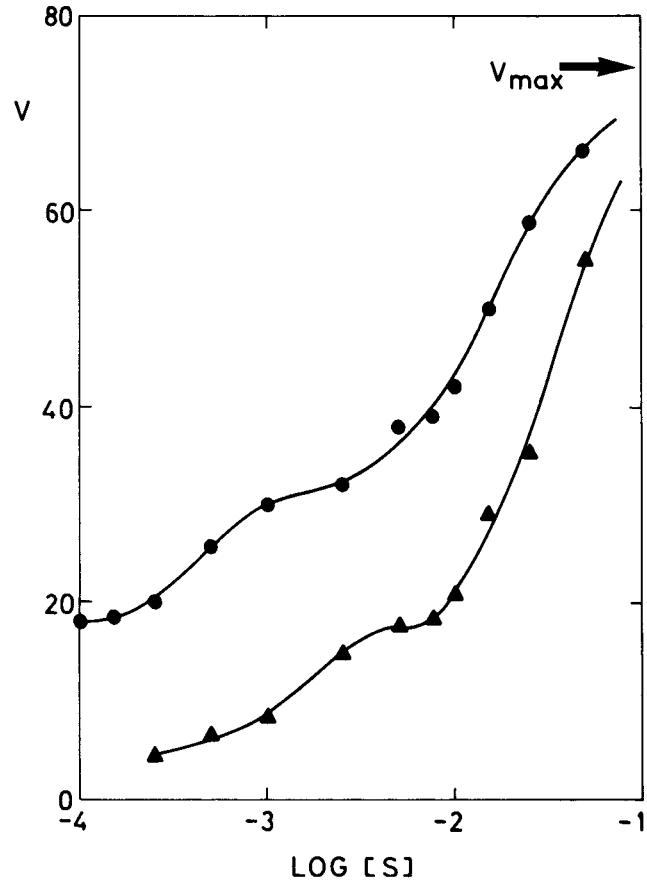

Fig. 3. Dependence of initial velocity on substrate concentration for free (●) and immobilized (▲) fumarase (L-malate as substrate) at pH 7.5 (A) and at pH 8.5 (B).

gel, display normal specific activities with either L-malate or fumarate as substrate. In an earlier publication, normal specific activities were also observed for immobilized fumarase (at enzyme concentrations between 25 and 50 μ/g gel) by Erekin and Friedman (*3*). However, they had activated their Sepharose less completely,

TABLE 1
Values of Michaelis Constants of Free and Immobilized Fumarase (2 mg/g Sepharose) at Different Values of pH[a]

	K^1_m, m*M*	$K^2_{m,app.}$, m*M*	$K_{0.5}$, m*M*
pH 7.5			
Free fumarase	0.049	6.85	10.5
Immobilized fumarase	0.395	22.50	30.0
pH 8.5			
Free fumarase	0.136	4.04	5.8
Immobilized fumarase	0.667	12.60	17.0

[a]L-Malate was the substrate and solutions were buffered with 10 m*M* Tris-acetate.

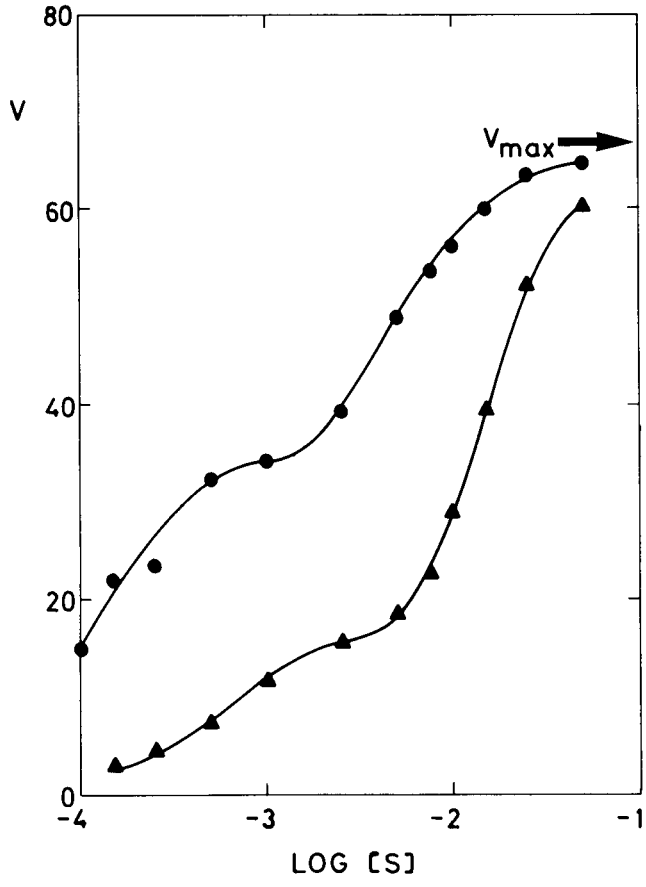

Fig. 3. (*continued*)

and consequently the number of attaching points between matrix and enzyme was reduced. Upon simply raising the concentration of immobilized fumarase, we observed that its specific activity drops to about 45% at 250 μg/g and to 30% at 2 mg/g gel. According to these results the lowering of the specific activity at higher enzyme concentration undoubtedly must be attributed to diffusional limitation. As a consequence of the presence of an unstirred layer of solvent molecules at the sur-

TABLE 2
Specific Activities of Immobilized Fumarase in Function of Concentration on the Sepharose Matrix

Fumarase concentration in gel, mg/g gel	L-Malate as substrate		Fumarate as substrate	
	Specific activity, U/mg	% from native	Specific activity, U/mg	% from native
2	150	27	235	30
0.250	225	41	375	48
0.050	540	98	745	95

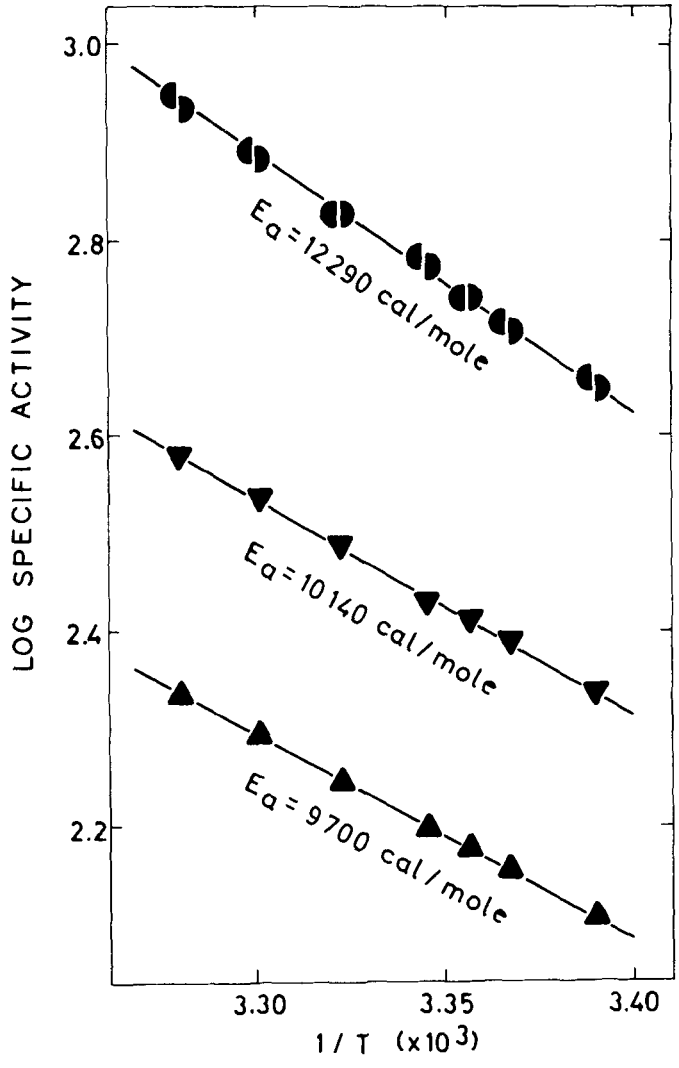

Fig. 4. Arrhenius plot for different concentrations of immobilized fumarase: 2 mg enzyme/g Sepharose (▲), 250 μg/g gel (▼) and 50 μg/g gel (◐), and comparison with free fumarase (◑). Experimental conditions are 50 m*M* potassium phosphate buffer, 50 m*M* L-malate, pH 7.9.

face of the solid support, a gradient in substrate as well as in product concentration is formed in the vicinity of the Sepharose beads: when L-malate is used as substrate in the fumarase catalyzed reaction, its concentration at the level of the active sites of immobilized enzyme will be lower, and the concentration of fumarate formed will be higher, than the respective concentrations in the bulk phase.

In the same way, we can explain the raise of $K^2_{m,\,app.}$ (i.e., the Michaelis constant at higher substrate concentrations) with a factor three upon immobilizing the enzyme. At lower substrate concentrations, diffusional factors will become still

more important and a little higher increase in K_m^1 consequently was observed for immobilized fumarase.

Activation energies for free and immobilized fumarases were estimated from Arrhenius plots. With L-malate as substrate, the activation energy of free and low concentrated immobilized enzyme (50 μg/g gel) were found to be identical between 22 and 32°C, again confirming that fumarase retains its native overall structure upon immobilization. The identity in activation energies also indicates that the flexibility in the enzyme, required for catalysis, is fully conserved. An increase in concentration of enzyme immobilized on the gel, results in a small decrease in activation energy. This observation is also consistent with the diffusional limitation hypothesis, since at higher temperature diffusion will become more limiting, thus giving a lower apparent activation energy. The increase in activity of free fumarase with temperature is indeed more important (E_a = 12,290 cal/mol at pH 7.9) than the acceleration of a diffusion-limited process [increase in diffusion coefficient is about 40% per 10 degrees of temperature (*24*), from which E_a = 6000 cal/mol).

From knowledge of the apparent K_m values in 50 m*M* potassium phosphate buffer at pH 7.9 (which are, respectively, $K_{m,M}$ = 1.7 m*M* and $K_{m,F}$ = 0.32 m*M*, the latter value being calculated from the Haldane equation $K_{eq} = [M]_{eq}/[F]_{eq} = 4.4 = (K_{m,M} \times V_F)/(K_{m,F} \times V_M)$ (6) with V_F = 460 U/mg and V_M = 550 U/mg), and assuming that in reality these values are unaffected by immobilizing the enzyme, the relative concentrations of substrate (L-malate) and product (fumarate) at the level of the active sites of fumarase can be estimated from the equations:

$$V_M \times [EM] - V_F \times [EF] = V_{\text{observed}}$$

$$[EM] + [EF] = 1$$

and from

$$[M]/[F] = (K_{m,M} \times [EM])/((K_{m,F} \times [EF])$$

$$[M] + [F] = 50 \text{ m}M$$

For Sepharose containing 2 mg immobilized fumarase per gram gel, these concentrations are calculated at 25°C as [*M*] = 44.4 m*M* and [*F*] = 5.6 m*M*; for the gel containing 250 μg fumarase/g they amount to [*M*] = 45.7 m*M* and [*F*] = 4.3 m*M*. These relative concentrations give an idea about the diffusional restrictions in our system. They only represent however mean values, since Sepharose beads will be heterogeneous both in structure and in distribution of the covalently attached enzyme.

When considering all the properties of immobilized fumarase we may conclude that the true catalytic properties are not essentially different from the properties of free fumarase. We suggest that our system can be considered as a good model for the in vivo situation, given that the citric acid cycle enzymes indeed are associated in some way with the inner mitochondrial membrane (*1*).

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