Role of diffusion of substrates on the apparent behaviour of immobilized malate dehydrogenase

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1. INTRODUCTION

As shown by electron microscopy and mild subcellular fractionation [1,2], many enzymes reported as soluble are in fact bound to membrane structures. The actual behaviour of such enzymes cannot easily be described by the classical laws of homogeneous enzymology. Among the different factors affecting kinetics, diffusional limitations which may lower the substrate concentration in the enzyme microenvironment are very important. Techniques of immobilization of enzymes onto the surface of artificial support provide a valuable approach, because they simulate such a situation [3].

Malate dehydrogenase and aspartate aminotransferase are enzymes for which the microenvironment may play an important role in the cellular behaviour. These coupled enzymes are required on both sides of the inner mitochondrial membrane to transfer reducing equivalent through NADH between cytosol and the matrix of mitochondria [4,5]. These enzymes can be immobilized on collagen films [6]. Kinetics obtained with aspartate aminotransferase after immobilization have shown that the enzyme exhibited a greater affinity for glutamate, but a lower affinity for oxaloacetate [7]. A mathematical model based on diffusional limitations for oxaloacetate demonstrated that diffusional resistance alone was responsible for the kinetic modifications [8].

This work aimed to determine the effect of diffusion of substrates on the behaviour of malate dehydrogenase immobilized on collagen films.

2. MATERIALS AND METHODS

Malate dehydrogenase (L-malate-NADH oxidoreductase, EC 1.1.1.37) from pig heart cytosol was purchased from Sigma. Its concentration was determined spectrophotometrically ($\varepsilon_{280}^{1\%} = 9.05$, [9]). NADH and oxaloacetic acid were of the highest purity available from Sigma. Collagen films (F 70 type) were a generous gift of the Centre Technique du Cuir (Lyon).

2.1. *Immobilization of malate dehydrogenase*

The enzyme was covalently bound to the surface of the collagen film activated by the acyl–azide method, as in [6]. Coupling was performed by soaking the activated film in 0.9 mg enzyme/ml solution in 0.1 M borate buffer (pH 8.4) for 1 h at 4°C.Then enzymatic films were thoroughly washed with 1 M KCl and stored in 0.05 M Tris–HCl buffer (pH 8.3) at 4°C.

2.2. *Enzyme assays*

Malate dehydrogenase activity was determined spectrophotometrically with oxaloacetate and NADH as substrates at 27°C in 0.05 M Tris–HCl buffer (pH 8.3). Kinetics were studied at 340 nm, by using the expanded scale of a Cary 16K spectrophotometer (0.1 *A* full-scale).

The activity of the immobilized enzyme was assayed in [8]; films measuring 1.25 × 1.5 cm (i.e., 3.75 cm^2 for both faces of the film) were immersed in a vessel containing 12 ml reaction mixture magnetically stirred. A circulation loop enabled the

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reaction mixture to pass through the spectrophotometer cuvette for continuous recording of the reaction rate. The reaction was initiated by pouring the active film into the reaction vessel and stopped by taking it out.

3. RESULTS AND DISCUSSION

3.1. *Kinetics of soluble malate dehydrogenase*

Experiments were performed by varying [oxaloacetate] at fixed [NADH] and vice-versa: 6 values were chosen for [oxaloacetate] between 10–1000 μM and 5 for [NADH] between 2–50 μM. Primary and secondary plots indicated that K_m for oxaloacetate was 50 μM and 2.5 μM for NADH; spec. act. was 33 × 10^3 min^{-1} for the dimer. These values agree with those reported within these substrate and coenzyme concentration limits [9–11].

3.2. *Kinetics of immobilized malate dehydrogenase*

Kinetic behaviour of immobilized and native enzyme were compared under saturating substrate levels as determined with the native enzyme. Activity was first studied as a function of [oxaloacetate] when [NADH] was held saturating at 50 μM and secondly for varying [NADH] when [oxaloacetate]

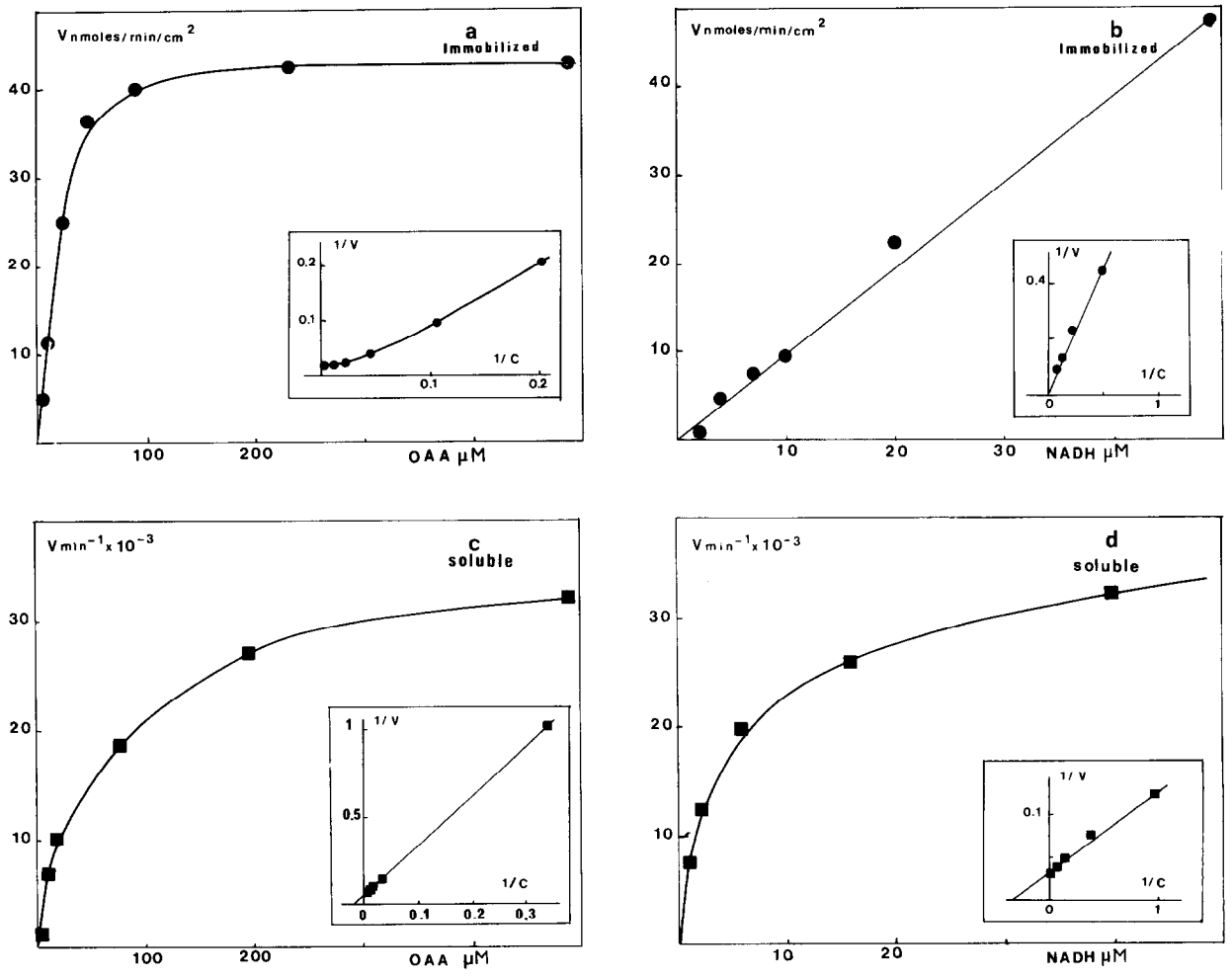

Fig.1. Compared kinetics of malate dehydrogenase bound to collagen film (a,b) and soluble (c,d), at 27°C in 0.05 M Tris–HCl buffer (pH 8.3): (a,c) saturating [NADH], 50 μM; (b,d) saturating [oxaloacetate], 1.1 mM. The insets show the double-reciprocal representation of the kinetics.

was held saturating at 1.1 mM. The curves thus obtained are given in fig.1 (a,b) and compared, in fig.1 (c,d) to those obtained in the same conditions for the native enzyme. Double reciprocal plots are given in the inserts.

Obviously, the apparent behaviour was strongly modified after immobilization. When comparing fig.1a and 1c, we see that at a fixed saturating [NADH] the [oxaloacetate] giving the half-maximum velocity, $S_{0.5}$, was lower for the immobilized enzyme than for the soluble enzyme: 20 μM instead of 50 μM. Furthermore the double reciprocal plot was no more linear.

At a fixed saturating [oxaloacetate] the exhibited activity of the immobilized enzyme was practically proportional to [NADH] up to 50 μM and it was not possible to determine $S_{0.5}$ for NADH; this observation suggested that the app. K_m was strongly displaced toward high [NADH]. As reported with aspartate aminotransferase and for sorbitol dehydrogenase immobilized on collagen films [8,12] this behaviour can be explained by taking into account the transport of substrates between the bulk solution and the enzymatic film surface.

The rate of substrate transport by molecular and convective diffusion is given by the product of the transport coefficient h and the difference of concentration between the bulk solution and the surface of the film, due to the substrate and coenzyme consumption at the active site:

$$V_{diff.} = h_{OAA}\,((OAA) - (OAA)_o)$$
$$= h_{NADH}\,((NADH) - (NADH)_o)$$

h_{OAA} and h_{NADH} are the transport coefficients for oxaloacetate and NADH ($h = D/l$, where D is the translation diffusion coefficient and l the thickness of the boundary layer at the membrane solution interface). (NADH) and (OAA) and $(NADH)_o$ and $(OAA)_o$ are the coenzyme and substrate concentrations in the bulk phase and at the film surface, respectively. For the immobilized enzyme, an intrinsic rate equation similar to that of the enzyme in solution [10] was assumed:

$$V_{enz.} = \frac{V_{max}\,(NADH)_o\,(OAA)_o}{K_{i,NADH}K_{OAA} + K_{NADH}(OAA)_o + K_{OAA}(NADH)_o + (NADH)_o(OAA)_o}$$

K_{NADH} and K_{OAA} are the Michaelis constant for NADH and oxaloacetate and $K_{i,NADH}$ is the dissociation constant for NADH. $K_{i,NADH}$ is approximately equal to K_{NADH} for malate dehydrogenase [10]. At steady-state $V_{diff.} = V_{enz.}$.

The diffusion coefficient and consequently the transport coefficient of oxaloacetate and NADH are of the same order of magnitude, so the concentration difference must be approximately the same for the 2 substrates. In the case of excess oxaloacetate (1.1 mM) this difference cannot exceed that of the bulk [NADH] which is very low compared to [oxaloacetate]. Thus diffusional resistances are negligible for oxaloacetate and only $h((NADH) - (NADH)_o)$ must be considered. If the enzymatic activity on the film surface is high, the NADH concentration at this surface must be very small compared to its bulk concentration and the rate is only given by $v = h(NADH)$; it is independent of enzymatic parameters. That is what was observed in fig.1b. From these experiments, a transport coefficient equal to $1.6\ 10^{-3}$ cm/s can be calculated. This value agrees with the data in [3,8,12–14]. The decrease of the app. K_m for oxaloacetate is also explained by taking into account the diffusional limitations.

The observed effect of diffusion on immobilized malate dehydrogenase: increased app. K_m for NADH and decreased app. K_m for oxaloacetate, may be of importance to the functional role of this enzyme. Mitochondrial malate dehydrogenase is partially bound to the internal membrane of mitochondria [2]. Furthermore, the cytosolic and mitochondrial forms of malate dehydrogenase and aspartate aminotransferase are specifically bound together [15]. These observations indicate that malate dehydrogenase is active in an heterogeneous medium. This work shows that the binding of soluble enzymes to artificial membranes represents an approach to simulate their behaviour in the heterogeneous environment.

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