THE INTERACTION OF SOME DEHYDROGENASES WITH N^6-(6-AMINOHEXYL)-ADENOSINE 5′-MONOPHOSPHATE SEPHAROSE

C. R. LOWE and M. G. GORE

Department of Physiology and Biochemistry, University of Southampton, Southampton S09 3TU, England

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1. Introduction

In recent years the importance of affinity chromatography as a technique for the purification of enzymes has increased significantly and is now well established [1,2]. The general utility of the technique has been greatly enhanced by the introduction of immobilised coenzymes as 'general ligand' adsorbents for binding a wide range of complementary enzymes [3,4]. More recently, however, a number of analytical applications of affinity chromatography have been proposed and applied to the exploration of binding mechanisms and kinetics [5], the investigation of enzyme–substrate interactions [6] and the determination of dissociation constants [7]. However, despite general appreciation of the utility of these applications of affinity chromatography, little is known about the effect of immobilisation of the ligand on subsequent interactions with the complementary macromolecule. It is almost invariably assumed that there is a close parallel between the interaction of the enzyme and free ligand in homogeneous aqueous solution with that between the enzyme and immobilised ligand. However, steric restrictions imposed by proximity of the ligand to the matric backbone and the possibility of non-specific interaction generated by the hydrophobic spacer molecule may compromise this simple assumption. Therefore it was considered imperative to re-evaluate the nature of the interaction between the immobilised ligand and complementary enzymes. To this end, the interaction of several dehydrogenases with N^6-(6-aminohexyl)-ado-5′-P–Sepharose was examined. The present paper demonstrates that several of these enzymes interact with immobilised ado-5′-P in a complex fashion.

2. Materials and methods

Pig heart mitochondrial malate dehydrogenase (EC 1.1.1.37), rabbit muscle lactate dehydrogenase (EC 1.1.1.27), pig heart lactate dehydrogenase (EC 1.1.1.27) and yeast alcohol dehydrogenase (EC 1.1.1.1) were purchased from Boehringer, London. Bovine liver glutamate dehydrogenase (EC 1.4.1.3.) was from Sigma, London. The enzymes were assayed according to the methods quoted in the Boehringer catalogue.

2.1. *Preparation of immobilised-ado-5′-P*

N^6-(6-Aminohexyl)-adenosine 5′-monophosphate was synthesised and characterised according to the methods of Guilford et al. [8] and coupled to CNBr-activated Sepharose 4B essentially as described by Axen et al. [9]. The immobilised ligand concentration was 2.5 μmol ado-5′-P/g moist wt gel measured by the methods described by Lowe and Mosbach [10]. C^8-(6-Aminohexyl)-adenosine 5′-monophosphate was prepared, characterised and covalently attached to Sepharose 4B as described previously [11].

2.2. *Measurement of interaction between enzyme and gel*

The interaction between pig heart lactate dehydrogenase (EC 1.1.1.27) and immobilised-ado-5′-P was investigated by incubating the enzyme (2.8 μg; 1 IU) with the gel (25–1000 mg moist wt) at 21°C in 0.1 M

potassium phosphate buffer, pH 7.0, total vol. 4.0 ml, for times up to 5 h. The gel suspensions were continuously stirred and allowed to settle under gravity before samples (20 μl) were withdrawn for measurement of the residual enzyme activity in the supernatant buffer. Other dehydrogenase enzymes (1 IU) were incubated in a similar fashion. Control incubations comprised all components of the incubation medium except that the immobilised ado-5′-P was replaced by underivatised gel. In some experiments the uptake of the enzymes by the gel was studied in the presence of various concentrations of free NAD^+ (0–10 mM), NADH (0–0.1 mM) or ado-5′-P (0–10 mM).

3. Results and discussion

Figure 1 illustrates the decrease in the percentage of lactate dehydrogenase remaining in free solution as the amount of ado-5′-P–Sepharose in the incubation vessel is increased. The curve (closed circles) was obtained by sampling the supernatant as close as possible to time zero after mixing; in practice after about 2 min. This curve appears to be hyperbolic and its shape is determined by the degree of interaction between enzyme and immobilised ado-5′-P. As has been noted before [12] the uptake of enzyme onto the gel is time-dependent and proceeds over a period of several hours. The other curves given in fig.1 describe the percentage of enzyme bound to the gel after incubations of 50, 100, 200 or 300 min. These curves suggest that the apparent operational capacity of the ado-5′-P–Sepharose increased over a period of at least 5 h and this clearly has important practical implications. The changing shape of the curves simulates a situation where the dissociation constant for the reaction between enzyme and immobilised ado-5′-P decreases with time. In the particular experiment described above the same curves could be described by dissociation constants of 93 μM (for time zero) decreasing to 6 μM (for time 300 min), these values were calculated using an assumed concentration of available immobilised ado-5′-P of 2.5 μmol/g Sepharose moist wt. This value is of course an over estimate since not all of the ado-5′-P bound to the Sepharose will be available for interaction with the enzyme [13]. Significantly the value of the dissociation constant given at time 0 is very close to the value of the dissociation constant for the reaction between enzyme and free N^6-(6-aminohexyl)-ado-5′-P [7].

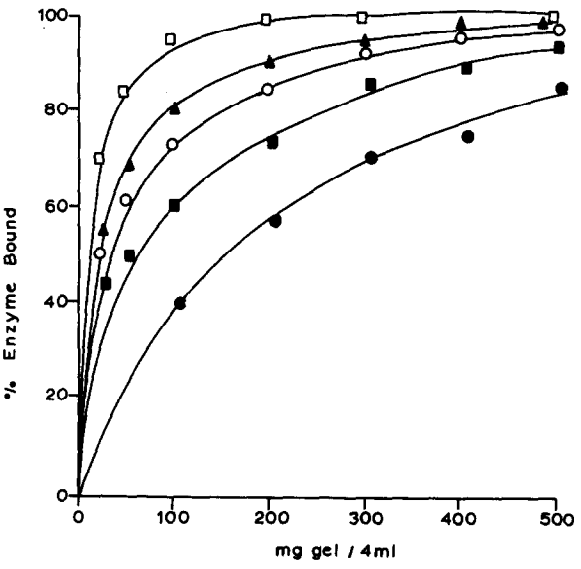

Fig.1. The binding of pig heart lactate dehydrogenase to N^6-(6-Aminohexyl)-ado-5′-P–Sepharose as a function of time. Samples of enzyme (2.8 μg; 1 IU) were incubated with 0–500 mg gel in total vol. 4 ml 0.1 M potassium phosphate buffer, pH 7.0, at 25°C and the residual activity in the supernatant was assayed at time 0 min (●), 50 min (■) 100 min (○), 150 min (▲) and 300 min (□).

The time-dependency of the interaction between enzyme and ado-5′-P–Sepharose is typical of any chemical reaction although in this instance the rate of reaction is much slower than would be anticipated. A more rigorous analysis of the reaction progress between lactate dehydrogenase and ado-5′-P–Sepharose is given in fig.2 in the form of a semi-logarithmic plot. Here the logarithm of the percentage of enzyme activity remaining in free solution is plotted against time. There are two clear stages in the disappearance of enzyme from free solution. First a very fast uptake of some of the enzyme onto the gel occurring at a rate too fast to be measured by the above technique. This process is followed by a much slower uptake of enzyme onto the gel at a rate dependent on the concentration of the ado-5′-P–Sepharose. These two processes may reflect the different environment or reactivities of at least two species of reactive ado-5′-P.

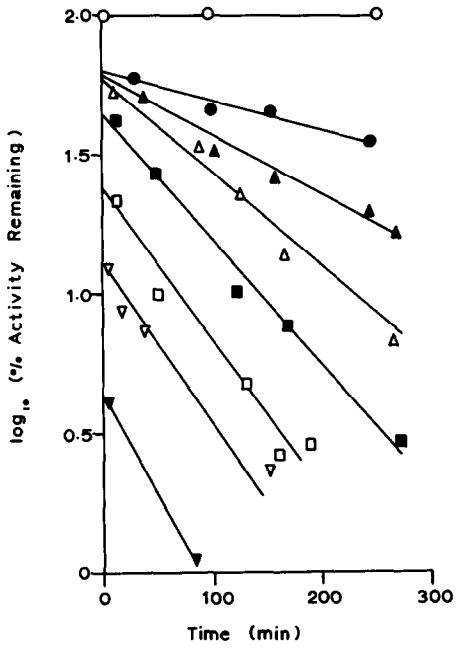

Fig. 2. A semilogarithmic plot showing the biphasic nature of the binding of lactate dehydrogenase to immobilised ado-5′-P at 25°C as a function of time. Amounts of gel present per total vol. 4 ml 0.1 M potassium phosphate buffer, pH 7.0, were: 0 mg (○), 25 mg (●), 50 mg (▲), 100 mg (△), 200 mg (■), 300 mg (□), 500 mg (▼), and 1000 mg (▽).

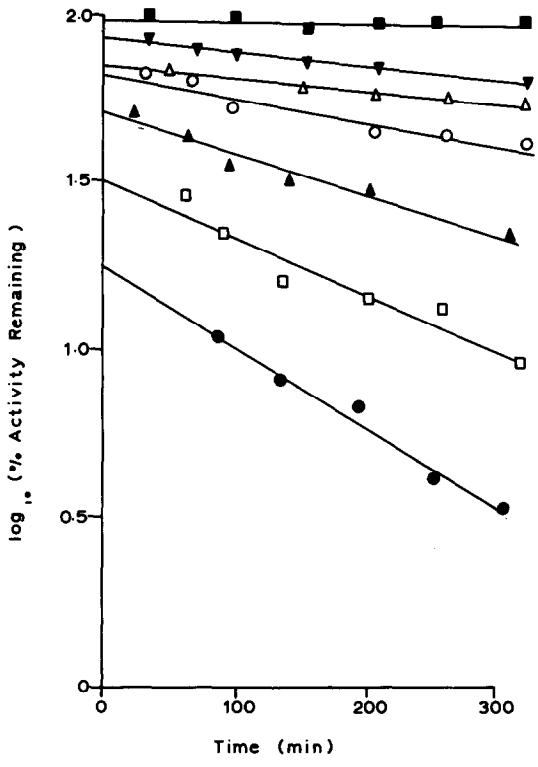

Fig. 3. A semilogarithmic plot showing the effect of several concentrations of free ado-5′-P on the binding of lactate dehydrogenase to immobilised ado-5′-P. The binding of pig heart lactate dehydrogenase (2.8 μg; 1 IU) to 300 mg gel-bound ado-5′-P in 4 ml total vol. 0.1 M potassium phosphate, pH 7.0, was measured as a function of time in the presence of several concentrations of free ado-5′-P; 0 mM (●), 1 mM (□), 2 mM (▲), 3 mM (○), 4 mM (△), 5 mM (▼) and 10 mM (■).

That the biphasic nature of the adsorption of rabbit muscle lactate dehydrogenase to N^6-(6-aminohexyl)-ado-5′-P–Sepharose is not a characteristic of the individual enzyme is shown by the fact that other enzymes, including malate dehydrogenase, alcohol dehydrogenase and pig heart lactate dehydrogenase also exhibit the same phenomenon although with different rates of uptake. Furthermore, the phenomenon is not a characteristic of this gel alone since similar observations can be noted when these enzymes are adsorbed to C8-(6-aminohexyl)-ado-5′-P–Sepharose.

The two distinct phases of the adsorption of these enzymes to the gel must be attributed to interactions between immobilised ado-5′-P and specific sites on the enzymes since both adsorption processes are readily inhibited by the presence of free nucleotides in the incubation media. Figure 3 illustrates the effect of several concentrations of free ado-5′-P on the percentage enzyme-bound in the fast phase and on the rate of uptake of enzyme in the slow phase. High concentrations of free ado-5′-P appear to inhibit binding of the enzyme in both phases. Similar results were obtained with NADH and NAD^+ although the concentrations required to give the same effect parallel the dissociation constants of the enzyme–nucleotide binary complexes in free solution.

The results of the experiments above suggest that in rigorous attempts to quantitate the interaction of enzymes with immobilised ligands in terms of dissociation constants [7] the time element should be closely regarded. The system under study is best described by the apparent dissociation constant calculated from the extrapolated values of enzyme uptake at time zero. Furthermore, the data suggest some practical implications for separation of enzyme

mixtures by affinity chromatography. For example, not only is the effective capacity of affinity adsorbents enhanced by contacting the enzyme with the gel for extended times of equilibration but also an increase in resolution of protein mixtures can be achieved by selecting a suitable time of pre-incubation. For example, most of the lactate dehydrogenase is adsorbed to ado-5′-P–Sepharose within a few minutes whereas glutamate dehydrogenase could be adsorbed to immobilised ado-5′-P only by equilibration for several hours.

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