ENZYMATIC BEHAVIOUR OF LIPOAMIDE DEHYDROGENASE ISOENZYMES IMMOBILIZED ON *N*-ALKYL SEPHAROSE MATRICES

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1. Introduction

Lipoamide dehydrogenase (EC 1.6.4.3), a component of the α-keto acid dehydrogenase complexes, is electrophoretically heterogeneous in eukaryotes [1–3]. The usual purification procedure [4] leads in the case of the pig heart enzyme to at least six distinguishable isoenzymes, some derived from PDC, others from α-KGDC [1–3]. These isoenzymes can be separated by a combination of ionic and hydrophobic effects both on substrate- and on *N*-alkyl Sepharose 4B matrices into two sets of three components each. The enzyme fraction which contains the isoenzymes derived from the α-KGDC is hardly bound by butyl-Sepharose but is tightly bound by hexyl-Sepharose [5]. The interaction is reversible at high ionic strength. The second fraction is bound reversibly to the butyl-matrix but irreversibly to the hexyl-matrix. As the interactions observed might be related to the behaviour of the isoenzymes within their respective multienzyme complexes, the kinetic activities of these noncovalently immobilized enzyme fractions were compared.

2. Materials and methods

2.1. *Preparation of NAD$^+$- and N-alkyl Sepharose 4B matrices*

The matrices were prepared by CNBr activation of Sepharose (Pharmacia, Uppsala) and subsequent coupling of the various ligands as described previously [5].

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2.2 *Preparation of the isoenzyme fractions*

Lipoamide dehydrogenase was purified as described elsewhere [4]. 60 mg of enzyme was separated on NAD$^+$-Sepharose into two fractions. The first fraction (I) was collected by elution with 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA and then concentrated by an ammonium sulphate precipitation (75%). The second enzyme fraction (II) has been eluted with a 100 mM sodium phosphate buffer (pH 7.2) also containing EDTA and was concentrated in the same manner. The final enzyme preparations have an absorbancy ratio 280/455 nm of 5.3 and 6.4 respectively. Disc gel electrophoresis, in order to characterize both fractions, was carried out on 8% polyacrylamide gels according to Davis [6].

2.3. *Enzyme activities*

The enzymatic activities of both free and immobilized enzymes were determined with an Aminco-Chance dual wavelength spectrophotometer (0–0.05 absorbance range) using 340 nm as measuring and 380 nm as reference wavelength [7]. Reduced lipoamide was prepared by reduction of the oxidized form with sodium borohydride.

2.4. *Preparation of the immobilized enzymes*

Sepharose 4B was activated with cyanogenbromide (200 mg/ml of packed gel) and coupled with aminoalkanes (20 mM). The substituted gels were diluted 8-fold in 30 mM sodium phosphate buffer (pH 7). Concentrated enzyme (800–1000 μg/ml of packed gel) was added under gentle stirring. After adsorption the gel was diluted approximately 5-fold. Free in solution this would have meant a final protein concentration of 0.020–0.025 mg/ml.

3. Results

Lipoamide fraction II which was bound to butyl-Sepharose in a low ionic strength buffer still retains its catalytic activity since it can be visibly reduced on the matrix by NADH. Only traces of enzyme are released upon this treatment (< 4%). It was also verified for enzyme fractions I and II bound to the hexyl-matrix that no release of enzyme occurred in a 30 mM sodium phosphate buffer containing EDTA. Thus this buffer was used to compare the kinetic properties of both enzyme fractions free in solution and upon noncovalent attachment to butyl- and hexyl-Sepharose. In fig.1 the results are shown for the isoenzymes from fraction I. L-B plots are shown for the variation of reduced lipoamide at two NAD^+ levels using free and hexyl-Sepharose bound enzyme. Non-linear kinetics is observed in both cases as is usually observed when the enzyme is assayed at 25°C [8]. In the case of enzyme fraction I bound to the hexyl-matrix, it is less obvious whether parallel lines are obtained at low substrate concentration.

The kinetic parameters have been summarized in Table I. K_m values, obtained by extrapolating the high

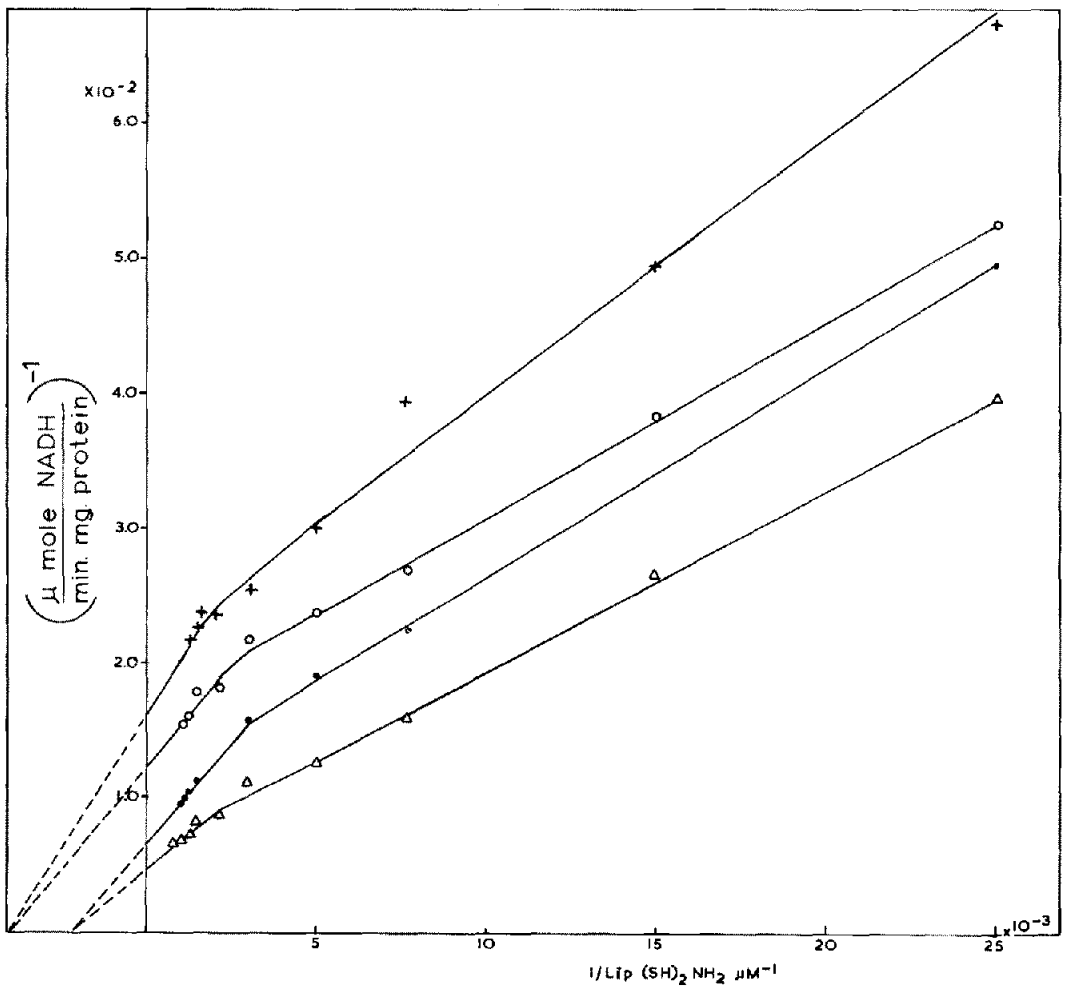

Fig.1. Lineweaver–Burk plots of lipoamide dehydrogenase isoenzyme fraction I at two different NAD^+ levels. Assays were performed at 25°C. 400 μM NAD^+: (ΔΔΔ) free lipoamide dehydrogenase; (●●●) hexyl-Sepharose immobilized lipoamide dehydrogenase. 67 μM NAD^+: (ooo) free enzyme; (+++) hexyl-Sepharose 4B immobilized enzyme.

Table 1
Kinetic parameters of lipoamide dehydrogenase fraction I and II

	400 μM NAD^+		67 μM NAD^+	
	K_m lip $(SH)_2NH_2$ (μM)	V	K_m (μM)	V
Enzyme I free	465	220 (100%)	250	85 (100%)
Enzyme I + hexyl-Seph	465	155 (70%)	250	65 (76%)
Enzyme II free	455	170 (100%)	230	65 (100%)
Enzyme II + butyl-Seph	455	65 (38%)	230	25 (38%)
Enzyme II + hexyl-Seph	910	20 (12%)	–	–

Velocities are expressed in μmoles NADH produced/minute/mg of protein and are obtained by extrapolation from fig.1 and 2.

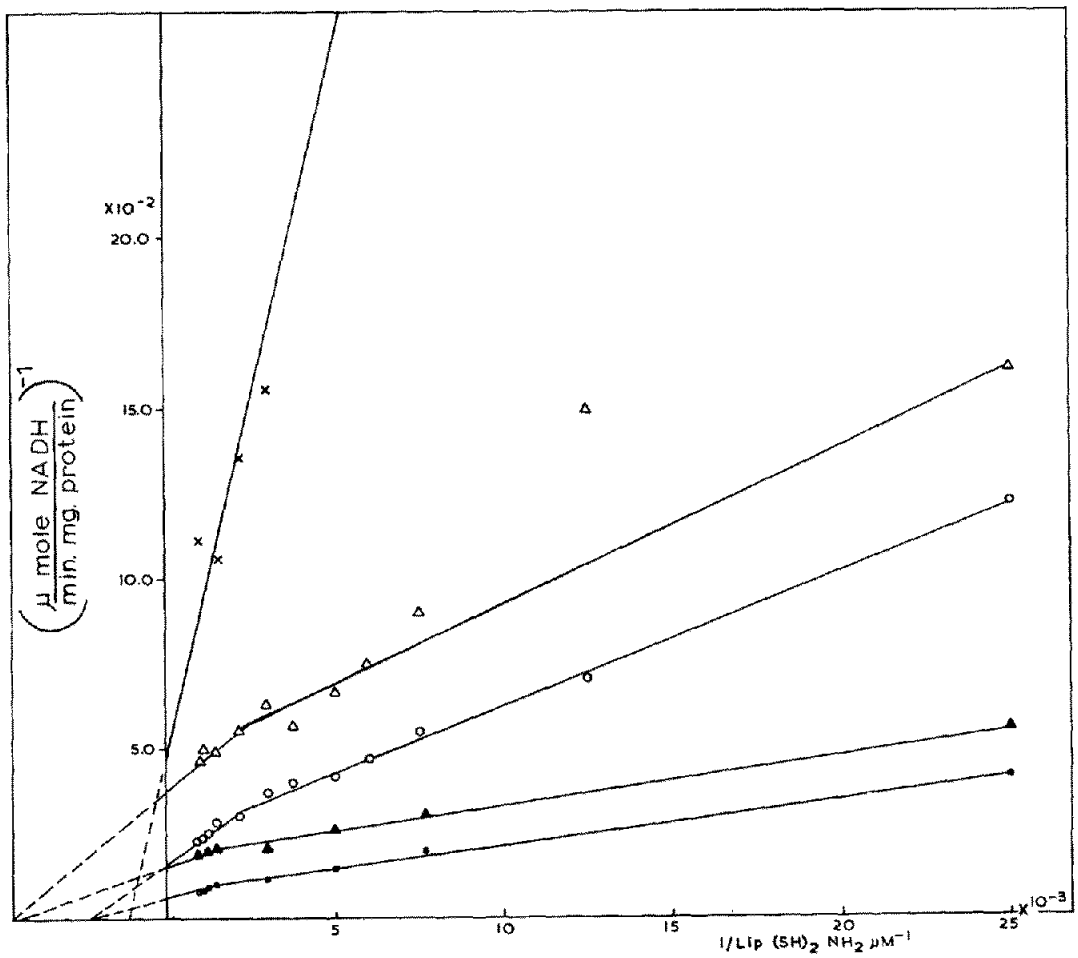

Fig.2. Lineweaver–Burk plots of lipoamide dehydrogenase isoenzyme fraction II. Conditions as in fig.1. 400 μM NAD^+: (●●●) free enzyme (II); (○○○) butyl-Sepharose immobilized enzyme (II). 67 μM NAD^+: (▲▲▲) free enzyme (II); (ΔΔΔ) butyl-Sepharose immobilized enzyme (II); (xxx) hexyl-Sepharose immobilized enzyme (II).

lip $(SH)_2NH_2$ concentration data, remain unchanged in fixed and free enzyme whereas the V values are reduced by approximately 30% at both NAD^+ levels.

Similar data is shown for the second isoenzyme fraction which has been immobilized on butyl-Sepharose beads (fig.2 and Table 1). In this case the V is strongly affected; a 60–65% reduction in velocity is obtained. This is even more the case when enzyme fraction II is tested with a hexyl-matrix. The extrapolated velocity is reduced to approximately 15% of the value for the free enzyme, whereas the K_m also changes. In Table I we have not compared the V values for both free isoenzyme fractions on the basis of their flavin content. A recalculation on this basis demonstrates that there is only an insignificant difference of 6–8% in turnover at both NAD^+ levels between both isoenzymes fractions.

4. Discussion

Two isoenzyme fractions of lipoamide dehydrogenase which are remarkably different with respect to their relative affinities for *N*-alkyl substituted Sepharose-4B matrices both show non-linear kinetics with reduced substrate. The kinetic parameters are nearly identical on a flavin basis when determined free in solution (cf. Table 1). Both enzyme fractions remain catalytically active upon attachment to the appropriate hydrophobic matrices but the enzymes behave quite differently from each other under these conditions. Fraction II which has a higher affinity for the alkyl-matrices, particularly those of low chain length, has much lower activities than isoenzyme fraction I. A noncompetitive type of inhibition is observed only with those matrices to which the enzymes are reversibly bound. This noncompetitive inhibition may result from a local distortion of the enzyme conformation due to a specific interaction with the ligand which slows the enzyme down. An alternative but less likely explanation is that part of the enzyme molecules become totally inactivated. Denatured protein has always been observed in our electrophoretical analyses of the enzyme after passage over *N*-alkyl matrices. Yost et al. [9] postulated already that such matrices lead to detergent-like interactions with proteins.

The combination of ionic and hydrophobic forces which are the basis of the interactions observed may well have biological significance as they may also operate distinctly in the multienzyme complexes. It is of particular interest that Imai and Tomita have recently reported the interaction of free lipoamide dehydrogenase from pig heart with basic polypeptides [10] however without a significant effect on the enzyme activity.

From the viewpoint of enzyme immobilization, the present method is, at least for this particular enzyme, an alternative for covalent linkage procedures (see also [11,12]). By direct covalent linkage we have always observed that the K_m values increase considerably whereas, even in our best preparations, the turnover number only reaches 30% of the value obtained with the free enzyme [7].

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