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LIPOAMIDE DEHYDROGENASE IMMOBILIZED ON POROUS GLASS

WILLIAM H. SCOUTEN, HARRY KNOWLES Jr., LISA C. FREITAG and WILLIAM IOBST

Chemistry Department, Bucknell University, Lewisburg, Pa. 17837 (U.S.A.)

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Summary

Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) isolated from pig heart and *Escherichia coli* was covalently coupled by both diazonium and amide bonds to controlled pore glass beads (96% silica). When the enzyme was immobilized in the presence of NAD^+, the enzyme no longer exhibited its normal requirement for NAD^+ for full activity. If the immobilized enzyme was then treated with NADase, the requirement for NAD^+ was restored. Enzyme immobilized in the absence of NAD^+ exhibited normal NAD^+ dependence both prior to an after NADase treatment. These results are discussed in terms of co-immobilization of NAD^+ at or near the allosteric site of the enzyme.

Introduction

The immobilization of enzymes on solid matrices imparts important new features to the enzyme systems. Generally, the enzymes are more stable and more easily handled. Often they may resemble in vivo multienzyme complexes or membrane-bound enzyme systems much more closely than highly purified in vitro enzymes.

Lipoamide dehydrogenase (NAD:lipoamide oxidoreductase, EC 1.6.4.3) is one of the component enzymes of the two α-keto acid dehydrogenase multienzyme complexes, pyruvate dehydrogenase and α-ketogluturate dehydrogenase. In vivo it is non-covalently bound to the other components of these macromolecular complexes. Several reports have been published [1,2] in which lipoamide dehydrogenase has been immobilized to agarose as a model of the multienzyme complex. Visser et al. [2] have noted that lipoamide dehydrogenase immobilized in the presence of its substrate, NAD^+ and lipoamide, is more active than enzyme immobilized in their absence. In this report we confirm this observation and provide evidence that the NAD^+ may actually be co-immobil-

ized at some site at or near the allosteric NAD^+ binding site which has been proposed [3] for lipoamide dehydrogenase.

Methods and Materials

Preparation of activated glass beads. Two forms of activated glass beads were employed, one with a diazonium function and one with a reactive *N*-hydroxysuccinimide function. The latter was purchased from Pierce Chemical Co., Rockford Ill., and used directly, while the former was synthesized from virgin glass beads obtained from the same firm.

To synthesize the glass beads with a diazonium reactive group the following procedure was employed. 10 g of glass beads (550 Å pore diameter, 30—120 mesh) were refluxed in 1 M HCl for 3 h, filtered and washed with deionized water. After washing a second time with 95% ethanol the beads were dried at 110°C overnight. The acid washed beads were refluxed for 12 h in 50 ml of 10% 3-aminopropyltriethoxy silane in toluene. The silylated beads were washed with hot ethanol until a negative ninhydrin test was obtained from the wash. The beads themselves will now give a strong positive ninhydrin reaction. 3 g of these beads were refluxed for 1 h in 100 ml of benzene saturated with *p*-nitrobenzoyl chloride. The beads were removed by filtration and washed as before. The beads should now be ninhydrin negative.

The resulting nitroaryl beads were reduced to aminoaryl beads by reflux in 10% $Na_2S_2O_4$ for 30 min. The aminoaryl beads were diazotized by adding 1 g of beads to 10 ml of ice-cold 2 M HCl in an ice bath. Next, 0.25 g of $NaNO_2$ were slowly added and the mixture was swirled in an ice bath for 20 min and then degassed for 30 min to ensure complete derivitization of the internal pores. The light yellow diazonium beads were washed on a Buchner funnel with cold, deionized water and used immediately.

Enzyme sources. Pig heart lipoamide dehydrogenase was obtained from Sigma Chemical Co. and bacterial lipoamide dehydrogenase was isolated from *Escherichia coli*, strain B (Grain Processing Co., Muscatine, Iowa) by the method of Williams et al. [4]. NADase from *Neurospora crassa* was obtained from Worthington Chemical Co. and was assayed as described by Kaplan [5]. Treatment of immobilized lipoamide dehydrogenase with NADase was performed essentially as described by Massey and Veeger [6] for the free enzyme, using 50—500 units of enzyme per g of immobilized enzyme (dry weight) in 5 ml of 0.1 M potassium phosphate, pH 8.0.

Enzyme coupling. Lipoamide dehydrogenase, either from pig heart or *E. coli*, was coupled via the diazonium function by adding 2 ml of lipoamide dehydrogenase (2—3 mg/ml) to 1.0 g (dry weight) of freshly diazotized and washed beads suspended in 1.0 ml of 1 M sodium borate buffer, pH 9.1. The mixture was reacted by agitation in a wrist action shaker for 12 h at 4°C. The coupled beads were filtered, washed with 50 ml of the same buffer and stored as wet cake. Where desired, NAD^+ (2.5 mg/ml) was added to the enzyme solution immediately prior to coupling.

To couple the enzyme via the *N*-hydroxysuccinimide method, 0.2 ml of enzyme (10 mg/ml) in 0.1 M potassium phosphate buffer, pH 8, 3 mM in EDTA, was added to 0.2 g (dry weight) of glass beads in 3 ml of the same

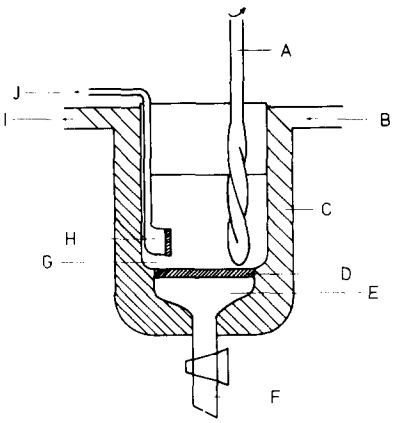

Fig. 1. Cross-section of the apparatus for enzyme assay. Assay medium and immobilized enzyme beads are placed in the center chamber and stirred continuously. Samples are withdrawn through a sampling tube fitted with a fine filter. After sampling the sample port is filled with air until the next sampling period. The exit port at the bottom permits the entire assay mixture to be removed via vacuum and the beads to be washed in preparation for different assay conditions. The fine sintered glass filter at the sample port and exit port enable one to keep these filled with air whenever the stopcocks are closed. A, electrically driven glass stirrer; B, cooling water input; C, water jacket; D, fine sintered glass filter; E, airpocket; F, exit port for washing enzyme beads prior to the next assay; G, assay chamber containing assay media and enzyme beads; H, fine sintered glass tip on sampling port; I, cooling water exit; J, sample port exit.

buffer. To couple in the presence of substrate, 2.5 mg NAD^+ and 0.6 mg lipoamide was added. The beads were tumbled in the reaction mixture at 4°C overnight on a Kendall-Fisher mixer and then collected by suction filtration. The beads were washed with 10 ml of the same buffer, followed by 10 ml of 0.1 M potassium phosphate, pH 7.0.

Activity of the immobilized enzyme. The immobilized enzymes were assayed following the oxidation of NADH by lipoamide spectrophotometrically at 340 nm. This was done at first on columns of immobilized enzyme by passing the assay mixture of Koike and Hayakawa [7] through a small (approx. 0.5 ml) column of immobilized enzyme at various flow rates. It was difficult, using this technique, to obtain fast enough flow rates to determine the initial velocity. Therefore a special apparatus was designed (see Fig. 1) such that a number of assays could be performed under various conditions on a single set of enzyme-glass beads.

The activity was converted to specific activity based on the knowledge that lipoamide dehydrogenase contains two FAD molecules per enzyme molecule. The FAD content of the immobilized beads was determined via perchlorate treatment [1] and comparing the fluorescence of the perchlorate extract of the beads with a standard curve of FAD fluorescence.

Results

Lipoamide dehydrogenase bound to diazonium glass beads

The initial observations that suggested co-immobilization of NAD^+ at or near the allosteric center were made using pig heart lipoamide dehydrogenase which has been coupled to diazonium glass and immediately afterwards has been

TABLE I

Method of enzyme immobilization	Amount of enzyme bound (mg/g dry weight boads)	Bound * (%)	Specific activity bound enzyme (1 units/mg)
Diazonium	1.1	44	1.25
N-Hydroxysuccinimide	2.5	100	4.0

* Based on FAD determinations.

assayed in the presence of the allosteric effector, NAD^+. The initial activity was determined using a column of immobilized lipoamide dehydrogenase by plotting flow rate against the reciprocal of the rate of oxidation of NADH (see Fig. 2). If, after once assaying in the presence of NAD^+, the same beads were reassayed in its absence, the initial rate did not decrease as expected. In fact, instead of stimulation, added NAD^+ caused a slight inhibition. These observations might suggest that the NAD^+, used in the first assay performed using this particular column, had become coupled at or near the allosteric centre, producing an immobilized enzyme which was always fully stimulated.

If this was true, removal of NAD^+ from the beads would cause the immobilized enzyme to once again become NAD^+ sensitive. In fact, when the enzyme-glass beads were treated with NADase, such treatment rendered them sensitive to NAD^+ stimulation. Further evidence that NAD from the first assay was coupled to the glass beads was obtained by treating freshly derivatized enzyme-glass beads with active aromatic compounds which would react with any excess diazonium function. For example, if the beads were treated with 0.1 M borate buffer, pH 9.1, saturated with either tyrosine or β-naphthol prior to their first exposure to NAD^+, the immobilized enzyme remained sensitive to NAD^+. The β-naphthol beads turned reddish-pink, indicating that indeed excess diazonium functions had been present.

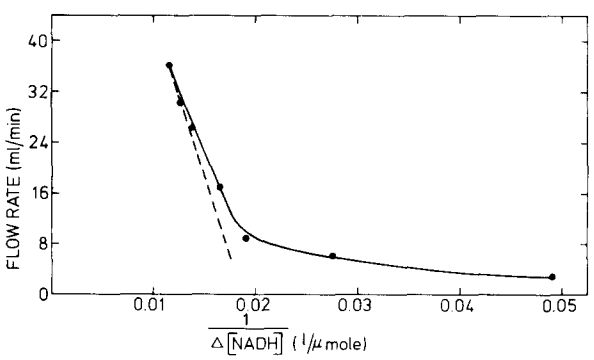

Fig. 2. Initial activity of lipoamide dehydrogenase-glass beads determined from column flow rates. The flow rate is plotted vs. the decrease in NADH concentration that occurs after the assay mixture passes through the column. The slope of the plot, $y = mx + b$, is a measure of activity (ΔNADH μmol/min). When $y = mx + b$, the slope $m = y/x$. Since y is flow rate (ml/min) and $x = 1/\Delta$NADH in units of ml/μmol, then slope $m = y/x$ = activity in μmol NADH oxidized/min. This slope, extrapolated to infinite column velocity, is the initial velocity of the reaction. The same enzyme column was used throughout employing the assay medium of Koike and Hayakawa [7] with appropriate concentrations of NAD^+.

Lipoamide dehydrogenase coupled to N-hydroxysuccinimide glass

Lipoamide dehydrogenase (pig heart) coupled to *N*-hydroxysuccinimide glass in the presence of NAD^+, as described previously, was totally insensitive to added NAD^+. Conversely, the enzyme which had been coupled to the beads in the absence of NAD^+, had at least a 5-fold increase in activity when assayed in the presence of NAD^+ (1 mM) than in its absence (see Fig. 3). Enzyme immobilized to glass beads in this fashion were assayed by the batch method using the apparatus shown (Fig. 1).

The *N*-hydroxysuccinimide—lipoamide dehydrogenase-glass beads which were immobilized in the presence of NAD^+, were treated with NADase. Immobilized enzyme treated in this fashion now exhibited sensitivity toward NAD^+ stimulation. The total activity remained higher, even after NADase treatment, than that of enzyme immobilized in the absence of added NAD^+. This would reflect some degree of real protection of the active site by the presence of substrate during the immobilization reaction. Additional treatment with NADase

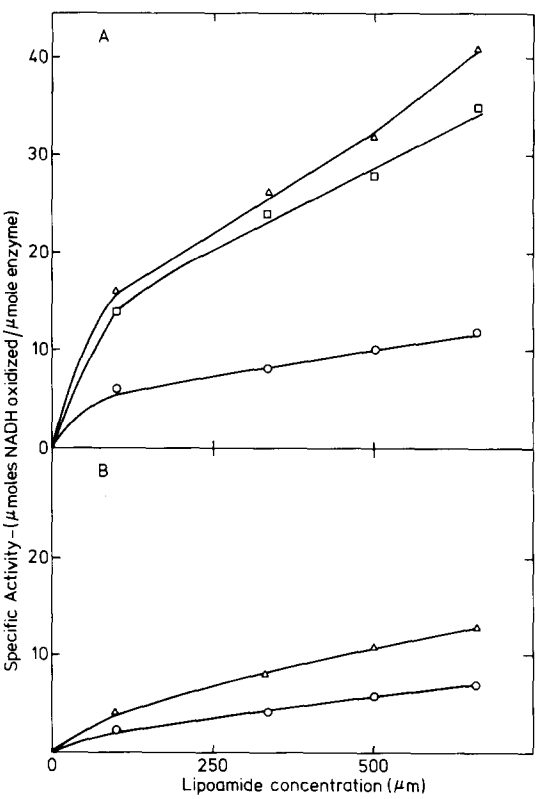

Fig. 3. Activity of lipoamide dehydrogenase-glass beads immobilized with/without added NAD^+. (A) Depicts the enzyme co-immobilized with NAD^+ assayed with added NAD^+ (△), without added NAD before NADase treatment (□), and without added NAD after NADase treatment (○), (B) Depicts the enzyme immobilized in the absence of NAD^+ and assayed in the presence of added NAD^+ (△) and in the absence of added NAD^+ (○). When this enzyme is reassayed after NADase treatment, the results are identical to that found prior to NADase treatment. A and B were prepared identically, except for the presence of NAD^+ in the immobilization media. Invariably higher activities are found when the immobilization takes place in NAD^+-containing media.

did not further change the activity, although it is possible that some of the immobilized NAD^+ molecules were not accessible to NADase.

These results could also be expected if the NADase-treated beads had some residual NADase activity. However, the NADase activity of the treated beads was below the levels of detection and thus residual NADase would be unlikely to account for the change in NAD^+ stimulability after NADase treatment.

Discussion

While alternate explanations for the observed results are not totally excluded, they strongly point toward the co-immobilization of NAD^+ at or near the allosteric site. There results are not totally unexpected, since Mosbach and Gestrelius [8] have co-immobilized glycogen phosphorylase *b* and its allosteric effector analogue, N^6-(aminohexyl)-AMP. The resulting co-immobilized glycogen phosphorylase *b*-AMP analogue was fully active, with or without added AMP. Conversely, either the free enzyme or enzyme immobilized in the absence of the AMP analogue, was dependent upon added AMP for full activity. These results are very similar to the results reported here.

Similarly, Gestrelius et al. [9] have prepared alcohol dehydrogenase immobilized in the presence of the coenzyme analogue, N^6-[(6-aminohexyl) carbamoylmethyl]-NADH. This preparation did not require added NADH for activity, again providing an example of a coenzyme immobilized sufficiently close to the active center such that it can function in a normal fashion.

The probable physical appearance of the lipoamide dehydrogenase-NAD^+-glass matrix is shown in Fig. 4. The NAD^+ is attached to be silylated glass matrix by an arm which permits the movement of the NAD^+ to the allosteric site or even to the active site. In the particular case of lipoamide dehydrogenase, the allosteric site binds NAD^+ much more tightly than the active site (NAD^+ is both product and effector molecule for lipoamide dehydrogenase) [3]. Therefore any particular NAD^+ molecule that has a choice of binding to either site will, on the average, be most likely to be found at the allosteric site. It is to be expected that some of the immobilized NAD^+ will be able only to reach the active site or only the allosteric site, while many of the effector molecules will be at neither site. Quite possibly the binding of NAD^+ to the glass surface will not be random but rather many effector molecules will be non-covalently bound to the enzyme allosteric site at the time of immobilization. Thus a higher number of effector molecules will have orientations at or near the allosteric site.

The results could also be explained by non-covalent attachment of NAD^+ to the glass matrix by ionic and/or hydrophobic forces. However, the immobilized enzyme-NAD^+-glass was stable for at least six months, with losses of less that 10% and it retained its lack of sensitivity in added NAD^+ over the same period. During this time it was repeatedly assayed and washed without added NAD^+. If the NAD^+ were covalently bound one would expect that its sensitivity to NAD^+ would be restored, at least in part, by such treatment.

The question of the point of attachment of NAD^+ to the porous glass particles is rather complex and was not investigated by us. Weibel et al. [10] have reported that NAD coupled via the diazonium method to glass beads was enzy-

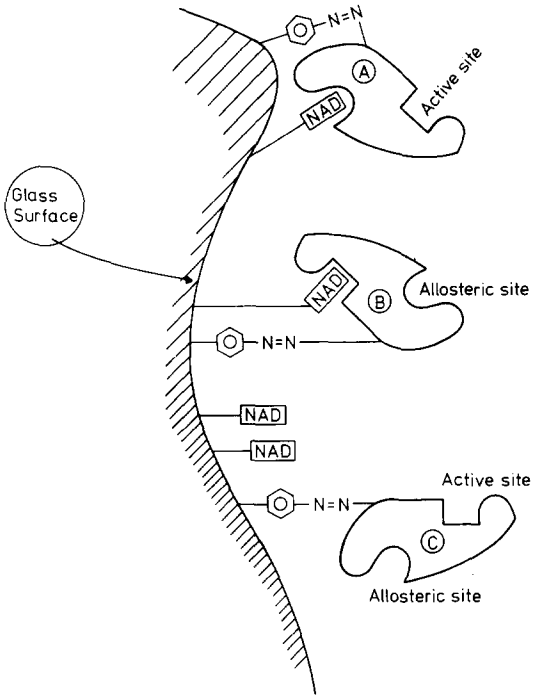

Fig. 4. Proposed appearance of the surface of a lipoamide dehydrogenase-NAD^+-glass bead. NAD^+ molecules as shown may reach the allosteric site as in A, where they serve as an effector molecule, or they may reach the active site, as in B, where they function as product inhibitors in the oxidation of NADH, or they may be unable to reach either site, as in C. Those molecules in which the NAD^+ can reach either site will have the NAD^+ primarily bound to the allosteric site as in A.

matically active, but the mode of attachment was not described. Lowe and Dean [11] have suggested that NAD^+ couples to diazonium groups chiefly at position 8, although the coupling appears to be heterogeneous. Similarly, the attachment of NAD^+ to carboxyl groups is heterogeneous [12], involving presumably both the free NH_2 and OH functions.

Co-immobilization of a molecule, such as NAD^+ in this case, which can bind near an allosteric site or an active site, and thereby serve as either effector or product inhibitor, will undoubtedly result in a very heterogeneous enzyme system. The kinetics of such a system will be complex and not easily interpreted and may, in fact, vary from preparation to preparation. Thus the kinetic analysis of the immobilized lipoamide dehydrogenase preparations of Visser et al. [1] in which NAD^+ was employed during immobilization, ostensibly as "substrate protection", will need to be further analyzed in terms of potential co-immobilization.

One general conclusion that can be drawn from these experiments is the need to carefully consider all factors present during immobilization, not only the matrix and functional arm, but the substrate(s) which may be present and possibly even the effect of particular solvent or buffer molecules employed during the immobilization process.

Acknowledgements

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