IMMOBILIZED ENZYMES: THE CATALYTIC PROPERTIES OF LACTATE DEHYDROGENASE COVALENTLY ATTACHED TO GLASS BEADS

Jack E. Dixon, Francis E. Stolzenbach,

Jeffrey A. Berenson and Nathan O. Kaplan

Department of Chemistry, University of California, San Diego

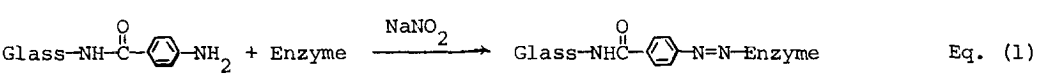
La Jolla, California 92037

Received April 18,1973

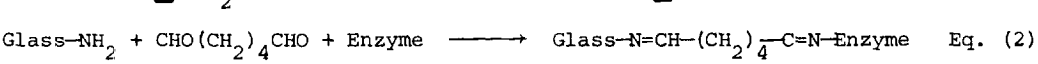
SUMMARY - Chick LDH (H_4 and M_4) has been covalently attached to aryl and alkyl amine glass using sodium nitrite and glutaraldehyde respectively. These immobilized enzymes remain active for months at 0°C and exhibit Km values similar to those of the soluble enzyme; however, they have pH-rate profiles that are independent of pH and show decreased substrate inhibition. Disaggregation followed by reassociation indicate the enzymes are bound by all four subunits and the resulting activity restored to the native, aryl amine and glutaraldehyde bound enzyme are 33, 25 and 90% respectively. At a pH of 3.2 and 25°, the soluble and aryl amine glass LDH's are rapidly denatured while the glutaraldehyde bound enzyme shows no loss of activity for at least 35 days.

INTRODUCTION - Enzymes bound to matrices have been used as model systems for enzyme reactions taking place *in vivo* or on membranes (1). In addition, these immobilized catalysts afford an opportunity for repeated use in enzymatic synthesis and may also serve in the detection and treatment of clinical abnormalities associated with enzyme deficiencies (2). The objective was to develop an immobilized enzyme system which would retain its catalytic activity while exhibiting extended stability toward denaturation. The aim of this work was to minimize substrate and product inhibition so that the matrix-bound enzyme would show a maximum efficiency in the presence of high concentrations of reactants.

Lactate dehydrogenase is a well studied multimeric enzyme whose sequence (3) and x-ray structure (4) are presently under investigation. We have attached to chick (H_4) lactate dehydrogenase both aryl amine glass and alkyl amine glass amine glass using the reactions shown in Equations 1 and 2 respectively:



Eq. (1)



Eq. (2)

The aryl amine glass and sodium nitrite exhibits a propensity towards coupling to aromatic residues such as tyrosine, whereas the alkyl amine glass and glutaraldehyde seems to involve certain lysines of the enzyme. These immobilized enzymes exhibit many of the advantageous characteristics mentioned above, as well as being extremely insensitive to changes in catalysis over a wide pH range.

MATERIALS AND METHODS - Enzyme activities were measured on a Perkin-Elmer Model 46 Spectrophotometer equipped with a special apparatus designed for determining kinetic parameters associated with immobilized enzyme systems[1]. Protein concentrations were determined by a modified Kjeldahl procedure (5) and by the procedure of Udenfriend et al. (6). The lithium chloride denaturation technique employed was essentially that reported by Chilson et al. (7). Renaturation was achieved by using a twenty-fold dilution of 0.1 M Tris buffer (pH 7.5), containing 0.1 M β-mercaptoethanol or by removing the denaturation medium and resuspending the protein on glass in the renaturation solution. The pH 4.1 denaturation was carried out in 0.1 M sodium acetate buffer while 0.05 M acetic acid was used for the pH 3.2 studies. Samples of the glass were withdrawn at given time intervals and checked for activity in 0.1 M phosphate at pH 7.5 using pyruvate as the substrate.

The chicken (H_4) lactate dehydrogenase was covalently attached to alkyl amine glass using the following procedure: one gram of glass was allowed to react in a dessicator at 25° for one hour with one ml of a five percent glutaraldehyde solution (0.1 M phosphate, pH 7.0). Upon completion of the reaction, the glass was washed with buffer to remove any excess glutaraldehyde. Following this procedure, the glass was then incubated with 30 mg of enzyme at 0°C for two hours (0.1 M phosphate buffer, pH 7.0). Then the glass bound enzyme was washed with distilled water to remove extraneous enzyme. Enzyme was covalently attached to the aryl amine glass using a procedure that has

[1] F. Widmer, J. E. Dixon and N. O. Kaplan. A description of this apparatus is in preparation.

been developed by Weetal (8). Non-covalently attached protein was removed by allowing the glass bound enzyme to stand overnight at 0°C in pyrophosphate, lithium chloride, or 0.1 % albumin solution and then filtering off the beads.

RESULTS AND DISCUSSION

Michaelis Constants - The kinetic behavior observed with immobilized enzyme systems may be the result of specific protein structural alterations of the enzyme as well as interactions between the enzyme and insoluble support. Changes in the nature of Michaelis constant give some insight into the nature of these alterations. Differences in the catalytic properties of soluble and immobilized enzyme may also arise as a result of limited rates of diffusion. This would be reflected by an increase in the Km of the substrate for the catalyst attached to a solid support (9). In order to assess the effect of immobilization on the function of the enzyme, Km values were determined. The Michaelis constants for the LDH (H_4) attached to the aryl amine and glutaraldehyde glass bound enzyme are 3.9×10^{-5} and 4.0×10^{-4} M, respectively, whereas the soluble enzyme has a Km of 1.25×10^{-4} M. These results seem to indicate that the affinity between substrate and enzyme is altered upon immobilization, but that these alterations are generally not large. A comparison of the Michaelis constant for the soluble lactate dehydrogenase and glass bound enzyme stystems would indicate that rates of mixing and diffusion have been minimized. We cannot, however, completely rule out the possibility that the slight decrease in Km observed for some of the porous glass bound enzymes may at least in part be attributed to a difference in the substrate concentration gradient.

pH Optimum of Immobilized LDH's - The pH-rate profiles for the reactivity of pyruvate with glass bound chick lactate dehydrogenase (H_4) and native enzyme are shown in Figure 1. The remarkable insensitivity of the immobilized enzymes to changes in pH is most striking. Although it may be argued that local concentration effects might account for this observed insensitivity, an

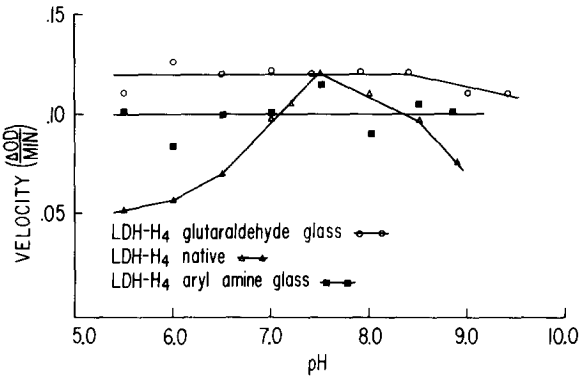


Figure 1: The effects of insolubilization on the pH-rate profile for chick heart lactate dehydrogenase. Assays were carried out in 0.1 M phosphate at the appropriate pH. Assays were performed using pyruvate + DPNH.

examination of Equation 3 indicates that acid is consumed in the formation of

$$DPNH + H^+ + \text{pyruvate} \rightleftharpoons \text{lactate} + DPN^+ \qquad \text{Eq. (3)}$$

lactate. This would generate a local microenvironment of higher basicity, thus amplifying the decrease in reaction velocity. The fact that this is not the case would seem to rule out the effect of changes in local concentrations as an explanation for the altered pH-rate profile. These observations are also substantiated by the observed values of Km, which are not influenced significantly by limiting rates of diffusion. The functional importance of this insensitivity allows the carrying out of reactions with little regard to buffer capacity or changes in pH.

pH Effects on Stability of Bound LDH's - Chick lactate dehydrogenase (H_4) is denatured quite rapidly at high acidity. Both the aryl amine glass bound and native soluble enzyme loose eighty percent of their activity after four hours at pH 4.0, whereas the glutaraldehyde glass bound LDH remains fully active. Lowering the pH to 3.2 results in a complete loss of native enzyme activity within one hour, but again the glutaraldehyde glass bound enzyme shows no detectable loss in activity for 35 days (Figure 2). The dramatic stability of the glutaraldehyde bound LDH reflects the importance of the value binding plays in determining the stabilities of the LDH's and

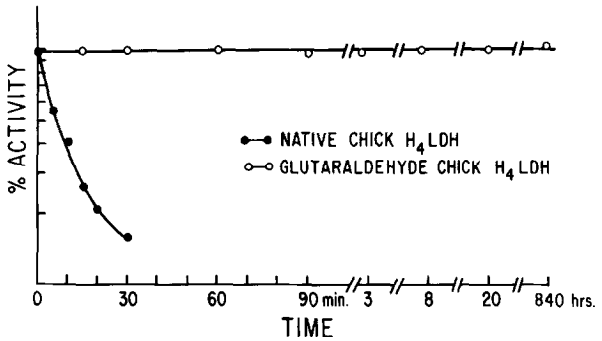


Figure 2: Effect of pH on the stability of soluble and glutaraldehyde glass-bound chick heart LDH in 0.1 M acetate at pH 3.2. Assays represent pyruvate reduction at pH 7.5.

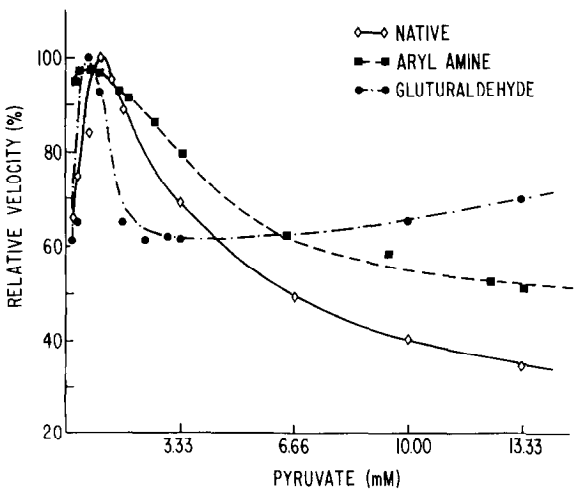


Figure 3: The inhibition exhibited by native, aryl amine glass-bound, and glutaraldehyde glass-bound, chick heart lactate dehydrogenase at various concentrations of pyruvate. The pH of the reactions was 7.5.

the significance of specific amino acids in determining stability.

Substrate Inhibition - Chick (H_4) lactate dehydrogenase exhibits substrate inhibition in the presence of high concentrations of pyruvate (10). Figure 3 indicates that both the glutaraldehyde and aryl amine glass bound enzymes are much less sensitive to substrate inhibition. Therefore it appears that an increase in the Km of pyruvate might also result in a decreased formation of the ternary DPN, pyruvate, LDH complex, which would then result in a decrease in substrate inhibition (11). This could explain the result

TABLE I

Subunit Disaggregation of Chick (H_4) Lactate Dehydrogenase with 5.3 M Lithium Chloride

Enzyme	Percent Activity			
	Filtrate	Suspension (glass beads and filtrate)	Refiltered[1] glass beads of suspension	Refiltered[1] filtrate of suspension
Aryl Amine Glass-H_4	0	18	18	0
Aryl Amine Glass-M_4	0	24	25	0
Glutaraldehyde Glass-H_4	0	90	90	0
Native Enzyme	33	--	--	33

[1]Protein content of the glass was found to be the same before and after treatment with lithium chloride. There were no detectable amounts of protein in the filtrates.

with the glutaraldehyde glass-bound enzyme, but other factors could be of importance in determining the lack of inhibition noted with aryl amine glass-bound enzyme. The fact that there is an insensitivity to substrate inhibition provides a distinct advantage in the design of bioreactors which could be used for the synthesis of large quantities of material by immobilized enzyme systems.

Denaturation by Lithium Chloride - High concentrations of salt and urea dissociate the tetrameric lactate dehydrogenase enzyme into monomeric subunits (10). We have treated our aryl amine and glutaraldehyde glass bound enzyme with 5.3 M lithium chloride and obtained the results shown in Table I. These observations indicate that the bound enzyme is also subject to loss of activity in the presence of high concentrations of salt; upon reassociation all enzymatic activity is associated with the glass matrix. The glutaraldehyde glass-bound enzyme regains ninety percent of its activity, whereas the aryl amine glass reassociates to only 25 percent activity. It is of interest that only 33 percent recovery of enzymatic activity is obtained after treatment

with lithium chloride. This observation implies that the method of coupling to the glass matrix plays a significant role in determining the degree of subunit reassociation. Protein determination also indicates that no significant quantity of enzyme was removed from the glass (see Table I) upon subunit disaggregation. The fact that neither protein nor enzymatic activity is lost from the glass upon treatment with 5.3 M LiCl and that no protein or activity appears in solution upon renaturation clearly indicates that all subunits are attached in such a way that they are not removed from the glass under conditions which normally produce subunit dissociation. Cho and Swaisgood (12) have reported that rabbit muscle lactate dehydrogenase bound to succinyl glass appears to have only one subunit attached to this support. Differences between this observation and our findings may reflect a change in the method of covalent attachment as well as the nature of the groups involved in binding[2].

There are several distinct advantages associated with the glutaraldehyde bound LDH which are evident from its stability toward pH denaturation, its insensitivity to substrate inhibition, and the ninety percent reactivation following lithium chloride denaturation. We are presently investigating the possibility that this is a general phenomenon associated with glutaraldehyde immobilization and may therefore serve as a valuable technique in stabilizing a wide variety of enzymes. We are further investigating additional characteristics of the immobilized lactate dehydrogenase enzymes to gain insight into their ability to produce antibodies, the amino acids involved in binding, and the effects of other denaturating agents.

ACKNOWLEDGEMENT - J.E.D. is a postdoctoral fellow with the National Science Foundation. This work was supported by grants from the National Science Foundation (GI-36249) and the American Cancer Society (BC-60-0).

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