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SURFACE-BOUND LACTATE DEHYDROGENASE: PREPARATION AND STUDY OF THE EFFECT OF MATRIX MICROENVIRONMENT ON KINET IC AND STRUCTURAL PROPERTIES*

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SUMMARY

Rabbit muscle lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) was covalently bound to porous glass beads using conditions which resulted in attachment of only one of the four subunits of each tetramer. Refolded, covalently bound subunits, prepared by washing with strong denaturants and re-exposure to more nearly physiological conditions, were capable of enzymatic activity and recombination with native subunits in solution. Kinetic properties of a similarly modified, soluble enzyme derivative and several immobilized derivatives were compared. An increase in the apparent Michaelis constant for NADH is suggested to be caused by intrapore diffusion limitation; whereas, the large decrease in the constant for pyruvate may reflect a conformational change induced by the matrix environment. The equilibrium dissociation constant for NAD also exhibited a large decrease compared to reported values for native enzyme suggesting a structural change resulting in more favorable binding.

INTRODUCTION

Demonstration that many enzymes function in vivo as an integral part of membrane surfaces has led to a growing interest in the behavior of enzymes attached to solid supports. Numerous studies have shown that the kinetic behavior of such derivatives is significantly altered from that observed in solution [1–8]. Various microenvironments such as cationic [2], anionic [3], and hydrophobic [7] have been shown to affect kinetic properties. Recently, we have examined microenvironmental effects on protein structure by investigation of refolding of chymotrypsinogen [9] and lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) subunits [10] which were covalently attached to a glass surface. Refolded lactate dehydrogenase exhibited activity and the ability to combine with free subunits in solution.

Abbreviation: EDC, 1-ethyl-3-dimethylaminopropyl carbodiimide.

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Water-soluble carbodiimides have been used to modify carboxyl groups of proteins [11–13] and to prepare immobilized enzymes [7, 10, 14]. However, addition of carbodiimide directly to protein solutions may result in several undesirable side reactions such as modification of tyrosyl or sulfhydryl residues.

We report here a method for covalent attachment of enzyme to glass surfaces without directly exposing the enzyme to carbodiimide. The kinetic properties of surface-bound preparations obtained by linkage through amino groups or carboxyl groups of lactate dehydrogenase are compared to those of enzyme with modified carboxyl groups and to native enzyme. Dissociation of unbound subunits is also examined further.

MATERIALS AND METHODS

Materials

Crystalline rabbit muscle lactate dehydrogenase (Type II, lot number OOC-9350), NADH (Grade III), dithiothreitol, and pyruvic acid (Type II) were obtained from Sigma Chemical Co. Guanidinium chloride (Sequanal grade) and 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) were purchased from Pierce Chemical Co. Porous γ-amino propylsilane glass beads (40–60 mesh, 522 Å pore diameter) were products of Corning Glass Works and ^{14}C-labeled NAD was obtained from Amersham/Searle Corp. All other chemicals were reagent grade.

Enzyme concentration was calculated from absorbance measurement at 280 nm using an absorptivity of 1.44 cm^2/mg [15] and that for NADH by measurement at 340 nm using a molar absorptivity of $6.22 \cdot 10^3$.

Attachment of the enzyme to glass beads

γ-Aminopropyl–glass beads were washed with distilled water and equilibrated for 24 h in phosphate buffer (pH 7.0) containing 0.1 M NaCl. Prior to reaction, the beads were degassed in a vacuum dessicator. To 0.5 g of the beads, 3 ml of 0.5 M succinic acid (pH 4.75) was added and the pH readjusted to 4.75. A 300-mg portion of crystalline EDC was added to the reaction vessel and the reaction proceeded for 2 h at 25 °C with the pH maintained at 4.75 in a Radiometer pH Stat. After reaction, the beads were washed with 500 ml of distilled water and the number of amino and carboxyl groups determined by titration.

Enzyme was covalently coupled to these carboxyl groups via its amino groups using a carbodiimide procedure briefly described in an earlier communication [10]. Preparation of succinylpropyl–glass enzyme was accomplished without exposure of the protein to EDC. Degassed beads (0.5 g) were packed in a 6-mm diameter column and 5 ml of freshly prepared 0.1 M EDC at pH 4.75 was recycled through the column with a peristaltic pump for 30 min at room temperature to produce a maximum concentration of *O*-acylisourea derivatives [13]. The column was rapidly washed with 200 ml of 0.1 M phosphate buffer at pH 7 and 0 °C. Immediately following the cold wash, 5 ml of 1 mg/ml enzyme solution in 0.1 M phosphate buffer was recycled through the column for 24 h at 4 °C.

Propyl–glass enzyme was prepared directly by reaction of the enzyme's carboxyl groups with the γ-aminopropyl group on the glass surface in the presence of EDC as previously described [10].

Enzyme not covalently bound to the glass was removed by washing the column of beads with 1000 ml of 0.1 M phosphate buffer containing 0.05 M NaCl at pH 8.5. Controls were treated in the same manner except the carboxyl groups were not activated.

The amount of enzyme attached to the glass was determined from the glycine content obtained by analysis using a Beckman Model 116 amino acid analyzer. Samples to be analyzed were added to 1 ml of glass-distilled, constant-boiling HCl, repeatedly evacuated to remove dissolved O_2, sealed under vacuum, and placed in a refluxing toluene bath for 24 h. After hydrolysis, HCl was removed under vacuum, over KOH pellets, followed by addition of an appropriate volume of sodium citrate buffer (pH 2.2), and filtration of the resulting amino acid solution. This solution was added directly to the analyzer column.

Measurement of thermal stability

A column (0.6 cm $\times$ 1.5 cm) of glass-bound enzyme was prepared in a Glenco precision bore, water-jacketed column and the temperature controlled with a Forma circulating temperature bath. A 10-ml volume of $6.8 \cdot 10^{-8}$ M native enzyme was placed in the temperature bath. Difference in absorbance at 340 nm for substrate solution was measured with a Cary Model 15 spectrophotometer before and after being passed through the column at a constant flow rate maintained with a peristaltic pump. Substrate concentrations in 0.03 M phosphate buffer at pH 7.0 were 505 μM pyruvate and 57 μM NADH. Activities of both native and glass-bound enzyme were determined at increasing temperatures, holding at each experimental point for 10 min before measurement and then increasing the temperature to the next point.

Dissociation of subunits of glass-bound enzyme

Glass-bound enzyme was incubated in 7 M guanidinium chloride, pH 5, or 1 % sodium dodecylsulfate, pH 7, for 1 h at room temperature. Following incubation, the dissociating agents were removed, the beads washed with 5 ml distilled water, and the two solutions combined. After dialysis of the washings to remove the dissociating agents, both the washings and the beads were subjected to amino acid analysis as previously described. Using glycine as a measure of protein content, the ratio of the amount remaining on the beads to that removed in the washings was calculated.

^{14}C-Labeled NAD binding studies

Equilibrium binding of ^{14}C-labeled NAD was determined to provide a measure of the number of active sites. ^{14}C-labeled NAD (50 μCi) was dissolved in a stock NAD solution from which dilutions were made with 0.1 M phosphate buffer at pH 8.0. Radioactivities were measured with a Packard Model 3002 Tri-Carb liquid scintillation spectrometer using an automatic external standard for quench corrections. Triton X-100 served as a jelling agent and PPO–POPOP was used as the phosphor [16].

Glass-bound enzyme was equilibrated for 1 h at room temperature with 3 ml of NAD solution having a known radioactivity. After equilibration a 2-ml portion of liquid supernatant was removed for counting. The beads were washed, dried, weighed, and hydrolyzed for amino acid analysis to determine the amount of enzyme.

An identical amount of plain glass beads were equilibrated with NAD solutions and treated in the same manner to serve as a control.

Modification of carboxyl groups

Enzyme in solution with varying degree of incorporation of glycine methyl ester was prepared by activation of the carboxyl groups with EDC [11]. By controlling pH, temperature, time of reaction and the concentrations of the reagents, glycine methyl ester and EDC, the extent of modification could be selected [13]. The degree of modification was determined by amino acid analysis.

Kinetic studies

The rate of pyruvate reduction and NADH oxidation was determined in 0.1 M phosphate buffer, pH 7.0, from the decrease in absorbance at 340 nm as a function of time as recorded with a Cary 15 spectrophotometer. This direction of the reaction was followed since the equilibrium greatly favors NAD and hence the initial stages of the reaction are essentially irreversible. Glass-bound enzyme was analyzed in both column and stirred-vessel reactors as described previously [10].

The initial rate was measured at less than saturating concentrations for both substrates and at a saturating concentration of NADH. Two integrated forms of the Michaelis–Menten equation were used to analyze the column data; one for saturating levels of NADH and the other for less than saturating concentrations of both substrates. To facilitate comparison of the results, the same amount of glass-bound enzyme was used in both the stirred-vessel and column reactors. Sufficient "wet" glass–enzyme was weighed out to give 0.18 g of dry beads (1.6 nmoles of enzyme) for each experiment.

RESULTS

Covalent coupling parameters

A value of 47 mmoles of amino groups per g of glass beads was found by titration (24 mmmoles NH_2/g less than the value given on the label). Similar titration of carboxyl groups with the succinylpropyl–glass indicated that 65% of the aminopropyl groups had reacted with succinic acid. As shown in Table I, more enzyme was covalently attached to the succinylpropyl–glass than to the aminopropyl–glass.

Thermal stability of glass-bound enzyme

Results of a cumulative temperature effect study are given in Fig. 1. These

TABLE I

AMOUNT OF ENZYME COVALENTLY ATTACHED TO GLASS

Preparation	Amount bound		
	Mean* (mg/g)	Range (mg/g)	Mean (nmoles/g)
Succinylpropyl–glass	1.3	0.8–1.8	8.90
Propyl–glass	0.5	0.3–0.8	3.42

* Average of three trials

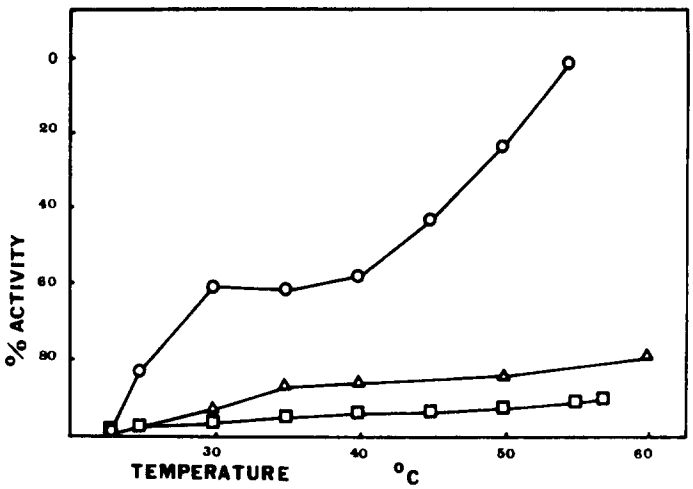

Fig. 1. Thermal inactivation of surface-bound lactate dehydrogenase. In all cases the enzyme was held at the specified temperature for 10 min prior to activity measurement. The temperature of the same preparation was then increased to the next higher value. ○, native enzyme; △, propyl–glass enzyme; □, succinyl–propyl–glass enzyme.

data indicate that both surface-bound preparations are considerably more stable than native lactate dehydrogenase.

Dissociation of the surface-bound tetramer

Ratios of the amount of enzyme remaining on the beads washed with dissociating agents to that removed in the washing solutions are shown for fresh and aged preparations, see Table II. Control beads, treated in exactly the same manner except for the addition of coupling reagent (EDC), did not contain a detectable amount of protein either before or after treatment with dissociating agent. Three-fourths of the freshly prepared surface-bound enzyme is removed in the dissociating solvents whereas slightly more than half (57%) is removed in the case of the aged preparation.

Coenzyme binding to the surface-bound enzyme

Data obtained for binding of ^{14}C-labeled NAD to succinylpropyl–glass enzyme

TABLE II

RATIO OF THE AMOUNT OF ENZYME REMAINING ON THE GLASS TO THAT REMOVED BY STRONG DISSOCIATING AGENTS

Error is based on a 3% error for amino acid analysis.

Preparation	Dissociating agent	Ratio (amount remaining/amount in the wash)
Fresh succinylpropyl–glass enzyme	Guanidinium chloride (7 M)	0.33 ± 0.02
Fresh succinylpropyl–glass enzyme	Sodium dodecylsulfate (1%)	0.37 ± 0.02
2-month-aged succinylpropyl–glass enzyme	Guanidinium chloride (7 M)	0.74 ± 0.04

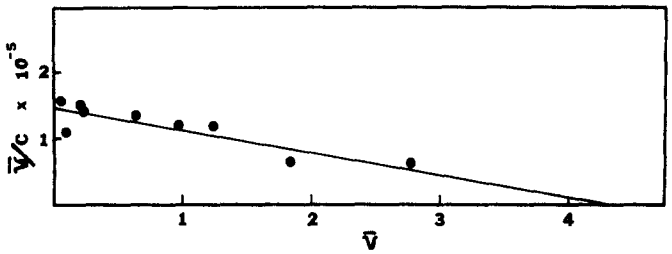

Fig. 2. Scatchard plot of ^{14}C-labeled NAD binding data. Noting the equivalence of the equation describing Michaelis–Menten kinetics and that for binding equilibria of one set of sites, the number of sites and the dissociation constant were evaluated by computer fitting to a hyperbola using a program similar to that described by Cleland [31]. The line represents the best fit of the data.

are shown in Fig. 2. The Scatchard equation was fitted with a computer program giving the number of binding sites, $n = 4.27 \pm 0.53$, and the dissociation constant, $K_{NAD} = 29 \pm 7$ μM. A value of 0.25 mM has been reported for the Michaelis constant [17] and 0.91 mM for the dissociation constant [18] of native rabbit muscle enzyme.

Initial rate kinetic studies

Kinetics of lactate dehydrogenase with an average of five carboxyl groups modified, succinylpropyl–glass enzyme, and propyl–glass enzyme were analyzed by the initial rate method and the results compared to those of the native enzyme. Primary reciprocal plots for modified enzyme are shown in Figs 3A and 3B and the

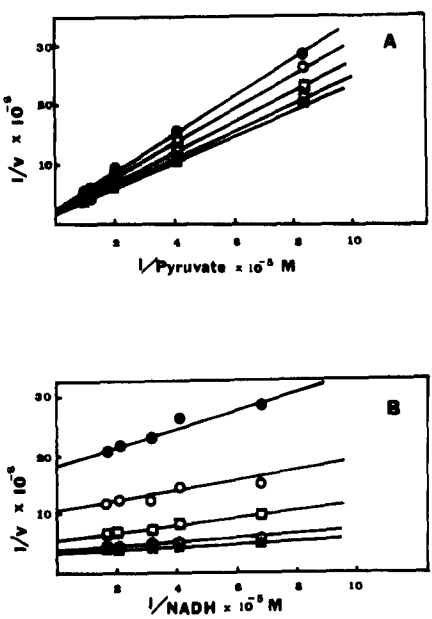

Fig. 3. Primary reciprocal plots of initial velocity data for modified lactate dehydrogenase. (A) NADH concentrations were held constant at: ●, 14.5 μM; ○, 24.1 μM; □, 30.5 μM; ○, 46.5 μM, and △, 56.2 μM. (B) Pyruvate concentrations were held constant at: ●, 11.9 μM; ○, 23.8 μM; □, 47.6 μM; ○, 75.8 μM, and △, 95.2 μM.

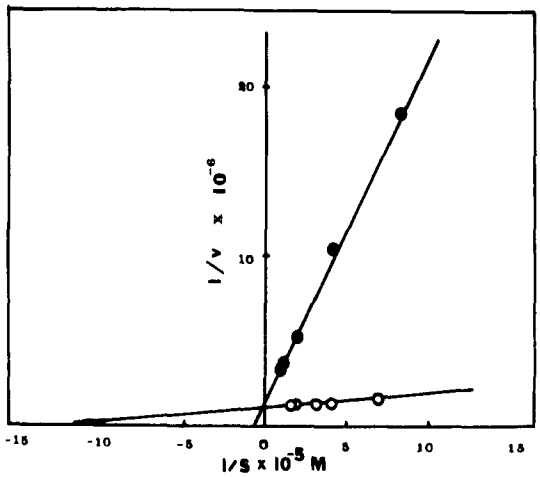

Fig. 4. Secondary reciprocal plot of maximal velocties obtained from primary plots of initial velocity data for modified lactate dehydrogenase. Values for V for varying concentrations of pyruvate (○) and NADH (●).

secondary reciprocal plot from which the Michaelis constants were evaluated is given in Fig. 4. Initial rate data for surface-bound lactate dehydrogenase obtained with a stirred-vessel reactor were analyzed similarly as shown by representative plots for propyl–glass enzyme given in Figs 5 and 6. Michaelis constants, K_{NADH} and K_p

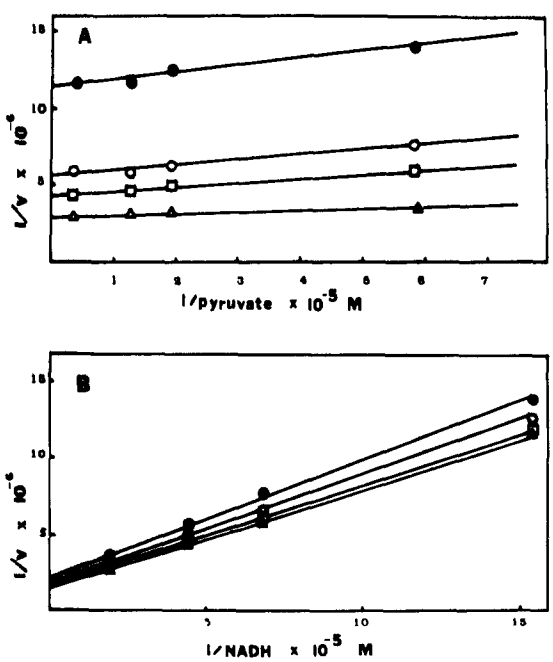

Fig. 5. Primary reciprocal plots of initial velocity data for a preparation of propyl-glass lactate dehydrogenase. (A) NADH concentrations were held constant at: ●, 6.4 μM; ○, 14.5 μM; □, 22.2 μM, and △, 48.2 μM. (B) Pyruvate concentrations were held constant at: ●, 17.0μ M; ○, 50.9 μM; □, 67.9 μM, and △, 229 μM.

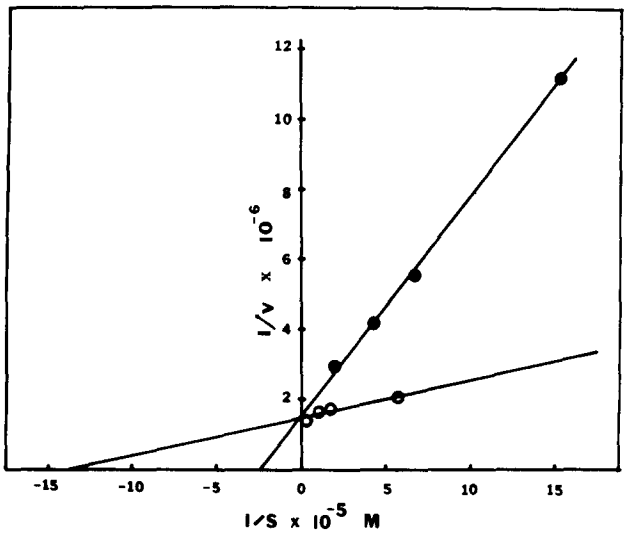

Fig. 6. Secondary reciprocal plot of maximal velocities obtained from primary plots of initial velocity data for propyl–glass lactate dehydrogenase. Values for V for varying concentrations of pyruvate (○) and NADH (●).

(pyruvate), and the turnover numbers for all three enzyme derivatives are compared to those of native enzyme in Table III.

The Michaelis constant for pyruvate and turnover number were also determined from a reciprocal plot of data obtained using a single concentration of NADH much greater than its Michaelis constant. Results for native and succinylpropyl–glass enzyme are listed in Table IV. These conditions and method of analysis are similar to those used by Wilson et al. [2] for a preparation of lactate dehydrogenase bound to a cellulose anion exchanger. In 0.24 M NaCl at pH 7.4 and 25 °C, they obtained values of 71 and 55 μM for the K_p of native and immobilized enzyme, respectively.

Integrated Michaelis–Menten analysis of column reactor data

If it can be assumed that the concentration of NADH does not affect evaluation of K_p when the NADH concentration is well above its Michaelis constant as proposed by Wilson et al. [2], then a simple integrated form of the Michaelis–Menten

TABLE III

SUMMARY OF KINETIC PARAMETERS FOR NATIVE, MODIFIED, AND GLASS-BOUND LACTATE DEHYDROGENASE OBTAINED FROM INITIAL RATE MEASUREMENTS

All measurements were made in 0.1 M phosphate buffer at pH 7.0 and 25 °C

Preparation	K_{NADH} (μM)	K_p (μM)	$V_r \times 10^7$ (M/s)	V_r/e (s^{-1})
Native	7.8	185	8.84	529
Modified	6.4	154	8.42	457
Succinylpropyl–glass	39	5.1	6 76	12.2
Propyl–glass	55	3.6	6.91	10.9

TABLE IV

KINETIC PARAMETERS OBTAINED AT HIGH CONCENTRATIONS OF NADH

Concentration of NADH was 93 μM Activities were determined at 25 °C in 0.1 M phosphate buffer, pH 7.0.

Preparation	K_p (μM)	$V_r \times 10^7$ (M/s)	V/e (s^{-1})
Native	48	11.4	570
Succinylpropyl–glass	6.7	4.5	11.2

equation obtains

$$S_0 - S_e = K_m' \ln (S_e/S_0) + C/Q \tag{1}$$

where S_0 is the initial substrate concentration, S_e; the substrate concentration in the emergent solution; K_m', the apparent Michaelis constant; C, the reaction capacity of the column (mass/unit time), and Q, the flow rate. Values for K_p' and C at a range of flow rates were evaluated from plots of $S_0 - S_e$ vs. $\ln (S_e/S_0)$ and are listed in Table V. Unlike the data for lactate dehydrogenase bound to cationic cellulose paper discs obtained by Wilson et al. [2], these plots for succinylpropyl–glass enzyme were linear. A systematic variation of these parameters with flow rate is not obvious. The reaction capacity may increase slightly with increasing flow rate, but not as greatly as that observed by Wilson et al. [2].

TABLE V

KINETIC PARAMETERS OBTAINED FROM COLUMN REACTOR STUDIES USING AN INTEGRATED MICHAELIS–MENTEN EQUATION

Data obtained for succinylpropyl–glass enzyme in 0.1 M phosphate buffer at pH 7.0 and 25 °C using a concentration of NADH of 101 μM.

Flow rate (ml/min)	K'_p (μM)	$C \times 10^4$ (M/min)
4	45.2	3.4
8	55.1	4.6
10	46.3	4.5
15	59.2	5.1

Schwert [19, 20] has obtained a solution for the two-substrate equation

$$v = \frac{V_r}{1 + \frac{K_p}{P^\circ} + \frac{K_R}{R^\circ} + \frac{K_{RP}}{P^\circ R^\circ}} \tag{2}$$

for the case of an irreversible reaction which can be written as

$$t = \frac{n\Delta x}{V_r} + \frac{K_R}{V_r} \ln \left(\frac{1}{1 - F_R}\right) + \frac{K_p}{V_r} \ln \frac{1}{1 - F_p} + \\ + \frac{K_{RP}}{V_r} \left[\frac{\ln \left(\frac{1}{1 - F_R}\right) - \ln \left(\frac{1}{1 - F_p}\right)}{P^0 - R^0}\right] \tag{3}$$

In these equations n is an integer; Δ_X, an arbitrarily selected increment in product concentration; V_r, the maximum velocity; F_R and F_p, the fraction of NADH and pyruvate converted to product; P° and R°, the initial concentrations of pyruvate and NADH, and K_P and K_{RP}, the Michaelis constant for pyruvate, and a mixed constant, respectively. From this expression a relation can be obtained for the time difference required to reach the same value of $n\Delta_x$ at two different pyruvate concentrations for the same concentration of NADH. Noting that for a column reactor of large-pore glass beads, time is given by V_0/R where fh_0 is the liquid volume and R the flow rate or the column, this relation becomes

$$\frac{\Delta(V_0/Q)}{\Delta Y} = \frac{K_{RP}}{V_r} + \frac{K_p}{V_r}\frac{\Delta \ln\left(\frac{1}{1-F_p}\right)}{\Delta Y} \tag{4}$$

where Y is the bracketed quantity in Eqn 3. Hence a plot of this equation yields K_{RP}/V_r and K_p/V_r as the intercept and slope, respectively. Further manipulation of these equations allows evaluation of K_R, K_p, and K_{RP}; however, the error is substantially greater and this information is not necessary for our purpose.

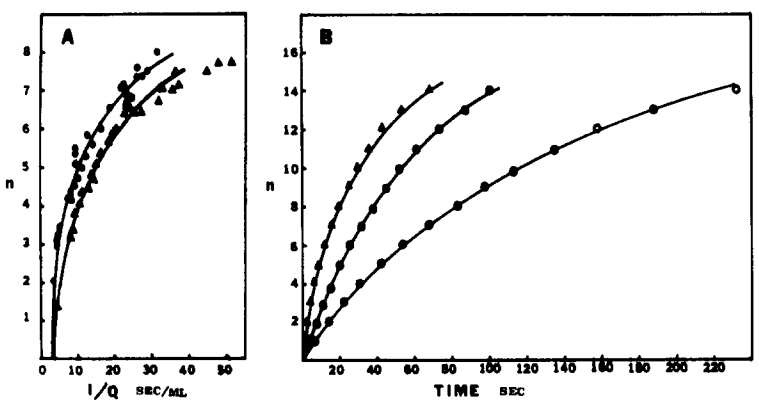

Fig. 7. (A) Plot of n against the reciprocal flow rate for two pyruvate concentrations: ●, 101.8 μM, and ▲, 25.6 μM. Succinylpropyl-glass enzyme in 0.1 M phosphate buffer, pH 7.0 and 25 °C; NADH concentration was 22.5 μM. Column dimensions were approx. 0.6 cm × 1.0 cm. (B) A similar plot for native enzyme (approx. 1.67 nM) at pyruvate concentrations of: △, 109.1 μM; ●, 54.5 μM, and ○, 27.3 μM. NADH concentration was 12.5 μM.

Data are plotted in Fig. 7A for columns of succinylpropyl–glass enzyme and in Fig. 7B for solutions of native enzyme by analogy to Eqn 3. An increment of 0.8039 μM was used in these experiments. Results of this plot are plotted in Fig. 8 according to Eqn 4 and the parameters obtained from this analysis are given in Table VI. In order to make comparisons of the ratio K_p/V_r with results from initial rate experiments, these data were obtained using the same enzyme concentration. Agreement between the two methods appears to be very good. The ratio K_{RP}/K_p is not dependent on enzyme concentration. Our value is in good agreement with that obtained by Schwert [20] ($3.8 \cdot 10^{-6}$) for bovine heart lactate dehydrogenase using this method.

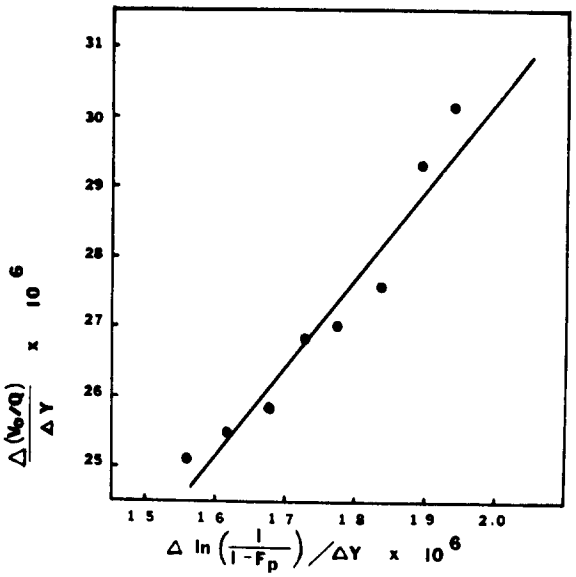

Fig. 8. Plot of the data from Fig. 7 for succinylpropyl–glass enzyme according to Eqn 4 in the text. V_0 was approx. 1 ml and thus neglected.

TABLE VI

EVALUATION OF KINETIC PARAMETERS USING AN INTEGRATED FORM OF THE MICHAELIS–MENTEN EQUATION PROPOSED BY SCHWERT

All measurements were made in 0.1 M phosphate buffer at pH 7.0 and 25 °C.

Preparation	K_p/V_r* (s) (initial rate)	K_p/V_r** (s) (integrated)	$K_{RP}/V_r \times 10^5$** (mole·s/l)	$K_{RP}/K_p \times 10^6$*** (M)
Native	209	193	84.0	4.4
Succinylpropyl–glass	7.5	12.8	4.7	3.7

* Calculated from data given in Table III.

** Evaluated from data plotted in Fig. 8. The same preparations of native and surface-bound enzyme and the same amount (approx. 1.67 nM) of native and the same weight (0.18 g, dry weight) of surface-bound enzyme was used in this experiment as was used to obtain the data shown in Table III.

*** For a compulsory pathway mechanism this is equal to the dissociation constant of the enzyme–NADH complex.

DISCUSSION

Succinylpropyl–glass enzyme was prepared without exposing the protein to EDC. Actually these preparations contained a higher concentration of enzyme than propyl–glass enzyme prepared by reaction in the presence of EDC probably because less EDC was used in the latter procedure in order to prevent excessive side reaction.

Dissociation of covalently bound enzyme

Release of three-fourths of the original protein by dissociating agents indicates that tetramers were initially covalently bound to the matrix by only one of the four

subunits. Binding of a smaller oligomer or by more than one subunit would result in a value of the ratio greater than 0.33, the theoretical value for binding one of the four subunits.

In an earlier publication [10], we reported that incubation of the unfolded glass-subunit with substrate solution resulted in a return of 10–20% of the original activity. This observation combined with the above results indicates that individual subunits of lactate dehydrogenase are capable of exhibiting enzymatic activity. If this activity was, in fact, due to the presence of 10% tetramer remaining on the washed beads, the rest being monomer, then the ratio would be 0.48. This value seems well above the magnitude of error for determination of the ratio.

Interaction of the remaining subunits on the glass to form an active oligomer also seems improbable. The surface area of these beads calculated from the pore volume by assuming a cylinder of the average pore diameter is approx. 107 m^2/g. Since the amount of enzyme bound is 1.3 mg/g or $56 \cdot 10^{14}$ molecules/g and using a molecular diameter of 70 Å [21], we can calculate the surface area occupied by "touching spheres", i.e. $A = (N^{\frac{1}{2}}d)^2$ where N is the number of molecules. This calculation gives roughly 0.27 m^2/g, therefore, the enzyme per se occupies only $<1\%$ of the total surface area.

Chan [22] has reached a similar conclusion concerning the activity of rabbit muscle aldolase subunits from studies of this enzyme bound to Sepharose. In our opinion, glass should provide a more rigid matrix than Sepharose and thus should further insure the prevention of interaction of bound subunits.

From results of solution studies some investigators [23–25] have suggested that oligomers smaller than the tetramer may be active; whereas, others [26] have concluded that only the tetramer exhibits activity. Some of the discrepancy may result from the difficulty in finding the proper conditions to bring about dissociation to an active form as recently demonstrated for yeast enolase [27]. The effect of aging on the ratio (Table II) is particularly striking. This can only result from dissociation of non-covalently bound subunits. The observation is congruent with the recent illustration of a slow concentration-dependent dissociation of rabbit muscle lactate dehydrogenase [28]. A ratio of 1.0 could be explained by complete dissociation to a dimer (mixtures of monomers, dimers, trimers and tetramers could also account for this value). Attribution of the increased ratio to dissociation to dimer is appealing since solution studies have suggested dissociation to this species [28]. It is also consistent with the hypothesized "dimer of dimers" structure for this enzyme [26, 28]. These results and those of previous studies [10, 28] suggest that tetramer could be reformed by incubation with free enzyme and that aging dissociation could be prevented by storage in the presence of coenzyme.

Kinetic parameters

The kinetic behavior resulting from the attachment of an enzyme to an insoluble matrix has been the subject of a number of recent investigations [1–8]. Changes in kinetic parameters may be attributed to: (1) conformational changes in the enzyme structure, (2) changes in the reaction environment, (3) specific interactions of the substrate and the matrix, (4) specific interactions of protons and the matrix, (5) the effect of an unstirred surface layer, (6) the effects of intrapore diffusion for porous solids, and (7) attachment of enzyme to a matrix pore inaccessible to macrosubstrates.

For these considerations it is assumed that the modification reactions per se do not affect the kinetic parameters; an assumption that has not been tested in many studies of immobilized enzymes. Comparison of lactate dehydrogenase having an average of five derivatized carboxyl groups per mole with propyl–glass enzyme which was attached via its carboxyl groups using the same concentration of reagent shows that the kinetic parameters of the modified enzyme in solution is essentially identical to that of native enzyme vis-a-vis the surface-bound enzyme (Table III). Hence, we conclude that the observed changes are caused by the presence of the surface and its effect on the micro-environment of the enzyme.

In this study the K_m for pyruvate was observed to decrease roughly 40-fold whereas that for NADH increased about 6-fold. Wilson et al. [2] observed a decrease in the K_m for pyruvate of 1.3 times in 0.24 M NaCl and 2 times in 0.98 M NaCl for lactate dehydrogenase bound to a positively charged matrix. They attributed the decrease to electrostatic attraction of the substrate to the matrix. In our case, there should be approx. 24 mmoles/g of excess positive charge for propyl–glass enzyme and 7 mmoles/g of excess negative charge for succinylpropyl–glass enzyme, disregarding charges inherent to the glass; hence, it is not a highly charged surface. Perhaps even more convincing evidence for the absence of an electrostatic effect is the similarity of the kinetic parameters for both types of glass surfaces and furthermore, the Michaelis constant for NADH is increased. This same argument suggests that a local pH effect, factor (4) above, is not responsible for the observed changes.

Pore size, in relation to the size of substrate molecules, indicates that item (7) would not be a factor in this case. The effects of diffusion, however, most likely influence the apparent values for the kinetic parameters. An unstirred layer effect is probably negligible since this can be eliminated by using high stirring or flow rates [29]. Experiments in the stirred-vessel reactor were performed at stirring speeds such that variation of the speed did not affect the observed activity. Also the reaction capacity, C, and K_p for the column reactor was not observed to vary significantly with the flow rate (Table V). However, intrapore diffusion is probably a significant factor and may be responsible for the increased value of K_m for NADH with respect to enzyme in solution. Approximation of an effectiveness factor according to relationships given by Bunting and Laidler [6] and Marsh et al. [30] suggests a value of the order of 0.1 which is consistant with the observed increase.

The large decrease in the apparent K_m for pyruvate is thus apparently caused by an enzyme-conformational change or a specific interaction with the matrix other than electrostatic. Measurement of equilibrium binding of NAD eliminates the diffusivity problem and corrects for specific interaction with the matrix. Hence, the 30-fold decrease in the dissociation constant is most likely caused by conformational changes resulting in greater affinity for this cosubstrate.

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