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The reactivation of an unfolded subunit enzyme covalently linked to a solid surface

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SUMMARY

Rabbit muscle lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) was covalently coupled to porous glass beads by two procedures. In Procedure I the enzyme was coupled directly to the surface by reaction with the enzyme's carboxyl groups. Procedure II involved coupling to succinyl-glass through the enzyme's amino groups in the absence of activating reagent. Following complete inactivation in 7.0 M guanidine·HCl, substantial reactivation of the bound enzyme, approx. 10–20%, could be obtained by simply exposing the enzyme to substrate solution after removal of the denaturing agent and washing with buffer. The enzyme could be reconstituted, as judged by total recovery of activity, by incubation of the bound enzyme with a solution of lactate dehydrogenase.

Numerous studies of the reversible unfolding of proteins and enzymes[1] have given experimental verification to the proposal that the amino acid sequence provides sufficient information to determine the native conformation[2]. The experimental approach usually followed in these studies has been to greatly dilute the enzyme from the unfolding solution into a more nearly physiological buffer, thus favoring intramolecular interactions and refolding. This may be followed by reconcentration to induce reformation of the quaternary structure. Following essentially this procedure, with addition of mercaptoethanol to prevent oxidation of exposed sulfhydryl groups, Epstein *et al.*[3] obtained 50–60% reactivation of rabbit muscle lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) from concentrated urea or guanidine·HCl solutions.
In a later study of various heart muscle lactate dehydrogenases, Chilson *et al.*[4] found the

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

rate of reactivation to be greatly accelerated by the presence of NADH although they observed only a 40% regain of activity. Concentrated urea or guanidine·HCl solutions have been commonly employed as unfolding agents. Study of many different proteins has shown that random coils are generated by non-cross-linked proteins in 6–10 M guanidine · HCl solution[5]. The present communication reports reactivation of rabbit muscle lactate dehydrogenase which has been covalently linked to glass beads.

Two procedures were used to link crystalline rabbit muscle lactate dehydrogenase (Sigma Chemical Co., lot No. L-2500) to porous alkylaminosilane-glass beads (40–60 mesh, 522 Å pore diameter, Corning Glass Works). In Procedure I, 5 mg lactate dehydrogenase was mixed with 0.5 g of previously equilibrated and degassed glass beads in 3 ml of sodium phosphate buffer, pH 7. At 0°C, 70 mg 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) was added directly to the mixture and coupling was allowed to proceed for 2 h. In Procedure II, succinic acid was first attached to the alkylaminosilane-glass surface in the presence of EDC. Stirring was accomplished by recycling the reagents through a column of the beads. After establishing the degree of linkage of succinic acid to the glass by titration, the carboxyl groups were activated by addition of EDC. When the activation of carboxyl groups had reached a maximum the pH was raised to 7 and the temperature lowered to 0°C in order to slow the hydrolysis rate (Swaisgood and Natake, submitted for publication) and the excess reagent was washed from the beads. A solution of lactate dehydrogenase was added to the washed beads and coupling was allowed to proceed for 24 h at 0°C.

Activities of the glass-bound enzyme were determined both by a stirred batch procedure similar to that described by Mosbach[6] and by a column procedure. For the stirred solution procedure, a Chromatronix 0.9-cm diameter column plunger fitted with a 40-μm pore diameter cloth was inserted into a beaker containing the bound enzyme and 10 ml of substrate solution. The solution was pumped through a 0.08-ml flow-through cell placed in a Cary Model 15 spectrophotometer using a Stalprodukter pump and a flow rate of 4 ml/min. In the case of the column procedure, a column of the bound enzyme, 0.9-cm diameter by 0.4-cm height, was prepared in a Glenco Precision Bore column. Substrate was pumped through the column with the peristaltic pump at a rate of 0.67 ml/min. The absorbance was monitored with the Cary Model 15 spectrophotometer. Activities were measured using $3.45 \cdot 10^{-4}$ M pyruvate and $1.929 \cdot 10^{-5}$ M NADH as the substrates in 0.03 M sodium phosphate buffer, pH 7.0.

Following the preparation of glass-enzyme and glass-succinyl-enzyme, both were washed along with a control with several liters of buffer (0.1 M sodium phosphate, 0.05 M NaCl, pH 8.5) to insure maximal removal of unbound enzyme. After washing, a portion of the preparation was transferred to the beaker or the column for activity measurements, unfolding and removal of subunits not covalently attached to the glass, and reconstitution by incubation with a dilute lactate dehydrogenase solution. After washing with 200 ml of distilled water, 10 ml of 7.0 M guanidine·HCl solution containing 0.05 M dithiothreitol was added and the mixture was allowed to stand for 1 h at room temperature. The guanidine·HCl solution was removed, the beads were washed with 1 l

of distilled water, and the activity was again determined. Following the activity measurements the beads were washed with 200 ml distilled water and then soaked in a solution containing 0.1 mg/ml lactate dehydrogenase and 0.05 M dithiothreitol for 2 h. After incubation the solution was removed and the beads were washed with 1 l of washing buffer, prior to activity measurements.

Numerous controls were examined to eliminate the contribution of adsorbed enzyme to the observed activity. Most controls exhibited roughly 10% of the activity of the corresponding preparation, however, the amount could be reduced by washing with larger volumes. Controls washed with 7.0 M guanidine·HCl did not retain any activity. Exposure of the guanidine·HCl-washed beads to lactate dehydrogenase solutions resulted in retention of the same level of activity as was initially observed for the control.

TABLE I

ACTIVITIES OF GLASS-BOUND LACTATE DEHYDROGENASE BEFORE AND AFTER EXPOSURE TO GUANIDINE·HCl AND AFTER RECONSTITUTION WITH LACTATE DEHYDROGENASE IN SOLUTION

Activities were determined from absorbance measurements at 340 nm.

Preparation	*Before treatment with guanidine·HCl*		*After removing guanidine·HCl*		*Reconstituted*	
	Stirred batch ΔA/min	*Column* ΔA*	*Stirred batch ΔA/min*	*Column* ΔA*	*Stirred batch ΔA/min*	*Column* ΔA*
Glass-enzyme	0.12	0.035	0.02	0.004	0.13	0.040
Glass-succinyl-enzyme	0.13	0.020 0.090	0.04	0.002 0.012	0.16	0.020 0.080

*Values were corrected by subtraction of control absorbance readings.

The average results of duplicate measurements by the stirred-batch procedure are shown in Table I expressed as the change in absorbance per min at 340 nm. The beads were washed with 200 ml of distilled water between the duplicate runs. Examples of the absorbance *vs* time profiles obtained with the Cary 15 spectrophotometer for the untreated, and the reconstituted forms of both types of bound enzyme preparations are shown in Fig. 1. Comparison of Curves A, B, and C and Curves D, E, and F shows the loss of activity after washing with guanidine·HCl and the dramatic return of activity following incubation of the treated enzyme with a dilute solution of lactate dehydrogenase Examples of data obtained by the column procedure for several preparations are also shown in Table I expressed as the change in 340-nm absorbance due to NADH oxidation.

These data indicate a 10–20% restoration of activity upon removal of guanidine·HCl from the bound enzyme preparations. Assays in the presence of 6 M guanidine·HCl exhibited no activity indicating unfolding was extensive in this solution.

The activity regained after removal of guanidine·HCl may be due to refolding of units smaller than the tetramer. The question of activity of smaller subunits has been raised previously[7]. Rabbit muscle aldolase subunits have been shown to be active by studies of the enzyme covalently bound to Sepharose[8].

Upon addition of substrate solution to the treated bound enzyme a short lag period was observed prior to return of maximal activity (Fig. 1). This phenomenon was also quite apparent in the column activity studies as shown by an initial gradual decrease in the 340-nm absorbance of the column effluent. Presumably the lag is the result of a more favorable folding of the protein structure induced by the binding of substrate. Others have observed structural effects due to binding of NADH[9,10].

Reconstituted enzyme exhibited nearly complete recovery of activity (80–100%) for every case studied. The extent and rapidity of recovery of activity was dependent upon concentration and age of the lactate dehydrogenase solution used for incubation. Enhanced interaction probably results from more extensive dissociation of lactate dehydrogenase subunits for aged or dilute solution. Millar[11,12] has reported dissociation of beef heart lactate dehydrogenase and the occurrence of hydridization in dilute solutions. Also we have observed a time-dependent dissociation of rabbit muscle lactate dehydrogenase. The lag period was not observed for reconstituted enzyme (Fig. 1).

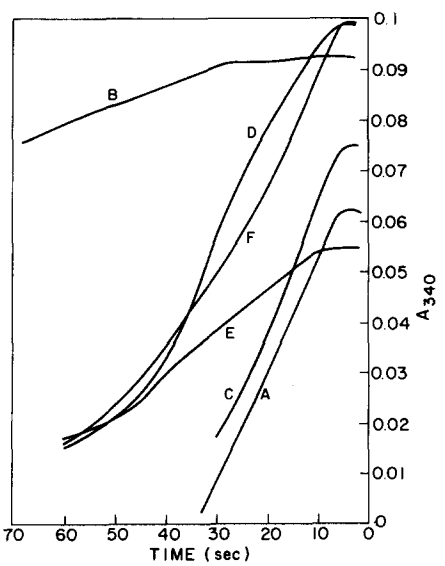

Fig. 1. Typical reproductions of the absorbance *vs* time tracings obtained with the Cary 15 Spectrophotometer. The values recorded are not absolute absorbance as the pen was simply adjusted on scale with the balance control. Zero time represents the time of addition of bound enzyme; therefore, a short lag (5 sec) was observed which represents the time required for solution to travel from the mixing vessel to the cuvette. This value should be constant since the same flow rate was used for each measurement. A, untreated glass-enzyme; B, treated glass-enzyme; C, reconstituted glass-enzyme; D, untreated glass-succinyl-enzyme; E, treated glass-succinyl-enzyme; F, reconstituted glass-succinyl-enzyme.

Comparison of these results with those obtained from free solution studies[3,4] shows the effectiveness of subunit attachment to a solid surface in preventing undesirable interchain interactions during refolding. Reconstitution of complete activity by addition of free enzyme shows that refolded subunits contain the necessary information for reformation of quaternary structure. It is tempting to draw an analogy of this reaction to that which may occur as individual subunits are synthesized on the ribosome.

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