Effects of Subunit Interactions on the Activity of Lactate Dehydrogenase Studied in Immobilized Enzyme Systems†

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ABSTRACT: Rabbit muscle lactate dehydrogenase (LDH) was coupled to Sepharose in such a way that each molecule is expected to be attached via only one subunit. Dissociation of the bound active enzyme by several methods all yielded immobilized subunit derivatives which were inactive. These derivatives were capable of regenerating activity by interacting specifically with subunits in solution formed transiently during renaturation. This ability to pick up soluble subunits is lost fairly rapidly upon storage of the immobilized subunits. Similarly, LDH subunits attached to Sepharose via disulfide bonds were found to be inactive. When these subunits were detached from the matrix by mild reduction with mercaptoethanol, activity was regenerated. The kinetics of this reactivation process suggests that reassociation is required for appearance of activity. All these results can be interpreted as showing that subunit interactions are essential for LDH activity.

One approach to understanding the function of quaternary structures in enzymes is to study the properties of single subunits and to compare them with those of the native oligomer. Several techniques have now been developed for this purpose and have been applied to rabbit muscle aldolase which normally exists as a tetramer. In this case, the results from examining immobilized subunits (Chan, 1970), subunits formed as intermediates during renaturation (Chan et al., 1973a), and subunits stabilized by chemical treatment during renaturation (Chan et al., 1974) are in good agreement and show that the monomeric form of aldolase is enzymically active but distinctly more susceptible to denaturation.

In contrast, similar studies with lactate dehydrogenase (LDH)[1] have given apparently conflicting results. Earlier workers had inferred from indirect evidence that dissociated forms of LDH might be enzymically active (Markert, 1963; Koen, 1967). Later results from inhibition studies (Griffin and Criddle, 1970), however, led to the opposite conclusion. Using a more direct approach similar to that used for aldolase, Cho and Swaisgood (1972) found rabbit muscle LDH monomers immobilized on porous glass beads to give appreciable activity. On the other hand, Jaenicke (1974) has shown that the reactivation kinetics of acid-denatured pig-heart LDH is second-order and has suggested that the monomer is inactive.

The present work was undertaken to attempt to clarify the question of possible activity of LDH subunits. We have studied the subunits of rabbit muscle LDH linked covalently to CNBr-activated Sepharose[2] and their interaction with LDH subunits added in solution. We have also prepared subunits of LDH attached to Sepharose via disulfide bonds and have observed the effects obtained when the subunits are detached from Sepharose by thiol treatment.

Materials and Methods

Rabbit muscle lactate dehydrogenase and the corresponding pig-heart enzyme (mainly the H_4 isozyme) were both obtained as crystalline suspensions from Boehringer Mannheim Corporation. Guanidine hydrochloride and urea were both of ultra-pure grade and supplied by Schwarz/Mann. NADH and bovine serum albumin were products of Sigma Chemical Company. Sepharose 4B was obtained from Pharmacia Fine Chemicals. Other chemicals were of reagent grade.

Analytical Procedures. Protein concentration of soluble lactate dehydrogenase was determined from the absorbance at 280 nm using a factor of 1.44 (Jaenicke and Knof, 1968) for the absorbance of 1 mg/ml of the rabbit-muscle enzyme and 1.40 (Jaenicke, 1974) for the corresponding absorbance of the pig-heart enzyme. The protein content of Sepharose derivatives was determined by 24-h acid hydrolysis of the washed and lyophilized samples followed by amino acid analysis. The values for Ala, Val, Leu, and Lys were corrected for background levels in this lot of Sepharose 4B (per ml of gel: Ala, 7 nmol; Val, 4.2 nmol; Leu, 6.2 nmol; Lys, negligible) and then used to work out the amount of bound protein. The number of residues of these amino acids per mol of LDH (mol wt 142 000) was determined to be: Ala, 91.2; Val, 137.6; Leu, 148.3; and Lys, 109.8.

Sepharose and its derivatives were generally measured as previously described (Chan et al., 1973b). Values for lyophilized Sepharose derivatives were converted to those for the swollen gel by assuming 40 mg of dry weight per ml of Sepharose.

Lactate dehydrogenase activity was measured at 25 °C with the reagents at the following final concentrations: sodium phosphate, pH 7.0, 0.1 M; bovine serum albumin, 0.5 mg/ml; NADH, 0.15 mg/ml; and sodium pyruvate, 0.5 mM. The commercial preparations of rabbit-muscle and pig-heart lactate dehydrogenases used in this work had specific activities of about 380 and 290 U/mg, respectively, when assayed under these conditions.

Sepharose-bound enzyme was assayed by continuous stirring in a 1-cm^2 cuvette using a 2-ml final assay volume as described

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[1] Abbreviations are: BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoate); LDH, lactate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; CNBr, cyanogen bromide; Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane.

[2] In a paper (Levi, A. S. (1975), *Arch. Biochem. Biophys. 168,* 115–121) which appeared after this work was completed, the Sepharose-bound subunits of dogfish muscle LDH were also reported to be inactive. The paper, however, was mostly concerned with properties of the immobilized LDH oligomer and did not present extensive data on the immobilized subunit.

elsewhere (Mort et al., 1973; see also Mattiasson and Mosbach, 1976). Whenever possible, after obtaining the required recorder tracing of absorbance change, the assay mixture was centrifuged and the supernatant checked for soluble activity. Freshly washed Sepharose-enzyme derivatives in this work contained no detectable soluble activity. However, soluble activity appeared upon storage at 4 °C at a rate of about 1% per day.

Preparation of LDH-Sepharose. Washed Sepharose 4B (80 ml packed volume) was activated in 160 ml of 0.25 M sodium carbonate solution by adding 160 mg of CNBr dissolved in 1 ml of acetonitrile (Axén et al., 1967, modified according to March et al., 1974). After 2 min, the gel was filtered and washed on the filter with 800 ml of cold 1 M $NaHCO_3$, pH 9.7, and then with 160 ml of 0.1 M sodium phosphate buffer, pH 8.0. A portion of this product (50 ml packed volume) was made up to 75 ml with 0.1 M sodium phosphate buffer (pH 8.0) and 5 ml (50 mg) of the crystalline suspension of rabbit muscle lactate dehydrogenase was added. Part of the remaining activated gel was used for coupling bovine serum albumin and the rest was left as "control Sepharose".

After allowing the protein to couple for 24 h, aniline (pH adjusted to 6.5 with HCl) was added to a final concentration of 0.1 M, in order to block the remaining activated groups on the Sepharose. After a further 18 h, the product was washed at least seven times by suspending with gentle stirring in 0.05 M sodium phosphate buffer, pH 7.0, containing 1 M NaCl and 1 mM EDTA followed by filtration. The product (LDH-Sepharose) was stored in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA. BSA-Sepharose and control Sepharose were treated in exactly the same way.

Preparation of Subunit-LDH-Sepharose. LDH-Sepharose (1 ml packed volume) was washed by suspension at 0 °C in 10 ml of 0.1 M sodium phosphate buffer, pH 3.0, containing 1 M NaCl and 1 mM EDTA followed by centrifugation and removal of supernatant. The procedure consisted of first adding a portion of the washing buffer (about 5 ml) to the Sepharose in a centrifuge tube and gently swirling the mixture on a vortex mixer. The remaining amount of buffer was then used to wash down the sides of the tube. After 5 min at 0 °C the sample was centrifuged and supernatant was discarded. A total of seven such washing cycles were performed. For the fourth cycle, the washing solution contained 6 M guanidine hydrochloride instead of 1 M sodium chloride in the same buffer. The product was then washed three times in the above manner at room temperature but using 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 10 mM mercaptoethanol and stored in the same buffer.

Preparation of LDH-S-S-Sepharose. Sepharose 4B was activated as described for the preparation of LDH-Sepharose except that 10 mg of CNBr was used per ml of Sepharose. After washing with 0.1 M sodium bicarbonate buffer (pH 9.7), the activated gel was added to 0.1 M 2-mercaptoethylamine (pH adjusted to 8.0 with HCl). Coupling was allowed to proceed for 24 h at 4 °C. The product was then treated with aniline and washed as described for LDH-Sepharose. Two washing cycles were conducted with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M 2-mercaptoethanol to ensure complete reduction of Sepharose-bound SH groups. Excess mercaptoethanol was then removed by washing with more Tris buffer. This Sepharose derivative contains 45 nmol of sulfhydryl groups per ml as determined by reaction with 5,5′-dithiobis(2-nitrobenzoate) (DTNB).

The above SH-Sepharose was allowed to react twice with excess DTNB in 0.1 M Tris-HCl (pH 8.0) containing 1 mM EDTA and then washed in the same buffer. LDH (20 mg) was allowed to couple to this treated Sepharose derivative at 0 °C in Tris-HCl buffer in a total volume of 30 ml. After 48 h, the product (LDH-S-S-Sepharose) was washed the same way as LDH-Sepharose.

TABLE I: Activity and Protein Content of LDH Derivatives.

	Act. (U/ml)	%	Protein Content (μg/ml)	%	Spec Act. (U/mg)
Native LDH					378
LDH-Sepharose	4.44	100	82.0	100	54.1
Subunit-LDH Sepharose	<0.004	<0.1	43.7	53.3	<0.1
Renatured LDH-Sepharose	2.59	58	61.6	75.1	42.0

Preparation of Subunit-LDH-S-S-Sepharose. Dissociation of the bound enzyme in LDH-S-S-Sepharose was achieved by washing seven times at 0 °C in 0.1 M sodium phosphate buffer (pH 3.0) containing 1 M NaCl. The product was further washed twice with 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and stored in this buffer.

Interaction of Sepharose Derivatives with Added Soluble Subunits. A 0.5-ml suspension (1 in 5) of subunit-LDH-Sepharose (or, in control experiments, other Sepharose derivatives) in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 M NaCl, 1 mM EDTA, and 10 mM mercaptoethanol at room temperature was gently stirred with a small magnetic bar in a centrifuge tube. To this was added 5 μl of a solution at 0 °C containing 2.5 μg of lactate dehydrogenase in 0.1 M sodium phosphate buffer (pH 3.0) and 10 mM mercaptoethanol. Two more additions were made at 5-min intervals. The lactate dehydrogenase was freshly mixed with the pH 3 buffer 1 min before the first addition. After the third addition, the mixture was allowed a further 5 min for interaction. The gel was then washed in the same tube by repeated suspension in the same phosphate buffer (containing NaCl, EDTA, and mercaptoethanol) followed by centrifugation and removal of supernatant. After seven such washing cycles, the volume was made up to the previously calibrated 0.5-ml mark and 50-μl aliquots were assayed.

Results

Activity and Protein Content of Immobilized Enzyme Derivatives. Rabbit muscle lactate dehydrogenase was coupled to CNBr-activated Sepharose 4B and then washed exhaustively to give a derivative (LDH-Sepharose) which contained significant, bound-enzyme activity (Table I). The specific activity of the bound enzyme corresponds to about 15% of that of the soluble enzyme. Possible reasons for the relatively low specific activity will be discussed in a later section.

Since a very low amount of CNBr (2 mg/ml of Sepharose) was used to activate the gel, it was expected that the great majority of the bound-enzyme molecules would be covalently linked to the polysaccharide matrix via only one subunit (Chan, 1970). This immobilized enzyme preparation was then washed repeatedly with pH 3 buffer to remove those subunits which were not covalently bound. Previous work had indicated that muscle lactate dehydrogenase would dissociate essentially completely into monomers at this pH (Anderson and Weber, 1966). A washing step with guanidine hydrochloride solution (also at pH 3) was included in order to ensure the removal of

TABLE II: Specific Interaction of Subunit-LDH-Sepharose with Soluble Subunits Generated in Situ.

Sample	Treatment	Bound Act. (U/ml)
Subunit-LDH-Sepharose	None	<0.02
	pH 3 dissociated LDH	2.47
	Control (buffer only)	<0.02
	Native LDH in pH 7 buffer	<0.02
	pH 3 dissociated LDH after 5′ reassociation at pH 7	0.13
LDH-Sepharose	None	4.44
	pH 3 dissociated LDH	4.86
	Control (buffer only)	4.86
BSA-Sepharose[a]	pH 3 dissociated LDH	<0.02
Sepharose control[b]	pH 3 dissociated LDH	<0.02

[a] Pretreated exactly the same way as subunit-LDH-Sepharose (see Materials and Methods). [b] Activated and pretreated exactly the same way as subunit-LDH-Sepharose (see Materials and Methods).

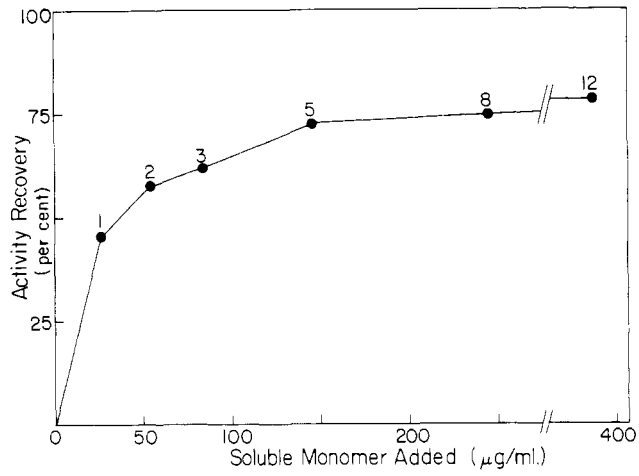

FIGURE 1: Extent of reactivation of subunit LDH-Sepharose by interaction with soluble subunits. A sample of freshly prepared subunit-LDH-Sepharose (2 ml) in 10 ml of buffer was treated with pH 3 dissociated rabbit muscle LDH as described in Materials and Methods. At 5-min intervals, 50 μg of LDH which had been exposed to pH 3 buffer (50 μl) for 1 min was added. The suspension was continuously stirred and aliquots (0.5 ml of the suspension) were removed prior to the next addition. The aliquots were then extensively washed and the bound activity was determined. The bound activity is expressed as percentage of the activity in the original sample of LDH-Sepharose from which the subunit derivative was prepared. The amount of soluble (pH 3 dissociated) monomer added has been calculated in terms of added protein per ml of packed volume of Sepharose, taking into account the amount of gel removed in successive aliquots. Reference to Table I shows that theoretically 82.0 − 43.7 or 38.3 μg of soluble monomer would be required to give complete recovery of activity if the bound subunits reassociated with added subunits with 100% efficiency. The number above each data point indicates the number of additions prior to removal of sample.

any denatured polypeptides which might be noncovalently entangled in the matrix. The product was finally washed with pH 7 phosphate buffer to allow the covalently bound enzyme subunit to refold. This derivative (subunit-LDH-Sepharose), however, possessed no detectable catalytic activity (Table I), although a considerable amount of protein remained bound. The protein content of subunit-LDH-Sepharose (about half the amount in LDH-Sepharose) was considerably higher than that expected (25%) on the basis of a conversion from the tetrameric to a monomeric form. The various possible explanations of this anomalous result will be dealt with in the Discussion. For considerations of the results here, it should be sufficient to regard this derivative as containing immobilized protein with an incomplete quaternary structure.

Specific Interaction with Subunits in Solution. The inactivity of subunit-LDH-Sepharose could indicate either that subunit interactions were essential for activity or that the particular experimental conditions employed did not allow correct refolding of the bound polypeptides to take place. In order to test for the presence of correctly folded subunits, we examined the ability of the derivative to associate specifically with soluble subunits generated in situ. This technique, which had been developed to study the Sepharose-bound subunits of transaldolase (Chan et al., 1973b) and aldolase (Chan, 1973), consisted of adding a small aliquot of the dissociated soluble enzyme to a much larger volume of nondissociating buffer containing the Sepharose-bound subunits. As the added polypeptides refold into soluble subunits, they can reassociate among themselves or with subunits in the Sepharose derivative. To favor the latter process, the dissociated soluble enzyme is added several times in small quantities. The treatment of subunit-LDH-Sepharose in this way with pH 3 dissociated soluble lactate dehydrogenase resulted in significant recovery of Sepharose-bound activity (Table II). Control experiments showed that addition of either the pH 3 buffer alone or native enzyme in pH 7 phosphate buffer did not produce any bound activity. If the pH 3 dissociated enzyme was added to pH 7 buffer 5 min prior to mixing with subunit-LDH-Sepharose, the recovery of bound activity decreased about 20-fold. These results indicate that the regain of bound activity depended on the presence of soluble subunits formed transiently as the dissociated enzyme is placed in pH 7 buffer.

It should be pointed out that, in these experiments, the Sepharose-derivative remained under nondissociating conditions. It was therefore possible to demonstrate that LDH-Sepharose did not interact with added soluble subunits (Table II). A slight increase (<10%) in activity was observed with either pH 3 dissociated enzyme or pH 3 buffer alone. Further controls showed that neither BSA-Sepharose nor Sepharose itself (both pretreated the same way as subunit-LDH-Sepharose) would yield an active derivative through treatment with pH 3 dissociated enzyme. All these experiments indicate that the regain of activity of subunit-LDH-Sepharose upon treatment with soluble subunits is a highly specific process. The most plausible explanation is that the Sepharose derivative contains subunits folded sufficiently correctly that reassociation between bound subunits and soluble subunits can take place.

Extent of Reactivation. The nature of the above interaction between subunit-LDH-Sepharose and soluble subunits was further studied by measuring the extent of regain in activity upon repeated treatment with pH 3 dissociated enzyme. Substantial bound activity was obtained even after a single addition of soluble subunits and this activity increased rapidly with further additions reaching a plateau level (Figure 1). The pick-up of soluble subunits appeared to be essentially complete after five additions when a total of approximately 150 μg of pH 3 dissociated enzyme had been added per ml of Sepharose. Little further increase was found after seven more additions. The final level of activity was approximately 80% of that of the original LDH-Sepharose preparation from which this subunit derivative was obtained. The finding that subunit-LDH-Sepharose can pick up only a limited amount of activity provides further evidence for the specific nature of the process. Compared with the original activity of LDH-Sepharose, the

TABLE III: Subunit-LDH-Sepharose Derivatives Prepared by Different Methods and Their Interaction with Various Kinds of Dissociated Enzyme.

Method of Dissociating the Subunits of LDH-Sepharose	Bound Act. (U/ml)	Soluble Enzyme Added	Bound Act. after Addition (U/ml)
pH 3 + 1 M NaCl	<0.02	pH 3 dissociated LDH	2.35
8 M urea, + 1 M NaCl, pH 7	<0.02	pH 3 dissociated LDH	2.17
6 M Gdn·HCl, pH 7	<0.02	pH 3 dissociated LDH	2.50
Standard[a]	<0.02	pH 3 dissociated LDH	2.47
Standard	<0.02	Gdn·HCl-dissociated LDH	0.37
Standard	<0.02	Urea-dissociated LDH	0.58
Standard	<0.02	pH 3 dissociated Pig-heart LDH	1.24

[a] The standard method consists of washing with pH 3 buffer containing 1 M NaCl and one washing step with 6 M Gdn·HCl at pH 7.

high degree of activity recovery indicates that the predominant part of the bound subunits was capable of reassociating with soluble subunits. The level of recovery, however, varied somewhat depending on the preparation and on the extent of storage. A different sample of subunit-LDH-Sepharose was similarly treated with excess soluble subunits, washed, and analyzed for protein content. This sample of renatured LDH-Sepharose showed 58% of the original activity of LDH-Sepharose and 75% of the protein content (Table I). Thus the renatured immobilized LDH had a specific activity similar to that of the LDH-Sepharose preparation from which it was derived.

Bound Subunits Prepared by Different Methods Are All Inactive. To ensure further that the inactivity of subunit-LDH-Sepharose was not an experimental artifact, we examined similar derivatives prepared by dissociating the Sepharose-bound enzyme in different ways (Table III). In all cases studied, the subunit-LDH-Sepharose did not show detectable activity when it was brought back into nondenaturing conditions. Thus the inactivity of subunit-LDH-Sepharose is apparently not caused by particular experimental procedures but is most probably due to the absolute requirement of subunit interactions for enzyme activity. These inactive derivatives, however, were capable of regaining activity by interacting with added pH 3 dissociated subunits. Therefore, they appear to contain correctly folded subunits which can pick up soluble subunits. Further experiments (Table III) showed that subunit-LDH-Sepharose would also regain activity when urea dissociated or guanidine hydrochloride dissociated enzymes were added to it. The considerably lower level of recovery in these cases suggests that either the refolding of these more extensively unfolded polypeptides (compared with pH 3 dissociated enzyme) was less efficient or that even the relatively low final concentrations of these denaturants interfered with the process of subunit interactions. It was interesting to find that regain of bound activity also occurred when pH 3 dissociated pig-heart enzyme was added. This result is consistent with the well-known ability of the two types of lactate dehydrogenases readily to form hybrids (Markert, 1963).

TABLE IV: Effect of Storage on the Ability of Subunit-LDH-Sepharose to Interact with Soluble Subunits.

Subunit-LDH-Sepharose	Incubation	Act. after Treatment with pH 3 dissociated LDH (U/ml)	(%)
Preparation A	None (freshly prepared)	1.78	100
	2 h at 37 °C	1.32	74
	24 h at 4 °C	1.11	62
	8 days at 4 °C	<0.02	<1
	10 days at 4 °C plus another dissociation treatment	3	
Preparation B	None	2.47	100
	8 days at 4 °C	0.74	30

Stability of the Bound Subunits. Because the subunits were inactive, their stability could be studied only by monitoring the ability of the derivative to regain activity by interaction with soluble subunits. It was found that the subunits fairly rapidly lost their ability to pick up soluble subunits (Table IV). After storage for 1 day, one sample of bound subunits showed only 60% of the extent of reactivation obtainable when it was freshly prepared. Further storage yielded an "incompetent" derivative which was completely incapable of reactivation by interacting with soluble subunits. Attempts to regenerate "competent" subunits by subjecting the derivative to another denaturation-renaturation treatment were also unsuccessful. Although some preparations of bound subunits lost their "competence" more slowly, it was necessary to use freshly prepared derivatives for the quantitative studies reported here (Tables II and III and Figure 1). To ensure valid comparison, each set of data was obtained by simultaneous experiments using the same fresh preparation.

Lactate Dehydrogenase Bound to Sepharose via Disulfide Linkages. The results described so far were all obtained using derivatives in which the enzyme was coupled to CNBr-activated Sepharose 4B. Previous work (Axén et al., 1967) has indicated that, in this method of coupling, the linkage to Sepharose probably occurs through exposed ϵ-amino groups of lysine residues on the protein. The possibility remained that the bound lactate dehydrogenase subunits were inactive because the coupling to the matrix involved an essential residue (presumably lysine) of the protein. In order to eliminate this possibility, we tested derivatives in which coupling to Sepharose involved a different type of amino acid side chains on the enzyme. Coupling via disulfide bonds seemed particularly suitable because rabbit muscle lactate dehydrogenase is known to contain reactive sulfhydryl groups which are not essential for activity (Cho and Swaisgood, 1973). Therefore, the covalent linkage between the enzyme and the Sepharose should not substantially affect the activity of the enzyme. Another advantage of the disulfide linkage is that it offers the possibility of detaching the bound enzyme by mild treatment with excess thiol thus allowing valuable controls to be made for effects which depend on the immobilized nature of the enzyme.

A suitable matrix containing sulfhydryl groups was prepared by coupling 2-mercaptoethylamine to CNBr-activated Sepharose. Any remaining activated groups not reacted with 2-mercaptoethylamine were blocked by treatment with aniline at neutral pH. The product, which contained 45 nmol of SH groups per ml, was allowed to react with excess 5,5′-dithio-

TABLE V: Effect of Mercaptoethanol Treatment on the Activity of Various LDH Derivatives.[a]

Derivative	Act. before Treatment (U/ml)	Act. after Treatment: Bound Act. (U/ml)	Act. after Treatment: Soluble Act. (U/ml)
LDH-S-S- Sepharose	0.89	0.14	1.13
Subunit-S-S-Sepharose	<0.01	0.05	0.45
Native LDH	393[b]		387[b]
LDH-Sepharose	4.08	4.02	<0.01
Subunit-LDH- Sepharose	<0.01	<0.01	<0.01

[a] Each sample was stirred in a cuvette in 1.95 ml of sodium phosphate buffer pH 7 (0.3 M) containing bovine serum albumin (0.5 mg/ml), EDTA (0.1 mM), NADH (0.15 mg/ml), and 2-mercaptoethanol (0.14 M). After incubation at 25 °C for 20 min, pyruvate (1 μmol in 50 μl) was added and the enzymic rate was followed for 1 min. The suspension was then rapidly centrifuged to determine the rate in the supernatant. [b] Per milligram

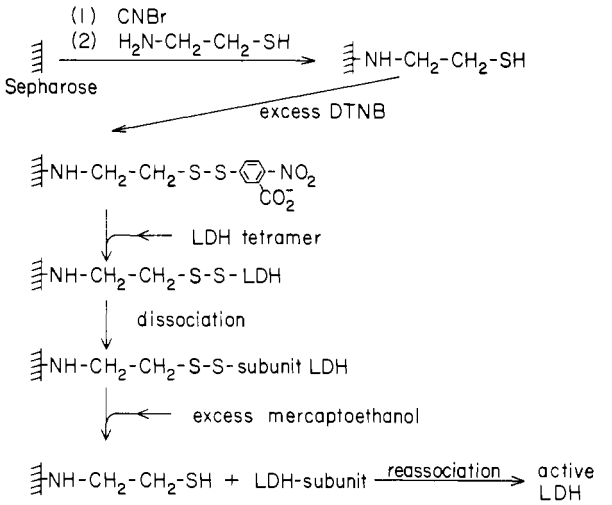

FIGURE 2: Reaction scheme for the coupling of LDH to Sepharose via disulfide bonds and the subsequent dissociation of the bound enzyme and detachment of the subunits.

bis(2-nitrobenzoate) to form a derivative containing bound mixed disulfide bonds (Figure 2). This derivative was then incubated with native LDH at pH 8. It was expected that coupling of the enzyme in the above manner would be favored because, out of the two possible ways by which a protein sulfhydryl group could attack the mixed disulfide bond, the reaction leading to coupling of the protein would produce a much better leaving group (the mercaptide ion of 5-thiol-2-nitrobenzoate).

LDH-S-S-Sepharose prepared in this way showed substantial bound-enzyme activity (Table V). As expected, even a relatively brief treatment of this derivative with excess 2-mercaptoethanol released most of the enzyme into free solution. This detachment of the enzyme from the matrix was accompanied by a small but significant increase in total activity. The bound enzyme was dissociated by treatment with pH 3 buffer and the product (subunit-LDH-S-S-Sepharose) showed no detectable activity. In this respect it was similar to the corresponding subunit derivative prepared from LDH coupled directly to CNBr-activated Sepharose. However, when this derivative, which presumably contained subunits bound via disulfide bonds, was treated with excess 2-mercaptoethanol, activity gradually appeared. Not unexpectedly, most of the regenerated activity was found in free solution rather than in the bound form. These results are consistent with the postulated existence in the above derivative of correctly folded but inactive subunits which were detached from the matrix by thiol treatment. As reported below, further experiments suggest that activity was generated upon reassociation of the detached subunits. Control experiments (Table V) showed that the same thiol treatment had no significant effect on native lactate dehydrogenase. This treatment also did not affect either the activity of enzyme bound directly to CNBr-activated Sepharose or its subunit derivative. The failure of mercaptoethanol to generate activity from the subunit derivative prepared with enzyme bound directly to CNBr-activated Sepharose suggested that detachment of the subunits was necessary for reactivation.

Dependence of Reactivation Rate of Subunit-LDH-S-S-Sepharose on Enzyme Concentration. The regain of enzyme activity accompanying the cleavage of disulfide bond linking bound subunits to the Sepharose could be explained in at least two ways. First, the bound subunits could be inactive because subunit interactions were required for activity and detachment then allowed reassociation into the active form. Alternatively, the bound subunits might be inactive because an essential cysteine residue has been modified in the coupling reaction. The latter possibility was unlikely because reaction of DTNB with this enzyme had been shown by earlier studies to have little effect on enzyme activity (Cho and Swaisgood, 1973). Distinction between the two explanations was, however, possible since reassociation would be expected to be dependent on subunit concentration. If interactions are absolutely required for activity or at least the reassociated form is significantly more active than the subunit species, then the rate of the reactivation process would be dependent on enzyme concentration. On the other hand, if the released subunits are as active as the reassociated form, then no kinetic concentration dependence could be observed.

The superimposed recorder tracings of the spectrophotometric assays of subunit-LDH-S-S-Sepharose at three different concentrations are shown in Figure 3. To facilitate comparison, the absorbance scale (ordinate) is adjusted to compensate for the differences in enzyme concentration so that equal slope would represent an equal extent of reactivation. In all three experiments, a steady absorbance was found prior to the addition of mercaptoethanol confirming that the bound subunits were inactive. The introduction of a large excess of mercaptoethanol at zero time then led to gradual appearance of activity. As judged by the curvature of the reaction course, the rate of reactivation was clearly faster at higher initial concentrations of the subunit-LDH derivative. Control experiments showed that, if the subunit derivatives at the above concentrations were preincubated with mercaptoethanol for 4 h before the addition of substrates, the extent of reactivation (for any given amount of the subunit derivative) was the same in all cases. Thus the above observed differences were solely due to unequal rates of reactivation. Further control experiments showed that the very large excess of mercaptoethanol used (0.14 M) led to a small constant decrease in absorbance even in the absence of enzyme. This background absorbance rate change was more noticeable in the experiments with lower enzyme concentrations because of the expansion of the absorbance scale. Thus, in these cases, the apparently considerable initial activity immediately after addition of the thiol can be attributed entirely to this nonenzymic rate.

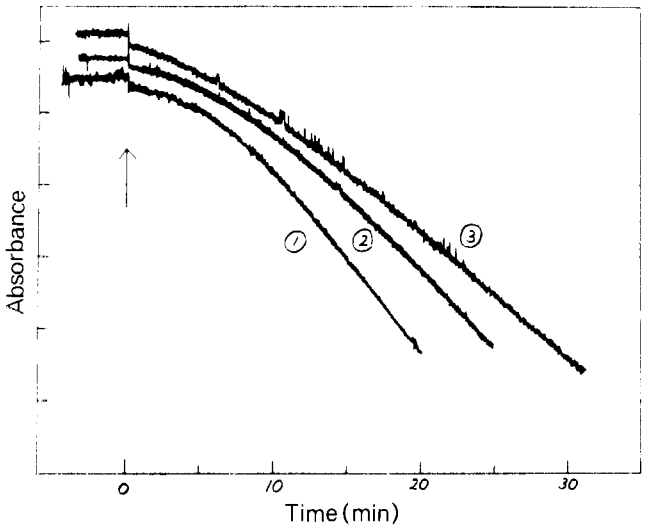

FIGURE 3: Spectrophotometric record showing the course of reactivation of subunit-LDH-S-S-Sepharose upon treatment with excess thiol. Results of three separate experiments have been superimposed. In each case the cuvette contained: sodium phosphate buffer pH 7.0, 0.3 M; bovine serum albumin, 0.5 mg/ml; NADH, 0.15 mg/ml; and sodium pyruvate, 0.5 mM. The aliquots of subunit-LDH-S-S-Sepharose (1 in 5 v/v suspension) added were: experiment 1, 100 μl; experiment 2, 50 μl; and experiment 3, 25 μl. The final volume was 2 ml in each case. After obtaining a steady trace, 2-mercaptoethanol (10 μl) was added at the position indicated by the arrow. The ordinate was adjusted in scale so that equal slope represents equal extent of reactivation: experiment 1, 0.1 (absorbance units) per division; experiment 2, 0.05 per division; and experiment 3, 0.025 per division. The considerable noise in the tracing was due to the turbidity of the stirred suspension.

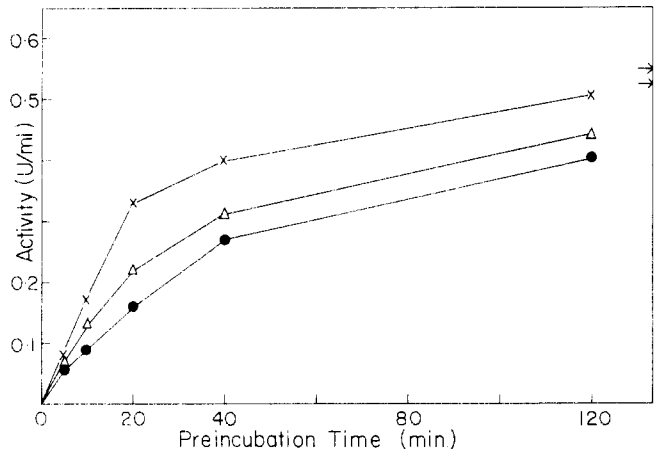

FIGURE 4: Kinetics of reactivation of subunit-LDH-S-S-Sepharose upon thiol treatment. Each experimental point represents a separate incubation mixture containing 200 μl of subunit-S-S-Sepharose in the same buffer as described in Figure 3 except that pyruvate was omitted. The preincubation volume was varied as follows to give different initial concentrations of bound subunits: (X) 0.8 ml; (△) 1.6 ml; and (●) 2.4 ml. At zero time, mercaptoethanol was added to a final concentration of 0.14 M. After incubation at 25 °C for the stated period, pyruvate and other components of the assay mixture were added so that their final concentrations (in 2.5 ml) would be equal to those in the normal assay. The two arrows indicate the range of activities reached by all three series of experiments after 4 h.

Because the spectrophotometric assay of lactate dehydrogenase was approximately linear only over a limited part of the reaction, we also studied the rate of reactivation in the absence of the substrate pyruvate. At various times after the addition of mercaptoethanol, substrate was added and the initial rate was measured. These studies, which were more accurate, also

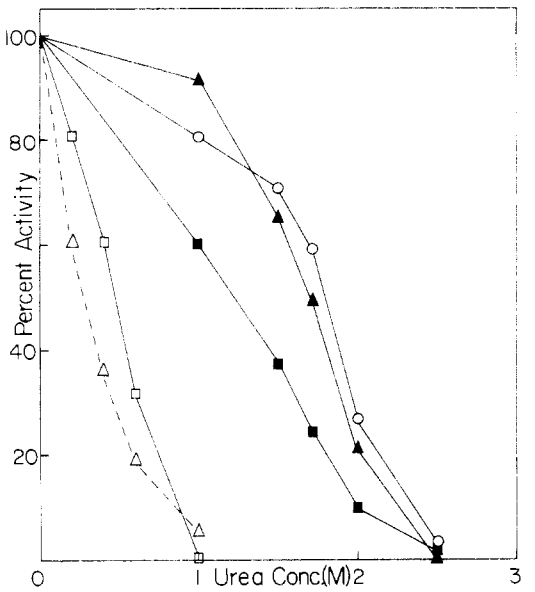

FIGURE 5: Urea sensitivity of various LDH derivatives. (■) Native LDH; (○) LDH-Sepharose; (▲) renatured LDH-Sepharose; (□) pH 3 dissociated LDH (upon renaturation); (△) subunit-LDH-S-S-Sepharose (upon thiol reactivation). The amount of sample used was adjusted so that, in the absence of urea, an activity of 10–20 mU would be obtained in the assay. Samples were preincubated for 45 min in a solution (1.95 ml) similar to the normal assay mixture except that pyruvate is omitted and urea is present at indicated concentrations. Pyruvate (1 μmol in 50 μl) was then added to start the assay. For subunit-LDH-S-S-Sepharose (△), after 5 min of preincubation, mercaptoethanol (final concentration 0.14 M) was added and incubation continued for a further 40 min.

showed the dependence of reactivation rate on the initial concentration of bound subunits (Figure 4). Since the extent of reactivation after 4 h was the same at all concentrations tested, the observed differences reflected only differences in reactivation rate. These results furnish further evidence for the importance of subunit interactions on the enzyme activity of lactate dehydrogenase.

Urea Sensitivity of Various Immobilized LDH Derivatives. To gain further insight into the nature of the molecular species present in the various LDH derivatives, we tested their sensitivity to denaturation by different concentrations of urea. In the case of two other enzymes studied previously, the immobilized subunit form (which was active) was considerably more urea sensitive than the immobilized oligomeric form of the same enzyme (Chan, 1970; Chan et al., 1973b). It has therefore been suggested (Chan et al., 1973a) that urea sensitivity may be generally of value in distinguishing between subunit and oligomeric forms of proteins since the presence of exposed subunit interface would usually make the subunit form more susceptible to denaturation.

As the concentration of urea in the assay mixture is increased from 1 to 2 M, the activity of native LDH decreases substantially (Figure 5). The urea concentration necessary to reduce the activity to half of the value in the control was approximately 1.3 M for native LDH. The corresponding value for LDH-Sepharose was significantly higher (1.75 M) indicating a stabilizing effect of the matrix on the immobilized enzyme. Of some interest is the similarity in urea sensitivity between LDH-Sepharose and renatured LDH-Sepharose suggesting that refolding and reassociation has regenerated enzyme of a conformation similar to that of the native enzyme.

Since we have found in this work that the subunit form of muscle LDH is inactive, it was not feasible to use activity to

monitor its sensitivity toward urea denaturation. However, an indirect test is provided by studying the effects of different urea concentrations on the renaturation of pH 3 dissociated enzyme. To prevent renaturation during assay, urea concentration was maintained in the assay mixture at the same level as in the renaturation medium. It was expected that correct refolding of the monomer was necessary before specific recognition of the subunit contacts and consequently reassociation can take place. Therefore, if the subunits were more susceptible to urea denaturation, then the renaturation of pH 3 dissociated enzyme would be inhibited at a concentration of urea lower than that necessary for the inactivation of native LDH. This was indeed found to be the case (Figure 5). The urea concentration required to inhibit renaturation by 50% was about 0.6 M. It was interesting to find that the process of reactivation of subunit–LDH-S-S-Sepharose by treatment with mercaptoethanol showed a similar urea sensitivity (50% inhibition at about 0.4 M urea). This result suggests that the reactivation process consists of the detachment of subunits and their reassociation into active enzyme.

Discussion

The results described above are all consistent with the interpretation that subunit interactions are essential for the activity of rabbit muscle lactate dehydrogenase. The bound subunit prepared from enzyme coupled to CNBr-activated Sepharose are most probably attached to the carrier via lysine residues. From numerous x-ray crystallographic studies, it is known that in general lysine residues are situated on the surface of the protein and in the case of LDH there are approximately 27 lysine residues per mol of monomer. Thus it seems highly unlikely that all the bound subunits could be inactive because of modification of an essential lysine residue. A variety of experimental conditions were used for dissociation of LDH-Sepharose and all of them yielded inactive bound subunits. These observations and the ability of the bound subunits to interact specifically with added soluble LDH subunits indicate that the inactivity of the bound subunits is not an experimental artifact. It is interesting to note that, using techniques identical with those employed here, one of us has previously detected the active immobilized subunits of two other enzymes (Chan, 1970; Chan et al., 1973b). The results therefore appear to depend on the nature of the enzyme rather than the technique.

When the immobilized subunits are inactive as in the case of LDH, an important control experiment is to test whether activity can be regenerated by allowing the inactive immobilized subunit to reassociate with soluble subunits whose active sites have been blocked. This experiment was carried out by Feldman et al. (1972) to show that the monomer of muscle phosphorylase was inactive. We have attempted a similar experiment using (pig-heart) LDH inactivated by 3-(2-bromoacetyl)pyridine (Woenckhaus et al., 1969). Although some activity was generated, the results were not unequivocal because the soluble enzyme could only be inactivated to about 92%. Owing to the low efficiency with which the bound subunits reassociated with subunits formed transiently in solution (especially between bound muscle LDH subunits and soluble heart LDH subunits), it was necessary to use an excess of the dissociated modified enzyme. Thus a certain amount of activity would be expected to arise simply from the renaturation of the approximately 8% residual unmodified protein.

The failure to obtain clear-cut results in the above control experiment was offset to some extent by results with subunits immobilized on Sepharose via disulfide bonds. Since these subunits are attached via cysteine rather than lysine residues, the results indicate that the inactivity of the immobilized subunits was most probably not due to coupling via an essential residue. From this work, it seems that the detachment of subunits bound via disulfide linkages and the accompanying kinetics of reactivation might offer a promising approach for analyzing the effects of subunit interaction.

The protein content of subunit–LDH-Sepharose (which amounts to about 50% of that of the corresponding LDH-Sepharose derivative) is anomalous because conversion of the bound tetramer to the bound monomer should reduce it to 25%. One obvious explanation is that some enzyme molecules were attached to Sepharose via more than one subunit. If this occurred, then from the results on protein content one would expect a certain statistical distribution of the various bound molecules with one, two, or three subunits covalently bound. Treatment of the derivative with 6 M guanidinium chloride followed by renaturation would therefore yield a mixture of the bound monomer, dimer, and trimer. Our results would then mean that all three species are inactive. However, from previous experience of coupling oligomeric proteins to Sepharose (Chan, 1970; Green and Toms, 1973), the low level of CNBr used for activation in these experiments should not lead to multiple attachment.

Another possibility is that the immobilized LDH derivative before dissociation consists of bound active dimers. However, this derivative is less urea sensitive than native LDH in solution whereas one might expect the dimer to be more urea sensitive. Moreover, this derivative does not increase in activity when treated with soluble LDH subunits suggesting that the bound (active) enzyme present cannot associate any further. Therefore, we believe that the most satisfactory explanation of the anomalous protein content is that the active component of LDH-Sepharose consists of bound tetramers contaminated by the presence of denatured monomers (and possibly some denatured dimers) of LDH. One possible indication for this is the specific activity of LDH-Sepharose which corresponds to only about 15% of that of the native enzyme, although diffusion limitations of the matrix may be partly responsible for the low activity. The formation of denatured monomers could have been caused by the very exhaustive washing procedure in which the LDH-Sepharose beads were also subject to mechanical stirring. It has been reported by other workers that rabbit muscle LDH undergoes slow dissociation (Cho and Swaisgood, 1973) and any tendency to dissociate would be accentuated by washing with large amounts of buffer. Evidence for the gradual dissociation of Sepharose-bound LDH is also provided by the observed spontaneous leakage of activity into the supernatant which occurs at a much faster rate (1% per day) than that reported for Sepharose-bound insulin (about 0.006% per day) under comparable conditions (Kolb et al., 1975). Once dissociation has occurred, the immobilized monomers are likely to denature rapidly as we have shown in this work. It is worthwhile to point out that many studies of the LDH-Sepharose derivative were made many days after its preparation. Thus it is not surprising that the postulated monomers present as contaminants in LDH-Sepharose are unable to interact with soluble subunits. Similarly if LDH-S-S-Sepharose contains significant amounts of monomeric contaminants, these must also consist mainly of denatured material since only a small increase in activity is observed when the bound enzyme is released into solution.

Irrespective of which of the above explanations is correct, our results indicate that subunit interactions are important for LDH activity. This is in contrast to work reported by Cho and Swaisgood (1972) with the same enzyme but immobilized on

porous glass beads. One of the results in that earlier work was that active immobilized subunits had apparently been obtained. However, it was not shown that the observed activity was physically different (e.g., in stability). The concentration of coupling points was also not regulated to prevent coupling of the enzyme via more than one subunit. Thus it is possible that the reported activity in that case was due to tetrameric LDH bound or adsorbed on the glass surface or in the tubing of the flow assay system. Further characterization of glass beads as a carrier for immobilized subunits would be needed to resolve the present discrepancy. The satisfactory agreement between our results with Sepharose-bound subunits and results of renaturation kinetics studies (Jaenicke, 1974) parallels similar agreement between the two methods in the case of aldolase, although the monomer is active in one case and inactive in the other. Provided that adequate control experiments are performed (as discussed by Chan, 1976), it appears that the study of subunits immobilized on Sepharose can provide information regarding the effects of alterations in quaternary structure on protein function.

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Purification of Thymidylate Synthetase from Enzyme-Poor Sources by Affinity Chromatography†

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ABSTRACT: The adsorption of thymidylate synthetase from *Escherichia coli* B to aminoalkyl-Sepharose with the increasing length of carbon chain (2–6 carbon atoms) was investigated. A correlation was found between the chain length and adsorption effectiveness, increasing from the two- to the six-carbon chain. A hydrophobic chromatography of the enzyme on aminobutyl-Sepharose gave about 20-fold purification. A new affinity chromatography carrier was synthesized containing tetrahydromethotrexate linked to aminoethyl-Sepharose via its carboxylic groups. The carrier adsorbed the enzyme from the crude preparation only in the presence of deoxyuridine 5′-monophosphate (dUMP) in a concentration of 2×10^{-5} M. The specifically adsorbed thymidylate synthetase was eluted with sacharose-containing buffers in which dUMP was omitted. The purification procedure was applied to a crude thymidylate synthetase preparation from resting *E. coli,* calf thymus, Sarcoma 180, and Gardner lymphosarcoma. The purified enzyme from all mentioned sources showed one protein band on disc electrophoresis corresponding to enzymatic activity. The formation of a reversible noncovalent complex enzyme–tetrahydromethotrexate–dUMP on the affinity column is supposed.

Thymidylate synthetase catalyzes the reductive methylation of deoxyuridine 5′-monophosphate (dUMP) to thymidylate with simultaneous conversion of N^5,N^{10}-methylenetetrahydrofolate to dihydrofolate. This enzyme is being intensively studied because of its key function in the DNA synthesis and, therefore, it has been purified from various microbial and animal sources by conventional methods (Greenberg et al., 1961; Mathews and Cohen, 1963; Friedkin et al., 1962;

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