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Preparation and Partial Characterization of a Soluble Site-to-Site Directed Enzyme Complex Composed of Alcohol Dehydrogenase and Lactate Dehydrogenase

NILS SIEGBAHN,* MATS-OLLE MAÅNSSON, AND KLAUS MOSBACH

Pure and Applied Biochemistry, Chemical Center, University of Lund, PO Box 124, S-221 00 Lund, Sweden

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ABSTRACT

A soluble, bifunctional enzyme complex has been prepared by crosslinking lactate dehydrogenase and alcohol dehydrogenase with glutaraldehyde. The crosslinking was performed on a solid phase while the active sites of alcohol dehydrogenase and lactate dehydrogenase were held adjacent to one another with the aid of a bis-NAD analog. Subsequently, the enzyme complex was released from the solid phase. The soluble enzyme complex was then purified by using NAD-Sepharose as an affinity adsorbent. Based on gel filtration experiments, the complex was estimated to consist of one of each dehydrogenase.

By using a third enzyme, lipoamide dehydrogenase, which competes with lactate dehydrogenase for NADH produced by alcohol dehydrogenase, the effect of site-to-site orientation was studied. It was found that about 83% of the NADH produced by alcohol dehydrogenase was oxidized by site-to-site oriented lactate dehydrogenase compared to a figure of only about 61% obtained in an identical system of separate enzymes. This indicates that given two alternative routes, the preference for the one to lactate dehydrogen-

*Author to whom all correspondence and reprint requests should be addressed.

ase over the one to lipoamide dehydrogenase is enhanced when lactate dehydrogenase and alcohol dehydrogenase are site-to-site oriented.

Index Entries: Reversible immobilization, of an enzyme complex; oriented enzyme complex, reversible immobilization of; alcohol dehydrogenase, reversible immobilization of the lactate dehydrogenase complex of; lactate dehydrogenase, reversible immobilization of the alcohol dehydrogenase complex of; substrate channeling, of an immobilized enzyme complex; Bis-NAD, and reversibly immobilized enzyme complexes; enzyme complex, reversible immobilization of; complex, reversible immobilization of an enzyme; dehydrogenases, reversible immobilization of complexes of; immobilized enzyme complex, reversible.

INTRODUCTION

A way to obtain a model system for membrane-bound enzymes or for enzymes that operate in close proximity to one another is to coimmobilize the enzymes. Several coimmobilized multistep enzyme systems have been described in the literature (*1–7*) and in general the efficiency of these systems exceeds that of the comparable soluble enzymes.

For example, the enzymes of a complete metabolic cycle, the urea cycle, have been coimmobilized on Sepharose (*6*). This immobilized enzyme system was found to be more efficient than the corresponding soluble system in terms of the overall rate. In fact, with the immobilized system a true operative cycle was found since the amount of fumarate produced was higher than that or arginine, with which the cycle had been initiated.

The observed kinetic properties of the coimmobilized multienzyme systems have been attributed to a closer proximity between the enzyme molecules immobilized on the solid phase, leading to a faster uptake of the intermediate by the next enzyme in sequence and/or to an enrichment of the intermediate product in the enzymes; microenvironment, caused by hindered out-diffusion from the gel phase (*2,8*). In an investigation by Koch-Schmidt et al. (*9*), an attempt was made to distinguish between proximity and changed microenvironment as contributors to the increased initial catalytic efficiency of the two consecutively working enzymes, malate dehydrogenase and citrate synthase. It was thus found that for a system with "at random" proximity between the two enzymes, i.e., when the two enzymes were crosslinked with glutaraldehyde, no advantage compared to separate enzymes in free solution was observed. However, it was found that immobilization of the consecutively working enzymes on the matrix caused a change in the microenvironment as reflected by a change in the catalytic rate of the system.

Coimmobilization of enzymes leads to heterogeneous preparations in which the active sites of the enzymes are found "at random" in rela-

tion to one another on the support. In a recent paper (*10*) an approach to immobilizing enzymes in a more defined matter, in this instance with their active sites facing one another, was described. Bis-NAD analogs (*11*) were here used for obtaining such a juxtapositioned orientation of the active sites of lactate dehydrogenase and alcohol dehydrogenase. Bis-NAD thus acted as a template for the formation of the immobilized two-enzyme complex before subsequent crosslinking with glutaraldehyde. By such an arrangement on the solid phase, the active sites were accordingly positioned against one another, even after removal of the template.

In this report we wish to describe our attempts to obtain a system comprising of a soluble enzyme complex with the active sites of the enzymes facing one another. The complex was immobilized on Sepharose with a cleavable disulfide bond and the complex was subsequently split off from the solid phase with the aid of 1,4-dithioerythritol (DTE).

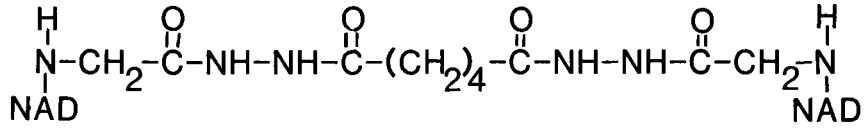
This kind of soluble complex may potentially prove to be valuable as model for naturally occurring enzyme complexes (*12*), which are believed to be important for metabolic regulation and in channeling of labile intermediate (*13*).

MATERIALS AND METHODS

Bovine heart lactate dehydrogenase (L-lactateNAD^+ oxidoreductase, EC 1.1.1.27; 560 units/mg of protein) (LDH), pig heart lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3; 136 units/mg of protein) (LiDH), β-NAD^+ (grade III), and oxalate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Horse liver alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1; 2.0 units/mg of protein) (ADH), AMP, pyruvate, β-NADH, and 1,4-dithioerythritol (DTE) were obtained from Boehringer Mannheim GmbH (Mannheim, FRG). Acetaldehyde, glutaraldehyde, cyanogen bromide, sodium cyanoborhydride, and pyrazole were purchased from Merck (Darmstadt, FRG). Sepharose 4B, Sepharose 6B, Sephacryl S300-SF, molecular weight gel filtration calibration kit, and *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Ethylenediamine was purchased from Riedel-de Haen AG (Hannover, FRG). Benzylalcohol (obtained from Merck, FRG) was distilled before use.

N^2,$N^{2'}$-[Adipodihydrazido-bis-(N^6-carbonylmethyl)]-NAD(bis-NAD) (Fig. 1) was synthesized according to the method of Larson and Mosbach (*11*). Bis-NAD can also be obtained from Sigma Chemical Co.

NAD-Sepharose was obtained by attaching N^6-[*N*-(6-aminohexyl)-carbamoyl methyl]-NAD [which was synthesized according to the method suggested by Lindberg et al. (*14*)] to Sepharose 4B using the cyanogen bromide activation method according to March et al. (*15*). A 0.5-mg sample (0.55 μmol) N^6-[*N*-(6-aminohexyl)carbamoyl methyl]-



Bis-NAD

Fig. 1. Bis-NAD. The connection with NAD is through exocyclic *N* of adenine.

NAD (which can be obtained from Sigma Chemical Co.) dissolved in 10 mL 0.1*M* sodium carbonate, pH 8.5, was added to 10 mL of activated Sepharose (50 mg CNBr/g of moist gel).

The coupling was allowed to proceed 16 h at 4°C under gentle stirring. The NAD-Sepharose was subsequently washed with several volumes of 0.2*M* Tris, pH 8.0, water and 0.1*M* NaCl/0.1*M* sodium phosphate, pH 7.5 (buffer A).

Preparation of a Soluble ADH–LDH Complex

Reversible Immobilization of ADH

This solid-phase approach was used in order to facilitate preparation and purification of the enzyme complex. ADH was immobilized in a reversible manner to Sepharose 4B using the method of Carlsson et al. (*16*) modified as follows. A 5-mL portion of moist Sepharose 4B was activated with 50 mg CNBr/mL of gel as described earlier (15) and 25 mL 1*M* ethylenediamine, pH 10, was added. After 16 h at 4°C the amino-Sepharose obtained was carefully washed with 0.5*M* NaCl, distilled water, and buffer A. In order to introduce thiol groups on to the gel, 2 mL 20 m*M* SPDP in ethanol was added to 5 mL of amino-Sepharose suspended in 5 mL buffer A (Fig. 2, step 1). After 2 h at 25°C under gentle stirring the SPDP-activated amino-Sepharose was washed with several volumes of buffer A and could easily be converted into the free thiol form by treating 5 mL gel with 5 mL 100 m*M* DTE dissolved in buffer A (Fig. 2, step 2) for 30 min at 25°C. The gel was then washed with several volumes of 10 m*M* pyrazole in buffer A. The yield of thiol groups on the gel can be determined by the amount of pyridine-2-thione released from the gel during the cleavage of the disulfide bond (*17*).

SPDP-activated ADH was prepared as follows (Fig. 2, step 3). A 25-mg portion (312 nmol) of ADH was dissolved in 6 mL 10 m*M* pyrazole in buffer A. In order to protect the active sites of ADH during the SPDP-activation step, about 1 μmol of bis-NAD was added. A ternary complex between the active sites of ADH, pyrazole, and bis-NAD was accordingly formed during 10 min of equilibration. A fivefold excess of 20 m*M* SPDP dissolved in ethanol (about 1.5 μmol SPDP) was added to the solution of protected ADH and the reaction was allowed to proceed for 30 min at

25°C. The unreacted SPDP was removed by dialyzing the ADH solution against 3 × 1 L of 10 m*M* pyrazole in buffer A.

The SPDP-activated and dialyzed ADH was coupled to the thiol-Sepharose (Fig. 2, step 4) for 16 h at 4 °C immediately after reduction of the 2-pyridyl disulfide-Sepharose. The gel was then carefully washed with several volumes of buffer A, 0.5*M* NaCl in buffer A, and once more with buffer A. In order to remove remaining affinity-bound bis-NAD, the 5 mL ADH-Sepharose was incubated for 30 min with 5 mL 10 m*M* AMP in buffer A. The AMP treatment was performed since AMP can competitively displace bis-NAD from the active sites of ADH. The gel was then thoroughly washed once more with 0.5*M* NaCl in buffer A and finally with buffer A.

It is not unlikely that some of the thiol groups of ADH could have reacted directly with the SPDP activated amino-Sepharose. However, the approach used minimized this possibility and produced a more easily defined number of bonds between ADH and the solid phase.

Affinity Immobilization of LDH to ADH-Sepharose

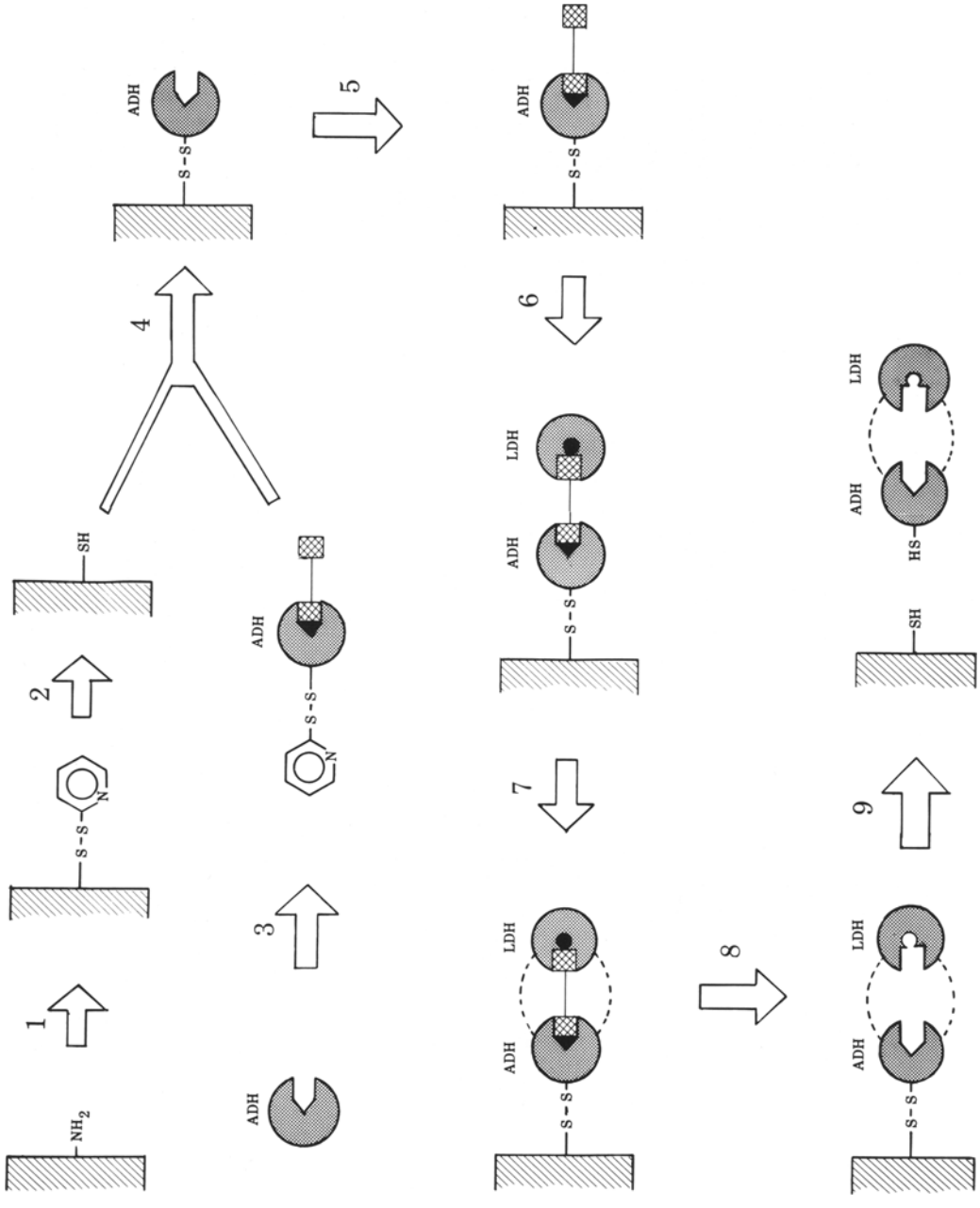
ADH-Sepharose, 5 mL, was equilibrated with 500 nmol bis-NAD in buffer A containing 50 m*M* oxalate, 10 m*M* pyrazole (Fig. 2, step 5). The gel was subsequently washed with buffer A containing 50 m*M* oxalate and 10 m*M* pyrazole to remove the bis-NAD that was not affinity-bound to ADH. The amount of affinity-bound bis-NAD was calculated as the difference between added and eluted bis-NAD.

Approximately 250 nmol LDH, dissolved in buffer A containing 50 m*M* oxalate and 10 m*M* pyrazole, was added to the gel and the mixture was incubated at 4°C for 5 min (Fig. 2, step 6). The 5 mL gel was then washed for 5 min with 4 × 5 mL cold buffer A containing 50 m*M* oxalate and 10 m*M* pyrazole to remove LDH that was not affinity-bound. The amount of affinity-bound LDH was determined spectrophotometrically by subtracting the amount removed during washing from that initially added.

In order to crosslink the ADH with the LDH, 12.5 μL 25% glutaraldehyde was added to 5 mL gel suspended in 5 mL buffer A containing 50 m*M* oxalate and 10 m*M* pyrazole (Fig. 2, step 7). After 2 h crosslinking at 25 °C, the gel was washed with several volumes of 0.2*M* Tris, pH 8.0, and 0.5*M* NaCl in 0.2*M* Tris, ph 8.0. Quenching of unreacted aldehyde groups was accomplished using 0.2*M* Tris buffer and 33 m*M* $NaCNBH_3$ for 2 × 24 h at 4°C (Fig. 2, step 8). AMP (10 m*M*) was added to the quenching solution to competitively displace the remaining affinity-bound bis-NAD. After this thorough quenching treatment, the gel was carefully washed to remove all AMP with several volumes of buffer A containing 0.5*M* NaCl and finally with buffer A.

Purification of LDH–ADH Complex

Cleavage of the disulfide bonds between ADH and the matrix (Fig. 2, step 9) was accomplished by incubation of 5 mL gel with 5 mL 100 m*M*


1
2
3
4
5
6
7
8
9
ADH
LDH
SH
HS
S-S
N
NH2

DTE in buffer A for 25 min at 25°C. After washing with 20 mL buffer A containing 50 m*M* DTE, the 25 mL eluate was added to 25 mL 50 m*M* oxalate in 0.05*M* sodium phosphate buffer, pH 7.5. This 50 mL oxalate–protein solution was subsequently applied to a 10 mL column of the lowly substituted NAD-Sepharose (Fig. 3). The complexes were, in the presence of oxalate, affinity-bound to the NAD column by formation of a ternary complex of NAD, oxalate, and the active sites of the LDH part of the enzyme complex. After the protein solution was applied to the column, the NAD gel was washed with 25 m*M* oxalate in 50 m*M* sodium phosphate, pH 7.5, until all protein not affinity binding was eluted. The LDH–ADH complex was then eluted from the affinity column by excluding oxalate from the elution buffer (buffer A). The protein fractions that contained both LDH and ADH activity were collected and used for further experiments.

Enzyme Assays

Immobilized Enzymes

All assays with immobilized enzymes (5–100 mg of moist gel) were carried out with recycling through a flow cuvet (*19*) in a total assay volume of 15 mL.

Buffer A, 1 m*M* NAD and 50 m*M* ethanol, were used for the determination of ADH activity, by measuring the formation of NADH at 340 nm. The ADH activity was also measured with a coupled substrate assay using 5 m*M* benzyl alcohol and 5 m*M* acetaldehyde as substrates (Fig. 4).

Fig. 2. (*opposite page*) Preparation of a soluble site-to-site directed alcohol dehydrogenase (ADH)–lactate dehydrogenase (LDH) complex.

Step 1: SPDP treatment of amino-Sepharose.
Step 2: Treatment of 2-pyridyl disulfide Sepharose with DTE.
Step 3: SPDP-activation of ADH in the presence of bis-NAD and pyrazole.
Step 4: Immobilization of ADH.
Step 5: Affinity binding of bis-NAD to immobilized ADH in the presence of pyrazole.
Step 6: Affinity binding of LDH to affinity-bound bis-NAD in the presence of oxalate and pyrazole.
Step 7: Crosslinking with glutaraldehyde.
Step 8: Removal of bis-NAD, pyrazole, and oxalate by washing.
Step 9: Cleavage of the disulfide bond between the site-to-site enzyme complex and the Sepharose with DTE.

The oligomeric nature of the enzymes has not been taken into account. ▩, bis-NAD; ▶; pyrazole, ●, oxalate.

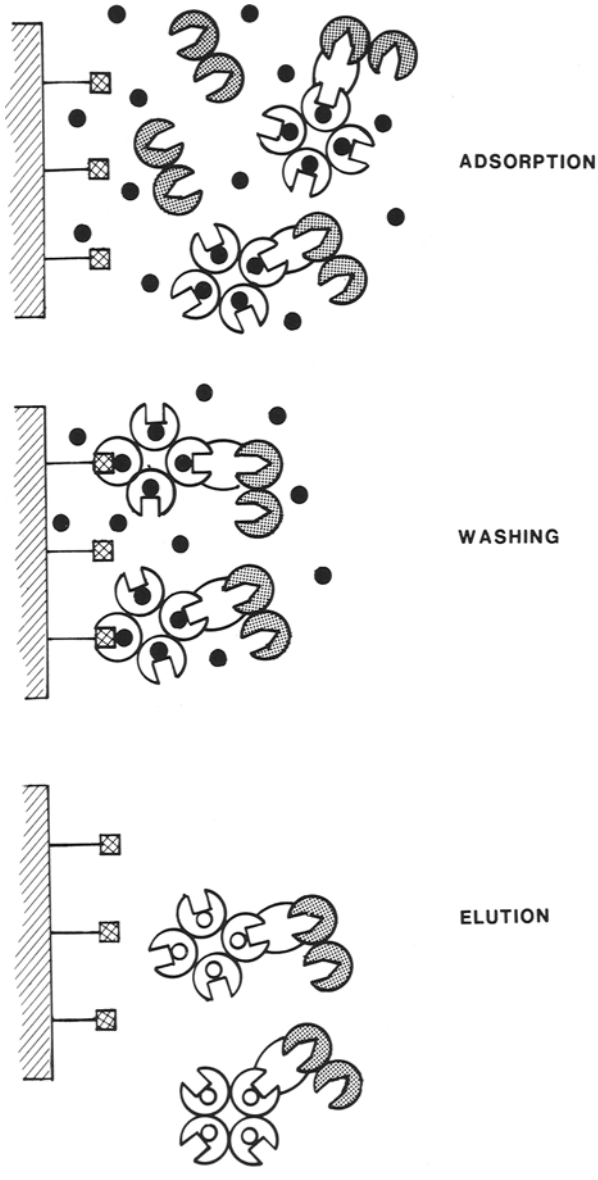


Fig. 3. Schematic representation of chromatographic purification of the soluble site-to-site directed alcohol dehydrogenase–lactate dehydrogenase complex.

Adsorption of the complex of NAD-Sepharose in the presence of 25 m*M* oxalate.

Washing. Removal of all protein not affinity bound.

Elution of the complex by excluding oxalate from the elution buffer.

The dimeric enzyme is alcohol dehydrogenase and the tetrameric enzyme is lactate dehydrogenase: ●; oxalate; ⊠ NAD.

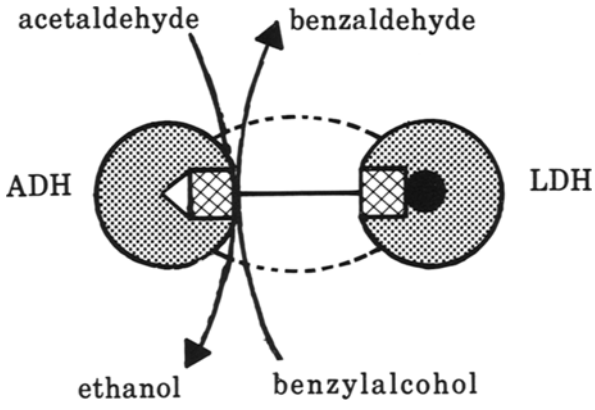


Fig. 4. Schematic representation of coupled substrate assay (*21*) used for determination of alcohol dehydrogenase activity (ADH). Addition of bis-NAD (⊠—⊠) in the presence of oxalate (●), giving affinity binding to lactate dehydrogenase (LDH) and activity in a coupled substrate assay for alcohol dehydrogenase. The oligomeric nature of the enzymes has not been taken into account.

Soluble Enzymes

LDH activity was determined by measuring the decrease in absorbance at 340 nm following the oxidation of 0.3 m*M* NADH with 5 m*M* pyruvate in buffer A as the second substrate.

LiDH was determined by the method suggested by Massey (*20*). The reduction of 0.75 m*M* ferricyanide was followed at 420 nm with 0.2 m*M* NADH as second substrate. The molar extinction coefficient for ferrocyanide at 420 nm is 1.040 $cm^{-1}M^{-1}$.

Soluble ADH activity was determined by measuring the formation of NADH at 340 nm using 50 m*M* ethanol and 1 m*M* NAD as substrates in a total volume of 1 mL buffer A. The ADH activity for the purified enzyme complexes, as well as for a comparable system consisting of soluble separate enzymes, was measured with a coupled substrate assay (total volume, 1 mL). Either 50 m*M* bis-NAD or 50 μ*M* NAD was used as a recycling coenzyme and 5 m*M* benzylalcohol and 5 m*M* acetaldehyde were utilized as substrates (Fig. 4). The extinction coefficient for benzaldehyde is 1.400 $cm^{-1}M^{-1}$.

In the "scavenger"-enzyme assay (Fig. 5) with LiDH, 100 μL enzyme complex solution or 100 μL solution of separate enzymes with the same total amount of enzyme activity was added to 2.45 mL buffer A. LiDH, 100 μL, 10 m*M* ferricyanide, 225 μL, and 2*M* ethanol, 75 μL, were then added and the formation of ferrocyanide was measured after NAD addition to a final concentration of 1 m*M*. In order to measure the competition between LiDH and LDH for the NADH formed by ADH, pyruvate was subsequently added to a final concentration of 5 m*M* (Fig. 5). The amount of NADH oxidized by LDH was measured indirectly as the decrease in LiDH activity. All assays were performed at 23°C.

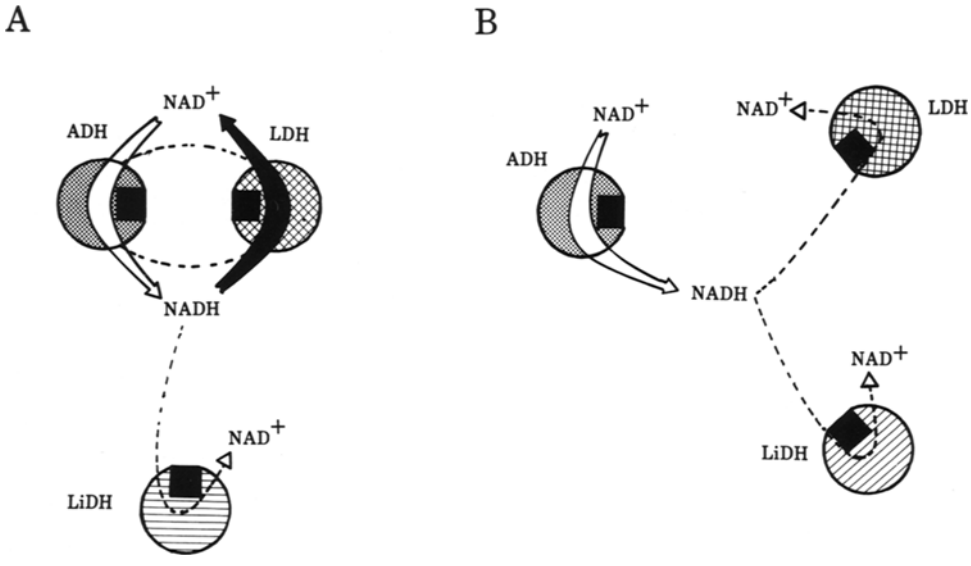


Fig. 5. Schematic representation of the three enzyme system used for the "scavenger enzyme" assay. (A) Site-to-site enzyme complexes and separate lipoamide dehydrogenase (LiDH). (B) Separate lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH) and lipoamide dehydrogenase.

RESULTS AND DISCUSSION

Preparation of a Reversibly Immobilized Site-to-Site Directed Two-Enzyme System

A fivefold molar excess of SPDP was used for the activation of ADH and it was found that about 3 mol SPDP were bound per mole of the dimeric enzyme. When this SPDP-activated ADH was immobilized to thiol-Sepharose, a reversible disulfide bond was introduced between ADH and the matrix (Fig. 2, step 4).

The immobilized ADH was determined by a spectrophotometric measurement of the amount of affinity-bound bis-NAD with the assumption that each of the two active sites of ADH would bind one bis-NAD molecule when excess bis-NAD was added to the gel in the presence of pyrazole. For one immobilization experiment, it was found that 27 nmol active ADH was immobilized.

After the addition of bis-NAD to reversibly immobilized ADH, LDH was added in excess compared with the amount of bis-NAD affinity-bound in ADH (Fig. 2, step 5 and 6). Approximately 25 nmol LDH was affinity-bound per milliliter of moist gel. The amount of LDH that was retained on the gel was less than the amount of affinity-bound bis-NAD (55 nmol). This could be the result of steric hindrance, since one dimeric ADH with its two affinity-bound bis-NAD can probably only bind one

molecule of tetrameric LDH. Adding LDH to a bis-NAD-ADH gel without oxalate present showed no LDH binding, indicating that the bis-NAD affinity-bound in ADH acts as an affinity agent for LDH.

In order to ensure that the ternary complexes were maintained during the crosslinking of the two enzymes, the reaction was performed in the presence of oxalate and pyrazole. Attempts to make a system of LDH randomly coupled to immobilized ADH using glultaraldehyde in the absence of bis-NAD were unsuccessful.

Glutaraldehyde was used as coupling agent for the crosslinking of the two enzymes (*6,9,22*). Because of the variable length of polymerized glutaraldehyde (*23*), the crosslinks between ADH and LDH probably occurred not only between closely adjacent amino groups, but also between amino groups that were situated further apart from each other on the two different enzymes.

Unreacted aldehyde groups remaining on the immobilized enzymes after the crosslinking were quenched with Tris and $NaCNBH_3$·$NaCNBH_3$ was introduced to irreversibly reduce the Schiff's bases formed to amines (*18*).

Release and purification of LDH–ADH Complex

A 50-m*M* portion of DTE was used for splitting the disulfide bonds between the enzyme aggregates and the matrix (Fig. 2, step 9). After the DTE-step, about 40% of the apparent ADH activity found on the gel prior to release was recovered in the eluate. This rather low recovery could be several factors for example: (1) the glutaraldehyde could have formed uncleavable bonds between the enzymes and remaining amino groups on the gel unmodified by SPDP; (2) crosslinking between different enzyme complexes on the gel might have occurred; or less likely (3) the number of disulfide bonds between the matrix and ADH may vary, making it more difficult to split off the fraction of immobilized ADH with more than one disulfide bond.

The eluted protein solution was subsequently applied to a NAD-Sepharose column in the presence of oxalate (Fig. 3). Complexes containing LDH were then affinity-bound to the NAD-Sepharose since a ternary complex was formed between the active sites of LDH, NAD, and oxalate. For this purification step a NAD-Sepharose with a low substitution degree was used. (Purification experiments were also performed with an NAD-column with a higher substitution degree, but then denaturing conditions were required to elute any protein from the column. This may be the result of strong nonspecific protein–protein interaction of the enzyme complexes sitting densely packed on the affinity material). After extensive washing with oxalate buffer, elution of the enzyme complex from the column with a low degree of substitution was performed with 0.1*M* NaCl in 0.1*M* sodium phosphate buffer, pH 7.5.

To characterize the complex further, it was subjected to gel filtration on Sephacryl S-300 SF and Sepharose 6B and it was found that the molec-

ular weight was in the range of 200,000–280,000. These results indicate that the enzyme complex consisted of one LDH and one ADH since such a complex would theoretically have a molecular weight of 222,000.

ADH Activity of the Enzyme Complex

ADH activity for the purified enzyme complex as well as for a comparable system of separate enzymes was determined by the coupled substrate assay outlined above. NAD functioned as a recyclable coenzyme with other substrates being benzyl alcohol and acetaldehyde (Fig. 4). The assay was performed at three different concentrations of coenzyme in the presence of oxalate, as shown in Table 1.

For the first measurement, no coenzyme was added to the assay solution. Nevertheless, some ADH activity (20%) was observed for the enzyme complex. This was probably a result of some remaining bis-NAD that had been physically entrapped between the active sites of the two enzymes on account of the crosslinking treatment, since adding more glutaraldehyde during the crosslinking treatment caused a higher coupled substrate activity prior to adding any coenzyme to the assay solution. For the comparable system of separate enzymes there was, as expected, no ADH activity observed unless NAD was added to the assay solution.

The subsequent NAD-concentration that was used for the coupled substrate assay was 50 n*M* with respect to bis-NAD. Based on enzyme activity calculations, this amount of bis-NAD corresponded approximately to the amount of available active sites of LDH. As shown in Table 1, the coupled substrate activity for the enzyme complex increased when the bis-NAD concentration was increased to 50 n*M* in the presence of oxalate.

For the corresponding system of separate enzymes, no detectable increase in benzaldehyde formation was observed when bis-NAD concentration was 50 n*M* in the presence of oxalate. The geometry of the enzyme complex was probably retained after the removal of the bis-NAD template and the bis-NAD will after readdition fit into the cavity formed. This readded bis-NAD could then substantially increase the concentra-

TABLE 1
Relative ADH Activity of Soluble Complex of ADH and LDH

	Relative ADH activity[a] at different NAD concentrations		
	No NAD	50 n*M* bis-NAD	50 μ*M* NAD
Site-to-site enzyme complex	20	55	100
Separate enzymes	0	<1	100

[a]ADH activity was determined with a coupled substrate assay (*21*) using three different coenzyme concentrations. Relative ADH activity = (ADH activity at different coenzyme concentration/activity with 50 μ*M* NAD) × 100.

tion of coenzyme in the proximity of juxtapositioned active site of ADH and thereby increasing the ADH. For the system with separate enzymes, no such proximity effects were observed.

When excess NAD was added, i.e., 50 μ*M*, the coupled substrate activity increased to the same value for the enzyme aggregates and the system with separate enzymes. The enzyme aggregates showed in the presence of oxalate, at 50 n*M* bis-NAD, about 55% of the ADH activity observed with an excess of NAD (50 μ*M*). The corresponding system of separate enzymes had, in the presence of oxalate, no measurable activity (<1%) at 50 n*M* bis-NAD compared with the maximal activity obtained at 50 μ*M* NAD. These results strongly suggest that the active sites of LDH and ADH are site-to-site oriented. These results also imply, for the enzyme complex, that at 50 n*M* bis-NAD about half of the active sites of ADH are saturated with an NAD molecule at the same time as most of the active sites of LDH are saturated with bis-NAD. The gel filtration experiments indicated that the enzyme aggregate was a complex consisting of LDH and one ADH, showing that one of the two sites of ADH can be facing one of the active sites of the tetrameric LDH. The other active site of ADH would then probably be too far away from any other affinity-bound bis-NAD, preventing interaction between the remaining active site of ADH and LDH-bound bis-NAD.

"Scavenger Enzyme"-Assay

In order to further investigate the effects of the orientation of the active sites of LDH and ADH, a third enzyme, LiDH, was introduced in an assay as a "scavenger enzyme." LiDH, which utilizes NADH and ferricyanide as substrates, was added to an assay solution consisting of either the enzyme complex or a comparable system of separate enzymes. When NAD and ethanol were added to such an enzyme solution NADH and acetaldehyde were formed. The NADH formed was subsequently oxidized by LiDH when ferricyanide was added. When pyruvate was subsequently added, LDH competed with LiDH for the NADH formed by ADH (Fig. 5). Only ADH operated under substrate saturation conditions and, since LDH and LiDH were in excess, it was ensured that all NADH molecules produced by ADH were rapidly oxidized. For the system of separate enzymes, the relative amount of NADH oxidized by either LiDH or by LDH was expected to be determined by the relative total numbers of enzyme units of the two competing enzymes. For a system consisting of LiDH and site-to-site coupled LDH and ADH (Fig. 5A), it was predicted that 37% of the NADH formed by ADH would be oxidized by LiDH. It was found, however, as is shown in Table 2, that LiDH oxidized only 17% of the NADH formed. For a system with separate enzymes that had the same total amount of enzymic units for the three different enzymes (Fig. 5B) as the system of site-to-site enzyme complexes, a value of 39% was obtained which was thus rather similar to the expected value (37%). This result indicates that the NADH formed by ADH

TABLE 2
Relative NADH Oxidizing Activities in Soluble Three-Enzyme Systems Comprising ADH, LDH, and LiDH

	NADH oxidized in coupled assay, %			
	By LDH		By LiDH	
	Expected from separate enzyme activities[a]	Found	Expected from separate enzyme activities[a]	Found
Site-to-site enzyme complex (Fig. 5A)	63	83	37	17
Separate enzymes (Fig. 5B)	63	61	37	39

[a]The expected percentage of NADH oxidized by either LDH or by LiDH was calculated from the obtained separate enzyme activities as the ratio of found V_{max} activities of LDH and LiDH, LDH/(LDH + LiDH), and LiDH/(LDH + LiDH), respectively. The decrease in LiDH activity was used for the determination of LDH activity in the coupled assay.

is preferentially channeled to LDH instead of to the "scavenger enzyme" when LDH and ADH are oriented in a juxtaposed position.

The results obtained then indicate that given two alternative routes, the one to LDH is preferred over the one to LiDH when LDH and ADH are site-to-site oriented. This observed effect likely arose because the distance between the active sites of LDH and ADH was much shorter than between ADH and LiDH, and that the former route was therefore preferred. It can not be excluded that the crosslinking bonds *per se* somewhat sterically prevent the NADH formed from leaving the cavity between ADH and LDH, thereby enhancing the effect.

The complex of ADH and LDH show both before and after splitting the disulfide bonds between ADH and the matrix properties similar to those of a corresponding complex irreversibly immobilized to Sepharose. This indicates that the nature of the covalent bond did not influence the enzymatic properties. The results obtained with the immobilized complex reflect the close proximity between the two active sites and not restricted diffusion caused by the Nernst layer around the enzyme bead.

CONCLUSION

A soluble enzyme complex with the active sites of ADH and LDH facing one another has been prepared. The active site orientation was performed on solid phase and the enzyme complex was subsequently split off from the matrix. The results of this study indicate strongly that the channeling observed with the soluble complex is caused by the site-to-site orientation of the two enzymes. This then would indicate that in the case of the corresponding immobilized system (*10*), matrix effects are negligible.

ACKNOWLEDGMENTS

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