RECYCLING BY A SECOND ENZYME OF NAD COVALENTLY BOUND TO ALCOHOL DEHYDROGENASE

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1. Introduction

The covalent coupling of an NAD-analogue to liver alcohol dehydrogenase (LADH) giving an enzyme–coenzyme complex that does not require any exogenous NAD for activity has been described [1]. We report here on the interaction of such an enzyme-bound NAD with a second enzyme, lactate dehydrogenase (LDH) or malate dehydrogenase. In contrast to [2] where the enzyme and the coenzyme had been co-immobilized on a matrix, which prevents proper interaction with other enzymes, our preparation allows recycling of the covalently-bound NAD by a second enzyme. The cycling of the covalently-bound coenzyme has been studied mainly by fluorimetry and advantage has been taken of the fluorescent properties of the reduced form of the coenzyme [3].

2. Materials and methods

2.1. *Materials*

Horse liver alcohol dehydrogenase (lyophilized; 1.85 U/mg), beef heart lactate dehydrogenase (type III, 550 U/mg) were obtained from Sigma (St Louis, Mo.) and pig heart malate dehydrogenase (1100 U/mg) from Boehringer (Mannheim) and Sepharose CL-4B from Pharmacia Fine Chemicals AB (Uppsala). Lactaldehyde was prepared from threonine [4] and N^6-[*N*-(6-aminohexyl)carbamoylmethyl]-NAD was synthesized as in [5]. Other chemicals used were of analytical grade and obtained from commercial sources.

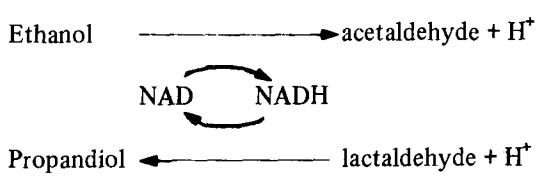
Abbreviations: NAD–LADH complex, N^6-[*N*-(6-aminohexyl)carbamoylmethyl]-NAD covalently bound to alcohol dehydrogenase from horse liver

2.2. *Coupling of* N^6-*[N-(6-aminohexyl)carbamoylmethyl]-NAD to horse liver alcohol dehydrogenase (formation of enzyme–coenzyme complex)*

The coupling was carried out as in [1] with slight modifications. The concentration of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysuccinimide was 250-times that of the enzyme subunit and the coupling proceeded for 20 h. The concentration of coenzyme and enzyme subunit in the covalent enzyme–coenzyme complex was determined from the ultraviolet spectrum of the complex.

2.3. *Determination of alcohol dehydrogenase activity*

The alcohol dehydrogenase activity of the enzyme-coenzyme complex was determined with a coupled-substrate assay using ethanol and lactaldehyde as substrates [1,4]:



2.4. *Immobilization of the enzyme–coenzyme complex*

Sepharose CL-4B (1ml) was washed with 0.5 M potassium phosphate (pH 12) and then suspended in 2 ml same buffer. Cyanogen bromide (25 mg) was dissolved in 25 μl acetonitrile and added to the gel;

the suspension was stirred for 10 min, then washed exhaustively with cold water. The activated gel was finally added to 1 ml enzyme–coenzyme complex (~1 mg) in 50 mM sodium bicarbonate (pH 7.5). The coupling proceeded overnight at 4°C and the gel was washed with 0.5 M NaCl and 50 mM sodium bicarbonate (pH 7.5) to remove any non-coupled enzyme–coenzyme complex.

2.5. *Fluorescence measurements*

The fluorescence measurements were carried out in a Perkin-Elmer spectrophotofluorimeter (MPF-2A) equipped with a thermostatted cuvette holder. The samples were excited at 340 nm (bandwidth 10 nm) and analyzed at 390–480 nm (bandwidth 10 nm). The measurements were carried out in 50 mM sodium bicarbonate (pH 7.5) at 18°C.

The fluorescence emitted from immobilized systems was measured in a flowcell developed for solid-phase fluorimetry [6]. About 100 μl substituted Sepharose was packed in the flowcell and the appropriate solution was pumped through the gel bed using a peristaltic pump. In order to diminish interference from scattered light, a filter with a cutoff at 390 nm was used.

2.6. *Lactate determination*

Lactate was determined enzymically using glutamic-pyruvic transaminase and lactate dehydrogenase [7].

3. Results and discussion

The coupling of the NAD-analogue, N^6-[*N*-(6-aminohexyl)carbamoylmethyl]-NAD, to LADH using a water-soluble carbodiimide gave a preparation that had 0.95 NAD molecules/enzyme subunit. The activity of the preparation in the coupled substrate assay [1] was 2.7 μmol acetaldehyde.min^{-1}.mg protein^{-1}, which was 22% of the activity obtained after addition of excess soluble NAD. Thus, the properties of the preparation were in accordance with that reported for a similarly prepared preparation [1].

To extend the range of possible applications for the covalent enzyme–coenzyme complex beyond that of substrate-coupled recycling of the coenzyme, recycling with the aid of a second enzyme was considered desirable. The feasibility of such recycling with lactate dehydrogenase was tested in a fluorimetric binding study, the results of which are given in table 1. Table 1 shows that addition of ethanol to the enzyme–coenzyme complex resulted as expected in a reduced coenzyme as seen from the increase in fluorescence. Subsequent addition of LDH further increased the fluorescence, indicating that a binary complex between the now reduced coenzyme and LDH, a complex which is known to increase the fluorescence of NADH, had been formed [3,8]. The alcohol dehydrogenase-bound nucleotide is thus capable of interacting with a second enzyme and this

Table 1
Relative fluoresence after consecutive additions of ethanol, lactate dehydrogenase, oxalate and pyruvate to a sample of enzyme–coenzyme complex (0.95 NAD/enzyme subunit) and to a reference system with the same amount of free NAD-analogue and free enzyme

Additions	Fluorescence at maximum (arbitrary units)	
	Enzyme–coenzyme complex	Reference system
1.2 μN LADH + 1.1 μN NAD-analogue	0.3	0.3
30 mM ethanol	1.0	1.0
14.3 μN lactate dehydrogenase	2.0	2.5
21.4 μN lactate dehydrogenase	2.1	2.6
10 mM oxalate	6.7	8.5
15 mM oxalate	6.6	8.2
0.05 mM pyruvate	0.5	0.4

The final volume was 1.5 ml in both cases. Excitation wavelength was 340 nm and emission wavelength 390–480 nm

was further substantiated by adding oxalate which is known to form a strongly fluorescent ternary complex with LDH and reduced coenzyme [8]. As can be seen, the fluorescence increased to about 3-times its former value.

Finally, addition of pyruvate decreased the fluorescence to its original value, the explanation being that the reduced coenzyme became reoxidized by LDH, proving that the LADH-bound coenzyme is not only able to bind to a second enzyme but is also functional in the catalytic process of that enzyme. It is apparent from table 1 that the enzyme–coenzyme complex behaves very much the same as the reference system which was prepared by mixing native LADH and NAD, indicating that the interactions are identical in the two systems.

It has been assumed above that only the properly attached coenzyme, i.e., a coenzyme bound in the immediate vicinity of an active site with which it can interact [1], can react with the second enzyme, leading to the fluorescence phenomena observed. To verify, the fluorescence experiment was repeated with the NAD–LADH preparation covalently bound to Sepharose-CL-4B in such a way that interaction between complexes should be minimal [9], thereby preventing reduction of randomly-attached coenzyme molecules upon addition of ethanol. As the fluorescence changes observed were substantially the same as for the corresponding system in free solution it is strongly indicated that indeed only the coenzyme molecules attached to the active site are reduced. These subsequently 'swing out' of the active sites and interact with added LDH.

In another experiment with the same constituents (omitting oxalate) the actual feasibility of recycling the LADH-bound coenzyme with LDH was investigated (fig.1). The original presence of excess of ethanol ensures a reduced coenzyme as seen from the strong fluorescence. When pyruvate is added (in an amount 20-times that of the coenzyme), the recycling commences and ethanol and pyruvate are consumed and acetaldehyde and lactate are produced. The enzyme-bound coenzyme is predominantly in its oxidized form during the first minutes (i.e., there is an immediate drop of the fluorescence to a level only slightly higher than the background). As the cycling of the coenzyme proceeds the concentration of pyruvate decreases and the 'oxidative half-cycle' of

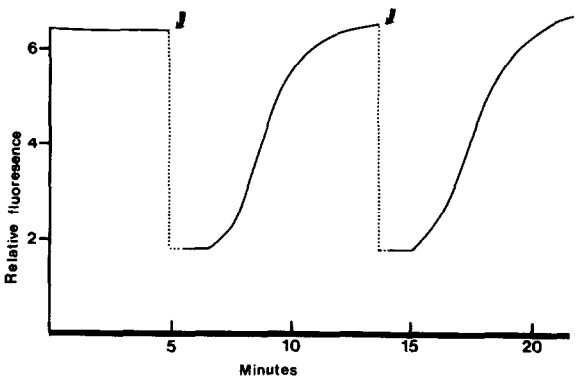


Fig.1. Fluorescence changes during enzymic cycling of NAD covalently bound to liver alcohol dehydrogenase. The assay mixture consisted of 50 mM $NaHCO_3$, pH 7.5, 1.2 μN of the enzyme coenzyme complex, 30 mM ethanol, 14.3 μN lactate dehydrogenase and 25 nmol pyruvate (additions are indicated by the arrows; the dotted line indicates the fact that no recorded response was obtained during the time needed for pyruvate additions) in 1.07 ml final vol. Excitation wavelength 340 nm and emission wavelength 425 nm.

the system gradually becomes less efficient. This has the consequence that the coenzyme will be finally in the reduced state, as demonstrated in the fig.1 by the gradual return of the fluorescence to its original level. New additions of pyruvate repeat the cycle. Based on the amount of pyruvate added, the time required for its consumption and the concentration of active enzyme-bound coenzymes in the system, a mean cycling rate of the coenzyme of ~300 h^{-1} is obtained. The same value was also found based on the amount of product formed (lactate analysis).

The turnover value of 300 h^{-1} is in parity of what has been reported for enzyme couple regeneration [5,10–12]. The outstanding efficiency of substrate couple recycling systems (40 000 h^{-1}), on the other hand, is probably due to the fact that the coenzyme does not have to leave the active site of the enzyme and is all the time engaged in binary or ternary complexes. In the present enzyme-couple recycling, the coenzyme has to dissociate from the active site to which it is bound, swing out, and interact with the second enzyme. This necessity of leaving the active site will slow down the cycling rate since this step is supposed to be the rate-limiting one in catalysis by dehydrogenase [13].

The turnover rate observed in the above experiments is comparatively low but enzyme-couple recycling of covalently bound enzyme–coenzyme complexes should be of interest since its use allows a whole new range of compounds to be produced/analyzed, provided that, of course, the NAD–LADH complex can interact properly with the pertinent enzymes. This is obviously the case with LDH and experiments with malate dehydrogenase show that this enzyme can also cooperate with NAD–LADH with approximately the same efficiency as LDH, i.e., a coenzyme cycling rate of 300 h^{-1} in the presence of ethanol and oxaloacetate.

The experiments described so far have been concerned with interactions between the covalent NAD–LADH complex and an additional soluble enzyme, e.g., lactate dehydrogenase or malate dehydrogenase. A logical extension of these studies would be to fix permanently the second enzyme to the NAD–LADH complex in such a way that the second enzyme could interact with the functioning coenzyme of the NAD–LADH complex. Ideally the two enzymes are covalently linked to each other and the coenzyme is equally accessible to the active sites of both enzymes, i.e., although the coenzyme would be 'physically' linked to one enzyme, functionally it would belong to both. The preparation of such a complex would of course be difficult. However, the observations made in the fluorescence studies, namely that addition of LDH to the reduced NAD–LADH complex apparently results in a binary complex between the LADH-bound coenzyme and LDH formed a promising basis for a coupling experiment. Thus, in one experiment Sepharose-bound NAD–LADH complex was after incubation with ethanol and addition of LDH treated with the crosslinking agent glutaraldehyde. The resulting complex used as substrates pyruvate and ethanol or, when driving the cycle in opposite direction, lactate and acetaldehyde. The efficiency of this complex, however, was very low and much work remains to be done before an efficient covalent two enzymes–one coenzyme complex is obtained.

4. Discussion

The experiments described show that it is possible to link permanently an enzyme and coenzyme to one another and that such preparations can exert their catalytic function either by the extremely efficient substrate-couple recycling (of which, however relatively few systems are known) or by aid of a second enzyme, i.e., enzyme-couple recycling. Chemical [14,15] or electrochemical [16] recycling of the enzyme-bound coenzyme could also be possible.

Initial attempts to bind covalently LDH to NAD–LADH preparations in such a way that the active sites of the two enzymes are permanently facing each other and share the covalently bound NAD, where hitherto only partly successful. The methods involved in the preparation of such 'self-contained' cataltyic units will be further studied as they appear of value for application in enzyme reactors, analytical devices ('reagent-less' electrodes, enzyme thermistors) or for the treatment of enzyme deficiency diseases involving coenzyme-dependent enzymes.

Acknowledgement

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