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Preparation of an Active Soluble Lactate Dehydrogenase—Nicotinamide Adenine Dinucleotide Complex Using Glutaraldehyde

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The cofactor analogue N^6-[(6-aminohexyl)-carbamoylmethyl]-NAD was bound to beef heart lactate dehydrogenase by the glutaraldehyde coupling method and the enzyme-coenzyme complex subsequently separated from excess of non-coupled cofactor by gel filtration chromatography. The number of cofactor molecules bound per enzyme molecule could be varied between 0 and 8 by altering the coupling time. The enzyme-coenzyme preparation could catalyze the reaction lactate → pyruvate in the absence of externally added free cofactor although with low efficiency.

The enzyme-coenzyme preparation was also immobilised on Sepharose in such a way that possible contact between complexes was minimized (low substitution of the gel) and the activity of the immobilised complex was found to be partly retained, indicating that a cofactor molecule can interact with the enzyme molecule to which it is bound.

One of the major problems to be overcome if enzymes are to be used to their full potential in industrial, analytical and medical applications is that of cofactor dependance. Many enzymes of interest in this field depend on the presence of a cofactor (in stoichiometric amounts) for their activity. Such cofactors are expensive, and because of their relatively small size are difficult to retain in a reaction vessel by use of a membrane, for example, in the same way as enzymes.

This report presents one approach to this problem by covalently binding a cofactor (in this case an analogue of nicotinamide adenine dinucleotide (NAD)) to the enzyme lactate dehydrogenase (LDH:E.C. 1.1.1.27). The method should also be applicable to other NAD-dependant enzymes, and possibly also to other classes of enzymes and other cofactors.

EXPERIMENTAL

Materials and methods. LDH (beef heart type III, 10 mg/ml suspension in $(NH_4)_2SO_4$), NAD (type III), NADH (type III), phenazine ethosulfate (PES), and 2,6-dichlorophenolindophenol (DCPIP) were obtained from Sigma (St. Louis, Mo., U.S.A.). Glutaraldehyde was obtained from BDH (Poole, England). Sephadex G-50 medium and Sepharose 4B CL were obtained from Pharmacia (Uppsala, Sweden), and N^6[(6-aminohexyl)-carbamoylmethyl]-NAD (N^6NAD) was prepared as described earlier.[1] Other chemicals were of analytical grade.

Coupling. LDH suspension (2.5 ml, 25 mg) was dialysed against 2 × 1 l standard buffer (potassium phosphate buffer, 0.1 M, pH 7.5) for 12 h. Eight mg of the dialysed LDH (60 nmol in 1.6 ml) were then equilibrated with N^6NAD (4.4 mg, 5 μmol) at 0 °C for 30 min. Glutaraldehyde (200 μl 0.25 % solution, 5 μmol) was then added and the volume was made up to 2.0 ml with standard buffer and the solution gently mixed. The coupling proceeded for up to 12 h at 4 °C. In a blank experiment, ε-aminocaproic acid (εACA) replaced the N^6NAD. After the reaction period, ethanolamine.HCl (50 μl 4 M solution, 200 μmol) was added to quench the reaction. After 1 h the mixture was separated on a column of Sephadex G-50 medium 1.0 × 100 cm using standard buffer as running buffer. Fractions were collected (1.3 ml; 1.0 ml/min) and subsequently analysed spectrophotometrically at 290, 280, and 266 nm. From these values the LDH and N^6NAD concentrations in the fractions could be determined using two simultaneous equations, with the absorption coefficients for the glutaraldehyde-treated LDH and N^6NAD as follows:

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LDH $A_{290nm} = 1.17$ mg ml^{-1} cm^{-1}
$A_{266nm} = 1.05$ mg ml^{-1} cm^{-1}

N^{6}NAD $A_{290nm} = 1750$ M^{-1} cm^{-1}
$A_{266nm} = 22500$ M^{-1} cm^{-1}

These values were calculated from the spectra of glutaraldehyde-treated LDH and N^{6}NAD preparations of known concentrations using suitable references.

Assay. The pooled protein fractions were assayed for activity, and their activities in the presence of excess NAD were also measured. The reaction assayed was the simultaneous conversion of lactate to pyruvate and NAD to NADH. The NADH formed was reoxidised, using PES, to NAD, and the reduced PES was reoxidised to PES by DCPIP. The decrease in concentration of oxidised DCPIP was followed at 610 nm, where it has an absorption maximum.[2,3]

The fraction to be assayed (10 – 100 µl) was pipetted into a 1 ml cuvette. DCPIP (50 nmol), PES (2 µmol), and standard buffer were added to a final volume of 950 µl. The blank rate was measured, and then lactate (50 µl, 50 µmol) was added to start the reaction. In the cases when NAD was added, its final concentration was 2 mM (2 µmol added).

When the immobilised preparation was assayed, 3 ml cuvettes were used (with magnetic stirring) with a final volume of 2 ml. The immobilised preparation (5 – 100 mg wet weight was weighed into the cuvette, and otherwise the assay mixture concentrations were identical to those of the free solution assay.

Immobilisation. The preparations were immobilised on Sepharose 4B CL (cross linked) by the cyanogen bromide method.[4] Sepharose 4B CL was washed extensively with water on a glass filter, excess water sucked off, and 20 g was then suspended in water (20 ml). Cyanogen bromide (1 g) was dissolved in acetonitrile (5 ml) and added to the stirred, cooled Sepharose suspension. The pH was raised, and maintained at 11.0 ± 0.2 by the careful addition of 1 M NaOH. After 8 min the activated gel was washed with $NaHCO_3$ (0.1 M, 500 ml), and 3.6 g sucked gel (equivalent to 5 ml of settled gel) was added to tubes containing 2.5 mg each of LDH, LDH-N^{6}NAD, or LDH-εACA in $NaHCO_3$ (0.1 M, 10 ml). After coupling overnight at 4 °C, the gels were exhaustively washed with $NaHCO_3$, standard buffer, NaCl (0.5 M), water and standard buffer again, and then assayed. The concentration of gel-bound protein was estimated from spectra of the settled gel in 2 mm light-path cells, using CNBr-treated blank gel as reference.

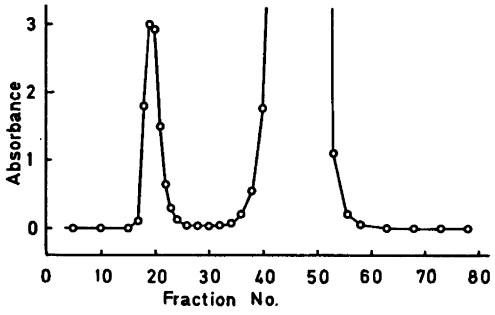

Fig. 1. Gel chromatography separation of N^{6}NAD and LDH-N^{6}NAD obtained from a 6 h coupling. The absorbance is measured at 280 nm (fractions 1 – 32) or 266 nm (fractions 33 – 80).

RESULTS AND DISCUSSION

A typical separation of the coupling mixture is shown in Fig. 1, in which it can be seen that the protein-containing fractions (first peak) are well separated from the fractions containing

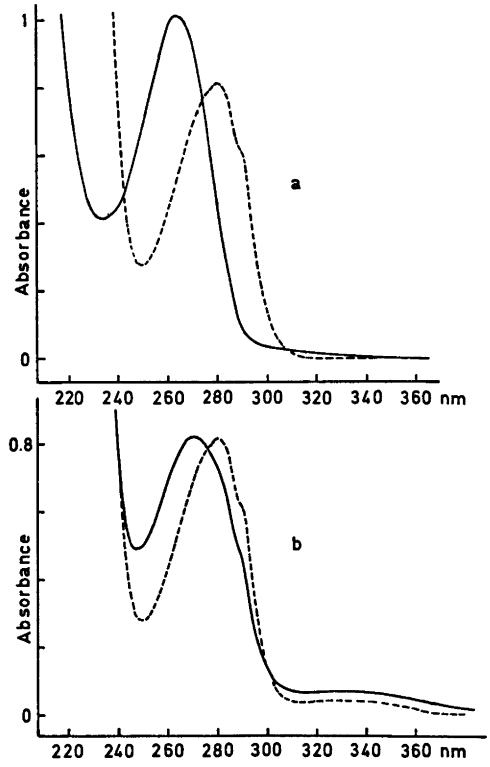

Fig. 2. (a) Spectra of N^{6}NAD (–) and LDH (---). N^{6}NAD 46 µM, LDH 530 µg/ml. (b) Spectra of LDH-N^{6}NAD (–) and LDH-εACA (---) from a 6 h coupling. LDH-N^{6}NAD 390 µg/ml, LDH-εACA 520 µg/ml.

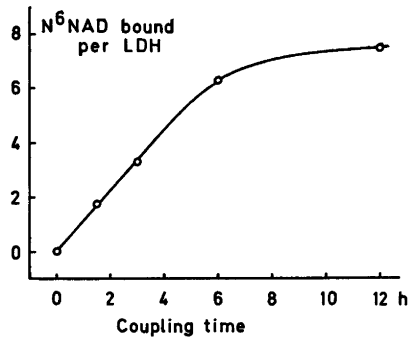

Fig. 3. Dependence on the reaction time of the number of N⁶NAD molecules bound per LDH molecule. Reaction conditions: LDH, 4 mg/ml; N⁶NAD, 2.5 mM; glutaraldehyde, 2.5 mM. After reacting up to 12 h, the reaction was quenched (ethanolamine.HCl 0.1 M, 1 h) and separated.

uncoupled N⁶NAD. A separation of the ε-aminocaproic acid-LDH (LDH-εACA) reaction mixture shows the same protein peak, and a separation of the LDH-εACA mixture to which N⁶NAD had been added after quenching also showed protein and N⁶NAD peaks in identical positions to those found for the separation of the LDH-N⁶NAD mixture. ε-Aminocaproic acid was chosen for the blank substituent because of the similarity it has to the "arm" of the N⁶NAD.

The spectra of LDH, N⁶NAD, LDH-εACA and LDH-N⁶NAD (Fig. 2) show that the wavelengths 290 and 266 nm are suitable for the determination of the concentrations of N⁶NAD and LDH in the coupled preparations. LDH-N⁶NAD shows a peak between 266 and 280 nm, whose exact location depends on the degree of substitution (higher N⁶NAD substitution shifts the peak towards 266 nm). The spectrum of LDH-εACA is almost identical to that of native LDH at wavelengths lower than 300 nm. Between 300 and 360 nm, on the other hand, the spectra of both LDH-εACA and LDH-N⁶NAD have a shoulder, which probably is caused by the glutaraldehyde treatment. This is substantiated by the fact that this shoulder increases with reaction time. Glutar-aldehyde itself shows no absorption in this region. After separation, the spectrum of LDH-εACA to which N⁶NAD was added, after quenching and before separation, is identical to that without N⁶NAD added; that is no N⁶NAD could be detected bound in any way to the LDH-εACA. This was substantiated by activity measurements (see below).

Fig. 3 shows the relationship between the coupling time and the calculated number of N⁶NAD molecules bound per LDH molecule. As can be seen, the coupling reaction is time-dependant, and this indicates that a covalently bound derivative is formed; the longer reaction time, the more N⁶NAD molecules bound per enzyme molecule. At higher concentrations of glutaraldehyde the coupling time must be shortened to avoid too high a substitution or protein denaturation.

In some cases a slight precipitation occurred during separation of the LDH-N⁶NAD preparations on the G-50 column. This never occurred with the LDH-εACA preparations. The pre-

Table 1. Activity of LDH derivatives.

Preparation	Activity[a] Intrinsic	+2 mM NAD
LDH-εACA	0.00(0.00)	530(100)
LDH-εACA[b]	0.06(0.01)	500(94)
LDH-εACA immobilised	0.00(0.00)	220(42)
LDH-εACA immobilised[c]	0.05(0.01)	–
LDH-N⁶NAD	1.7 (0.32)	200(38)
LDH-N⁶NAD immobilised	0.40(0.07)	180(34)

[a] Measured as ΔA min^{-1} mg^{-1} protein. Figures in brackets are percentages of the activity of the LDH-εACA preparation in the presence of 2 mM NAD. [b] N⁶NAD was added to the LDH-εACA reaction mixture after quenching, and after 30 min the mixture was separated. [c] The immobilised LDH-εACA preparation was steeped in N⁶NAD (2.5 mM) for 24 h and then washed (see text).

cipitate was not simply denatured protein, since it could be redissolved on addition of NADH, and this suggests that it was a complex of the LDH-N^{6}NAD which self-aggregated when excess, competing, N^{6}NAD was removed on the column. When this occurred, the pooled protein fractions were centrifuged (20 000 *g*, 10 min) and filtered (Millipore, 0.45 μm) to remove the precipitate. After this treatment, the precipitate did not reappear.

Table 1 summarizes the activities of the various preparations, both in the presence of excess NAD (2 mM), and in the absence of added NAD (the intrinsic activity). For these experiments, we used a preparation that had been coupled for 6 h, and which had 6 N^{6}NAD molecules bound per enzyme molecule. This preparation showed an activity of 1.7 Δ A min^{-1} mg^{-1} protein.

That the LDH and the N^{6}NAD are covalently bound by this procedure is suggested by the following facts:

1. LDH to which N^{6}NAD had been added in the absence of glutaraldehyde showed no activity in the protein peak fractions obtained on separation unless external NAD was added.

2. The activity shown by the LDH-εACA preparation to which N^{6}NAD had been added after quenching but prior to the separation is very low, more than twenty times lower than that of the LDH-N^{6}NAD preparations. This means that less than 5 % of the measured activity of such preparations is due to non-covalently bound N^{6}NAD which is not completely separated on the column.

3. The immobilised LDH-N^{6}NAD preparations retain activity despite rigorous washing conditions (identical to those described earlier), which are sufficient to remove non-covalently bound N^{6}NAD, as was demonstrated when N^{6}NAD was added to the immobilised LDH-εACA preparation, followed by the same washing conditions.

4. The relation between coupling time and substitution, which points to covalent binding.

The fact that the immobilised preparation is also active (although the activity was low) indicates that the reaction assayed is that between an N^{6}NAD molecule and the enzyme molecule to which it is bound ("internal" activity) rather than between one N^{6}NAD and another LDH molecule ("external" activity). The preparation was immobilised at a concentration of 430 μg/ml settled gel, at which concentration there is only a very small probability of external activity because the protein molecules are so far apart. This probability was calculated according to the method of Green.[5]

Although the activities reported here are low, the method is a step towards the solution of the problems of cofactor dependance and retention. The reason for the low activities is partly that the glutaraldehyde treatment is detrimental to the enzyme, considerably affecting its catalytic activity. This, we believe, can be overcome by the use of milder and more suitable coupling agents, and by using techniques that ensure that the coupled cofactor is in the most suitable position on the enzyme. We have found that the intrinsic activity is dependant on the degree of substitution of cofactor on the enzyme to a certain extent, and this indicates that only a proportion of the bound cofactor molecules are available to the active site. With more specific positioning of the N^{6}NAD molecule in the active site before coupling, higher intrinsic activities should be obtained. Preliminary results using bifunctional coupling reagents of differing lengths also indicate, as would be expected, that there is an optimum length.

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