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FACTORS AFFECTING RECOMBINATION OF LACTATE DEHYDROGENASE ISOENZYME SUBUNITS

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SUMMARY

The recombination of isoenzyme subunits of lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) present in heart and liver extracts was studied *in vitro*. Kinetic studies of the recombination after freezing revealed optimal recombination after 50 min and by addition of NADH before or after freezing.

The recovery of recombination was within certain limits proportional to the amount of NADH present in the reaction mixture. A high concentration (40 mM) of NADH gave optimal recovery. The electrophoretic mobilities of lactate dehydrogenase isoenzymes were slightly increased in the presence of a high concentration of NADH.

ADP did not influence the recovery. Nicotinamide in increasing concentrations inhibited recombination. The results suggest that NADH is essential for the formation of the active enzyme from the lactate dehydrogenase subunits. The results obtained are discussed on the basis of the literature available.

INTRODUCTION

Multiple molecular forms of enzymes are hybrids of parent subunits combinable to active enzymic polymers. The mechanism controlling the formation of these systems remains unknown. The lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) consists of five electrophoretically distinguishable fractions[1].

There are two primary parent subunits of lactate dehydrogenase which are combined together in groups of four to give the active enzymic structure[2–4]. These two primary parent proteins have been designated H and M (or A and B) as they are individually prominent in heart muscle and skeletal muscle respectively.

Evidence has been gained[5] in support of the subunit theory, *i.e.* freezing experiments in which equal amounts of the H_4 and M_4 tetramers were frozen in phosphate buffer containing NaCl. The isoenzyme pattern found after thawing of equal amounts of H and M subunits may be such that the isoenzyme activities are in the proportion 1:4:6:4:1—the binomial distribution. It has subsequently been found that guanidine · HCl and urea in high concentrations can also produce hybridization[6,7]. If dissociation

is assumed, it should then be possible to measure the recombination of subunits by measuring the recovery of activity after freezing. Also, if it is assumed that the NADH molecule plays an intimate role in the conformational state of the enzyme, it might be possible to ascertain more explicitly certain aspects of this relationship[8–11].

The present communication reports an investigation of the relationship between NADH and lactate dehydrogenase subunit recombination as determined by using the procedure of hybridization.

MATERIAL AND METHODS

Material

Macroscopically normal human tissue (heart and liver) was obtained at autopsy within 12 h after death*.

All chemicals used were of highest obtainable purity from British Drug Houses (Great Britain) with the exception of ADP, NAD^+ and NADH which were obtained from Boehringer, Germany.

Methods

Hybridization. To obtain optimal hybrid effect with an approximately binomial distribution following hybridization, liver and heart samples, *i.e.* tissues with predominant and high M_4 and H_4 concentrations, were taken together to make the extract. Samples of 1 g of both tissues were homogenized in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.01 M NaI (ref. 11). Homogenization was performed with a rounded glass stirring rod (40 rev./min) to crush the tissue in a Hostalen centrifuge tube. The homogenate was then centrifuged at 4° for 20 min at 18 000 $\times$ *g* in a Beckman Centrifuge Model L2. The supernatant was collected and diluted with the phosphate–iodide buffer (*vide supra*) to the desired enzymic activity. 20 ml of this dilution were then dialyzed for 16 h at room temperature against 500 ml of the phosphate–iodide buffer containing 5 g of activated charcoal. This was found to remove essentially 100% of all "free" NADH as determined by absorption at 366 nm of a pure NADH sample. Freezing was performed in a deep-freeze at −20° for approx. 30 min to 2 h (*vide infra*).

Assay of total lactate dehydrogenase activity. Total lactate dehydrogenase activity was measured as previously described[12] using NADH and sodium pyruvate as substrate. The enzyme units are expressed as μmoles NADH oxidized per min.

Assay of lactate dehydrogenase isoenzymes. The extent of hybridization of the lactate dehydrogenase isoenzymes from the frozen samples was followed by agar-gel micro-electrophoresis and staining (formazan method) as previously described[12–14]. The proportion between individual isoenzymes was determined by scanning in a Vitatron scanning device[12].

Effect of NADH on the rate of activity recovery following freezing. It was found necessary to work with the enzymic activity of the extracts within certain limits, as too high an enzyme concentration resulted in immediate recovery making kinetic studies impossible, whereas too low an activity resulted in complete loss of activity

* Gratitude is expressed to Professor H. POULSEN, the Institute of Pathological Anatomy, Municipal Hospital, Copenhagen, for these samples.

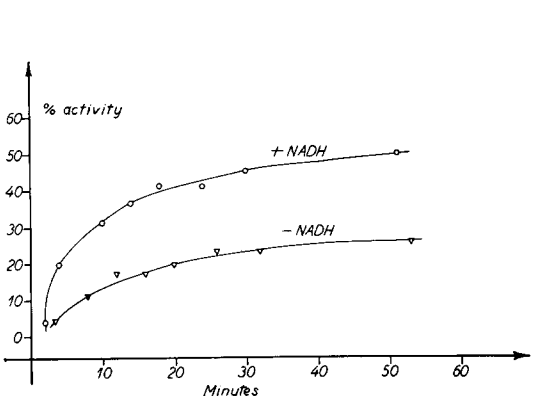
with no subsequent recovery. An enzyme activity level of approx. 0.554 unit per ml extract was found to be satisfactory.

Both undialyzed and dialyzed extracts were frozen for 2 h at −20°. At intervals (*vide infra*) during thawing samples of 500 μl were removed and added to 2000 μl 0.9% NaCl at room temperature, and total lactate dehydrogenase activity was measured. In the dialyzed extract, 250 μl were added to 1000 μl 0.9% NaCl, and 250 μl were added to 1000 μl 0.9% NaCl containing 2.82 μmoles NADH; total lactate dehydrogenase activity was then measured as above. At the time of optimal recovery, the isoenzyme pattern of each extract was determined.

Effect of prior addition of NADH, nicotinamide and ADP on the extent of activity recovery following freezing. The enzymic activity was so adjusted that the dialyzed extract exhibited complete loss of activity with no recovery after freezing. An original enzyme activity level of approx. 0.337 unit per ml extract was found to give rise to complete loss of activity with no recovery.

The binding of NADH was elucidated by studies of the influence of components possessing chemical groups identical with the end-groups of NADH. Thus, the following extracts were examined: the undialyzed and dialyzed extracts to which (a) NADH was added before freezing to final concentrations ranging from 1 to 30 mM; (b) nicotinamide was added to final concentrations ranging from 1 to 20 mM; and (c) ADP was added to final concentrations ranging from 1 to 20 mM. These extracts were then divided into two fractions: the one fraction was frozen as described above, and the other allowed to stand at room temperature. The frozen samples were thawed for 4 h at room temperature to allow for maximum activity recovery. Total activity and the isoenzyme patterns were determined on all samples.

Effect of pH. Hybridization was produced by rapidly decreasing the pH in an



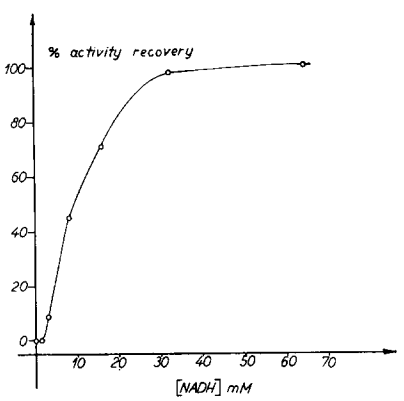


Fig. 1. The relationship between recovery of lactate dehydrogenase activity after freezing of equal amounts of H and M subunits of lactate dehydrogenase from heart and liver tissue as a function of time after freezing. Abscissa, time (min); ordinate, percentage of recovery of lactate dehydrogenase activity. The activity recovery was measured in an assay system lacking or possessing the coenzyme NADH. (For experimental conditions see the text.)

Fig. 2. The relationship between percentage of lactate dehydrogenase activity recovery after freezing of equal amounts of H and M subunits from heart and liver tissue respectively as function of concentration of NADH. Abscissa, percentage of activity recovery; ordinate, NADH concentration (mM). (For experimental conditions see the text.)

extract solution as follows. The pH of the undialyzed extract was lowered to 4.0 by the addition of conc. HCl to the sample during continuous shaking. NaOH (0.1 M) was then immediately added to bring the pH back to 7.0.

Effect of coenzyme (NAD^+ and NADH) on the mobility of isoenzymes. NAD^+ and NADH were added to the dialyzed extracts to final concentrations ranging from 1 to 65 mM. They were then run in agar-gel micro-electrophoresis[13], and their relative mobilities were determined[13] using albumin and dextran as reference markers.

RESULTS

Effect of NADH on the rate of activity recovery following freezing

As seen in Fig. 1, there was a 25% increase in the activity recovery in those dialyzed samples that were treated with NADH after freezing. Optimal activity was reached after 50 min.

The activity recovery in the undialyzed extract was equal to that found in the dialyzed extract to which NADH was added. In all instances, after freezing, the isoenzyme patterns showed complete hybridization.

Effect of prior addition of NADH, nicotinamide and ADP on the extent of activity recovery following freezing

When the dialyzed samples were frozen without prior addition of any exogenous NADH, there was complete loss of activity with no recovery. As seen in Fig. 2, the addition of NADH to the dialyzed extract prior to freezing resulted in activity recovery which was a function of the amount of coenzyme added. The extent of recovery approached that of the undialyzed extract at high coenzyme levels (above 40 mM).

The addition of NADH to the undialyzed extract before freezing had no significant effect on activity recovery. In the undialyzed unfrozen extract to whichNADH was added there was no significant activity change.

When ADP and nicotinamide were added to the dialyzed extract before freezing, there was no effect, *i.e.*, there was still total loss of activity without recovery. When ADP was added to the undialyzed extract before freezing, there was similarly no effect on activity recovery. The addition of nicotinamide to the undialyzed extract produced, however, a progressive loss of activity which was a function of concentration as seen in Fig. 3. This inhibition was not selective as it depressed all five isoenzymes to the same extent as determined by the H/M ratio.

Effect of pH change on hybridization

Lowering the pH of the extract to 4.0 with HCl and then immediately restoring it to 7.0 with 0.1 M NaOH produced hybridization without freezing. Furthermore, the pattern produced was unaffected by freezing.

Effect of exogenous NAD^+ and NADH on mobility of isoenzymes

As shown in Fig. 4, both NAD^+ and NADH had an enhancing effect on the mobility of all five isoenzymes. This effect is a function of the amount of coenzyme added, and isoenzymes H_4, H_2M_2, HM_3 and M_4 appeared to be affected to the same extent. Isoenzyme H_3M however, experienced a much greater initial effect after which it too exhibited a mobility shift paralleling the other isoenzymes.

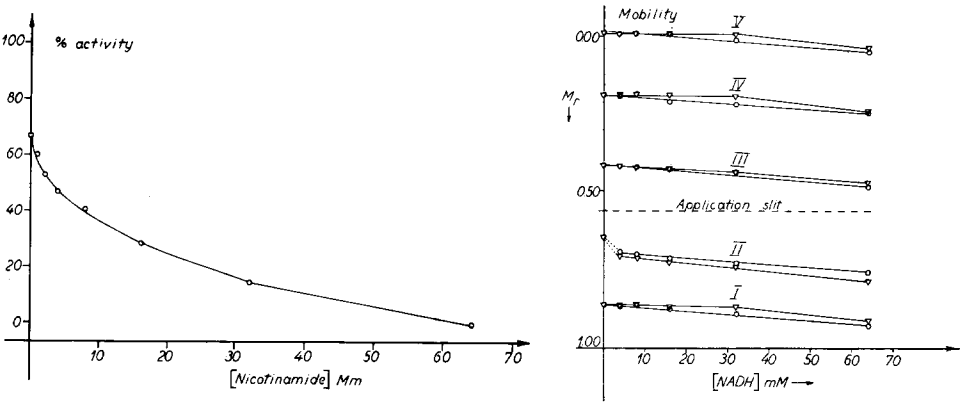


Fig. 3. The percentage of lactate dehydrogenase activity recovered after freezing of equal amounts of H and M subunits from heart and liver tissue respectively as function of concentration of nicotinamide. Abscissa, concentration (mM); ordinate, percentage of activity recovery.

Fig. 4. The relationship of the relative electrophoretic mobility of lactate dehydrogenase isoenzymes as function of concentration of NADH in an extract. Ordinate, relative electrophoretic mobility using albumin and dextran as reference markers possessing electrophoretic mobility M_r = 1.00 and 0.00, respectively. Abscissa, concentration of NADH (mM). I, H_4; II, H_3M; III, H_2M_2; IV, HM_3; V, M_4 isoenzymes.

DISCUSSION

The results of the present communication suggest that NADH plays a role in the enzymic reconstitution of lactate dehydrogenase activity from lactate dehydrogenase subunits. The addition of NADH to an enzyme extract of heart and liver either before or after freezing resulted in a more rapid and pronounced activity recovery. Indeed, it was possible by addition of NADH to restore activity in an extract which showed no activity without addition of NADH.

These results are supported by CHILSON *et al.*[6,10,15] who produced hybridization using guanidine·HCl or urea. They proposed that the NADH molecule served to stabilize the tertiary structure of the LDH molecule and that in the absence of NADH many subunits remained free in solution and/or as inactive polymers in equilibrium with the enzymically active tetramer. With the addition of NADH, the equilibrium shifted markedly towards the more stable tetramer structure with a corresponding increase in activity.

The fact that the addition of neither nicotinamide nor ADP resulted in recovery of activity suggests that the entire NADH molecule is necessary for the stabilizing effect.

The progressive loss of activity produced by increasing concentrations of nicotinamide in the undialyzed frozen extract furthermore suggests that it is the pyridine group which contains the site of attachment between NADH and the lactate dehydrogenase molecule. This was also suggested by SHIFRIN, KAPLAN AND CIOTTI[16] in fluorescence studies.

The exact implications of the phenomenon of hybridization produced by depressed pH is not as yet clear but it may be related to destruction of NADH at acid pH.

The observation that the exogenous addition of NADH to an extract produced a progressive increase in electrophoretic mobility suggests that there are additional sites of attachment in the tertiary structure for NADH molecules which are not essential for enzymic activity. Evidently a number of these sites is more readily available in isoenzyme H_3M possibly indicating a more unfolded active structure.

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