THE PRESENCE OF LACTIC ACID IN PURIFIED LACTATE DEHYDROGENASE

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1. Introduction

When mammalian lactate dehydrogenase [L-lactate: NAD oxidoreductase, EC 1.1.1.27] (LDH) from pig heart muscle or from rabbit or pig skeletal muscle is mixed with the oxidized form of the coenzyme an absorption peak is observed in the region 325 to 340 nm [1, 2]. The possibility that this peak derives from reduced coenzyme (NADH) formed from lactate present in the protein preparation was considered both by Winer [1] and by Vestling and Kunsch [2] who rejected this possibility on the basis of the extensive purification procedure used. Winer was unable to reduce significantly the extent of formation of this peak by pretreating the enzyme with NAD followed by dialysis. He concluded that no lactate was present in the enzyme preparation. Extensive examination of the properties of this lactate dehydrogenase–NAD binary complex spectrum and its similarities to the behaviour of binary complexes of lactate dehydrogenase and reduced coenzymes forced us to re-examine the possibility that the enzyme does indeed contain tightly bound lactate.

2. Materials and methods

Pig heart lactate dehydrogenase was either prepared by ammonium sulphate fractionation, DEAE cellulose chromatography and gel filtration – a modified procedure (White, unpublished results) after that of Pesce et al. [3] – or was purchased from the Boehringer Corporation. Both enzyme preparations had similar specific activities and showed only very faint traces of other proteins on polyacrylamide gel electrophoresis. Rabbit muscle LDH was purchased from both Boehringer and the Sigma Chemical Company. Dogfish muscle LDH was kindly provided by Professor M.G. Rossman (Purdue). Other proteins were Boehringer products.

Radioactive materials were Amersham products. Amide-[^{14}C]NAD was purified shortly before use by DEAE ion exchange chromatography using an ammonium bicarbonate gradient.

NADH (grade I) and NAD were from Boehringer. NAD was purified by ion exchange chromatography on DEAE cellulose columns.

All solutions were made up in double glass distilled water and other chemicals were Analar or equivalent grade.

Difference spectra were measured at 25°C using split compartment cells in a Cary 14 spectrophotometer equipped with a high intensity source and a pen period control to obtain low noise spectra at high background optical densities. Grade A pipettes were used.

LDH was freed of bound nucleotides by dialysis (in cellulose tubing boiled in 10 mM EDTA and then in glass distilled water) against 1 M NaCl in 25 mM phosphate buffer at pH 7 for 12 to 18 hr. This treatment increased the ratio of extinction at 280 nm to that at 260 nm from 1.44 to 2.00. Charcoal treatment of the enzyme produced equivalent results. A typical dialysis protocol (for 10–50 mg LDH) was 12 hr against 3 ℓ of 1 M NaCl : 25 mM phosphate, two changes each of 3 ℓ of 25 mM phosphate pH 7 for 6 hr each, followed by 6 to 8 hr against 3 ℓ of the buffer to be used in the experiment

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The extinction coefficient for pig heart LDH was determined (by drying constant weight) to be 1.2 at 280 nm and 0.9 at 290 nm for 1 mg per ml solution (molar extinction coefficients 168×10^3 and 126×10^3, respectively for a molecular weight of 140 000).

All solutions were passed through washed 0.45 μm pore diameter Millipore filters (Millipore (UK) Ltd.).

3. Results

3.1. *Binary complex spectra*

Fig. 1 shows difference spectra produced by mixing pig heart LDH with NAD at pH values of 7.2, 8.7 and 9.7 (pH was measured before and after mixing). The absorption maximum of the difference spectra at pH 7.2 lies between 325 nm and 330 nm and at the higher pH values between 333 and 338 nm. Controls with catalytic amounts of LDH produced no difference spectra (indicating there was no substrate in the buffers used) and controls with either LDH and buffer or NAD and buffer also showed no alterations after mixing. The high pH did not alter the NAD (as shown both by its spectrum and by its ability to react with alcohol dehydrogenase and ethanol) during the times required for the experiments. Charcoal treatment did not alter the binary difference spectrum.

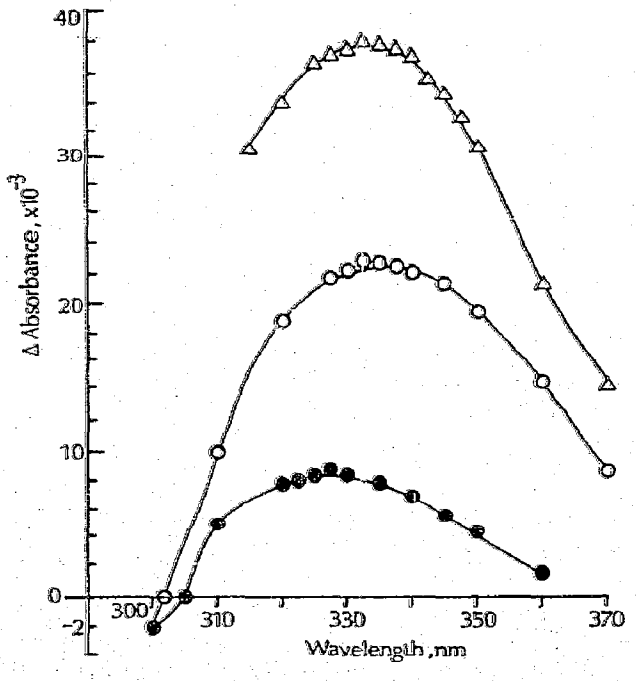

Fig. 1. Effect of pH on the difference spectrum produced upon mixing pig heart LDH and NAD. (●—●—●) pH 7.2 sodium potassium phosphate buffer, λ_{max} = 325 to 330 nm; (○—○—○) pH 3.7 ammonia–ammonium chloride buffer, λ_{max} = 333 to 337 nm; (△—△—△) pH 9.7 sodium carbonate–bicarbonate buffer, λ_{max} = 338 to 339 nm. All spectra were done at I = 0.1, [NAD] = 340 μM, [LDH] = 5.9 μM (1 mg/ml) (concentrations after mixing). The reference cell contained the same components unmixed. At pH 9.7 the enzymes loses activity and the experiment was performed after a shortened final dialysis, but had lost 20% of its original activity.

This dependence of the absorption maximum of the binary complex difference spectrum resembles the behaviour of enzyme bound NADH : absorption maximum at 340 nm at high pH but 325 nm at pH 7 [4]. The observed increase in the magnitude of the difference spectrum with increasing pH would be expected if L-lactate were being oxidized. Similar results were obtained for the skeletal muscle enzymes (rabbit, pig and dogfish).

The fluorescence emission spectrum of the pig heart LDH–NAD complex resembles that of enzyme bound NADH [5].

3.2. *Treatment to diminish the LDH/NAD difference spectrum*

Treatment of LDH with high concentrations of NAD at pH 8–9 followed by dialysis was found to produce a slight reduction in the magnitude of the difference spectrum. This suggested that repetitive treatments with NAD might have a larger effect. This was accomplished by using a gel filtration column equilibrated with NAD, but which would exclude the enzyme.

Pig heart LDH (90 mg) was passed through a column (93 × 140 cm) of Sephadex G-100 (Pharmacia Corp.) equilibrated with 1 mM NAD in I = 0.1, pH 8.9 ammonia–ammonium chloride buffer at 0.3 ml per min (taking about 18 hr). (The concentration of NAD as determined by assay with alcohol dehydrogenase and ethanol was unaltered during this time.) The protein eluate after concentration and dialysis to remove NAD gave a difference spectrum of 15×10^{-3} absorbance units at 340 nm ([LDH] = 0.7 mg/ml; [NAD] = 200 μM, I = 0.1, pH 9.0 ammonia–ammonium chloride buffer). A sample of the untreated enzyme gave a change of 53×10^{-3} absorbance units under identical conditions. Both samples had the same specific activity.

Controls with catalytic amounts of LDH mixed with the NAD solution produced no change from the unmixed baseline.

This reduction in the magnitude of the binary complex spectrum is consistent with the presence of an oxidizable substrate bound to the enzyme.

3.3. *Measurement of NADH produced from LDH and NAD*

Extensively dialyzed LDH was found to catalyze the equilibration of radioactivity between amide-[^{14}C]NAD and unlabelled NADH (mixing 9.3 mg of LDH with 10 μmoles [^{14}C]NAD at pH 9 and 25°C, gave equal specific activities for NADH and NAD after 75 min – a control with a catalytic amount of LDH had no effect over this time).

Radioactive NADH was isolated after mixing LDH with [^{14}C]NAD. In a typical experiment 26 mg dialyzed pig heart LDH in 6 ml pH 9 ammonia–ammonium chloride or (phosphate buffer) were mixed with 4 μmoles [^{14}C]NAD (46.6 mCi/mmole). After 20 min $HgCl_2$ was added (final concn. 1.7 mM). After a further 60 min urea was added (8 M final concn.). Carrier NADH was added 90 min after the urea and the mixture was passed down a Sephadex G-25 column after a further 30 min. The eluate following the protein peak was passed through a DEAE cellulose (carbonate form) column and the pyridine nucleotides were eluted with a gradient of ammonium hydrogen cabonate. The NADH peak was collected and the specific activity carefully measured. From the value obtained it was calculated that between one and two moles of oxidizable substrate had been present per mole of LDH. The [^{14}C]NADH could be converted back to [^{14}C]-NAD with acetaldehyde and horse liver alcohol dehydrogenase. This NAD had the same specific activity as the NADH; therefore the significant portion of the radioactivity was present as NADH and not as a contaminant.

3.4. *Extraction of lactate from the enzyme*

The ubiquity of lactic acid makes confirmation of its presence in a protein difficult. The following experiments were done with great care and polythene gloves were worn, but the possibility of adventitious lactate contamination cannot be entirely eliminated.

When freeze-dried 30–50 mg samples of carefully dialyzed LDH were treated with 2 N HCl for several hours the concentrated and neutralised acid extracts were found to contain substances capable of reducing NAD in the presence of LDH. Controls done with chymotrypsin which had been dialyzed in the same buffer as the LDH produced a change at 340 nm which was at most 15% of that produced with the LDH samples. Typically, 30 mg of LDH produced a total of 15 to 20 nmoles of NADH after subtraction of the control values. Such experiments involve considerable losses and indicate on the order of 0.1 mole of substrate per mole of LDH (140 000 molecular weight).

LDH, acidified to pH 1 with HCl was extracted with diethyl ether in a continuous extraction apparatus for 48 hr. The concentrated extract contained materials capable of being substrates for LDH in the presence of NAD. The LDH was treated with diazomethane [6] and the products were separated by gas liquid chromatography on both 15% polyethylene glycol on Chromosorb W (at 70° or 85°C) and 10% diethyl succinate on Chromosorb G (at 55° or 85°C) columns. Peaks having the same retention volumes as authentic methyl lactate were found with extracts from pig heart LDH (both prepared and purchased) and rabbit skeletal muscle LDH. Controls with no protein and controls with bovine serum albumin, chymotrypsin, and pig heart malate dehydrogenase did not show this peak. Controls with added lactate did give a peak in this position. When the extraction was done at pH 7 no methyl lactate peak could be found in LDH extracts.

3.5. *Exchange experiments with [^{14}C]L-lactate and [^{14}C]pyruvate*

Experiments in which LDH was incubated for several hours with NAD and [^{14}C]L-lactate or with NADH and [^{14}C]pyruvate did not show any incorporation of radioactivity into the protein after gel filtration on Sephadex G-25 which was significantly above that found with control proteins. Even under conditions known to dissociate reversibly the LDH tetramer [7, 8] no significant radioactivity was incorporated into the protein.

4. Discussion

All of these results may be explained by the hypothesis that the LDH preparations used contained tightly bound L-lactate which was not removed by dialysis. Oxidation of some of this produces enzyme bound NADH – the origin of the spectrum of the binary complex with NAD. NADH can be isolated after incubation of the enzyme with NAD. The amounts of "bound lactate" are difficult to determine exactly but appear to not exceed one mole per monomer of molecular weight 35 000.

The bound lactate is not exchangeable with free substrate and is removed only under acid conditions. This indicates that it must be very tightly bound – perhaps covalently. As this supposedly bound lactate can be oxidized; tightly bound pyruvate may also exist and we have some experimental evidence in support of this. Passage of LDH through a Sephadex G-100 column equilibrated with NAD at high pH should produce "high pyruvate" LDH.

These results strongly indicate that purified pig heart muscle and skeletal muscle LDH preparations contain small amounts of lactate which cannot be removed by conventional dialysis. The nature and function of this bound substrate are at present unknown.

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