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NAD RECYCLING IN THE COLLAGEN MEMBRANE

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Summary

NAD recycling in the collagen membrane was investigated as follows:

(1) Alcohol dehydrogenase and lactate dehydrogenase were co-immobilized in the collagen membrane and the rate of lactate production by immobilized enzymes was compared with that of free enzymes by using free NAD. An increased rate was observed in the case of immobilized enzyme.

(2) The soluble high molecular weight derivatives of NAD (dextran-NAD) were immobilized in the collagen membrane with the two dehydrogenases and recycling of dextran-NAD in the membrane was examined. Lactate was produced by the membrane without adding free NAD.

The interaction between the high molecular weight NAD derivatives and enzymes are also discussed.

Introduction

Considerable world-wide interest has arisen in the preparation and properties of immobilized enzymes and these have been applied to industrial production and clinical analysis [1,2]. Immobilization of coenzymes and recycling of coenzymes are desirable for the practical use of immobilized enzymes and recycling of coenzyme is a good model for studies on metabolic processes [3]. The reaction of two immobilized dehydrogenases with a soluble high molecular weight derivative of NAD was reported previously [4]. However, no report on immobilization of a coenzyme and enzymes within the same carrier has been published. A kinetic advantage (a microenvironmental effect) can be expected by use of immobilized coenzyme and enzymes [5].

In present study, the two enzymes, alcohol dehydrogenase and lactate dehydrogenase and the soluble high molecular weight NAD derivatives, are immobilized together in a collagen membrane and the recycling of coenzyme are examined.

Materials and Methods

Materials. Alcohol dehydrogenase (from yeast 420 units/mg protein) and lactate dehydrogenase (from pig heart 484 units/mg protein) were purchased from Oriental Yeast Co. Ltd., and NAD was obtained from Kyowa Hakko Kogyo Co. Ltd. Dextran T-70 (mol. wt. = 70 000) was purchased from Pharmacia Fine Chemicals. Analytical reagents or laboratory grade materials and deionized water were used through this work.

Preparation of dextran-NAD. Dextran-NAD was prepared by coupling NAD-N^6-[*N*-(6-aminohexyl)acetamide] through its terminal amino group to a CNBr-activated soluble dextran (T-70) according to the methods described by Mosbach et al. [6,7] 13 μmol of nucleotide per g dextran-NAD (about one cofactor per dextran molecule) was used. The amount of enzymatically-available nucleotide was approximately 79%.

Preparation of enzyme-collagen membrane. The solution containing various amount of alcohol dehydrogenase, lactate dehydrogenase and dextran-NAD was added to 30 g of 0.85% collagen fibril suspension (pH 5.0). The collagen membrane containing enzymes and dextran-NAD was prepared by casting the suspension on a Teflon plate and drying at room temperature [8]. The dried membranes were cut into 1 cm squares and these were tanned by dipping in 1% (w/v) glutaraldehyde solution (pH 7.0) for 1 min. The squares were then washed thoroughly with 0.1 M phosphate buffer (pH 7.0) for 1 h and dried at room temperature. The collagen membrane containing alcohol dehydrogenase and lactate dehydrogenase was also prepared by a similar method (these membranes were 50 μm thick).

Assay of dehydrogenase. The Michaelis constant (K_m) and the maximum velocity (V) were determined spectrophotometrically at 30°C with a assay medium of 3 ml of 0.1 M phosphate buffer (pH 7.0) containing 1 mM glutathione, 0.5 M ethanol and various amounts of oxidized cofactor or 50 mM pyruvate and various amounts of reduced cofactor. Immobilized dehydrogenases (the dextran-NAD · alcohol dehydrogenase · lactate dehydrogenase-collagen membrane or the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane) were assayed under the same conditions as for free dehydrogenases.

A unit of the enzymes was defined as the increasing or decreasing amount of μmol of NADH/min under the conditions described above.

Recycling experiments. Lactate produced was measured as follows: the standard assay mixture contained 20 ml 0.1 M phosphate buffer (pH 7.0)/1 mM glutathione/0.5 M ethanol/0.05 M pyruvate and NAD. The concentrations of cofactor and amounts of free and immobilized enzymes are indicated in the Results section. For the assay, 0.2 ml reaction mixture was withdrawn at intervals. 0.2 ml 0.3% H_2O_2 was added to stop the reaction and the mixture was heated at 100°C for 10 min. The lactate concentration was determined with 3.0 ml standard reaction mixture containing 1.5 ml 0.4 M hydrazine (in 0.5 M glycine/NaOH buffer, pH 9.5)/0.2 ml 0.1 M NAD/0.4 ml reaction mixture and 50 μg lactate dehydrogenase. After incubation for 30 min at 30°C, a change in absorbance at 340 nm was measured and the lactate concentration was calculated from a standard curve. NADH concentration was determined spectrophotometrically (A_{340} nm).

Results

The activity yields of the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane was 7% for alcohol dehydrogenase and 40% for lactate dehydrogenase respectively. No leakage of the enzymes from the membrane was observed during experiments.

Apparent K_m values of immobilized dehydrogenase (alcohol dehydrogenase for NAD and lactate dehydrogenase for NADH) were much higher than that of free enzymes (Table I).

The recycling of free NAD in the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane is shown in Fig. 1. The recycling activity of the membrane was determined by measuring lactate produced in the presence of excess amounts of ethanol and pyruvate. Fig. 2 represents the time-course of the lactate production by the immobilized enzymes and also by free enzymes. The activities of the free dehydrogenases and the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane was adjusted on the basis of V respectively. The rate of lactate production by the immobilized enzyme system was higher than that by the free enzyme system. In particularly, the initial rate of lactate production by the immobilized enzyme system was about 4 times higher than that of the free system at a high alcohol dehydrogenase to lactate dehydrogenase ratio (Fig. 2a). At a low alcohol dehydrogenase to lactate dehydrogenase ratio, a slight lag of lactate production was observed in the case of the free enzyme system (Fig. 2b).

TABLE I

K'_m VALUES FOR FREE AND IMMOBILIZED ENZYMES

Apparent K'_m values were obtained from Lineweaver-Burk plots. Cofactor concentrations were: (a) $0.1—0.2 \cdot 10^{-3}$ M NAD for free alcohol dehydrogenase and $0.5—5 \cdot 10^{-3}$ M NAD for immobilized alcohol dehydrogenase, (b) $0.2—2 \cdot 10^{-4}$ M NADH for free lactate dehydrogenase and $0.6—3 \cdot 10^{-4}$ M NADH for immobilized lactate dehydrogenase.

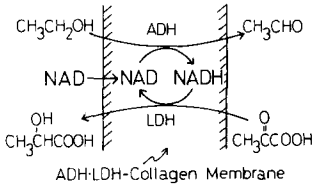
	K'_m of NAD for alcohol dehydrogenase (μM)	K'_m of NADH for lactate dehydrogenase (μM)
Free	0.29	29
Membrane	2.2	420

TABLE II

LACTATE PRODUCED BY VARIOUS RECYCLING SYSTEMS

Lactate production by using various NAD-recycling systems were carried out in the standard assay mixture containing 64 nmol NAD or dextran-NAD, 4.2 U of alcohol dehydrogenase and 0.51 U of lactate dehydrogenase for 60 min.

System			Lactate produced		NAD reused
Alcohol dehydrogenase, lactate dehydrogenase	Dextran-NAD	NAD	μmol	%	(cycles/h)
Membrane	membrane	—	1.84	194	29
Membrane	—	free	2.32	224	36
Free	—	free	0.95	100	15
Free	free	—	0.44	46	6.9



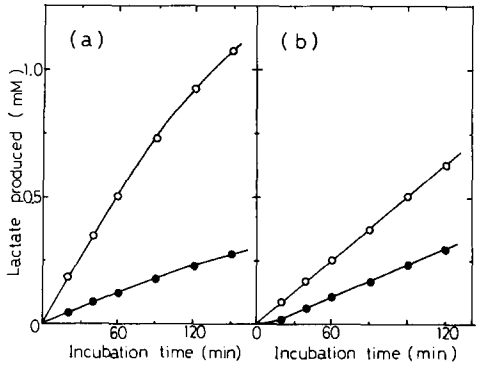
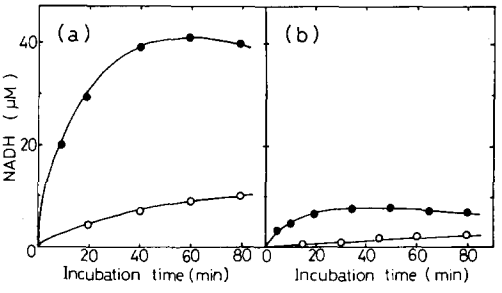


Fig. 1. Schematic diagram of the recycling of coenzymes in the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane. ADH: alcohol dehydrogenase. LDH: lactate dehydrogenase.

Fig. 2. Time courses of lactate produced by free and immobilized alcohol dehydrogenase · lactate dehydrogenase. The concentrations of NAD and amounts of free and immobilized enzymes (12 cm^2) used were (a) 100 μM, 1.25 U of alcohol dehydrogenase and 0.29 U of lactate dehydrogenase, and (b) 200 μM, 0.35 U of alcohol dehydrogenase and 0.85 U of lactate dehydrogenase, respectively. The other experimental details were given in the text. ●———●, free enzyme system. ○———○, immobilized enzyme system.

The amount of NADH produced by both systems was determined spectrophotometrically and the amount of NADH produced by the membrane system was much lower than that produced by the free enzyme system (Fig. 3). Furthermore, the amount of NADH produced by the system at a high alcohol dehydrogenase to lactate dehydrogenase ratio was higher than that at a low alcohol dehydrogenase to lactate dehydrogenase ratio.

Dextran-NAD, alcohol dehydrogenase and lactate dehydrogenase were co-immobilized in the collagen membrane and dextran-NAD recycling was attempted. The quantity of the dextran-NAD entrapped was 5.3 nmol/cm^2 membrane. Therefore, the concentration of the entrapped dextran-NAD in



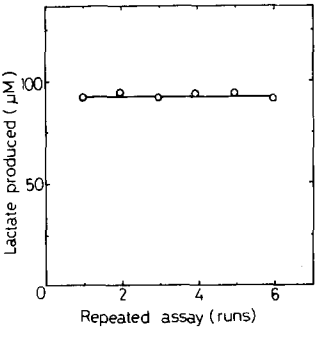


Fig. 3. Time course of NADH produced free and immobilized alcohol dehydrogenase · lactate dehydrogenase. Experimental conditions were the same as described in Fig. 2. ●———●, free enzymes system. ○———○, immobilized enzymes system.

Fig. 4. The reusability of the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane containing dextran-NAD. Lactate produced by the dextran-NAD · alcohol dehydrogenase · lactate dehydrogenase-collagen membrane in the standard assay mixture without containing free NAD was measured. The membrane washed with 0.1 M phosphate buffer (pH 7.0) and the same procedure was repeated. Other conditions were the same as described in Table II.

reaction mixture corresponded to 3.2 μM. The dextran-NAD · alcohol dehydrogenase · lactate dehydrogenase-collagen membrane produced lactate without adding free NAD to the reaction mixture (Table II). The activity of this membrane was about 4 times higher than that of the free enzyme system (soluble alcohol dehydrogenase, lactate dehydrogenase and dextran-NAD). However, the activity of this system was lower than that of the system described above (free NAD and the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane). The rate of dextran-NAD recycling in the collagen membrane was 29 cycles/h (calculated from the produced lactate and the dextran-NAD content).

The reusability of the dextran-NAD · alcohol dehydrogenase · lactate dehydrogenase-collagen membrane is shown in Fig. 4.

Discussion

The immobilization of enzymes in a collagen membrane was suggested as a useful technique [1,2] and alcohol dehydrogenase and lactate dehydrogenase were co-immobilized in the collagen membrane and recycling of free NAD in the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane was examined in this study. The rate of lactate produced by immobilized enzymes was higher than that produced by free enzymes. The same phenomenon has also been observed in the system of DEAE-cellulose-lactate dehydrogenase-alcohol dehydrogenase [4] and three immobilized enzymes [5]. The increasing NADH concentration around immobilized enzymes was said to explain this phenomenon. NADH was used as cofactor of lactate dehydrogenase. Therefore, the enhancement of the rate of lactate production in the immobilized enzyme system is due to effective recycling of the cofactor as compared to free enzyme system.

When free NAD was added to the reaction mixture, the amount of NAD permeating into the membrane was small and it was assumed that NAD recycling was ineffective in this situation. Effective NAD recycling is observed when matrix-bound NAD is employed for the experiments, yielding on rate of recycling 4 times that of a free enzyme system, presumably due to the local increase of dextran-NAD concentration in the membrane. On the other hand, the activity of this system was lower than that of free NAD and the alcohol dehydrogenase · lactate dehydrogenase-collagen membrane system, and the recycling rate of the dextran-NAD was lower than that of free NAD. This result may be caused from steric hindrance of dextran-NAD in the collagen membrane.

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