Journal of Solid-Phase Biochemistry, Vol. 1, No. 2, 1976

PHOTOCONTROL OF LACTATE DEHYDROGENASE–SPIROPYRAN COLLAGEN MEMBRANE

YOSHIAKI NAKAMOTO,[1] ISAO KARUBE,[2] SEIKICHI TERAWAKI,[1] and SHUICHI SUZUKI[2]

[1]*Faculty of Engineering*
Department of Industrial Chemistry, University of Kanazawa
Kodatsuno, Kanazawa-city, Ishikawa-Ken, Japan
[2]*Research Laboratory of Resources Utilization*
Tokyo Institute of Technology
Ookayama Meguro-ku, Tokyo, Japan

Accepted May 17, 1976

Collagen fibrils were modified with β-1-[3,3-dimethyl-6'-nitrospiro-(indoline-2,2'-2*H*-benzopyran)] propionic anhydride. The spiropyran collagen membrane showed reverse photochromism. Lactate dehydrogenase (LDH, E.C. 1.1.1.27) was entrapped in the spiropyran collagen membrane. The activity of the LDH–spiropyran collagen membrane decreased under visible-light irradiation, and then increased again after incubation in the dark. The optimum pH of the LDH–spiropyran collagen membrane was displaced toward lower pH values under visible-light irradiation. The activity change of the LDH–spiropyran collagen membrane under visible-light irradiation depended on the LDH content.

INTRODUCTION

Substances that undergo reversible color formation under light irradiation are called *photochromic compounds.* A number of spiropyran compounds show photochromism (1). Colored spiropyrans are converted into colorless form under visible-light irradiation or when they are placed in the dark. The authors previously found that photosensitive enzymes could be prepared by modifying enzymes with such photochromic spiropyran compounds (2). The substrate affinity of enzymes was found to be changed by the modification, possibly because it drastically changes the polarity of the spiropyran compound. Photosensitive immobilized enzymes could be prepared by entrapping urease modified with spiropyran compounds in a collagen membrane (3). It is well known that the diffusion of substrate affects the apparent activity of immobilized enzymes (4). Therefore, activity control of immobilized enzymes can also be accomplished by control of the rate of diffusion of the substrate to enzyme.

In this paper, the collagen fibrils were modified with β-1-[3,3-dimethyl-6′-nitrospiro-(indoline-2,2′-2*H*-benzopyran)] propionic anhydride. The photocontrol of LDH entrapped in spiropyran collagen membranes was studied.

MATERIALS AND METHODS

Materials. Purified collagen was prepared from Holstein calf skin as described previously (5). Lactate dehydrogenase (from pig heart, 4.36 U/mg protein) was obtained from Oriental Yeast Co. The β-1-[3,3-dimethyl-6′-nitrospiro-(indoline-2,2′-2*H*-benzopyran)] propionic anhydride was prepared by the method reported previously (7). Nicotinamide adenine dinucleotide (NAD) was obtained from Tokyo Kasei Co.

Modification of Collagen. For this procedure, 1 g collagen fibril was added to 100 ml acetone solution containing 180 mg of the spiropyran anhydride. The reaction mixture was allowed to stand at room temperature for 20 h with stirring. Then the collagen fibrils were separated by centrifugation at 2000*g* for 10 min, and thoroughly washed with acetone to remove unreacted compound. At total of 39 mg spiropyran was bound to 1 g collagen fibrils.

Preparation of LDH–Spiropyran Collagen Membrane. To 11.5 mg LDH was added 38 g 1.0% spiropyran collagen fibril suspension. The LDH solution was dialyzed against a large volume of distilled water for 6 h at 4°C to remove salts. The LDH–spiropyran collagen membrane was prepared by casting the suspension on a Teflon plate and drying it at room temperature.

Enzyme Assay. Unless otherwise noted, standard assays of the native LDH and the LDH–collagen membrane were carried out as follows: A reaction mixture was 3 ml 0.1 M phosphate buffered solution (pH 8.0) containing 10 μg native LDH or 1 mg LDH–collagen membrane (15 μg LDH), 25 mM sodium lactate, and 1 mM NAD. The reaction mixture was irradiated with visible light (Olympus Slide Projector KP-8) during the reaction or placed in the dark. The NADH produced was determined by spectrophotometry (7).

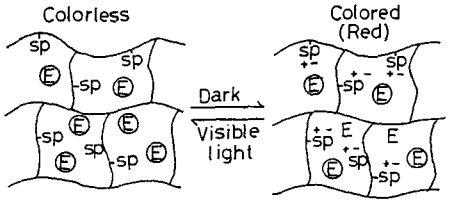

FIG. 1. Scheme of photoreversible change of LDH–spiropyran collagen membrane. Ⓔ: LDH; sp: spiropyran compound

Diffusion Coefficient Measurement. The coefficients of diffusion of lactate and NAD through the modified membrane were determined under visible-light irradiation or in the dark. The apparatus and procedures for measurement of solute fluxes were similar to those used previously (4). Lactate was determined colorimetrically with *p*-hydroxydiphenyl.

RESULTS

The spiropyran collagen membrane showed reverse photochromism; i.e., the membrane was colored in the dark and bleached immediately under visible-light irradiation (Fig. 1). This reverse photochromism was similar to that observed in the case of the free spiropyran compound in dioxane–water mixture (6).

The specific activity of the LDH–spiropyran collagen membrane was approximately 10% that of native LDH.

Figure 2 shows pH–activity profiles of the LDH–spiropyran collagen membrane in the dark and under visible light. The activity of native LDH was not affected by visible-light irradiation. The activity of the LDH–spiropyran collagen membrane was decreased to 63% under visible-light irradiation. Therefore, the activity decrease of the LDH–spiropyran collagen membrane was correlated with bleaching of the spiropyran membrane. The degree of activity decrease of the LDH–spiropyran collagen membrane was reproducible (63 ± 5%) and similar to that of spiropyran–urease in a collagen membrane (3). The activity of the membrane increased again after incubation in the dark. The optimum pH of the LDH–spiropyran collagen membrane was displaced toward lower pH values under visible-light irradiation. This result was also similar to that observed with the spiropyran–urease in a collagen membrane (3). As the spiropyran compound bound to collagen fibrils underwent irreversible isomerization above

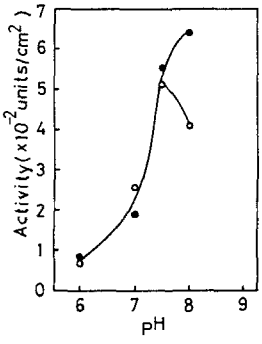

FIG. 2. pH–activity profiles of the LDH-spiropyran collagen membrane. For the determination, 48 ml 0.1 *M* phosphate buffered solution containing 75 mM lactate, 1 mM NAD, and the LDH–spiropyran collagen membrane (2 × 1 cm, 88 μm, 135 μg LDH/cm^2) was employed. The activity of the LDH–spiropyran collagen membrane was determined in the dark (•) and under visible light (○).

TABLE 1. **Apparent Diffusion Coefficients of Lactate and NAD**[a]

Conditions	$D_{lactate}$ (cm^2/sec)	D_{NAD} (cm^2/sec)
In the dark	1.29×10^{-6}	6.52×10^{-7}
Under visible light	1.57×10^{-6}	8.88×10^{-7}

[a] A 5 mM solution of lactate or a 1 mM solution of NAD (pH 8.0, 0.1 M phosphate buffer solution) was employed. The apparent diffusion coefficient, D, was evaluated from the following equation: $J = (A/l) \cdot D \cdot \Delta C \cdot t$, in which J is the solute flux, A is the membrane surface area, l is the membrane thickness, ΔC is the concentration difference between the two compartments, and t is the diffusion time.

pH 8.0, the activity of the LDH–spiropyran collagen membrane could no longer be determined.

Table 1 shows the apparent diffusion coefficients of lactate and NAD. Both lactate and NAD diffused more slowly in the colored membrane than in the colorless one. This slower diffusion may have been due to electrostatic interaction between the charges of the spiropyran and the substrate.

Table 2 shows the relationship between the amount of entrapped LDH and the activity of the LDH–spiropyran collagen membrane. The activity of the membrane containing 123 μg LDH/cm^2 in the dark was higher than the activity under visible light. When the LDH content in the membrane was 15 and 2.8 μg/cm^2, the activity of the membrane in the dark was lower than that under visible light. The activity change of the spiropyran collagen membrane under visible-light irradiation depended on the LDH content.

Figure 3 shows Lineweaver–Burk plots of the LDH–spiropyran collagen membrane under visible light and in the dark.

Table 3 shows the kinetic parameters of the LDH–spiropyran collagen membrane. As shown, the apparent Michaelis constant ($K_{m'}$) of the LDH–spiropyran collagen membrane with lactate in the dark was identical to that

TABLE 2. **Relationship Between the Amount of Entrapped LDH and the Activity of the LDH–Spiropyran Collagen Membrane**[a]

Amount of LDH (μg/cm^2)	Activity (U/cm^2)		
	Dark	Vis[b]	Vis[b]/dark (%)
123	0.059	0.034	57.6
15.0	0.015	0.017	113
2.80	0.003	0.005	166

[a] Solution: 24 ml, 25 mM lactate and 1 mM NAD in 0.1 M phosphate buffered solution (pH 8.0); membrane: 20 mm × 10 mm × 90 μm.
[b] Under visible light.

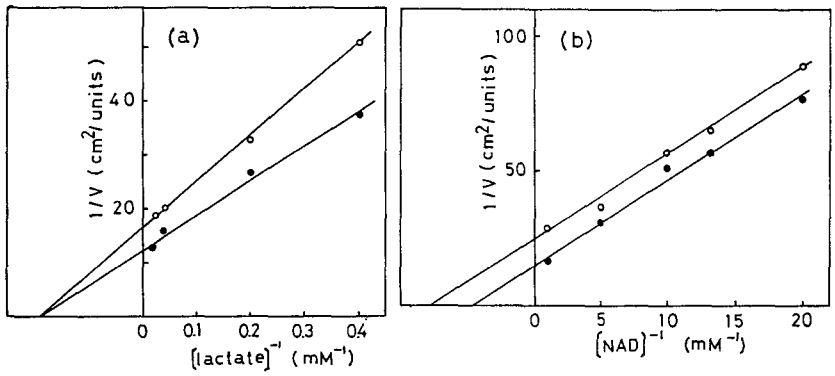

FIG. 3. Lineweaver–Burk plots of the LDH–spiropyran collagen membrane, using: (a) 24 ml phosphate buffered solution (pH 8.0) containing 1 mM NAD, lactate, and the LDH–spiropyran collagen membrane (2×1 cm, 82 μm, 123 μg LDH/cm^2); and (b) 24 ml phosphate buffered solution (pH 8.0) containing 25 mM lactate, NAD, and the LDH–spiropyran collagen membrane (2×1 cm, 82 μm, 123 μg LDH/cm^2). The activity of the LDH–spiropyran collagen membrane was determined in the dark (•) and under visible light (○).

of the membrane under visible light. However, the $K_{m'}$ with NAD in the dark was decreased under visible light. The maximum velocity (V) of the LDH–spiropyran collagen membrane with lactate and NAD in the dark decreased under visible-light irradiation.

DISCUSSION

Spiropyran compounds change in polarity with isomerization. The activity of the LDH–spiropyran collagen membrane in the dark was higher than that under visible light (see Table 2). The maximum velocity (V) of the LDH–spiropyran collagen membrane was decreased under visible-light irradiation. Lineweaver–Burk plots were different from those of the

TABLE 3. **Kinetic Parameters of the LDH–Spiropyran Collagen Membrane**

	$K_{m'}$ (mM)		V(μmol/min per cm^2)	
	Dark	Vis[a]	Dark	Vis[a]
Lactate	5.31	5.31	0.070	0.060
NAD	0.291	0.132	0.068	0.040

[a]Under visible light.

spiropyran–urease in a collagen membrane (3). Since the spiropyran collagen membrane showed reverse photochromism, the spiropyran compound bound to collagen fibrils must change in the dark. Also, the spiropyran collagen membrane is hydrophilic in the dark and hydrophobic under visible light. It was also assumed that the diffusion of lactate and NAD, substrates of LDH, in the dark was greater than that under visible light. The results obtained from the diffusion experiments (Table 1), however, did not bear out these speculations. The diffusion rates of lactate and NAD under visible light respectively higher than the rates in the dark. Therefore, the reaction of the LDH is not limited by diffusion of the substrates. As reported previously (4), the reaction of the enzyme occurred mainly in the surface of the enzyme–collagen membrane. It was concluded that the hydrophilic microenvironment around the immobilized LDH near the surface of the spiropyran collagen membrane in the dark may accelerate the velocity of the enzymatic reaction.

As described above, the LDH–spiropyran collagen membrane used in these experiments contained a large amount of LDH. However, in the case of the spiropyran collagen membrane containing a small amount of LDH, the activity in the dark was lower than that under visible light (see Table 2). The reaction of LDH in this spiropyran collagen membrane may be limited by diffusion of the substrates. Since the spiropyran collagen membrane has charges in the dark, lactate and NAD may interact with these charges of the spiropyran bound to the collagen membrane. Furthermore, the interaction of pyruvate, an inhibitor of LDH, with the charges on the spiropyran compound causes the accumulation of pyruvate in the membrane. These interactions may decrease the activity of the LDH–spiropyran collagen membrane. The effect of light irradiation on the activity was different, depending on the LDH content in the spiropyran collagen membrane.

The activity of the LDH–spiropyran collagen membrane increased again after incubation in the dark. However, the initial activity was not restored. It is known that spiropyran compounds undergo irreversible isomerization under visible-light irradiation (1). A part of the spiropyran compound bound to the collagen may undergo irreversible isomerization. These spiropyran compounds could not have charges again after incubation in the dark. Therefore, the LDH–spiropyran collagen membrane showed low activity even in the dark.

REFERENCES

1. INOUE, E., KOKADO, H., SHIMIZU, and KOBAYASHI, H. (1972) Bull. Chem. Soc. Jpn. 45 : 1951.
2. NAMBA, K., and SUZUKI, S. (1975) Bull Chem. Soc. Jpn. 48 : 1323.

3. KARUBE, I., NAKAMOTO, Y., NAMBA, K., and SUZUKI, S. (1976) Biochim. Biophys. Acta 429 : 975.
4. NAKAMOTO, Y., KARUBE, I., and SUZUKI, S. (1975) J. Ferment. Technol. 53 : 595.
5. KARUBE, I., SUZUKI, S., KINOSHITA, S., and MIZUGUCHI, J. (1971) Ind. Eng. Chem. Procl. Res. Dev. 10 : 160.
6. NAMBA, K., and SUZUKI, S. (1975) Chem. Lett. 9 : 949.
7. BERNT, E. (1974) *In* Methods of Enzymatic Analysis, Vol. 2, BERGMER, H. U. (ed.), Academic Press, New York, p. 574.
8. EEGRIWE, E. (1933) Z. Anal. Chim. 95 : 323.