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Hg^{2+}-STIMULATED NADH-OXIDASE ACTIVITY OF ASCARIS MUSCLE MICROSOMAL LIPOAMIDE DEHYDROGENASE

Sadayuki Matuda and Fujio Obo
Department of Biochemistry, Faculty of Medicine,
Kagoshima University, Kagoshima 890, Japan

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SUMMARY

In the presence of Hg^{2+} Ascaris lipoamide dehydrogenase stimulated the reduction of oxygen, ferricyanide, and 2,6-dichlorophenolindophenol with NADH, which was inhibited by lipoic acid. On the other hand, Cu^{2+} stimulated the reduction of the artificial dyes, but only a little the reduction of oxygen. Hg^{2+} changed the visible absorption spectrum of the lipoamide dehydrogenase, but did not change the fluorescence curve. Lipoic acid decreased the fluorescence, but did not change the visible absorption spectrum. The Ascaris lipoamide dehydrogenase have two SH groups per one subunit and 5-6 moles of $HgCl_2$ and 3-4 moles of $CuSO_4$ per one subunit were required for the maximal activity.

The parasitic roundworm, Ascaris lumbricoides var.suum resides in the small intestine of swine where the oxygen tension is low. We have found a large amount of lipoamide dehydrogenase in Ascaris muscle microsomes, and have obtained the enzyme in a homogenous state[1]. The enzyme did not participate in the decarboxylation of α-keto acids and the catabolism of glycine. The physiological function of this lipoamide dehydrogenase is obscure at present.

As already described by Casola et al[2,3] in pig heart lipoamide dehydrogenase, Cu^{2+} and phenylmercuri acetate stimulate the reduction of artificial electron acceptors. In Ascaris lipoamide dehydrogenase Cu^{2+} and Hg^{2+} also stimulate the reduction of these electron acceptors. In addition we observed that Hg^{2+} stimulates NADH-oxidase activity of Ascaris lipoamide dehydrogenase, but Cu^{2+} does not. At the present study, we report the effect of Hg^{2+} and Cu^{2+} on the lipoamide dehydrogenase from Ascaris muscle microsomes.

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METHODS

Preparation of Ascaris Muscle Microsomes —— Adults of Ascaris suum were obtained at a public slaughterhouse. The muscle obtained above was homogenized in 4 volumes of 1.15 % KCl solution with a Waring blender. The resulting homogenate was centrifuged at 700 x g for 7 min. The supernatant was again centrifuged at 20,000 x g for 30 min and the precipitate was discarded. The post-mitochondrial supernatant was centrifuged at 105,000 x g for 60 min. Microsomal pellets were washed three times with 150 mM KCl solution, and then finally suspended in 50 mM Tris-HCl buffer(pH 8.0)[4].

Purification of Ascaris Lipoamide Dehydrogenase——The purification of lipoamide dehydrogenase from Ascaris muscle microsomes[1] was carried out as follows; Ascaris microsomes were solubilized with trypsin and centrifuged at 105,000 x g for 60 min. The supernatant was fractionated with ammonium sulfate(40-70 % saturation), dissolved in a minimal volume of Tris-HCl buffer (pH 8.0), and then column-chromatographyed on Sephadex G-200 and DEAE-23 cellulose.

Assay of Enzyme Activities —— NADH-ferricyanide and NADH-2,6-dichlorophenolindophenol reductase activities were measured by the method of Takesue and Omura[5]. Lipoamide dehydrogenase activity was measured as described by Massey[2]. NADH-oxidase activity(in 50 mM Tris-HCl buffer, pH 8.0) was measured by following the decrease in absorbance at 340 nm, taking a millimolar extinction coefficient of NADH to be 6.2[6].

Measurement of Fluorescence —— Fluorescence was measured in quartz cuvetts (3 ml, 1-cm light) at room temperature in a Hitachi 203 fluorescence spectrophotometer.

Measurement of Hydrogen Peroxide ——Hydrogen peroxide was measured by the method of Synder and Hendly[7].

Protein Determination —— Protein was determined by the method of Lowry et al[8] using bovine serum albumin as a standard.

The Number of SH of the Native and Denatured Enzymes——The number of SH of the enzyme in the absence or presence of 1 % SDS were carried out as described by Glazer et al[9].

MATERIALS

Sephadex and DEAE-23 cellulose were obtained from Pharmacia Fine Chemicals, Uppsala, and Whatman Biochemicals, Ltd., respectively. NADH(grade II) and trypsin(type III) were purchased from Boehringer Mannheim GmbH and Sigma, respectively. Mercuric chloride and DL-lipoic acid were purchased from Wako Pure Chemical Industries, Ltd and Nakarai Chemical Industries, Ltd

(Japan), respectively. 2,6-Dichlorophenolindophenol was obtained from Merk, and other chemicals used were commercial products of reagent grade.

RESULTS

Electron Acceptors of Ascaris Lipoamide Dehydrogenase and Effect of Metal Ions on It —— When metal ions were absent from the reaction medium, the lipoamide dehydrogenase catalyzed the reduction of the electron acceptors with NADH in the decreasing order of magnitude as follows; lipoic acid, ferricyanide, 2,6-dichlorophenolindophenol and oxygen. Hg^{2+} increased about ten-fold the NADH-oxidase activity. The activities of NADH-2,6-dichlorophenolindophenol and NADH-ferricyanide reductase were also stimulated by Hg^{2+}. Cu^{2+} also stimulated NADH-ferricyanide and NADH-2,6-dichlorophenolindophenol reductase activities, but only a little the NADH-oxidase activity(Table I). These observations suggest that the stimulating mechanisms of Hg^{2+} and Cu^{2+} are different from each other. p-Chloromercuribenzoate only a little stimulated the NADH-oxidase, NADH-ferricyanide and NADH-2,6-dichlorophenol-

Table I. Electron acceptors of lipoamide dehydrogenase and effect of metal ions.

Donors	Acceptors	Activity
NADH(0.1 mM)	Lipoic acid(2 mM)	24
	+ $HgCl_2$	0.8
	+ $CuSO_4$	0.7
	Ferricyanide(0.8 mM)	8
	+ $HgCl_2$	20
	+ $CuSO_4$	25
	DCPI(0.1 mM)	2
	+ $HgCl_2$	10.3
	+ $CuSO_4$	13.6
	Oxygen	0.2
	+ $HgCl_2$	3.3
	+ $CuSO_4$	0.4

The final concentration of $HgCl_2$ and $CuSO_4$ was 0.05 mM.
DCPI; 2,6-dichlorophenolindophenol.

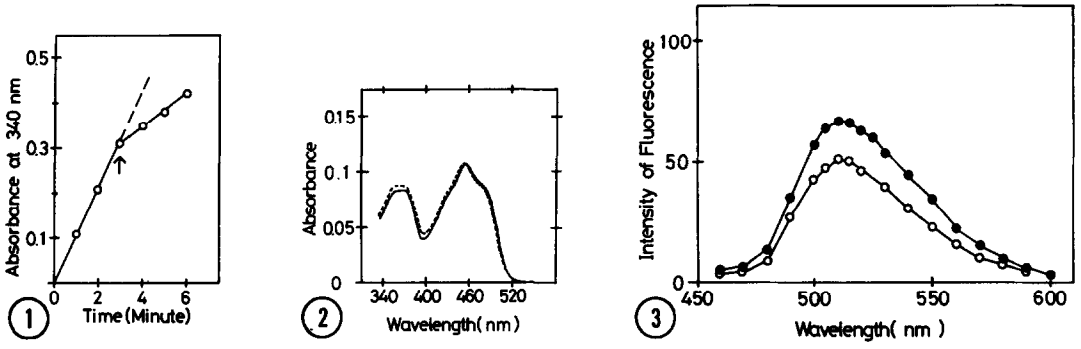


Fig. 1. Inhibition of NADH-oxidase activity with lipoic acid.
The lipoic acid was added at the arrow.

Fig. 2. Effect of Hg^{2+} or lipoic acid on the absorption spectrum of Ascaris lipoamide dehydrogenase.
——; None or lipoic acid, ----; Hg^{2+} or Cu^{2+} plus lipoic acid.

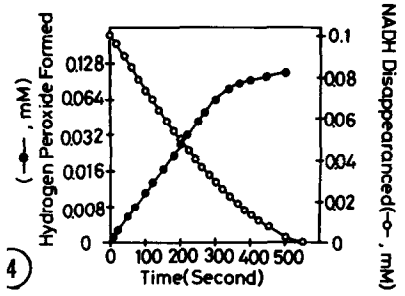
Fig. 3. Effect of Hg^{2+} or lipoic acid on the fluorescence of Ascaris lipoamide dehydrogenase.
—●—; None or Hg^{2+}, —○—; Lipoic Acid or lipoic acid plus Hg^{2+}.

indophenol reductase activities. As shown in Table I and Fig.1, lipoic acid inhibited the NADH-oxidase activity in the presence of Hg^{2+}.

Absorption and Fluorescence Spectra —— As shown in Fig.2, a slight change of the absorption spectrum was induced by Hg^{2+}, but lipoic acid had no effect. On the other hand, lipoic acid decreased the fluorescence of lipoamide dehydrogenase, but Hg^{2+} did not(Fig.3).

Formation of Hydrogen Peroxide by NADH-Oxidase Reaction of the Lipoamide Dehydrogenase —— As shown in Fig.4, accompanying the NADH-oxidase reaction 1 mol hydrogen peroxide was formed from 1 mol NADH in the presence of Hg^{2+}, but this reaction proceeded very slowly in the absence of Hg^{2+}.

Titration of the Lipoamide Dehydrogenase with Cu^{2+} or Hg^{2+} —— In order to estimate the amount of metal ions, Hg^{2+} and Cu^{2+}, required for the maximum activity of the enzyme, the enzyme was titrated with Hg^{2+} and Cu^{2+}. As shown in Fig.5 A and B, maximum activities of the enzyme were observed in molar ratio of Hg^{2+} to the enzyme of 10-12:1, and in a molar ratio of Cu^{2+} to the enzyme of 6-8:1, respectively.



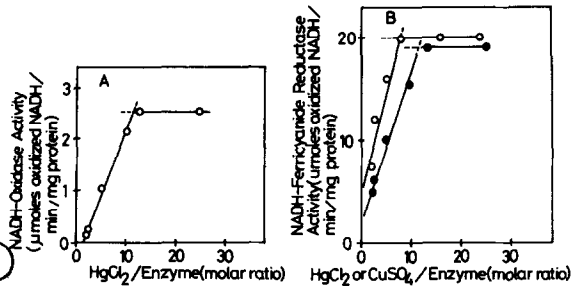


Fig. 4. Formation of hydrogen peroxide by NADH-oxidase reaction of Ascaris lipoamide dehydrogenase.

Fig. 5. Titration of Ascaris lipoamide dehydrogenase with Cu^{2+} or Hg^{2+}. A. Titration of NADH-oxidase activity with Hg^{2+}. B. Titration of NADH-ferricyanide reductase activity with Hg^{2+}(—●—) or Cu^{2+}(—○—).

Estimation of Number of SH Groups in Native and Denatured Lipoamide Dehydrogenase — As shown in Table II, the number of SH groups per one subunit was 0.3-0.4 for native enzyme and 1.5-1.7 for denatured enzyme, respectively. This observation suggests that SH groups in the native enzyme are present in the inside part of the enzyme and have the poor reactivity with thiol reagents. Ascaris lipoamide dehydrogenase contains four half-cystines[1]. Therefore, it appears that the lipoamide dehydrogenase contains two SH groups and one disulfide bond, because lipoamide dehydrogenase from pig heart and E.coli are known to have one disulfide bond in the active center[10].

Table II. Estimation of number of SH groups in native and denatured lipoamide dehydrogenase

Thiol Reagents		Thiol titer/One subunit
DTNB[a]	Native	0.3
	Denatured	1.5
p-CMB[b]	Native	0.4
	Denatured	1.7

a; 5,5'-Dithio-bis(2-nitrobenzoic acid), b; p-Chloromercuri-benzoic acid.

DISCUSSION

Although the treatments of Ascaris lipoamide dehydrogenase with Hg^{2+} and Cu^{2+} stimulate the reduction of the artificial electron acceptors with NADH, significant differences were observed between them. Hg^{2+} stimulated the reduction of oxygen, ferricyanide and 2,6-dichlorophenolindophenol, while Cu^{2+} stimulated the reduction of artificial dyes, but only a little the reduction of oxygen. According to Casola et al[2,3] phenylmercuri acetate enormously stimulates the reduction of 2,6-dichlorophenolindophenol in pig heart lipoamide dehydrogenase, but we observed that p-chloromercuri-benzoate only a little stimulates the reduction of artificial dyes or oxygen in Ascaris lipoamide dehydrogenase. Lipoamide dehydrogenase from pig heart contains 10 half-cystines in one subunit(51,000)[11]. Ascaris lipoamide dehydrogenase consists of two subunits and each(53,000) contains four half-cystines[1]. As shown in Table II, SH groups in Ascaris native lipoamide dehydrogenase have the very poor reactivity with thiol reagents, such as p-chloromercuribenzoate and 5,5'-dithiobis-(2-nitrobenzoic acid). As shown in Fig.3, 5-6 moles $HgCl_2$ per one subunit of the lipoamide dehydrogenase were required for the maximum activity, while 3-4 moles $CuSO_4$ were required. Although it is difficult to clearly explain the mechanism of the stimulating effect of Cu^{2+} and Hg^{2+} on the lipoamide dehydrogenase, the metals may modify amino acid residue(s) other than cystein or cystine, because larger amounts of metals are required for the maximum activity compared with SH groups in the enzyme.

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