Studies on Metmyoglobin Reducing Enzyme Systems in the Muscle of Blue White Dolphin—I Purification and Properties of Diaphorase

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Diaphorase was isolated from the skeletal muscle of blue white dolphin by DEAE-cellulose column chromatography, Sephadex G-100 gel filtration, starch block electrophoresis, etc. The diaphorase obtained showed an apparent homogeneity in both ultracentrifugal and electrophoretic analyses. Its sedimentation and diffusion coefficients were 6.28 S and 4.95×10^{-7} cm² sec⁻¹, respectively, and a molecular weight ($M_{s,d}$) of 120,000 was calculated. This enzyme was found to contain two moles of FAD per mole. NADH₂, but not NADPH₂, served as an electron donor. Reducing activities of dolphin diaphorase for metmyoglobin (MMb), dichlorophenol indophenol (DPIP), lipoamide, and lipoic acid were 0.55, 0.81, 11.5, and 1.20 U per mg protein, respectively. The Michaelis constants in these reactions were calculated to be 2.2×10^{-5} M, 1.3×10^{-5} M, 1.0×10^{-3} M, and 4.2×10^{-3} M in the above order, respectively. The lipoamide reducing activity was strongly inhibited by dithiol inhibitors. On the contrary, the MMb and DPIP reducing activities were activated by these inhibitors. Both activities were, however, effectively inhibited by PCMB. It was demonstrated that the MMb reducing activity is accelerated by lipoamide and lipoic acid to the same extent as by methylene blue.

After death of an animal, the color of its muscle gradually turns into brown due to the formation of metmyoglobin (MMb). MMb is hardly detected in the muscle of living animals, and it has been considered that there are some MMb reducing enzyme systems in the living muscle. It is the case with hemoglobin in the erythrocyte. The enzymes participating in methemoglobin (MHb) reduction such as diaphorase¹⁾ (EC 1. 6. 4. 3), methemoglobin reductase,^{2,3)} and ferrihemoglobin reductase⁴⁾ have already been isolated.

Recently, SHIMIZU *et al.*⁵⁾ have successfully isolated from the dolphin muscle "metmyoglobin reductase", an enzyme comparable to methemoglobin reductase. On the other hand, BODWELL *et al.*⁶⁾ and WATTS *et al.*⁷⁾ reported the detection of diaphorase in the bovine muscle, indicating its participation in the reduction of MMb. However, their finding was obtained by a qualitative method and seemed to require further examination, because it is thought that there are several kinds of enzyme having the characters similar to diaphorase in the muscle. Such situation aroused us to undertake the present study. This paper deals with the isolation and characterization of diaphorase from the muscle of blue white dolphin.

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Materials and Methods

Materials The skeletal muscle of blue white dolphin, *Stenella caeruleo-alba*, which was stored frozen below -20°C , was used for purification of the enzyme. MMb and MHb were prepared by the methods of SHIMIZU *et al.*³⁾ from the same animal. Ferricytochrome c (FerriCyt. c) was purchased from Sigma. All the other chemicals were obtained from the usual commercial sources. Concentrations of MMb and MHb, were determined spectrophotometrically, after conversion into the cyanmet form, and that of FerriCyt. c after conversion into the reduced form.

Analytical methods

Enzyme activity MMb reducing activity was assayed according to the method of SHIMIZU *et al.*³⁾ The reaction mixture contained 0.1 ml of 15 mM MMb (MMb or FerriCyt. c), 0.1 ml of 60 μM methylene blue, 1.5 ml of 0.2 M $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ (pH 7.2), 0.1 ml of enzyme solution, 1.1 ml of water and 0.1 ml of 15 mM NADH_2 in a total volume of 3 ml. The reaction was started by the addition of NADH_2 and the reduction of MMb was measured by the increase of absorbance at 582 nm (MHb: 577 nm, FerriCyt. c: 550 nm) with a Hitachi recording spectrophotometer model 124. Dichlorophenol indophenol (DPIP) reducing activity, lipoamide and lipoic acid reducing activity, and pyridine nucleotide transhydrogenase (EC 1. 6. 1. 1) activity were assayed by the methods of SAVAGE,⁸⁾ MASSEY,⁹⁾ and STEIN *et al.*¹⁰⁾, respectively. Incidentally, one enzyme unit (U) was defined here as the activity reducing 1 μmole of substrate per min.

Ultracentrifugation Ultracentrifugal analyses were performed with a Hitachi UCA-1 analytical ultracentrifuge at 12°C . The protein solution to be applied was previously dialyzed at 4°C against 0.2 μ $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$ (pH 7.0) containing 0.18 M NaCl. Sedimentation and diffusion runs were carried out at 60,000 and 12,400 rpm, respectively. Molecular weight determinations by the sedimentation equilibrium method were conducted using the same machine at 9,800 rpm.

Electrophoresis The starch block electrophoresis was carried out using a constant current of 20 mA per vessel ($5 \times 2 \times 30$ cm), for 15 hr at 4°C . Polyacrylamide disc electrophoresis was carried out by the method of ORNSTEIN¹¹⁾ and DAVIS¹²⁾ using 7.5% gel at a constant current of 3 mA per tube for 1.5 hr. SDS-polyacrylamide disc electrophoresis was conducted by the procedure of WEBER and OSBORN,¹³⁾ using bovine serum albumin, ovalbumin, cytochrome c, chymotrypsinogen A, and myoglobin as molecular markers. After electrophoretic runs, proteins were stained with amido black 10 B.

FAD content FAD was assayed according to the method of BEINERT and PAGE¹⁴⁾. Trichloroacetic acid was added to the enzyme solution up to 5% and centrifuged. The supernatant was adjusted to pH 7 with addition of crystalline Tris, and measured for the absorbance at 450 nm before and after reduction with a trace amount of dithionite. The

FAD content was calculated from the data obtained by use of the difference in molar extinction coefficient between the oxidized and reduced types of FAD, 10.3×10^3 .

Results and Discussion

Purification of the enzyme After preliminary examinations, the following procedures were adopted for purification of the enzyme. All the experimental operations were carried out around 4°C . The washed minced muscle was homogenized with 5 volumes of $0.02\text{ M Na}_2\text{HPO}_4$ for 2 min in a blender. The homogenate was centrifuged at $1,500 \times g$ for 10 min and the cloudy supernatant thus obtained was adjusted to pH 5.4 by addition of 1 N acetic acid. The precipitates formed were collected by centrifugation and suspended in cold 1% $(\text{NH}_4)_2\text{SO}_4$ (1 l per kg of washed mince) and homogenized for a few sec. After stranding overnight, the homogenate was centrifuged.

Ammonium sulfate fractionation The supernatant above obtained was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitates formed between 0.35 – 0.80 saturation were collected.

Sephadex G-100 gel filtration The precipitates dissolved in $0.05\text{ M Na}_2\text{HPO}_4$ – KH_2PO_4 (pH 7.0) and dialyzed overnight against the same buffer. The inner solution was applied to a Sephadex G-100 column ($5 \times 100\text{ cm}$) pre-equilibrated with the same buffer. In Fig. 1, a typical profile is presented for this gel filtration. Roughly, three peaks appeared in respect of DPIP activity, while three peaks also appeared in respect of lipoamide activity, the main peak mostly overlapping with the first peak of DPIP activity. Thus this peak was judged to be diaphorase. The fractions of the range indicated by arrows were combined and fractionated with $(\text{NH}_4)_2\text{SO}_4$ as described before.

DEAE-cellulose column chromatography The precipitates were dissolved in $0.02\text{ M Na}_2\text{HPO}_4$ – KH_2PO_4 (pH 7.2) and dialyzed against the same buffer. The inner solution was chromatographed on a DEAE-cellulose column ($2.5 \times 50\text{ cm}$) pre-equilibrated with the same buffer. For elution was used a linear gradient of phosphate buffer concentration

Fig. 1. Sephadex G-100 gel filtration of partially purified dolphin muscle diaphorase. Fractions shown by arrows were combined and further purified.

Fig. 2. Purification of dolphin muscle diaphorase by DEAE-cellulose column chromatography. Fractions shown by arrows were combined and further purified.

from 0.02 to 0.2 M. Fig. 2 shows an elution profile of this chromatography. Two peaks appeared in respect of the absorbance at 280 nm, one well coinciding with both lipoamide and DPIP activities. The fractions as indicated by arrows were combined, and concentrated by the precipitation with $(\text{NH}_4)_2\text{SO}_4$. The precipitates obtained were dissolved in 0.05 M Na_2HPO_4 - KH_2PO_4 (pH 7.5) and dialyzed against the same buffer.

Starch block electrophoresis The inner solution was applied to starch block electrophoresis in the same buffer as above, resulting in separation into two bands, one of which exhibited a strong fluorescence. The fluorescent band was scraped out and extracted with the same buffer. The diaphorase solution thus obtained was stored at -20°C until used for the following analyses.

Table 1. Purification of dolphin muscle diaphorase

Purification steps	Total activity* (U)	Specific activity* (U/mg protein)	Yield %
1% $(\text{NH}_4)_2\text{SO}_4$ extract	173	0.03	100
$(\text{NH}_4)_2\text{SO}_4$ fractionation (35–80% saturation)	103	0.05	59
Sephadex G-100 gel filtration	64	0.05	37
DEAE-cellulose column chromatography	64	0.60	37
Starch block electrophoresis	12	0.81	7

* DPIP reducing activity.

In Table 1 is shown the progress of purification of the enzyme. The enzyme preparation finally obtained showed a specific activity thirty times as high as that of the crude extract. However, the actual degree of purification could be much higher, since the extract is assumed to contain also some non-diaphorase enzymes having DPIP activity, as seen in Fig. 1. The yield at the final step was about 5 mg per 1 kg of skeletal muscle.

Physico-chemical properties

1. *Ultracentrifugation* The sedimentation pattern of the purified enzyme showed a symmetrical single peak with a sedimentation coefficient of 6.28 S (Fig. 3). The diffusion pattern showed also a symmetrical single peak with a diffusion coefficient of $4.95 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ (Fig. 4). These data are considered to show a high homogeneity of the enzyme preparation. Based on the above coefficients, the molecular weight of this enzyme was calculated as 123,000 assuming that its partial specific volume was 0.75. On the other hand, the molecular weight was determined to be 116,000 by the sedimentation equilibrium method. Thus, the molecular weight of this diaphorase is considered to be near 120,000. This value is rather comparable to the molecular weights of diaphorase from various sources: *e.g.*, 102,000–114,000 for pig heart,^{15,16)} 116,000 for human liver,¹⁷⁾ and 105,000 for bovine liver diaphorase.¹⁸⁾

Fig. 3. Sedimentation pattern of purified diaphorase. at 80 min after the top speed 60,000 rpm was reached at 12°C.

Fig. 4. Diffusion pattern of purified diaphorase at 80 min after the fixed speed of 12,400 rpm was attained at 12°C.

2. *Electrophoresis* Disc electrophoresis was performed at pH 8.3 and 3 mA for 90 min. As shown in Fig. 5, an apparent homogeneity was recognized. However, in SDS-disc electrophoresis one main and two very faint bands appeared. A molecular weight of 63,000 was obtained for the main band from the calibration curve. Hence, the enzyme possibly consists of two identical subunits.

Fig. 5. Polyacrylamide disc electrophoregram of purified diaphorase.

Fig. 6. Absorption spectrum of purified diaphorase.

3. *Absorption spectrum* As seen in Fig. 6, the enzyme showed three absorption maxima at 275, 355, and 455 nm and a shoulder around 480 nm. This type of absorption spectrum is of flavoprotein, and very similar to those from various sources. The ratio, $E_{355 \text{ nm}}/E_{455 \text{ nm}}$, was 0.98, well agreeing with 0.97 for pig heart diaphorase⁸⁾ but somewhat differing from 0.86 for human liver diaphorase.¹⁷⁾

4. *Flavin content* The flavin moiety which was liberated from the enzyme by a heat treatment at 80°C for 15 min, was concentrated *in vacuo* and subjected to paper chromatography using 5% Na_2HPO_4 as a solvent. The results are given in Fig. 7. The chromatogram showed a single spot under UV irradiation, the R_f value coinciding with that of the authentic FAD. The content of FAD was two moles per mole of the enzyme, agreeing to diaphorases hitherto reported.^{15,17,18)}

Fig. 7. Paper chromatograms of the prosthetic group of purified diaphorase, and the authentic FAD and FMN.

Enzymic properties

1. *Specific activity* The purified diaphorase showed a reducing activity for DPIP, lipoamide, and the three ferric hemoproteins examined here. The pyridine nucleotide transhydrogenase activity was also observed. The specific activities for various substrates are summarized in Table 2. As seen in the table, the enzyme is strictly specific for NADH_2 .

Table 2. Specific activity of purified diaphorase for various substrates

Substrates	pH	Coenzyme	Specific activity (U/mg protein)
MMb	7.2	NADH_2	0.55
		NADPH_2	0
MHb	"	NADH_2	0.50
		NADPH_2	0
FerriCyt. c	"	NADH_2	0.50
		NADPH_2	0
Lipoamide	6.0	NADH_2	11.5
		NADPH_2	0
Lipoic acid	5.6	NADH_2	1.20
		NADPH_2	0
Acetylpyridine-NAD	7.3	NADH_2	1.26
		NADPH_2	0
DPIP	7.2	NADH_2	0.81
		NADPH_2	0

NADPH₂ did not serve as the electron donor at all. The values for MMb, MHb, and FerriCyt. c were almost identical, 0.50–0.55 U per mg protein. The values for DPIP, lipoic acid, and lipoamide were 0.81, 1.20, and 11.5, respectively. The specific activity was 1.26 U per mg protein when acetylpyridine-NAD was used as the substrate. The reducing activities of this enzyme for various substrates are much lower than those of mammalian diaphorase.^{15,17,18)}

2. *Optimum pH* pH-Activity curves of the enzyme for various substrates are shown in Fig. 8. The MMb reducing activity increases with the rise of pH. In this connection, SCOTT *et al.*¹⁾ have reported that the MHb reducing activity of human erythrocyte diaphorase increases with the fall of pH. The optimum pH of lipoamide and lipoic acid reducing activities were near 6.0 and 5.6, respectively. The optimum pH for lipoamide is considerably lower than the values reported for pig heart¹⁶⁾ and human liver diaphorase,¹⁷⁾ although that for the lipoic acid reducing activity was roughly comparable to the results in those literature. On the other hand, the DPIP reducing activity linearly decreases with the ascent of pH, just like human erythrocyte diaphorase.¹⁾

Fig. 8. pH-Activity curves of purified diaphorase when MMb, lipoamide, lipoic acid, and DPIP were used as substrates.

3. *Michaelis constants* The effect of concentrations of various substrates on the reaction rate was examined. The apparent Michaelis constants (K_m) are shown in Table 3. The K_m values were 2.2×10^{-5} M and 1.3×10^{-5} M for MMb and DPIP, respectively, being much smaller than 1.0×10^{-3} M and 4.2×10^{-3} M for lipoamide and lipoic acid, respectively. Comparable values have so far been reported for mammalian diaphorases: *e. g.*, 5.0×10^{-3} M for lipoamide and 2.0×10^{-3} M for lipoic acid in pig heart diaphorase⁹⁾

Table 3. Michaelis constants of purified diaphorase for various substrates

Substrates	Accelerator	Km
MMb		$2.2 \times 10^{-5} \text{ M}$
NADH ₂		$1.3 \times 10^{-5} \text{ M}$
	methylene blue	$4.0 \times 10^{-5} \text{ M}$
Lipoamide		$1.0 \times 10^{-3} \text{ M}$
NADH ₂		$1.3 \times 10^{-5} \text{ M}$
Lipoic acid		$4.2 \times 10^{-3} \text{ M}$
NADH ₂		
DPIP		$1.3 \times 10^{-5} \text{ M}$
NADH ₂		$1.7 \times 10^{-5} \text{ M}$

and $1.33 \times 10^{-3} \text{ M}$ and $0.81 \times 10^{-3} \text{ M}$, respectively, in human liver diaphorase.¹⁷⁾ It is noteworthy that in dolphin diaphorase the Km for lipoamide is significantly smaller than that for lipoic acid, in contrast to the case of mammalian diaphorases. The Km values for NADH₂, $1.3\text{--}1.7 \times 10^{-5} \text{ M}$ were roughly identical irrespective of reaction systems. In the reduction of MMb, Km for methylene blue was $4.0 \times 10^{-5} \text{ M}$.

4. *Effect of inhibitors* Effects of various compounds on the enzyme activity were tested. As shown in Table 4, MMb and DPIP reducing activities were clearly activated by NADH₂. In contrast, lipoamide reducing and transhydrogenase activities were inhibited by NADH₂, in particular strongly in the presence of Cu and Cd. On the other hand, the former two activities were strongly inhibited by PCMB, and tended to be activated by

Table 4. Effects of various compounds on the reducing activities of diaphorase for several substrates

Compounds	Concentration (mM)	Relative activity (%)			
		MMb	Lipoamide	Acetylpyridine-NAD	DPIP
None	—	100	100	100	100
NADH ₂	0.2	108	61	48	153
Ateburin	0.25	95	100	104	96
" + NADH ₂	0.2	102	108	84	146
Cu ⁺⁺	0.25	76	13	20	204
" + NADH ₂	0.2	0	0	0	20
Cd ⁺⁺	0.25	139	88	100	170
" + NADH ₂	0.2	141	6	24	240
Arsenite	0.25	96	152	120	106
" + NADH ₂	0.2	102	117	74	159
DTNB*	0.25	107	100	110	100
" + NADH ₂	0.2	141	77	68	227
Ethylmaleimide	0.25	106	104	108	124
" + NADH ₂	0.2	106	55	50	114
PCMB	6×10^{-3}	24	98	103	23
Iodoacetoamide	0.5	102	150	114	134
" + NADH ₂	0.2	136	16	96	229

* 5, 5-dithiobis (2-nitrobenzoic acid)

dithiol inhibitors. These results are essentially the same as those with mammalian diaphorases.

5. *Acceleration of MMb reduction by lipoamide and lipoic acid* In the presence of NADH₂, dolphin diaphorase catalyzed the reduction of MMb. However, when methylene blue was added as an accelerator, the enzyme exhibited fifty times as high activity as that in the absence of methylene blue. When lipoamide or lipoic acid was added instead of methylene blue, the activity was similarly accelerated. With either lipoamide or lipoic acid, the optimum pH was 5.0. When lipoamide was used as an accelerator, the specific activity was 0.62 and 0.30 U per mg protein at pH 5.0 and 6.8 respectively. The latter activity is close to the level attained in the presence of methylene blue. On the other hand, the corresponding values were 0.43 and 0.03 when lipoic acid was used. The K_m value for lipoamide as accelerator at pH 6.8 was calculated to be 0.5×10^{-5} M. The value was much smaller than the value for methylene blue, indicating that lipoamide has a stronger affinity to the enzyme than does methylene blue. Lipoic acid and lipoamide might be natural accelerators which take the place of methylene blue.

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