Studies on Metmyoglobin Reducing Enzyme Systems in the Muscle of Blue White Dolphin—II Purification and Some Physico-chemical Properties of Ferrimyoglobin Reductase

Takashi MATSUI,*¹ Chiaki SHIMIZU,*² and Fumio MATSUURA*¹

(Received April 19, 1975)

Using a procedure essentially according to HEGESH's isolation method for human ferrihemoglobin reductase (FerriHb R), its corresponding enzyme, tentatively named ferrimyoglobin reductase (FerriMb R), was isolated in a highly pure state from the skeletal muscle of blue white dolphin, and compared physico-chemically with the FerriHb R isolated from the erythrocytes of the same animal. The sedimentation and diffusion coefficients were 3.70 S and $6.67 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$, respectively, for the FerriHb R, and 3.91 S and $5.85 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$, for the FerriMb R. The molecular weights, $M_{s, D}$ of the FerriHb R and FerriMb R were 51,000 and 65,000, and M_e were 61,000 and 64,000, respectively. Their pH-mobility curves also differed from each other. These two reductases were, however, hardly distinguishable from each other, both in possessing an isoelectric point near pH 5 and in exhibiting absorption maxima at 279 and 407 nm.

As the enzymes participating in metmyoglobin (MMb) reduction in the mammalian erythrocytes, diaphorase, methemoglobin reductase, and ferrihemoglobin reductase (FerriHb R) have already been isolated.¹⁻³⁾ In the preceding paper,⁴⁾ we reported the isolation and characterization of diaphorase from the dolphin muscle. On the other hand, SHIMIZU *et al.* have isolated metmyoglobin reductase, an enzyme corresponding to methemoglobin reductase, from the same source.⁵⁾

This time we searched for the enzyme corresponding to erythrocyte FerriHb R in the dolphin muscle, and succeeded in its isolation by employing HEGESH's method for human FerriHb R. The enzyme obtained, tentatively named ferrimyoglobin reductase (FerriMb R), was analyzed for physico-chemical properties and compared with those of FerriHb R isolated from the erythrocytes of the same animal.

Materials and Methods

Materials The blood and skeletal muscle used were obtained from a live blue white dolphin, *Stenella caeruleo-alba*, caught at Huto Bay in Shizuoka Pref. Immediately after quick transportation to our laboratory with ice, red cells were separated by centrifugation,

*¹ Lab. of Marine Biochem., Fac. of Agr., The Univ. of Tokyo, Bunkyo-ku, Tokyo (松居 隆・松浦文雄: 東大農学部)

*² Fisheries Lab., Fac. of Agr., The Univ. of Tokyo, Shizuoka Pref. (清水千秋: 東大農学部附属水産実験所)

washed several times with saline, and kept frozen below -20°C until used. Muscle blocks were provided and similarly kept frozen. The substrate pigments and other reagents were the same as described in the previous paper.⁴⁾

Analytical methods

Enzyme activity The assay was performed by the method of HEGESH *et al.*⁶⁾ with slight modifications. The reaction mixture contained 0.1 ml of 15 mM MMb, 0.1 ml of 3 mM ferrocyanide, 1.5 ml of 0.2 M $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$ (pH 5.2), 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 addition of NADH_2 and the reduction of MMb was measured by the increase of absorbance at 582 nm with a Hitachi recording spectrophotometer model 124. 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 Electrophoretic analyses were performed with a Hitachi Tiselius electrophoresis apparatus model D under a constant current of 5 mA at 4°C . A 1.3 ml cell, and Miller and Golder's buffers⁷⁾ (0.2 μ , pH 5.5 to 8.5) were used.

Spectrophotometry A Hitachi recording spectrophotometer model 124 was employed. In calculation of the specific activity of both enzymes, the protein concentration as determined by the biuret method was used.

Results and Discussion

Purification of FerriHb R The method of HEGESH *et al.* was adopted with several modifications as follows. All the operations were conducted around 4°C .

Extract To the frozen red cells was added two volumes of cold water and the mixture was adjusted to pH 7.0. The lysate was centrifuged and the clear red supernatant was diluted with 5 volumes of cold water and adjusted again to pH 7.0.

DEAE-cellulose adsorption The DEAE-cellulose (200 g on dry basis per litre of wet red cells) pre-equilibrated with 5 mM $\text{Na}_2\text{HPO}_4-\text{KH}_2\text{PO}_4$ (pH 7.0) was added batchwise to the above extract and the mixture was left standing for 30 min under sufficient stirring. The DEAE-cellulose was collected and washed with the same buffer until the washing was colorless. The protein was eluted from the DEAE-cellulose with 50 mM citrate buffer (pH 5.5) containing 60 mM KCl. To the eluate, solid ammonium sulfate was added up to 100% saturation. The precipitates formed were collected by filtration, dissolved in a

small volume of 20 mM $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$ (pH 5.2) containing 15 mM KCl, and dialyzed overnight against the same buffer. By this treatment, the hemoglobin present in the extract was for the most part eliminated.

CM-cellulose column chromatography The dialyzed solution was applied onto a column (5×60 cm) of CM-cellulose which was pre-equilibrated with 3.75 mM $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$ (pH 5.2) containing 15 mM KCl. The adsorbed protein was eluted stepwise with the above acetate buffer containing KCl at 15, 65, 115, and 165 mM, as indicated by arrows in Fig. 1. The active fractions as indicated by arrows were combined and saturated with ammonium sulfate. The precipitates obtained were collected and dissolved in 0.05 M $\text{Na}_2\text{HPO}_4-\text{KH}_2\text{PO}_4$ (pH 7.0) and dialyzed against the same buffer.

Sephadex G-100 gel filtration The dialyzed solution was passed through a Sephadex G-100 column (2.5×100 cm) pre-equilibrated with the above phosphate buffer. Fig. 2 shows a typical elution profile of the gel filtration. The peak in respect of MMb reducing activity appeared after the peak in respect of the absorbance at 280 nm, both peaks partly

Fig. 1. Purification of dolphin erythrocyte FerriHb R by CM-cellulose column chromatography. Fractions shown by arrows were combined and further purified.

Fig. 2. Purification of FerriHb R by Sephadex G-100 gel filtration. Fractions shown by arrows were combined and further purified.

Fig. 3. Purification of FerriHb R by the second Sephadex G-100 gel filtration. Fractions shown by arrows were combined and analyzed.

overlapping each other. The active fractions as indicated by arrows were combined and subjected again to the same gel filtration. As seen in Fig. 3, both peaks coincided satisfactorily this time. The active fractions as shown by arrows were combined, concentrated by ultrafiltration using a collodion bag, and stored at -20°C until subjected to the following analyses.

Purification of FerriMb R After the preliminary examinations, essentially the same procedure as that for FerriHb R was adopted for purification of FerriMb R. The frozen dolphin muscle was thawed, minced, and homogenized with 5 volumes of cold water for 2 min with a blender. The homogenate was centrifuged at $8,000 \times g$ for 15 min and the clear extract obtained was adjusted to pH 7.0 with 1 N NaOH. The solution was subjected to DEAE-cellulose adsorption, CM-cellulose column chromatography, and Sephadex G-100 gel filtration in the same way as described for purification of the FerriHb R. Figs. 4, 5, and 6 show typical chromatogram or gel filtration patterns at these steps. The FerriMb R solution thus obtained was concentrated by ultrafiltration and stored at -20°C

Fig. 4. Purification of FerriMb R by CM-cellulose column chromatography. Fractions shown by arrows were combined and further purified.

Fig. 5. Purification of FerriMb R by Sephadex G-100 gel filtration. Fractions shown by arrows were combined and further purified.

Fig. 6. Purification of FerriMb R by second Sephadex G-100 gel filtration. Fractions shown by arrows were combined and analyzed.

Table 1. Purification of FerriHb R

Purification steps	Total activity* (U)	Specific activity* (U/mg protein)	Yield (%)
Hemolyzed solution	31	0.00014	100
DEAE-cellulose adsorption	248	0.11	801
CM-cellulose column chromatography	259	2.22	836
Sephadex G-100 gel filtration	110	4.59	356

* MMb reducing activity was used.

Table 2. Purification of FerriMb R

Purification steps	Total activity* (U)	Specific activity* (U/mg protein)	Yield (%)
Water extract	2,300	0.0165	100
DEAE-cellulose adsorption	632	0.070	27
CM-cellulose column chromatography	164	1.54	7
Sephadex G-100 gel filtration	128	3.84	6

* MMb reducing activity was used.

until analyzed.

In Tables 1 and 2 are summarized the processes of purification including the changes in total and specific MMb reducing activities for both reductases. The specific activity of the FerriHb R finally obtained was about 30,000 times as high as that of the hemolyzate. A considerable increase in its total activity after DEAE-cellulose adsorption, seems to suggest the presence of some inhibitor or the substance disturbing the activity assay in the hemolyzate. On the other hand, the specific activity of the FerriMb R was 230 times as high as that of the aqueous extract. The specific activities of both enzymes did not differ so widely. The yield of FerriHb R was approximately 10 mg per litre of wet red cells, while that of FerriMb R 10 mg per kg of minced muscle.

Table 3. Physical properties of purified FerriHb R and FerriMb R

		FerriHb R	FerriMb R
Sedimentation coeff.	$s_{20, w}$ (S)	3.70	3.91
Diffusion coeff.	$D_{20, w}$ ($\times 10^{-7}$ cm ² sec ⁻¹)	6.67	5.85
Molecular weight	$M_{s, D}$	51,000	65,000
" "	M_e	61,000	64,000

The enzymes here obtained from the erythrocytes and the muscle of dolphin exhibited a single symmetrical peak in both sedimentation and diffusion patterns, indicating an apparent homogeneity. In Table 3 are summarized some physical properties of both enzymes. Sedimentation coefficients of the FerriHb R and FerriMb R were determined to be 3.70 and 3.91 S, respectively. Their diffusion coefficients were 6.67 and 5.85×10^{-7} cm² sec⁻¹, respectively. On the basis of those sedimentation and diffusion coefficients, the molecular weights of dolphin FerriHb R and FerriMb R were calculated to be 51,000 and 65,000, respectively. On the other hand, the values of 61,000 and 64,000 were obtained by the sedimentation equilibrium method for the FerriHb R and FerriMb R, respectively. Thus, the molecular weights of both enzymes seem to be somewhat different from each other. In this connection, SUGITA *et al.*⁸⁾ have reported a sedimentation coefficient of 2.77 S and a molecular weight of 28,000 for human FerriHb R. Hence, there seems to be a marked difference between the two enzymes from dolphin and human erythrocytes.

Both enzymes of dolphin behaved homogeneously in Tiselius electrophoresis over the pH range covered. As shown in Fig. 7, their pH-mobility curves clearly differed from each other. However, their isoelectric points were both near pH 5. SUGITA *et al.*⁸⁾ have reported a value of pH 6.2 for human FerriHb R.

As given in Fig. 8, the absorption spectra of both dolphin enzymes were very similar, showing a large maximum at 279 nm and a small and dull maximum at 407 nm. These spectra are hardly distinguishable from that of human FerriHb R.⁸⁾ The specific extinction, $E_{279 \text{ nm}}^{1\%}$, was calculated to be 11.6 and 10.6 for dolphin FerriHb R and FerriMb R, respectively.

Fig. 7. pH-Mobility curves of dolphin FerriHb R and FerriMb R.

Fig. 8. Absorption spectra of dolphin FerriHb R and FerriMb R.

The two enzymes obtained from the different origins of dolphin are by no means an identical enzyme. Their resemblance in various physico-chemical properties, however, might indicate the presence of organ specificity in one and the same enzyme. On the basis of the results obtained in the present investigation, it could be concluded that metmyoglobin reductase, diaphorase, and FerriMb R at least are participated in the reduction of MMb in the muscle.

This work was supported in part by a grant from the Ministry of Education.

References

- 1) E. M. SCOTT and J. C. MCGRAW: *J. Biol. Chem.*, **237**, 249-252 (1962).
- 2) C. SHIMIZU and F. MATSUURA: *Agr. Biol. Chem.*, **32**, 587-592 (1968).
- 3) E. HEGESH and M. AVRON: *Biochim. Biophys. Acta*, **146**, 91-101 (1967).
- 4) T. MATSUI, C. SHIMIZU, and F. MATSUURA: *This Bull.*, **41**, 761-769 (1975).
- 5) C. SHIMIZU and F. MATSUURA: *Agr. Biol. Chem.*, **35**, 468-475 (1971).
- 6) E. HEGESH and M. AVRON: *Biochim. Biophys. Acta*, **146**, 397-408 (1967).
- 7) G. L. MILLER and R. H. GOLDER: *Arch. Biochem.*, **29**, 420-427 (1950).
- 8) Y. SUGITA, S. NOMURA, and Y. YONEYAMA: *J. Biol. Chem.*, **246**, 6072-6078 (1971).