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Biliverdin Reductase from the Liver of Atlantic Salmon (*Salmo salar*)

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Abstract—Biliverdin reductase was characterized and purified from the liver of Atlantic salmon (*Salmo salar*) using a novel enzymatic staining method. The properties of the enzyme are quite different from those of mammals. The purified enzyme is a monomeric protein with a molecular weight of approximately 68 kD and an isoelectric point of around 3.8. The enzyme can utilize both NADH and NADPH as coenzyme, but the kinetic properties of the NADH-dependent and the NADPH-dependent enzyme activities are different: K_m value for biliverdin IXα is 0.6 μM in the NADPH system, while it is 6.8 μM in the NADH system. Both enzyme activities are inhibited by excess biliverdin IXα, but the NADPH-dependent enzyme activity is far more susceptible. The optimum pH for activity is 5.5 with NADPH and 6.0 with NADH. The optimum reaction temperature is 35°C.

Key words: biliverdin reductase, biliverdin, bilirubin, heme degradation, salmon

Biliverdin reductase is an important enzyme in heme metabolism. Biliverdin must be reduced to bilirubin by biliverdin reductase and is then excreted, although biliverdin is the final product of physiological heme degradation in mammals [1-3]. In practice, biliverdin reductase is used to obtain bilirubin from biliverdin [3-6].

So far biliverdin reductase has been mostly studied in mammals [3, 7, 8] since the activity of biliverdin reductase was insignificant or even not detectable in fish, amphibia, reptiles, and birds [9-11]. However, by using a modified assay, the activity of biliverdin reductase was found to exist in many marine fish [12, 13]. Biliverdin can be directly purified from fish as well as amphibia, reptiles, and birds [6, 14]. If biliverdin reductase used in the reduction of biliverdin is isolated from a related animal, the reduction of biliverdin may be more effective.

Biliverdin has been purified from Atlantic salmon in our laboratory. To get more effective reduction of biliverdin, biliverdin reductase was also purified from the liver of Atlantic salmon (*Salmo salar*) using a novel enzymatic staining method. This study presents the characterization and purification of biliverdin reductase from the liver of Atlantic salmon.

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MATERIALS AND METHODS

Reagents. Biliverdin IXα was purified from the bile of Atlantic salmon [6]. Bilirubin, NADH, NADPH, NAD^+, bovine serum albumin (BSA), DEAE cellulose, and Sephadex G-100 were from Sigma (USA). A molecular weight calibration kit was bought from Pharmacia (Sweden). All other reagents were of analytical grade.

Fresh liver of salmon. Fifty Atlantic salmons with live weight of approximately 2 kg each were sacrificed by cutting heads or anaesthetized using tricaine methanesulfonate (MS 222, 0.42 g/liter). Livers were excised, rinsed in ice-cold saline and perfused with fresh cold saline to clean their blood. The livers were blotted dry and weighed for using immediately or storing at −80°C.

Isolation of biliverdin reductase from the liver of salmon. Biliverdin reductase was prepared according to the modified method of Ding and Xu [3], and all operations were carried out at 4°C. Briefly, 500 g of liver were homogenized in a blender with 4 volumes of cold 0.02 M potassium phosphate buffer with 0.134 M KCl, 0.1 mM EDTA, pH 7.0. The homogenate was treated for 2 min by sonication (W-375, Ultrasonics Inc.) and centrifuged at 56,000*g* for 2 h. The pellet and lipid clumps were discarded.

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Solid ammonium sulfate was added to the supernatant to 30-70% saturation with stirring, after which it was centrifuged at 8,000*g* for 30 min. The precipitate was dissolved in a small volume of 1 mM potassium phosphate buffer with 0.1 mM EDTA, pH 6.5, and centrifuged at 12,500*g* for 10 min to remove denatured proteins. The supernatant was dialyzed against 10 volumes of the same buffer, which was changed every hour for 6 h. This fraction was called the solid ammonium sulfate fraction (SASF).

The dialyzed solution was loaded on a column of Sephadex G-100 (5 × 100 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The column was eluted with the same buffer at a flow rate of 0.5 ml/min and collected with a fraction collector. The eluate that showed the enzyme activity was pooled and called the Sephadex G-100 fraction (SGF).

The SGF was passed through a DEAE-cellulose column (2.5 × 45 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The column was washed with 200 ml of the same buffer and eluted with a 400 ml linear gradient of 20-250 mM potassium phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min. The eluate was collected with a fraction collector, and the enzyme activity emerged in the early portion of the gradient. The fractions that showed biliverdin reductase activity were combined and concentrated by ultrafiltration (molecular sieve at 10 kD). This fraction was called the DEAE-cellulose fraction (DCF).

Purification of biliverdin reductase. The DCF was subjected to disc electrophoresis (7.5% polyacrylamide gel, Tris-glycine buffer, pH 8.3, 4 mA/rod). After finishing the run, the gel rods (0.5 × 8.0 cm) were immediately soaked in test tubes containing 0.08 M phosphate buffer, pH 6.28, with 50 μM biliverdin IXα and 1 mM NADH. Thirty minutes later, the gel was taken out and a faint yellow band (where enzyme reaction took place) would appear on the green color stained rod. The yellow band was cut off and the enzyme protein in it was eluted. This procedure is called enzymatic-staining method and can be repeated to achieve pure sample if the pH of the running buffer did not change to 7.0.

The purified enzyme preparation could be stored in 50 mM potassium phosphate buffer, pH 7.0, at –20°C, for eight months without loss of activity.

Determination of molecular weight and purity of biliverdin reductase. To assay the purity of the biliverdin reductase preparations, the relative optical densities of the protein bands on the gel were measured with a Hitachi (Japan) gel scanner, recording the absorbance difference at 550 and 400 nm after the gel was stained with Coomassie blue.

The molecular weight of the biliverdin reductase subunit was estimated by 10% polyacrylamide gel electrophoresis in the presence of SDS according to the method of Weber et al. [15]. The proteins used as standards for calibrating molecular weight were: phosphorylase *b* (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), α-lactalbumin (14.4 kD).

Assay of biliverdin reductase activity. The activity of biliverdin reductase from the liver of Atlantic salmon was determined according to the modified method of Ding and Xu [6]. Briefly, a cuvette was placed in a constant temperature (37°C) chamber of a Hitachi 200-20 double-beam spectrophotometer (Japan). The reaction was conducted in the cuvette under dim light. The standard assay mixture with final volume of 2 ml contained 0.1 M potassium phosphate buffer (pH 7.0), 10 μM biliverdin IXα, 1 mg/ml bovine serum albumin (BSA), 100 μM reduced pyridine nucleotide, and an appropriate amount of the enzyme preparation. Reduced pyridine nucleotide was omitted in the reference cuvette. The reaction was started by the addition of reduced pyridine nucleotide after preincubation for 5 min at 37°C. The enzymatic conversion of biliverdin to bilirubin was monitored in terms of the increase in absorbance at 468 nm.

One unit of biliverdin reductase was defined as the amount of the enzyme which catalyzed the reduction of 10 nmol of biliverdin in 1 min under the standard assay conditions.

Stoichiometry of biliverdin reductase reaction. Stoichiometry of biliverdin reductase reaction was measured according to the method of Ding and Xu [6].

Determination of protein. Protein was determined according to the method of Bradford [16] using bovine serum albumin (BSA) as a standard.

Estimation of isoelectric point. Isoelectric point (p*I*) is the pH where an amphipath, such as protein, is neutrally charged and usually precipitates out from aqueous solution under this condition. Therefore, a partially purified enzyme sample was incubated in different pH solution (range 2.5-6.0) and then centrifuged to separate the supernatant and the precipitate. The precipitate was redissolved in a pH 6.5 buffer to examine its enzyme activity. All the supernatants were also tested for enzyme activity after the pH was adjusted to pH 6.0-7.0.

Measurement of biliverdin reductase K_m. There was approximately 150 μg of protein in the assay solution. Either 5-100 μM of NADPH or 100-1000 μM of NADH was introduced into the assay solution to trigger the enzymatic reaction. The results were plotted by the Lineweaver–Burk method to obtain the K_m for NADH or NADPH.

In the test of K_m for biliverdin, 0.2-1.2 μM of biliverdin IXα was used to react with 50 μM NADPH and 1-10 μM of biliverdin IXα with 1 mM NADH.

RESULTS AND DISCUSSION

K_m of biliverdin reductase. To ensure that the reaction is linear during the initial 1 min and avoid possible sub-

strate inhibition, low biliverdin IXα concentrations and relatively small amounts of the enzyme were used to determine the K_m of biliverdin reductase from the liver of Atlantic salmon. The K_m for NADPH and NADH was 5 and 545 μM, respectively. The K_m for biliverdin IXα reacted with NADPH and with NADH was 0.6 and 6.8 μM, respectively. These properties are different from those of the biliverdin reductase from mammals. The K_m of biliverdin IXα for the biliverdin reductase from cow spleen was 0.4 μM in the NADPH system and 1.5 μM in the NADH system [3]. For the biliverdin reductase from rat liver, the K_m for biliverdin was 3.0 μM with NADPH and 5.0 μM with NADH; the K_m was 3.0 μM for NADPH and 270 μM for NADH [7]. By contrast, Colleran and O'Carra [11] reported that there was no significant difference between NADPH and NADH for the reaction of biliverdin reductase from the liver of perch, *Perca fluviatilis*. These differences may be attributed to the different kinds of animals.

Inhibition of biliverdin reductase by excess biliverdin. The activity of the biliverdin reductase from mammals was inhibited by excess biliverdin [3, 7]. The same effect occurred in biliverdin reductase from the liver of Atlantic salmon, and the inhibition concentrations were also similar. The reaction with NADPH was markedly inhibited by 4 μM biliverdin IXα, whereas the reaction with NADH was inhibited over 18 μM (Fig. 1). Ding and Xu [3] explained the difference as follows: a dead-end ternary complex consisting of enzyme, biliverdin, and oxidized nucleotide may be formed to cause inhibition in the biliverdin reductase reaction. This would occur more readily in the reaction with NADPH because $NADP^+$ has a higher affinity for biliverdin reductase than NAD^+.

Molecular weight and isoelectric point of biliverdin reductase. When the finally purified biliverdin reductase preparation was run on SDS-polyacrylamide gel, the distinct major band showed a monomeric protein with an apparent molecular weight of around 68 kD (Fig. 2). This is different from that of the analogous enzymes purified from mammals, which are monomeric proteins with a molecular weight about 34 kD [3, 7].

When biliverdin reductase from the liver of Atlantic salmon was examined in a series of pH buffers, the isoelectric point (p*I*) of the enzyme was approximately 3.8. This is very different from that of biliverdin reductase from cow spleen, for which p*I* was about 6.2 [3].

Optimum pH and temperature of biliverdin reductase. The optimum pH of biliverdin reductase from the liver of Atlantic salmon was 6.0 in the reaction with NADH and 5.5 with NADPH (Fig. 3). These are different from those of the analogous enzyme from mammals. The optimum pH of biliverdin reductase from cow spleen was 7.0 with NADH and 6.8 with NADPH [3]. That of rat liver was 7.0 with NADH and 8.6 with NADPH [7]. The optimum pH of the biliverdin reductases from mammals is neutral or slightly alkaline, that is, the expected pH range of an

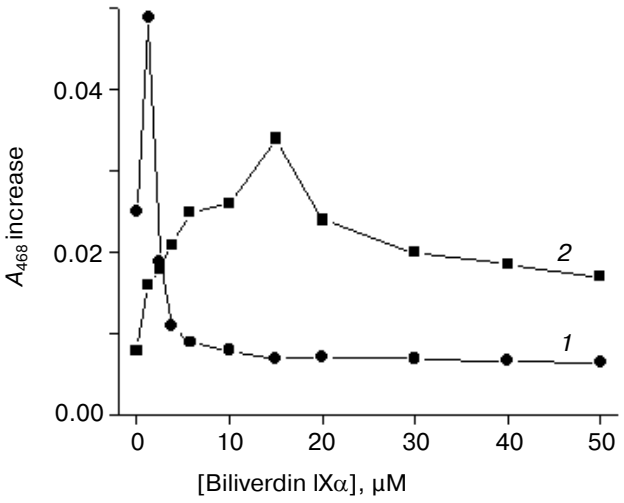

Fig. 1. Inhibition of biliverdin reductase from the liver of Atlantic salmon by excess biliverdin IXα. Concentrations: *1*) 50 μM NADPH; *2*) 1 mM NADH.

enzyme reaction. However, the optimum reaction pH of biliverdin reductase from the liver of Atlantic salmon is slightly acid.

The optimum reaction temperature of biliverdin reductase from Atlantic salmon was 35°C (Fig. 4) although Atlantic salmon lives in water bellow 20°C. Bend and James [17] pointed out that with marine species the *in vitro* temperature optima are often sub-

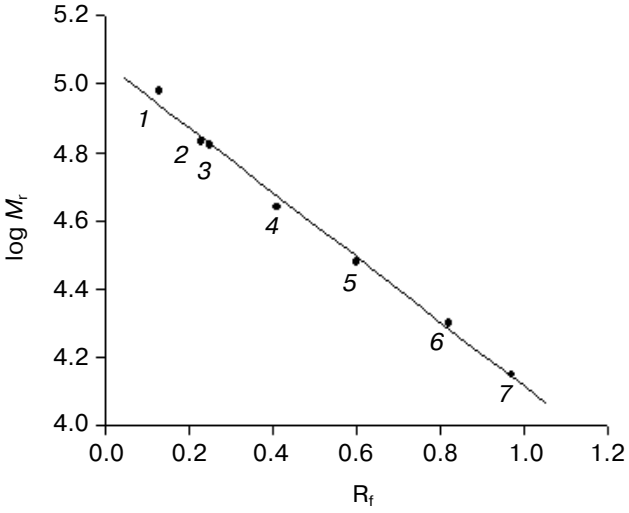

Fig. 2. Estimation of molecular weight for biliverdin reductase from the liver of Atlantic salmon. Protein standards: phosphorylase *b* (94 kD) (*1*), biliverdin reductase (~68 kD) (*2*), albumin (67 kD) (*3*), ovalbumin (43 kD) (*4*), carbonic anhydrase (30 kD) (*5*), trypsin inhibitor (20.1 kD) (*6*), α-lactalbumin (14.4 kD) (*7*).

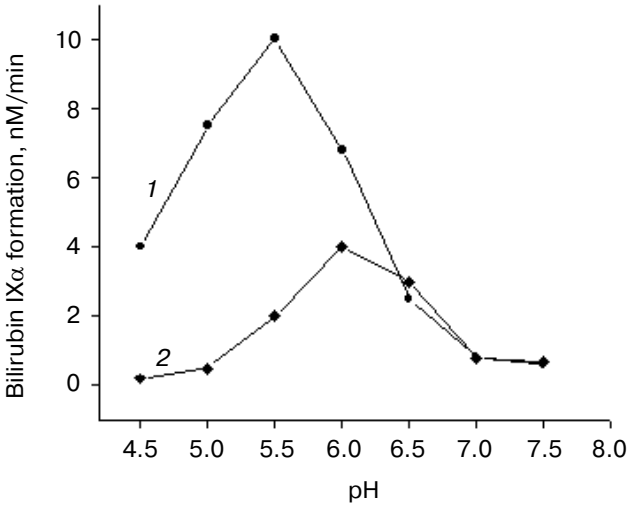

Fig. 3. Optimum pH of biliverdin reductase from the liver of Atlantic salmon. Concentrations: *1*) 50 μM NADPH; *2*) 1 mM NADH.

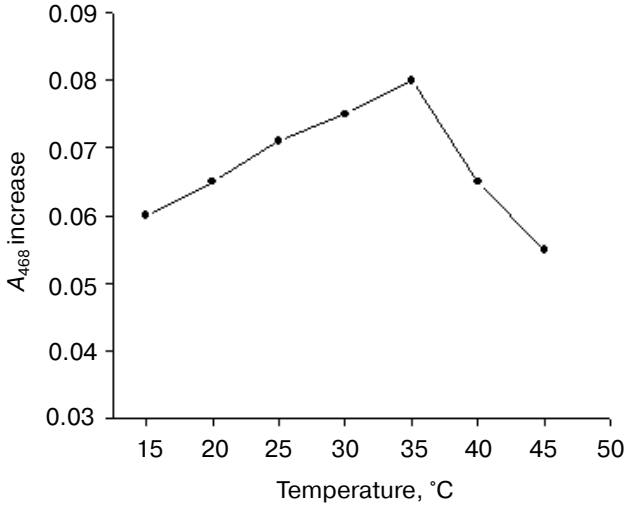

Fig. 4. Optimum temperature of biliverdin reductase from the liver of Atlantic salmon with 50 μM NADPH.

stantially greater than the environmental temperatures to which the fish are acclimated. Thus, temperature optima for aniline hydroxylase, benzo(a)pyrene hydroxylase, benzphetamine N-demethylase, and 7-ethoxycoumarin-*o*-deethylase activities occur near 30°C in hepatic microsomes from the little skate, *Raja erinacea*, even though sea temperature is only 12-15°C. Hepatic microsomes from the sheepshead, *Archosargus probatocephalus*, and the Atlantic stingray, *Dasyatis sabina*, acclimated to Florida water temperature in excess of 20°C, did not show any decrease in *in vivo* mixed-function oxidase (MFO) activities until temperature was elevated to 40°C [17]. Since *in vivo* metabolic rates are substantially lower than expected by extrapolation from *in vitro* studies at optimal temperatures, hepatic microsomal MFO activity in selected marine fish with several substrates were determined at temperature between 30-35°C [17], as well as most of fish enzymatic reactions were incubated at 30°C [18-20]. In addition, temperature has been known to affect enzyme activity and enzyme activity increases with increasing temperature under certain conditions [21-23].

Time course of biliverdin reductase reaction. When NADPH was used as coenzyme, the biliverdin reductase from Atlantic salmon completed the reaction in approximately 80 min under standard assay condition. The enzyme took much longer time to complete the reaction compared to the analogous enzymes from mammals [3, 24] in that the biliverdin reductases completed the reactions within 15 min. This may be attributed to Atlantic salmon living in the cold environment and usually the enzymes react slowly at lower temperature in the sea. Under the experimental conditions, the biliverdin reductase would have longer time to fit and to complete the reaction.

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