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Identification of a new *b*-type cytochrome from the whitefish *Coregonidae* eggs

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A new *b*-type cytochrome, termed cytochrome b_{560} according to the wavelength maximum of its α-band in the reduced minus oxidized difference spectrum, was isolated from eggs of whitefish and partially purified by fractionation of the water-soluble moiety of yolk. The absolute absorption spectrum of reduced cytochrome b_{560} has maxima at 426, 529 and 560 nm and that of the oxidized form at 416 nm. The reduced minus oxidized difference spectrum has maxima at 428, 529 and 560 nm. The midpoint potential of this cytochrome is +193 mV. Based on the MCD spectra of reduced cytochrome and the optical absorption spectra in the visible region of the oxidized cytochrome, it is suggested that the heme iron in cytochrome b_{560} has two histidine imidazoles as the 5th and 6th axial ligands.

Cytochrome b_{560}; Yolk of egg; Whitefish

1. INTRODUCTION

It is known that the pigmentation of eggs of most fish species at early stages of development is determined by the presence of carotenoids and lipids [1]. Investigation of the pigment composition of eggs of different fish species revealed a hemoprotein with spectral characteristics of the *b*-type cytochrome [2]. Further studies proved that the hemoprotein occurred only in eggs of whitefish and was a biochemical marker for the *Coregonidae* family [3].

In this paper we report the results of partial purification of a new *b*-type cytochrome obtained from eggs of whitefish and discuss some of its properties in comparison with other *b*-type cytochromes.

2. MATERIALS AND METHODS

Mature unfertilized eggs of *Coregonus lavaretus* from lakes Issyk-Kul and Sevan were washed from the blood and cavital fluid, frozen and stored at −20°C.

All purification steps were performed at 4°C. The eggs (10 g) were homogenized in 50 mM phosphate buffer, pH 6.5 and centrifuged at 22,000 × *g* for 40 min. The supernatant (5 ml) was chromatographed on a Sephadex G-75 column (1.6 × 50 cm) equilibrated with the same buffer. The fractions with cytochrome *b* were pooled, concentrated to a minimal volume by ultrafiltration through an Amicon UM-10 membrane and applied to a Sepharose 6B-CL column (1.6 × 150 cm). The fractions having the highest cytochrome *b*/protein ratio were pooled, concentrated and separated by gel filtration on Sephadex G-75 as described above.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [4]. Spectral measurements were made with a Hitachi-557 spectrophotometer. MCD measurements were made in a dichrograph constructed by Dr. A. Arutjunjan interfaced to an IBM PC and equipped with an electromagnet (magnetic field, 1 T for a 1 cm cell). The traces in the direct and reversed magnetic field direction were subtracted from each other to eliminate the contribution of CD. Protoheme was determined by the formation of alkaline pyridine hemochrome according to Folk [5]. The porphyrin obtained from the heme of the cytochrome was analysed by HPLC on a Brucker chromatograph with a Spheriosorb ODS RP18 column in the acetonitrile/1 M ammonium acetate/methanol (10:45:45) system. The redox potential of cytochrome was measured by registering the difference (red-ox) spectra of cytochrome at room temperature in the presence of dichlorophenolindophenol (DCPIP) as a redox indicator. The standard redox potential of DCPIP was taken to be +217 mV.

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3. RESULTS

To determine cytochrome localization in the eggs, the homogenate was centrifuged at 22,000 × *g* for 1 h. The resultant fractions of fat drops, the water-soluble moiety of yolk and embryo cells and egg envelopes were spectrally examined. Cytochrome *b* was only found in the water-soluble fraction of yolk. Centrifugation of the water-soluble fraction at 144,000 × *g* for 3 h did not alter the cytochrome *b* concentration in the supernatant. Thus, cytochrome b_{560} seems to be a water-soluble hemoprotein.

The absolute absorption spectra of the reduced and oxidized forms of the most-purified cytochrome b_{560} are shown in Fig. 1. The spectrum of the reduced form has maxima at 426, 529 and 560 nm and that of the oxidized form at 416. Less pronounced peaks at 529 and 560 nm are also seen in the spectrum of the oxidized form.

The absolute absorption spectra of the oxidized forms of cytochrome b_{560} and, for comparison, of the bovine cytochrome *c* in the region of the 695 nm charge

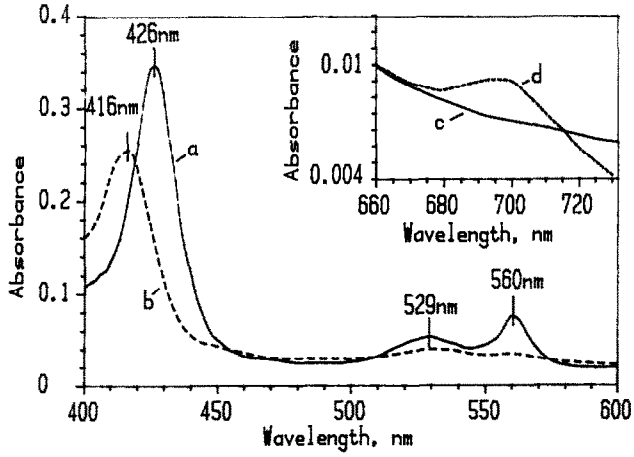

Fig. 1. Absorption spectra of partially purified cytochrome b_{560} in 50 mM phosphate buffer pH 6.5 at 20°C. The reduced form (a) was prepared by the addition of crystalline dithionite, and the oxidized one (b) by the addition of 0.1 mM H_2O_2. Inset: absorption spectra of the oxidized forms of cytochrome b_{560} (c) and bovine cytochrome c (d) in the region of a band at 695 nm.

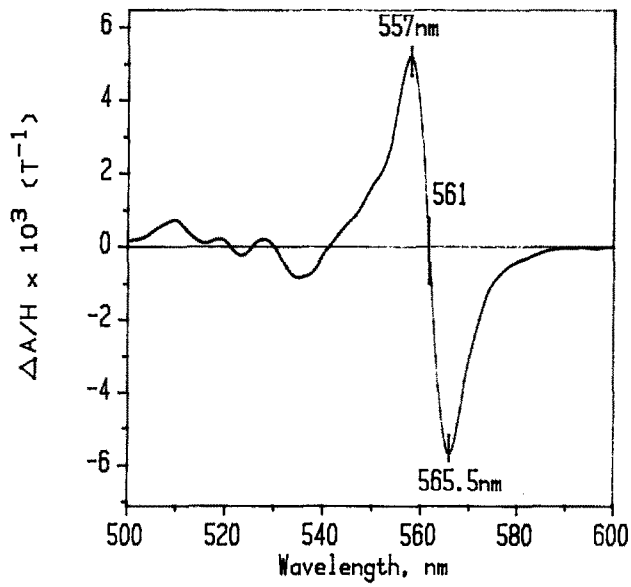

Fig. 3. MCD spectrum of dithionite reduced cytochrome b_{560} in 50 mM phosphate buffer pH 6.5 at 20°C.

transfer band are shown in Fig. 1 (inset). It is clearly apparent that cytochrome b_{560} in contrast to bovine cytochrome c has no peak at 695 nm which rules out methionine ligation of heme iron.

The reduced minus oxidized difference spectrum of purified cytochrome is shown in Fig. 2. It has peaks at 428, 529 and 560 nm.

The pyridine hemochrome spectrum of the heme prosthetic group isolated from the purified cytochrome b_{560} was indistinguishable from the pyridine hemochrome spectrum of protoheme 1X (not shown). The porphyrin derived from the prosthetic group of cytochrome comigrated with protoporphyrin when being chromatographed using HPLC. Thus we may conclude that the cytochrome contains protoheme 1X as a prosthetic group.

The MCD spectra of reduced cytochrome b_{560} (Fig. 3) is characteristic of low-spin ferrous hemoproteins with an intense A-term with a maximum at 557 nm, minimum at 565.5 nm, zero-crossing point at 561 nm in the α-band region and a well-resolved vibronic structure in the β-band region.

The redox titration of cytochrome b_{560} is shown in Fig. 4. The experimental points fit well to the theoretical curve for a one-electron carrier with a midpoint potential of +193 mV.

The cytochrome b_{560} does not combine with CO and CN^- during the CO bubbling through a solution of cytochrome for 20 min and on addition of 5 mM KCN^-. The hemoprotein is easily reduced by ascorbic acid and sodium dithionite and oxidized by ferricyanide and

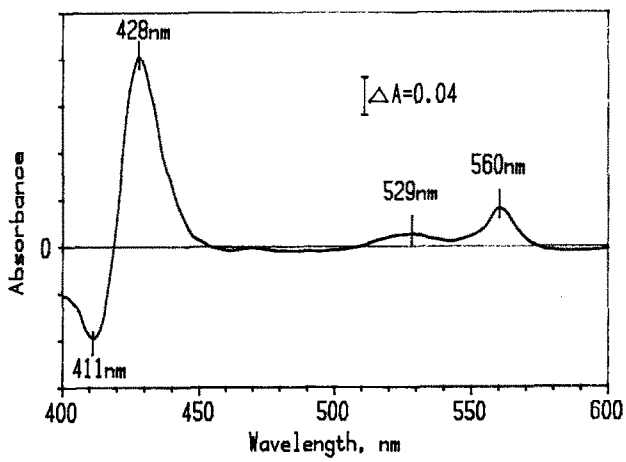

Fig. 2. Reduced minus oxidized difference spectrum of cytochrome b_{560}. The sample and conditions are the same as in Fig. 1.

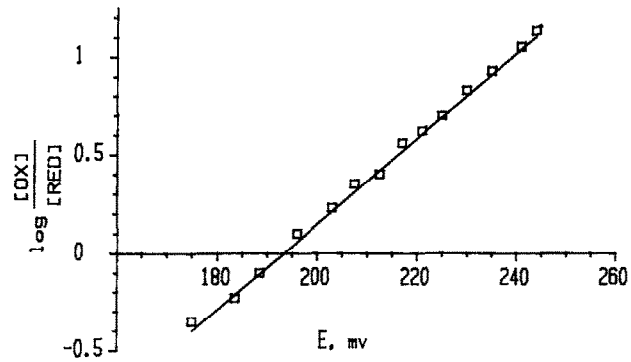

Fig. 4. Redox titration of partially purified cytochrome b_{560}. The sample (2 ml) contained 20 μM DCPIP, 50 mM phosphate buffer, pH 6.5, and 2.15 nmol cytochrome b_{560} (E^{mm}_{560} = 20 was used to calculate the concentration of cytochrome b_{560}). The sample was reduced by adding of small aliquots of 0.1% dithionite. The reduced cytochrome was determined from the absorbance difference between 560 and 540 nm, and the ratio of oxidized to reduced DCPIP was determined from the 600 nm absorption peak.

H_2O_2. The reduced form is oxidized by water-dissolved oxygen with an initial rate corresponding to a half-time of 28 min.

4. DISCUSSION

We have isolated and partially purified a new hemoprotein from eggs of whitefish and studied some of its physico-chemical properties.

The pigment has protoheme 1X as a prosthetic group and shows the absorption and MCD spectra characteristic of *b*-type cytochromes. The hemoprotein has been named cytochrome b_{560} (*Coregonidae*) according to the wavelength maximum of the α-band in the reduced minus oxidized difference spectrum.

The MCD spectra of ferrous cytochrome b_{560} in the visible region (Fig. 3) testify that both axial positions of the coordination shell of the Fe(II) ion are occupied by strong field ligands.

The line shape of vibronic structure of the magneto-optical activity spectra of low-spin ferrous hemoproteins and model compounds in the β-band region is sensitive to the nature of axial ligands of the heme iron [6]. The line shape of vibronic structure of the MCD spectra of cytochrome b_{560} in the β-band region is very similar to those of cytochrome *c* and cytochrome b_5 which are typical hemoproteins with His-Met and His-His coordinated hemes, respectively, and differs from those of hemoproteins and model compounds having other pairs of axial ligands. The His-Met and bis-histidinyl heme axial ligations are often indistinguishable in MCD spectra asa well as in EPR spectra [6–8]. The nature of these similarities is still unknown. However, the absence of a band at 695 nm in the absorption spectra of the ferricytochrome b_{560} (Fig. 1c), indicates that it does not contain a methionyl residue bound to the heme iron [9].

Thus, it may be suggested that the heme iron in cytochrome b_{560} has bis-histidinyl heme axial ligation.

The M_r of cytochrome b_{560} was estimated preliminarily to be 20,400 Da by SDS-polyacrylamide gel electrophoresis [10].

The cytochrome b_{560} is found in the water-soluble moiety of the egg yolk. Most of known *b*-type cytochromes are located in membrane structures [11]. Only a few of them have been extracted from cells or tissues in the soluble form, as in the case of cytochrome b_{562} from bacteria [12] and cytochrome b_5 from erythrocytes [13]. However, these cytochromes differ from cytochrome b_{560} in spectral properties and from cytochrome b_{562} in the 6th axial ligand [7]. Incidentally, cytochrome b_{560} resembles in many respects membrane-bound cytochrome b_{561} from chromaffin granules (M_r, similar E_m, absorption and MCD spectral characteristics [14]. Cytochrome b_{561} has the same 5th and 6th axial histidine ligands, does not combine with CO and CN^- and is easily reduced by ascorbic acid [7,14].

Based on the present study of cytochrome b_{560} we may conclude that this hemoprotein is a new animal *b*-type cytochrome, found only in eggs of whitefish.

Preliminary results show that cytochrome b_{560} alters its degree of reduction in the course of development and in response to changes in oxygen concentration in water. Thus, cytochrome b_{560} is unlikely to be an inert reserve substance of the yolk, but rather it may be involved in some redox processes.

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