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Occurrence of NADH-specific and NADH/NADPH-bispecific cytochrome b_5 reductases in the ascidians, *Styela plicata and Ciona intestinalis*

Toshitsugu YUBISUI, Takashi YAMANAKA*, Ken-ichi KURITA*,

Shigeki FUJIWARA* and Kazuo KAWAMURA*

Department of Biochemistry, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan

**Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, Kochi University, Akebono-cho, Kochi 780-8520, Japan*

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Occurrence of two types of cytochrome b_5 reductases, NADH-specific and NADH/NADPH-bispecific enzymes were found in the ascidians, *Styela plicata and Ciona intestinalis*. To characterize both types of the enzymes, crude extracts prepared were directly applied on a 5'-AMP-Sepharose column to trap NADH-cytochrome b_5 reductase. The adsorbed NADH-cytochrome b_5 reductase on the column was eluted specifically with NADH. The pass through fractions showed the cytochrome b_5 reductase activity with both NADH and NADPH as the electron donor. In other ascidians so far we examined, only NADH-dependent enzyme activity was detected. The apparent *Km* values of the NADH-specific enzyme of the *Ciona* and *Styela* were determined to be 1.1 μM and 0.6 μM, respectively, for NADH. The apparent *Km* values of the NADH/NADPH-bispecific enzyme of *Ciona* were determined to be 0.48 μM and 0.8 μM for NADH and NADPH, respectively. Those of *Styela* enzyme were to be 0.24 μM, 0.39 μM for NADH, NADPH, respectively. The apparent *Km* values for cytochrome b_5 with both the NADH-specific and NADH/NADPH-bispecific enzymes were 4-10 μM, respectively, with both the ascidian enzymes. Physiological significance of both the NADH-specific and NADH/NADPH-bispecific enzymes in the ascidians, *Styela plicata* and *Ciona intestinalis* was discussed.

Keywords: NADH/NADPH-specific cytochrome b_5 reductase, ascidians, kinetic properties.

INTRODUCTION

Ascidians, the marine invertebrates, are useful experimental sources to study on the developmental processes (Sato, 1994). We have studied on the bud development of the ascidian, *Polyandrocarpa misakiensis* (*P. misakiensis*), and characterized various genes (Shimada *et al.*, 1995; Harafuji *et al.*, 1996; Hisata *et al.*, 1998; Ohashi *et al.*, 1999; Kamimura *et al.*, 2000) and proteins (Suzuki *et al.*, 1990; Harafuji *et al.*, 1996; Ohashi *et al.*, 1999). During the studies on the mitogenic factors of the ascidian, we found that some phospholipids of the ascidian show the mitogenic activity (Arai *et al.*, 2004) against the cultured cells derived from the atrial epithelium of the ascidian (Kawamura and Fujiwara, 1995). Then we analyzed the fatty acids in the ascidian, and found that an extraordinary large amount of triacylglycerol is

accumulated in buds of the ascidian, and cytochrome b_5 is expressed at a high level in the developing buds (Yubisui *et al.*, 2001). These findings prompted us to study on the redox enzymes related to the fatty acid metabolisms in the ascidian. In this study, we studied on cytochrome b_5 reductase, which is known to participate in the fatty acid metabolism in vertebrates (Oshino *et al.*, 1971; Hildebrandt and Estabrook, 1971; Reddy *et al.*, 1977; Keyes and Cinti, 1980; Fukushima *et al.*, 1981; Onoda and Hall, 1982; Shinzawa *et al.*, 1985), to reveal the biochemical roles of the enzyme in the ascidians.

NADH-cytochrome b_5 reductase of vertebrates (EC 1.6.2.2) (b5R) is a flavoprotein, which catalyzes the reduction of cytochrome b_5 via FAD bound to the enzyme (Strittmatter, 1965; Yubisui and Takeshita, 1980). The enzyme receives two electrons from NADH, and then transfers one electron each to two molecules of cytochrome b_5 to reduce the hemoprotein, thereby participates in many metabolic reactions as described above. Mammalian b5Rs are already well characterized on the gene (Tomatsu *et al.*, 1989), cDNA (Yubisui *et al.*, 1987), amino acid sequence (Yubisui *et al.*, 1986), and kinetic properties (Yubisui *et al.*, 1980; Yubisui *et al.*, 1982).

The enzymes purified from various mammalian were all NADH-specific enzymes, and the enzyme activity with NADPH was less than 5% of that with NADH (Strittmatter, 1965; Yubisui *et al.*, 1982). The enzymes from human erythrocytes or liver have the unique FAD-binding (RxYT/S) and NADH-binding (GxGxxP) motifs (Karplus *et al.*, 1991), which are different from the classical Rossmann-fold (GxGxxG) (Rossmann *et al.*, 1974). The NADH-binding site of human b5R has a distinct structure for NADH-binding and a very hydrophobic environment as revealed from the X-ray crystallographic studies (Takano *et al.*, 1994). Human b5R belongs to the FNR family (Karplus *et al.*, 1991), which was identified as a new flavoprotein family different from those with the Rossmann-fold. In the FNR-family, the NADPH-dependent enzymes have NADPH-binding motif (GxGxxA) (Karplus *et al.*, 1991).

As described above, the substrate specificity of the cytochrome b_5 reductase in vertebrates is strictly NADH-dependent based on the distinct structure of the enzyme protein (Strittmatter, 1965; Yubisui *et al.*, 1982; Yubisui *et al.*, 1986). Therefore, the existence of the NADH/NADPH-bispecific enzymes in the ascidians, *Styela plicata* (*S. plicata*) and *Ciona intestinalis* (*C. intestinalis*) is very interesting findings. The analysis of the NADH/NADPH-bispecific b5Rs in the ascidians may reveal a new insight of the enzyme. The finding of the NADH/NADPH-bispecific b5R in the ascidians is the first case.

MATERIALS AND METHODS

β-NADH and β-NADPH were purchased from Roche (Tokyo, Japan), and 5'-AMP-Sepharose was obtained from Amersham/Pharmacia (GE healthcare). Other reagents used in this study are all reagent grade. DEAE-Toyopearl 650M was the product of TOSOH Corporation (Tokyo, Japan) and Sephacryl S-100 was obtained from Pharmacia (Uppsala, Sweden).

Ascidians were harvested at the Uranouchi Inlet near the Marine Biological Institute of Kochi University, Kochi, Japan.

Crude extracts of the ascidians were prepared after brief washing of the animals with saline, by homogenizing the chopped animal tissues by a Wahring blender with 4 volume of Tris-HCl buffer (pH 8) containing 0.1 mM EDTA and 0.1 mM dithiothreitol (DTT). To the homogenate Triton X-100 was added at a final concentration of 0.5%, and stirred at 4°C for 30 min. Then the homogenates was centrifuged at 12,000 x g for 30 min. Resulted supernatant was then centrifuged again at 100,000 x g for 30 min. The supernatant obtained was used as the crude enzyme solution. Cytochrome b_5s of the respective ascidians were not obtained as the substrate for cytochrome b_5 reductases, therefore, the enzyme activity was determined with rat recombinant cytochrome b_5. Soluble form of rat cytochrome b_5 was expressed in *Escherichia coli* as described previously (Shirabe *et al.*, 1989), and purified by chromatographies on a DEAE-Toyopearl 650M (2 x 15 cm) and a Sephacryl S-100 gel filtration column (3 x 80 cm). Cytochrome b_5 reductase activity was determined as described previously (Yubisui and Takeshita, 1980; Yubisui *et al.*, 1982) by following the increase of the absorbance of the reduced cytochrome b_5 at 424 nm. The

absorbance change of cytochrome b_5 was determined with a Union Spectrophotometer SM401 (Union Giken, Osaka, Japan). The enzyme activity was calculated using the difference of extinction coefficients between the reduced and oxidized form of cytochrome b_5 at 424 nm (Yubisui and Takeshita, 1980).

RESULTS AND DISCUSSION

Separation of two types of b5Rs

During the attempts to determine b5R activity of various ascidians with crude extracts, we found that *S. plicata* and *C. intestinalis* have about 40-70% enzyme activities with NADPH of those with NADH as the electron donors. NADH/NADPH-bispecific b5R was first found in human cells by Zhu *et al.* (2004), but not in ascidians. Therefore, we studied on the unique enzymes in the ascidians based on the present findings.

Initially we tried to purify the enzymes from both the ascidians, but the attempts were unsuccessful due to the limited tissues and also due to the instability of the enzymes. Subsequent many attempts done in this study to isolate a cDNA clone for cytochrome b_5 reductase from various cDNA libraries of the ascidians, *P. misakiensis*, *S. plicata*, or *C. intestinalis*, were all unsuccessful. Finally, kinetic properties of the enzymes from both the ascidians were characterized in the present study with the partially purified preparations.

Chromatography of the enzymes on a DEAE-Toyopearl column did not separate well the two types of the enzymes, and resulted in a significant loss of the enzyme activity during the procedures. Then, the crude extract was directly applied on a 5'-AMP-Sepharose column to trap the NADH-specific b5R, and the adsorbed enzyme on the column was eluted specifically with NADH after washing the column as shown in Fig. 1. The void volume fractions contained a significant b5R activity, which reacted with both NADH and NADPH as electron donors as shown in Fig. 1. Thus the two types of b5Rs with different substrate

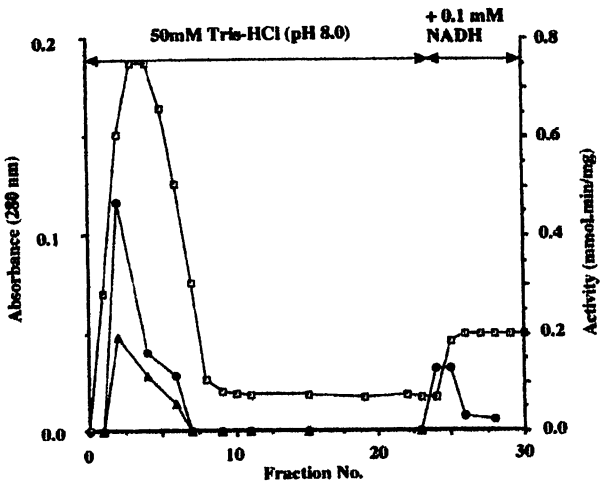

Figure 1. Affinity chromatography of cytochrome *b5* reductase of *C. intestinalis*.

Crude extract of *C. intestinalis* was applied on a 5'-AMP-Sepharose column (1.5 x 5 cm), which Was previously equilibrated with 50 mM Tris-HCl (pH 8) containing 0.1 mM EDTA and 0.1 mM DTT. The column was washed with the same buffer, and then the adsorbed enzyme on the column was eluted with 0.1 mM NADH in the same buffer. (□) show the absorbance at 280 nm, (●) indicate the NADH-specific b5R activities, and (▲) are the NADPH-specific b5R activities. The high absorbance after fractions 24 are mainly due to the absorbance of NADH added to elute the NADH-specific enzyme.

specificity were clearly separated. This is the first finding of the NADH/NADPH-bispecific b5Rs from ascidians. The enzyme activity with NADPH was about 50% with NADH. These findings suggest that in both the ascidians two types of enzyme are working separately in the cells.

Kinetic studies

Kinetic properties of b5Rs of *S. plicata* and *C. intestinalis* were examined with the partially purified preparations with the affinity chromatography on a 5'-AMP-Sepharose column. Those of various ascidians were also examined with crude extracts. As shown in Table 1, b5Rs in *S. plicata* and *C. intestinalis* showed both NADH-specific and NADH/NADPH- bispecific b5R activities. Other ascidians such as *Dideumnum moseleyi, Perophora japonica, Symplegma reptans, Polyandrocarpa misakiensis, and Herdmania momus* showed only NADH-specific enzyme activity. In these ascidians, b5R activities with NADPH were less than 5% of that with NADH or undetectable.

For the determinations of the enzyme activity of the NADH/NADPH-bispecific b5R in the void volume fractions from the 5'-AMP-Sepharose column, the assay was carried out as soon as possible to avoid the loss of the enzyme activity. For the determinations of kinetic properties of the NADH-specific b5R, fractions eluted from the column with NADH were pooled, and were concentrated by Centricon 10. Then the concentrated enzyme was dialyzed against the buffer before the kinetic experiments to remove excess NADH and NAD^+ used to elute the enzyme from the affinity column.

Table I summarized the kinetic parameters of the enzymes from various ascidians as determined by the Lineweaver-Burk's plots. The apparent *Km* values with the NADH-specific enzymes of both the *C. intestinalis* and *S. plicata* were determined to be 1.1 μM and 0.6 μM for NADH, respectively. The *Km* values of the NADH/NADPH-bispecific enzyme of *C. intestinalis* were determined to be 0.48 μM and 0.8 μM for NADH and NADPH, respectively. Those of *S. plicata* were 0.24 μM and 0.39 μM for NADH, NADPH, respectively. The apparent *Km* values for cytochrome b_5 were 4-10 μM in any cases. Total activity for cytochrome b_5 reductions by *Ciona* enzymes with NADPH was about 50% of that with NADH.

Physiological significance of the existence of both the NADH-specific and NADH/NADPH-bispecific enzyme in the ascidians, *S. plicata* and *C. intestinalis* is not clear at present. As we could not detect NADPH-specific cytochrome *c* reductase activity in *S. plicata*, the NADH/NADPH-dependent enzyme may work as the cytochrome *c* reductase via cytochrome b_5 in the metabolism of some substrates. Zhu *et al.* (2004) found NADH/NADPH-bispecific b5R in human cells, and similar bispecific b5Rs were also found in mouse and other animal species. Physiological functions of those enzymes are expected to clarify.

Table 1. Kinetic properties of cytochrome b_5 reductases from various ascidians.

Ascidians	*Km* (μM)[a]		
	NADH	NADPH	cytochrome b_5
Ciona intestinalis	0.48	0.8	10.4
	1.1[b]	-[c]	7.7
Didemnum moseleyi	0.71	36.5	12.1
Perophora japonica	0.83	41.9	1.9
Styela plicata	0.24	0.39	4.73
	0.6[b]	-[c]	
Symplegma reptans	43.0	-[c]	9.61
Herdmania momus	3.3	65.8	3.71

a: The enzyme activities were determined with the crude extracts. The values are those of averages from at least three determinations.
b: NADH-specific cytochrome b_5 reductase separated from NADH/NADPH-specific b_5 reductase by a 5'-AMP-Sepharose column.
c: Not detectable.

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