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PROPERTIES AND FUNCTION OF THE RESPIRATORY CHAIN OF FRESHWATER MUSSEL SPERMATOZOA IN RELATION TO MOTILITY

SATORU HIGASHI* AND KIYOZO KAWAI

Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560 (Japan)

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SUMMARY

1. The spermatozoa of the freshwater mussel (*Hyriopsis schlegelii*) contain cytochromes aa_3, *b* and *c*, flavoproteins and nicotinamide nucleotides in molar ratios of 1.0:0.9:1.8:1.8:8.7. Cytochrome c_1 is not detectable even at liquid-N_2 temperature, but a c_1-like cytochrome with an α-band at 550 mμ is found at liquid-N_2 temperature in a cell preparation from which cytochrome *c* is completely removed.

2. The near-ultraviolet difference spectrum of whole cells reveals an absorption peak at 315 mμ with a shoulder around 350 mμ.

3. Both the endogenous respiration and motility of spermatozoa are completely blocked by 0.2 mM CN^- and by 0.2 μM antimycin A. 2,4-Dinitrophenol and pentachlorophenol completely inhibit motility at the maximal stimulation of respiration. Rotenone strongly inhibits NADH oxidase of spermatozoa, although it has no effect on the respiration of whole cells.

4. It is concluded that the motility of mussel spermatozoa is tightly coupled to respiration, and the respiratory chain phosphorylating process is the only energy-supplying system for motility.

INTRODUCTION

It is generally accepted that mammalian spermatozoa can obtain motile energy from both respiration and glycolysis[1,2]. Spermatozoa of invertebrates, however, utilize only respiratory substrates for motility[3]. Recently we have shown that primary respiratory substrates in spermatozoa of the freshwater mussel are fatty acids derived from the breakdown of intracellular phospholipids[4]. The cytochrome components of mussel spermatozoa have been observed by KAWAI[5] using visual spectroscopy at liquid-air temperature. The present study was undertaken in order to characterize the respiratory chain components of mussel spermatozoa in more detail and to evaluate the role of the respiratory chain in motility.

* Present address: Department of Biology, Faculty of Education, Shiga University, Otsu, Shiga 520, Japan.

EXPERIMENTAL

Materials

Spermatozoa of the freshwater mussel (*Hyriopsis schlegelii*) were collected from the punctured gonads of mature animals obtained from a culture farm on the coast of Lake Biwa. The spermatozoa were suspended in a physiological solution[6] buffered with 0.05 M phosphate (pH 7.2), and washed once by centrifuging at 300 × *g* for 10 min. The washed cells were diluted to a required cell density with the physiological solution or other suspending media.

Cell homogenates for measuring enzyme activities were prepared in a Teflon homogenizer with 5 vol. of 0.05 M phosphate buffer (pH 7.2) to 1 portion of packed cells and a little glass powder, and the homogenates were centrifuged at 300 × *g* to remove intact cells and other heavy particles. Cytochrome *c*-free disintegrated cells for low-temperature spectrophotometry were obtained as follows. After being frozen and thawed with liquid N_2, spermatozoa suspended in 0.2 M phosphate buffer (pH 7.2) were homogenized in the Teflon homogenizer, and the homogenates were centrifuged at 20000 × *g* for 30 min. The extraction of cytochrome *c* from the residue was repeated 3 times by rehomogenization in 0.2 M phosphate buffer (pH 7.2) and recentrifugation. The final residue thus obtained was suspended in a small portion of 0.1 M phosphate buffer (pH 7.2).

Methods

Difference spectra were obtained using a Cary Model-14 recording spectrophotometer equipped with the 0–0.2 absorbance slide wire. Spectra at liquid-N_2 temperature were recorded employing a low-temperature apparatus and procedures described by KAWAI[7], and no glycerol was supplemented to the medium. The concentrations of respiratory carriers in spermatozoa were obtained from the room temperature difference spectra ($S_2O_4^{2-}$-reduced *minus* aerobic) using absorption coefficients given by CHANCE[8] for cytochromes *b* and *c*, flavoproteins and nicotinamide nucleotides. As to cytochrome aa_3, the coefficient of 24 for the α-region recently recommended by VAN GELDER[9] was adopted.

Cytochrome oxidase (EC 1.4.3.1), NADH oxidase, NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were spectrophotometrically measured as previously described[5]. Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of DENNIS AND KAPLAN[10].

The O_2 consumption of spermatozoa was measured at 25° using a Clark oxygen electrode in a 2.5 ml stirred cell as described elsewhere[11]. The medium contained 0.15 M mannitol, 0.02 M NaCl and 0.01 M Tris–HCl buffer (pH 7.2). The anaerobic incubation of spermatozoa was carried out in Thunberg tubes under N_2 with continuous shaking at 25°, and the physiological solution provided with 0.05 M glucose was used as the medium. Lactate was measured by the method of BARKER AND SUMMERSON[12].

The grade of cell motility was estimated by microscopic examination and expressed as follows: very vigorous movement, +3; vigorous movement, +2; faint movement, +1; motionless, 0. Spermatozoa counts were made with the aid of a Thoma ruling haemocytometer.

RESULTS

Respiratory enzymes of mussel spermatozoa

The activities of respiratory enzymes in the homogenates and the effects of various inhibitors on them are summarized in Table I. Cytochrome oxidase and NADH oxidase were strongly inhibited by CN^-, and NADH oxidase was also very sensitive to rotenone. Antimycin A was a potent inhibitor for NADH oxidase, NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase.

TABLE I

RESPIRATORY ENZYME ACTIVITIES AND EFFECT OF INHIBITORS

Enzyme	*Enzymatic activity (nmoles/min per mg N)*	*Inhibition (%)*		
		Cyanide (1 mM)	*Antimycin A (120 μM)*	*Rotenone (0.8 μM)*
Cytochrome oxidase	29.3	94	—	—
NADH oxidase	63.5	96	96	100
NADH–cytochrome *c* reductase	21.4	—	80	—
Succinate–cytochrome *c* reductase	14.2	—	98	—

Lactate production and lactate dehydrogenase activity

No lactate production was observed during the 3 h incubation of spermatozoa under anaerobic conditions. Furthermore, no lactate dehydrogenase activity was found in the homogenates. These negative results suggest that no glycolytic process exists in mussel spermatozoa.

Spectral properties of mussel spermatozoa

Fig. 1 shows the difference spectra of reduced *minus* oxidized respiratory chain components of spermatozoa at room temperature. Under anaerobic conditions, the electron carriers are readily reduced by endogenous substrates, and one can observe α-maxima due to cytochromes aa_3, *b* and *c* at 603, 562 and 550 mμ, respectively (Fig. 1 A). A trough around 460 mμ is indicative of flavoproteins, and two Soret bands at 446 and 420 mμ can be mainly attributed to cytochromes aa_3 and *c*, respectively. These absorption bands were intensified by reduction with $S_2O_4^{2-}$, the Soret band at 446 mμ being shifted a few mμ toward the ultraviolet (Fig. 1 B). CN^- inhibition gave a difference spectrum similar to those of anaerobic or $S_2O_4^{2-}$-reduced cells in the α-region, but the Soret band at 442–446 mμ shifted to around 450 mμ, and the intensity of this band decreased to approximately one-half (Fig. 1 C). A similar decrease of the trough at 460 mμ was also observed. A treatment with antimycin revealed the absorption bands of cytochrome *b* alone (Fig. 1 D).

The low-temperature spectrophotometry of spermatozoa suspensions gave sharpened and complicated spectra. Fig. 2 A shows a typical example of low-temperature difference spectra obtained with intact cells. The absorption maxima or shoulders at 601, 560 and 548 mμ in the α-region represent cytochromes aa_3, *b* and *c*, respectively. In the Soret region, the peaks at 418, 431 and 446 mμ can be mainly attributed to cytochromes *c*, *b* and aa_3, respectively. This spectrum also

shows a shoulder at 556 mμ, which will be discussed later. The phosphate extracts from disintegrated cells, on reduction with ascorbate, gave a typical spectrum of cytochrome *c* (Fig. 2 B). This spectrum shows an intense α-peak at 548 mμ (α_1) with a shoulder at 545 mμ (α_2), a β-peak at 518 mμ and a Soret peak at 415 mμ. With intact cells, the 415-mμ peak of cytochrome *c* did not appear, but a combined Soret maximum of *c*-type cytochromes appeared at 418 mμ (Fig. 2 A).

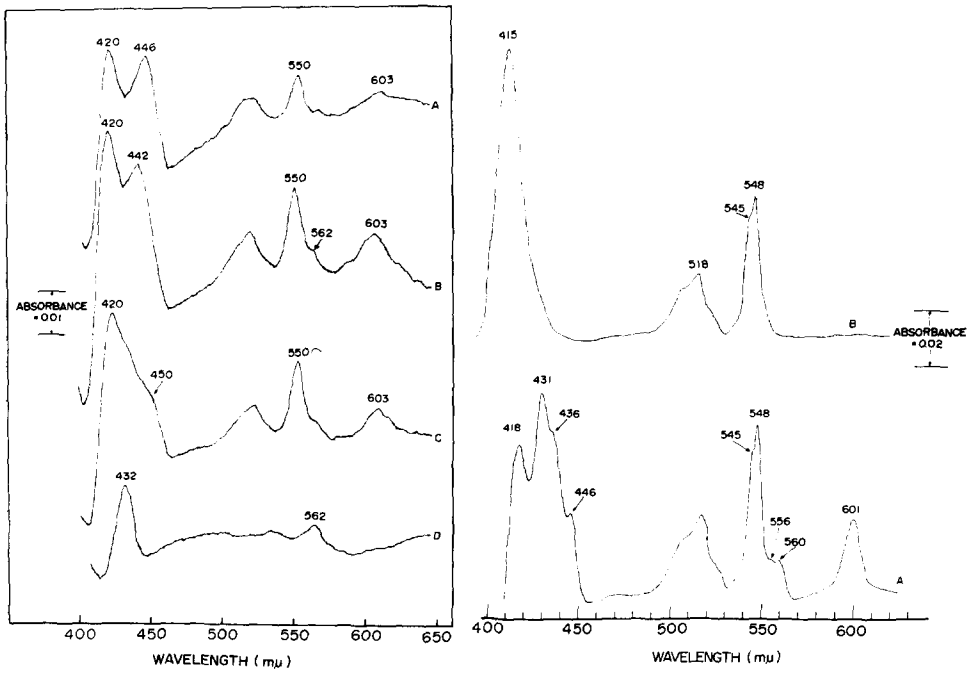

Fig. 1. Difference spectra of mussel spermatozoa at room temperature. Optical path, 10 mm. Cells suspended in the physiological solution (pH 7.2) at $2.6 \cdot 10^9$ cells per ml. A, anaerobic *minus* aerobic cells. A Thunberg tube was used for anaerobiosis. B, dithionite-reduced *minus* aerobic. C, 1 mM CN^--inhibited *minus* aerobic. D, 1 μM antimycin A-inhibited *minus* aerobic.

Fig. 2. Spectral properties of mussel spermatozoa at liquid-N_2 temperature. Optical path, 2 mm. A, 1 mM CN^--inhibited intact cells ($1.3 \cdot 10^9$ cells per ml) *minus* aerobic cells. B, ascorbate-reduced phosphate extracts of disintegrated cells *minus* aerobic.

The disintegrated cells, from which cytochrome *c* was completely removed by washing with 0.2 M phosphate buffer, were examined under various conditions of reduction. Fig. 3 A is a difference spectrum of antimycin-A-inhibited succinate aerobic preparation *minus* the aerobic preparation, and the absorption bands appearing at 560, 531 and 431 mμ represent cytochrome *b*. In the ascorbate-reduced spectrum, cytochrome aa_3 shows an α-peak at 601 mμ and a typical double-Soret peak at 439 and 446 mμ (Fig. 3 B). In this spectrum, the absorption peaks at 550, 518 and 422 mμ indicate the presence of a c_1-like cytochrome which is strongly bound to the cell structure. In the $S_2O_4^{2-}$-reduced preparation, an absorption peak newly appeared at 556 mμ (Fig. 3 C), which did not appear when the preparation was reduced with succinate. Presumably the 556-mμ peak is due to a *b*-type cytochrome, and a 556-mμ

shoulder which appears in the spectrum of intact cells seems to belong to this component.

The near-ultraviolet difference spectrum of anaerobic or CN^--inhibited cells *minus* aerobic cells revealed an absorption peak at 315 mμ with a shoulder around 350 mμ (Fig. 4). The 350-mμ shoulder may be attributed to reduced nicotinamide nucleotides, but the entity of the 315-mμ peak is not known at present.

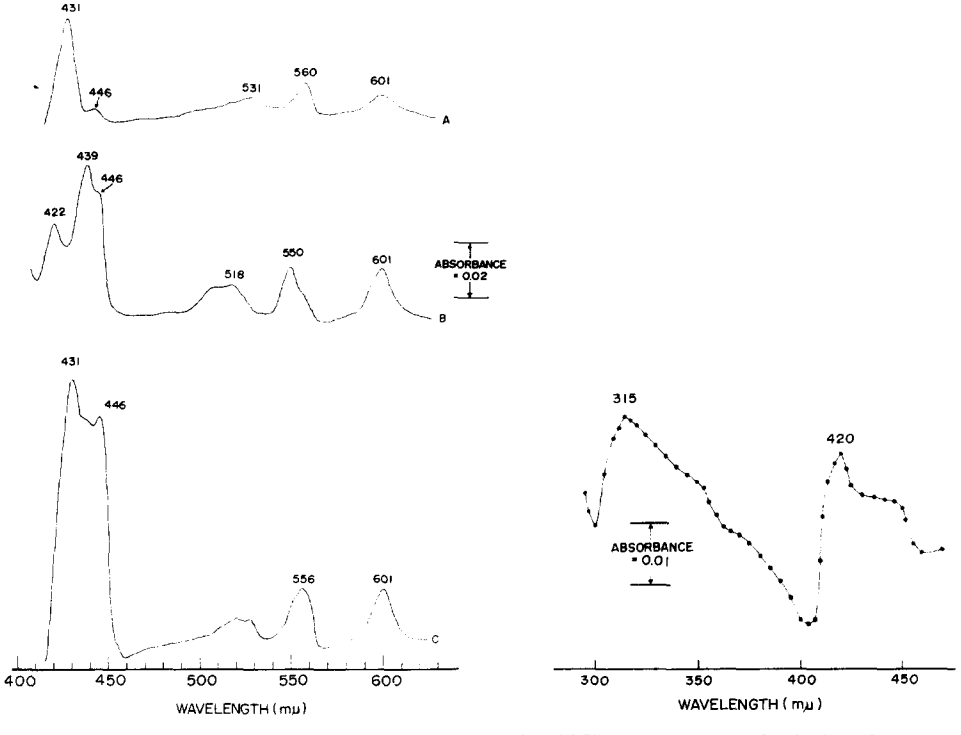

Fig. 3. Spectral properties of disintegrated cells at liquid-N_2 temperature. Optical path, 2 mm. Cytochrome *c*-free disintegrated cells were suspended in 0.1 M phosphate buffer (pH 7.2), 10.8 mg protein per ml. A, 10 mM succinate and 1 μM antimycin A *minus* the aerobic preparation. B, 20 mM ascorbate and 1 mM CN^- *minus* aerobic. C, $S_2O_4^{2-}$-reduced *minus* aerobic.

Fig. 4. Near-ultraviolet difference spectrum of mussel spermatozoa at room temperature. Optical path, 10 mm. Cells in the physiological solution (pH 7.2) at $3.2 \cdot 10^9$ cells per ml. 1 mM CN^--inhibited *minus* aerobic.

Concentration of respiratory carriers

The content of various electron carriers in mussel spermatozoa are given in Table II. Concentrations per unit volume of mitochondria were obtained from the content of the carriers per cell and the total volume of mitochondria included. The midpiece of the mussel spermatozoon constitutes five spherical mitochondria having a diameter of approx. 0.8 μ as described elsewhere[13,14], and the average volume of a mitochondrion is calculated to be 0.28 μ^3. The relative concentrations of cytochromes and other respiratory carriers in mussel spermatozoa are comparable to those obtained in mammalian cells[15] and the spermatozoa of the sea-urchin[16] and the clam[17]. However, the values found for the concentrations of each cytochrome

TABLE II

CONCENTRATION OF RESPIRATORY CARRIERS IN MUSSEL SPERMATOZOA

The concentrations of electron carriers were determined as described in the text. The value of cytochrome *c* was calculated assuming that one-fifth of the absorbance at 550 mμ was contributed by the c_1-like (550) component. Those of the c_1-like and 556-mμ components were obtained from the low-temperature spectra of cytochrome *c*-free preparations assuming that their absorption coefficients and low-temperature enhancements were equal to those of cytochromes *c* and *b*, respectively.

Component	*nmoles/10¹⁰ cells*	*nmoles × 10⁻¹⁰/μ³ of mitochondrion*	*Relative conc.*
Cytochrome aa_3	5.5	3.9	1.0
Cytochrome *b*	5.0	3.6	0.9
Cytochrome *c*	10.0	7.1	1.8
550-mμ component	5.4	3.9	1.0
556-mμ component	6.0	4.3	1.1
Flavoproteins	10.0	7.1	1.8
Nicotinamide nucleotides	47.5	33.8	8.7

per unit volume of mitochondrion are approx. 19–23 times larger than those of rat liver mitochondria[18].

Respiratory activity and motility

Concurrently with the enzymic and spectroscopic studies, experiments were performed on the effect of the same inhibitors on respiration and motility. CN^- completely blocked both respiration and motility at a concentration of 0.2 mM. Antimycin A also completely inhibited both respiration and motility at the very low concentration of 0.2 μM. Rotenone was a potent inhibitor for NADH oxidase of mussel spermatozoa, although it had no effect on the respiration and motility of intact cells in the range 0.4–200 μM.

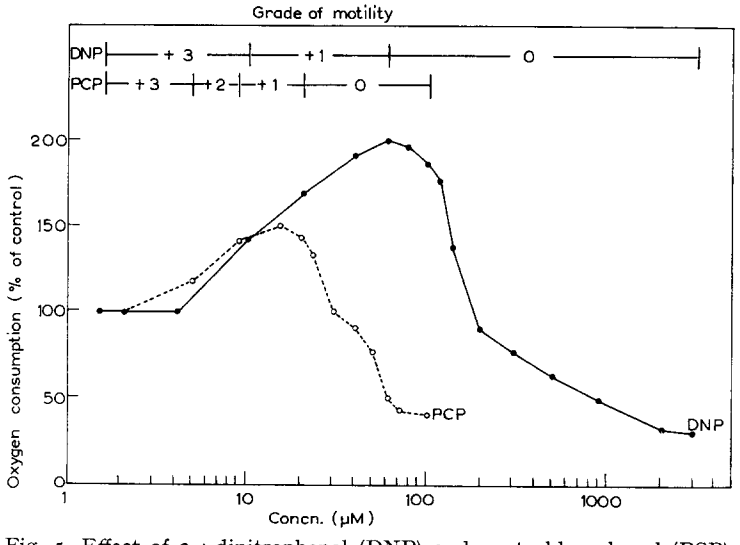

Fig. 5. Effect of 2,4-dinitrophenol (DNP) and pentachlorophenol (PCP) on the respiration and motility of mussel spermatozoa. O_2 consumption of control: 31 natoms oxygen per $1.2 \cdot 10^9$ cells per min. The grade of motility is as described in EXPERIMENTAL.

The uncoupling agents, pentachlorophenol and 2,4-dinitrophenol, maximally augmented respiration, to one and a half times to twice normal respiration, at concentrations of 16 and 60 μM, respectively, and motility was completely blocked at the slightly higher concentrations of 20 and 80 μM, respectively (Fig. 5). These uncouplers also inhibited respiration at higher concentrations than 30 μM for pentachlorophenol and 170 μM for 2,4-dinitrophenol.

DISCUSSION

It is well known that cytochrome c_1 is extensively distributed in mammalian cells, yeast cells and sea-urchin spermatozoa[19]. Recently a cytochrome component having an α-band at 556 mμ was found in sea-urchin spermatozoa by WILSON AND EPEL[16], who designated the component as cytochrome c_1. Although cytochrome c_1 was not detected in mussel spermatozoa, an ascorbate-reducible component having an α-band at 550 mμ was found at liquid-N_2 temperature in the disintegrated cells from which cytochrome *c* was completely removed. This component may play a role corresponding to cytochrome c_1 in mussel spermatozoa. In addition to that of cytochrome *b*, an absorption band appeared at 556 mμ in the same cell preparation when reduced with $S_2O_4^{2-}$. This band may be due to a *b*-type cytochrome, although its entity remains to be resolved.

The near-ultraviolet difference spectrum of intact cells revealed an absorption peak at 315 mμ with a shoulder around 350 mμ. A similar spectrum showing a 315-mμ peak with a shoulder at 340 mμ was described by HARMEY *et al.*[20]. They attributed the 340-mμ shoulder to reduced nicotinamide nucleotides, but distinguished the 315-mμ peak from the bound nicotinamide nucleotides by spectroscopic kinetic studies. We have also attributed the 350-mμ shoulder to reduced nicotinamide nucleotides. However, the origin of the 315-mμ peak in mussel spermatozoa is not known at present.

The respiratory enzymes of spermatozoa are sensitive to CN^-, antimycin A and rotenone (Table I). These respiratory inhibitors, except rotenone, are also effective in blocking the respiration and motility of intact cells. Although rotenone strongly inhibits NADH oxidase of spermatozoa, it has no effect on the respiration of whole cells. This phenomenon may be due to lower permeability of the outer sheath of spermatozoon mitochondria to rotenone. The uncouplers, 2,4-dinitrophenol and pentachlorophenol, completely blocked motility at the maximal stimulation of respiration. The effective concentrations of these uncouplers and of antimycin A are comparable to those obtained in isolated mammalian mitochondria[21, 22].

An early visual spectroscopic observation[5] that mussel spermatozoa are characterized by their high content of cytochromes *a*, *b* and *c* was reconfirmed in the present investigation. The concentrations of cytochromes per unit volume of spermatozoon mitochondria were calculated to be 19–23 times larger than those obtained in rat liver mitochondria[18]. The large values for cytochrome concentrations may be due in part to the peculiar structure of spermatozoon mitochondria in which the cristae are very profuse compared with rat liver mitochondria[13, 14]. On the other hand, the calculation has been made on the assumption that all respiratory carriers are located in mitochondria only. If the cytochromes are located at sites other than mitochondria, the values will be overestimated. We cannot deny such a possibility. Recently it

has been reported that considerable quantities of cytochromes are contained in the nuclei of calf thymus[23].

From enzymic, spectroscopic and physiological results, it can be concluded that the motility of mussel spermatozoa is tightly coupled to respiration, and the respiratory chain phosphorylation process is the only energy-supplying system for motility.

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