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Does Light-Induced Relief of Cytochrome *c* Oxidase from CO-Induced Inhibition Result in Photo-Reactivation of CO-Inhibited Respiration in Sperm of Sea Urchin, Oyster and Fish?

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ABSTRACT—Light irradiation, at a light fluence rate sufficient for the strong photo-reactivation of the CO-inhibited cytochrome *c* oxidase in mitochondria isolated from the sperm of fish, oyster and sea urchin, weakly activated the CO-inhibited respiration only in the sea urchin sperm, with peaks of photo-reactivation corresponding to those in the absorption spectrum of CO-bound cytochrome aa_3. NADH cytochrome *c* reductase was inhibited by CO, weakly in mitochondria from sea urchin sperm and completely in those from fish and oyster sperm. The CO-induced complete inhibition of cytochrome *c* reduction in fish and oyster sperm probably does not allow the photo-reactivation of CO-inhibited cytochrome *c* oxidase to augment CO-blocked respiration. At a light fluence rate higher than that mentioned above, photo-activation of NADH cytochrome *c* reductase, found in the sperm of oyster and sea urchin, occurred even in the presence of CO in mitochondria isolated from sea urchin sperm and strongly activated CO-inhibited respiration in sea urchin sperm, with peaks corresponding to those in the absorption spectrum of reduced cytochrome *b*. The acceleration of cytochrome *c* reduction due to the photo-activation of this complex enzyme in sea urchin sperm probably induces another activation of CO-inhibited respiration at the high light fluence rate.

INTRODUCTION

We recently reported that the enzymes NADH cytochrome *c* reductase and succinate cytochrome *c* reductase are activated by light irradiation in mitochondria isolated from the viscera and gametes of abalone, echiuroid, oyster and sea urchin but are not activated in those isolated from the sperm of several fish species (Tazawa *et al.*, 1996). The action spectra for the photo-activation of these two complex enzymes show peaks of photo-activation at the wavelengths of 430, 530 and 570 nm, corresponding to those in the absorption spectrum of reduced cytochrome *b* (Tazawa *et al.*, 1996). The photo-activation of NADH cytochrome *c* reductase and succinate cytochrome *c* reductase results from the absorption of photon energy by cytochrome *b*, which is involved in these complex enzymes. The photo-activation of these complex enzymes in mitochondria, however, does not cause any increase in the respiratory rate in gametes of echiuroid, oyster or sea urchin; respiration in these gametes depends solely on electron transport though the mitochondrial respiratory chain (Tazawa *et al.*,1991; Fujiwara *et al.*,1991, Yasumasu *et al.*, 1991).

It has been reported that light irradiation reactivates CO-inhibited respiration in the eggs and sperm of echiuroid (Tazawa *et al.*, 1991; Fujiwara *et al.*, 1991) and in the sperm of sea urchin and starfish (Yasumasu *et al.*, 1991). The CO-induced inhibition of cytochrome *c* oxidase, cytochrome aa_3, is well known to be released by light irradiation. In the gametes of echiuroid, sea urchin and starfish, the action spectra for the photo-reactivation of CO-inhibited respiration differ from the absorption spectrum of CO-bound cytochrome aa_3 but are very similar to the absorption spectrum of reduced cytochrome *b*. Hence, it seems probable that the photo-reactivation of CO-inhibited respiration results from the photo-activation of cytochrome *b* reaction and is not due to the light-induced reactivation of CO-inhibited cytochrome *c* oxidase in these gametes.

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However, light irradiation does not augment the rate of CO-inhibited respiration in oyster sperm (Yasumasu *et al.*, 1991), the mitochondria of which exhibited a strong photo-activation of NADH cytochrome *c* reductase and succinate cytochrome *c* reductase (Tazawa *et al.*, 1996).

Thus, we decided to examine the effects of light irradiation on cytochrome *c* oxidase and NADH cytochrome *c* reductase in CO-exposed mitochondria isolated from the sperm of fish, oyster, sea urchin and other species, to determine the roles of the photo-activation of NADH cytochrome *c* reductase and the photo-reactivation of CO-inhibited cytochrome *c* oxidase in the photo-reactivation of CO-blocked respiration.

MATERIALS AND METHODS

Gametes of oyster, sea urchin and teleosts

Sperm of the teleost *Oncorhynchus masou ishikawae* (amago salmon) and the oyster *Crassostrea gigas* were obtained from isolated testes. Sperm of the sea urchin *Hemicentrotus pulcherrimus* were obtained by an injection of 0.5 M KCl into the body cavity. The dry sperm of these animals were stored in an ice bath until use. Sperm of the abalone *Nordotis discus*, echiuroid *Urechis unicinctus*, the starfish *Asterias pectinifera*, and the teleosts *Oncorhynchus masou masou* (masu salmon), *Oplegnathus fasciatus* (striped knifejaw) and *Pagrus major* (red seabream) were obtained according to the procedures described in previous papers (Tazawa *et al.*, 1991, 1996; Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991).

Crude mitochondrial fraction

Dry sperm suspended in ice cold homogenizing medium (0.5 M sucrose solution containing 10 mM EDTA, 50 mM Tris-HCl pH 7.2 and 20 mM $MgCl_2$), were homogenized in a glass homogenizer with a motor-driven Teflon pestle in an ice bath. These homogenates were centrifuged at 7,000 × g for 15 min at 4°C. The precipitate was resuspended in the homogenizing medium. The suspension of the precipitate was centrifuged at 500 × g for 10 min and the supernatant obtained was centrifuged at 7,000 × g for 15 min. The precipitate obtained was also resuspended in the homogenizing medium and was used as the crude mitochondrial fraction. The crude mitochondrial suspensions isolated from these sperm were adjusted in their concentrations to be equivalent to about 2×10^{10} cell/ml.

Estimation of the respiratory rate

The respiratory rate of sperm was estimated by a polarographic method. To 2 ml ASW (artificial sea water) in a closed glass vessel kept at 20°C, 20–30 μl of dry sperm was added through a small hole in the vessel stopper. The decrease in the O_2 concentration in the sperm suspension in the closed vessel was monitored by an oxygen electrode (Yellow Spring Co., Yellow Springs, Ohio, USA) while the suspension was stirred with a magnetic stirrer; the O_2 concentration was recorded (model U-125 MU, Shimadzu Co., Kyoto). The respiratory rate, calculated on the basis of the oxygen decrease in sperm suspension, is expressed as nmol $O_2/10^8$ cell/min. A mixture of 0.2 ml air-bubbled ASW and 1.8 ml CO-bubbled ASW was used to estimate the respiratory rate of sperm in the presence of CO. The respiratory rate of the sperm was also estimated in a mixture of 0.2 ml air-bubbled ASW and 1.8 ml N_2-bubbled ASW. These mixtures contained about 50 μM O_2 in the presence and absence of CO. ASW was bubbled with CO gas, N_2 gas or air for 15 min at 20°C. Stock solutions of KCN and TMPD (tetramethyl p-phenylenediamine) in deionized water and AMA (antimycin A) in 95 % ethanol were also added to the sperm suspensions to make the final concentrations 0.1 mM, 30 μM and 50 μg/ml, respectively.

The activities of cytochrome *c* oxidase and NADH cytochrome *c* reductase

The activity of cytochrome *c* oxidase was estimated by the polarographic method described by Rafael (1983). The closed vessel was also used to measure the cytochrome *c* oxidase activity. The reaction mixture (2.35 ml) was composed of 10 μl of 50 mM TMPD, 150 μl of 100 mM Na-ascorbate, 90 μl of 25 mg/ml cytochrome *c*, 2 ml of 50 mM K-phosphate EDTA buffer pH 7.2 and 100 μl of mitochondrial suspension. The activity of cytochrome *c* oxidase was also estimated in the presence of CO. In this case, the reaction mixture (2.35 ml) contained 1.8 ml of the CO-bubbled EDTA phosphate buffer and 0.2 ml of the air-bubbled buffer. The reaction was initiated at 20°C by adding the mitochondrial suspension to the reaction mixture in the closed vessel. The activity of cytochrome *c* oxidase is expressed as nmol $O_2/10^8$ cell eq./min. The activity of NADH cytochrome *c* reductase was estimated essentially according to the method of Mahler (1955). The reaction mixture (2.2 ml) was composed of 90 μl of 25 mg/ml cytochrome *c*, 2 ml of 50 mM K-phosphate EDTA buffer (pH 7.2) containing 50 mM $MgCl_2$ and 100 mM KCN, 10 μl of 5 mM NADH, and 100 μl of mitochondrial suspension. The reaction at 25°C was initiated by adding the mitochondrial suspension and was terminated at 2 min of the reaction by chilling the reaction mixture in an ice bath for 2 min and then in dry ice. The difference in the absorbance between 550 and 540 nm (ΔA 550-540) was estimated by a dual-beam two-wavelength spectrophotometer (Model 557, Hitachi Co., Tokyo). On the basis of an increase in the ΔA 550-540 value during the reaction, the amount of reduced cytochrome *c* was calculated and expressed as the amount of O_2 to be utilized for the oxidation of reduced cytochrome *c*. The activity is expressed as nmol O_2 eq./10^8 cell eq./min. The activity was also estimated in the presence of CO. In these experiments, 50 mM phosphate EGTA buffer was bubbled with CO for 15 min, and the activities were estimated in the reaction mixture containing CO-bubbled EGTA buffer.

Light irradiation at various wavelengths

The respiratory rate of the sperm and the activities of cytochrome *c* oxidase and NADH cytochrome *c* reductase in the mitochondria were estimated in the presence and absence of CO under light irradiation or in the dark. Light irradiation was performed with the Okazaki Large Spectrograph, with which the light fluence rates of about 150–200 μmol/cm²/sec were obtained at wavelengths between 400 and 620 nm. The light fluence rate was monitored by a photon density meter (HK-1), custom made at the Institute for Physical and Chemical Research, Wako, Saitama, Japan. Neutral density filters were used to alter the light fluence rate.

Chemicals

Cytochrome *c*, NADH and AMA were obtained from Sigma Chemical Co., St Louis, MO. TMPD, K-cyanide were from Kanto Chemicals, Tokyo. ASW was purchased from Jamarin Laboratories, Osaka. The other chemicals were of analytical grade.

RESULTS

Figure 1 shows the effect of light irradiation at the wavelength of 430 nm on cytochrome *c* oxidase (A-C) and NADH cytochrome *c* reductase (D-F) in mitochondria isolated from sperm of the fish *Oncorhynchus masou ishikawae* (A,D), the oyster *Crassostrea gigas* (B, E) and the sea urchin *Hemicentrotus pulcherrimus* (C, F), which are representative of the results obtained with the sperm of the other fish species, *Oncorhynchus masou masou*, *Oplegnathus fasciatus* and *Pagrus major*, the abalone *Nordotis discus*, the starfish *Asterias pectinifera*, and the echiuroid *Urechis unicinctus*. The

effect of light irradiation at 430 nm, shown in Fig. 1, was the strongest among those obtained at the wavelengths between 410 and 610 nm.

As shown in Fig. 1, cytochrome *c* oxidase was almost completely inhibited by CO and was released from CO-induced inhibition by light irradiation in the mitochondria isolated from the sperm of fish, oyster and sea urchin, as well as from all of the other species examined, in the same manner as has been shown in other cell types. In the absence of CO, light irradiation did not enhance the activity of cytochrome *c* oxidase (Fig. 1A-C). The CO-inhibited cytochrome *c* oxidase was weakly reactivated by light irradiation at the light fluence rate of 1 μmol/cm^2·s, and the photo-reactivation of the CO-inhibited enzyme reached its maximum level (more than 85% reactivation) at above 20 μmol/cm^2·s at 430 nm in mitochondria isolated from sperm of fish, oyster and sea urchin, as well as those from all other species examined. The sensitivity of CO-inhibited cytochrome *c* oxidase to light irradiation was the same among the species examined.

As shown in Fig. 1D-F, NADH cytochrome *c* reductase was not affected by light irradiation in the mitochondria isolated from fish sperm but was activated by light in those isolated from the sperm of oyster and sea urchin, as well as those isolated from the sperm of abalone, echiuroid and starfish. CO inhibited this complex enzyme in the mitochondria isolated from the sperm of fish, oyster and abalone more strongly than in those isolated from sea urchin, echiuroid and starfish. In the presence of CO, no photo-activation of this complex enzyme in mitochondria was detectable in the sperm of oyster and abalone but evidently occurred in the sperm of sea urchin, echiuroid and starfish. In the sperm of all of the species examined, this complex enzyme was strongly blocked by

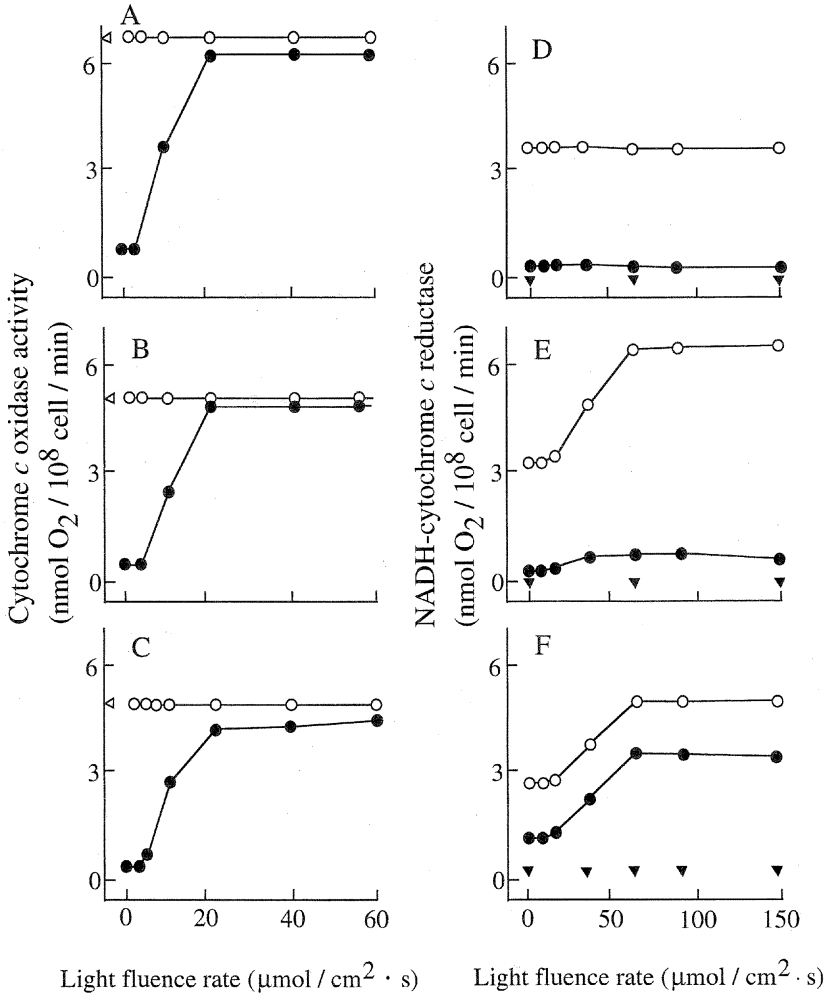

Fig. 1. Effect of light irradiation at 430 nm on cytochrome *c* oxidase (**A-C**) and NADH cytochrome *c* reductase (**D-F**) in mitochondria isolated from sperm of the fish *Oncorhynchus masou ishikawae* (**A**, **D**), the oyster *Crassostrea gigas* (**B**, **E**) and the sea urchin *Hemicentrotus pulcherrimus* (**C**, **F**) in the presence and absence of CO. Experimental procedures are described in Materials and Methods. Values shown by ● are activities of cytochrome *c* oxidase (**A-C**) and NADH cytochrome *c* reductase (**D-F**) in the presence of CO, and those shown by ○ are those in its absence, under light irradiation at 430 nm at various light fluence rates. Values shown with ▼ in **D-F** are NADH cytochrome *c* reductase activity in the presence of 50 μg/ml AMA under light irradiation at various fluence rates. These results are typical of 3-6 experiments made on different sperm batches of each species.

50 μg/ml AMA (antimycin A), a specific inhibitor of electron transport in a span of the mitochondrial respiratory chain between cytochrome *b* and cytochrome *c*, and the AMA-inhibited enzyme was not activated by light irradiation (Fig. 1D-F). The inhibition of this complex enzyme by CO, which is as strong as that shown by 50 μg/ml AMA in the mitochondria from sperm of oyster and abalone, probably blocks the photo-activation of this enzyme. In the presence of 5 μg/ml AMA, NADH cytochrome *c* reductase was weakly inhibited in the mitochondria isolated from the sperm of all of the species examined, and was augmented by light irradiation in those isolated from the sperm of abalone, echiuroid, sea urchin and starfish (data not shown). In the mitochondria isolated from the sperm of sea urchin, echiuroid and starfish, the CO-induced inhibition of this complex enzyme was as weak as that induced by 5 μg/ml AMA and hence did not completely block the photo-activation of this complex enzyme.

The photo-activation of NADH cytochrome *c* reductase in the mitochondria isolated from the sperm of oyster and abalone in the absence of CO and in the sperm of sea urchin, echiuroid and starfish in the presence and absence of CO was detectable at the light fluence rate of 5 μmol/cm^2·s, and reached a maximum level at above 60 μmol/cm^2·s. This photo-sensitivity of NADH cytochrome *c* reductase in the sperm of all of the species examined except the fish sperm is very similar to one another species (Fig. 1E, F) and was markedly lower than the sensitivity of CO-inhibited cytochrome *c* oxidase (Fig. 1A-C) in all of the species examined. At the wavelengths other than 430 nm, larger light fluence rates than those effective at 430 nm were necessary for the maximum photo-reactivation of CO-inhibited cytochrome *c* oxidase in the mitochondria isolated from the sperm of all of the species examined, and for the maximum photo-activation of NADH cytochrome *c* reductase in the mitochondria from the sperm of all of the species examined except fish species (data not shown).

Figure 2 shows the action spectra for the photo-reactivation of CO-inhibited cytochrome *c* oxidase and for the photo-activation of NADH cytochrome *c* reductase in the mitochondria isolated from the sperm of fish, oyster and sea urchin. The activities of cytochrome *c* oxidase and NADH cytochrome *c* reductase under light irradiation at 20 and 50 μmol/cm^2·s were calculated on the basis of the relationships between the light fluence rate and the activities of these enzymes (such as those shown in Fig. 1 for those at 430 nm) at various wavelengths between 410 and 610 nm. As shown in Fig. 2, cytochrome *c* oxidase was not affected by light irradiation at all

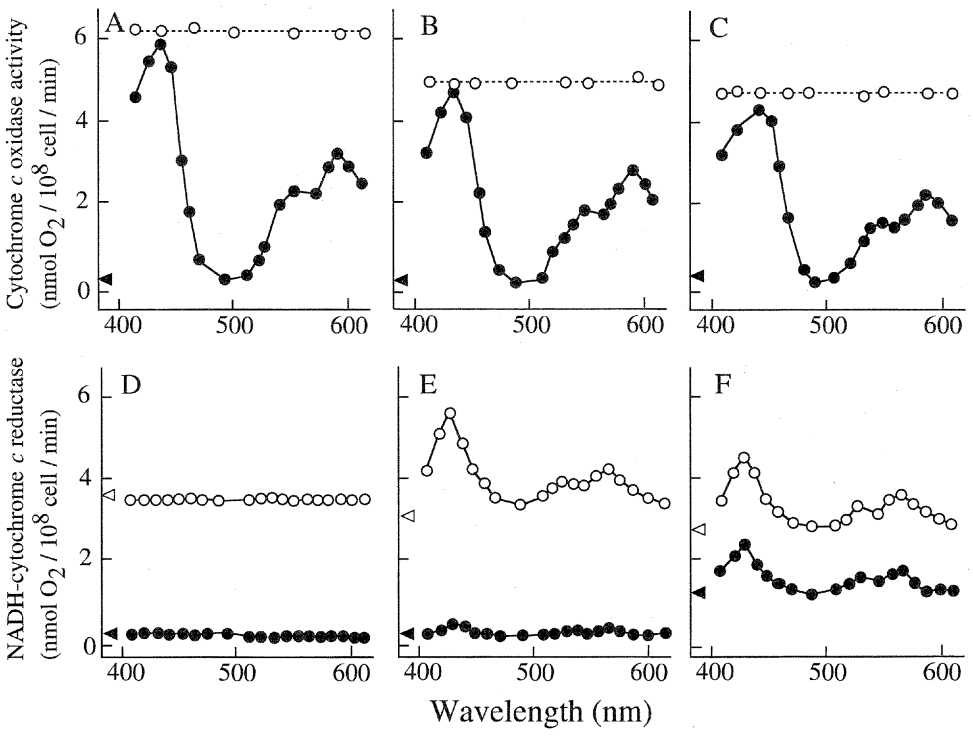

Fig. 2. Action spectra for photo-reactivation of CO-inhibited cytochrome *c* oxidase (**A**-**C**) and for photo-activation of NADH cytochrome *c* reductase (**D**-**F**) in mitochondria isolated from sperm of fish (**A**, **D**), oyster (**B**, **E**) and sea urchin (**C**, **F**). Sperm were obtained from animals of same species experiment described in used in Fig. 1. Cytochrome *c* oxidase activity under light irradiation at 20 μmol/cm^2·s (**A**-**C**) and NADH cytochrome *c* reductase activity at 50 μmol/cm^2·s (**D**-**F**) were calculated on basis of relationships between activities of those enzymes and light fluence rate (such as that shown in Fig. 1 for effects of light irradiation at 430 nm) obtained at various wavelengths (data not shown). Values shown with ● are these enzyme activities in presence of CO, and those shown with ○ are activities in the absence of CO under light irradiation at 20 (**A**-**C**) and 50 μmol/cm^2·s (**D**-**F**). These enzyme activities in dark are shown in ordinate of each figure with ◀ in the presence of CO (**A**-**F**) and with ◁ in its absence (**D**-**F**). In **A**-**C**, dashed holizontal line indicates cytochrome *c* oxidase activity in the absence of CO in dark. Values shown are typical of 3-6 experiments made on different sperm batches of each species.

examined wavelengths between 410 and 610 nm, as found at 430 nm (Fig. 1), unless this enzyme was strongly inhibited by CO, in the mitochondria isolated from the sperm of fish (A), oyster (B), sea urchin (C), abalone, echiuroid and starfish (data not shown). In the mitochondria isolated from the sperm of fish, oyster and sea urchin as well as those from all of the other species examined, the action spectra for the photo-reactivation of CO-inhibited cytochrome *c* oxidase, drawn on the basis of the calculated activity at 20 μmol/cm²·s, showed peaks at 430, 550 and 590 nm, at which wavelengths peaks are also found in the absorption spectrum of CO-bound cytochrome aa_3, or cytochrome *c* oxidase (Yonetani and Kidder, 1963). The absorption of photon energy by CO-bound cytochrome aa_3 probably releases cytochrome *c* oxidase from the effect of CO-induced inhibition.

In the mitochondria isolated from fish sperm, NADH cytochrome *c* reductase was not affected by light irradiation at the wavelengths between 410 and 610 nm, in the presence or absence of CO, as was found at 430 nm (Fig. 2D). This complex enzyme was activated by light irradiation at all wavelengths examined, as at 430 nm, in the mitochondria isolated from the sperm of oyster (Fig. 2E), sea urchin (Fig. 2F), abalone, echiuroid and starfish (data not shown) with peaks at 430, 530 and 570 nm, as reported previously (Tazawa *et al.*, 1996). These peaks in action spectra, drawn on the basis of calculated enzyme activity under light irradiation at 50 μmol/cm²·s, are found at the same wavelengths as those in the absorption spectrum of reduced cytochrome *b*. The absorption of photon energy by reduced cytochrome *b* probably activates the redox reaction by this cytochrome to enhance the activity of this complex enzyme, in which cytochrome *b* is involved. NADH cytochrome *c* reductase was weakly inhibited by CO in the mitochondria isolated from the sperm of sea urchin, echiuroid and starfish, and while weakly inhibited, this complex enzyme was activated by light irradiation with peaks at the same wavelengths as those found in the absence of CO. In the mitochondria isolated from the sperm of oyster and abalone, NADH cytochrome *c* reductase, strongly inhibited by CO, was not activated by light irradiation at the wavelengths between 410 and 610 nm (Fig. 2E).

Respiration in the sperm of echiuroid, sea urchin and starfish is strongly inhibited by AMA and CN^-, as well as by CO (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991). The same was the case in the sperm of fish and abalone (data not shown). These observations indicate that respiration in these sperm depends completely on electron transport from cytochrome *b* to cytochrome *c* in AMA-sensitive reactions (such as that catalyzed by NADH cytochrome *c* reductase), and finally to molecular oxygen in the reaction catalyzed by CN^--sensitive cytochrome *c* oxidase. To determine the contribution of the photo-activation and photo-reactivation of these reaction steps to the respiration in these sperm, the action spectra for the effects of light irradiation on respiration were drawn based on the respiratory rate at the light fluence rates of 10, 20 and 50 μmol/cm²·s (Fig. 3). The respiratory rates under light irradiation at these fluence rates were calculated from the relationships between the light fluence rate and the respiratory rate (data not shown) in the sperm of fish, oyster and sea urchin, by the same procedures those described previously (Tazawa *et al.*, 1991; Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991). In the absence of CO, respiration in the sperm of oyster and sea urchin was not activated by light irradiation as in fish sperm (Fig. 3A), though NADH cytochrome *c* reductase was activated by light irradiation in the mitochondria isolated from the sperm of oyster and sea urchin (Fig. 1).

In the presence of CO, the respiration was almost completely inhibited in the sperm of all of the species examined and was not affected by light irradiation in the sperm of fish, oyster and abalone, at the light fluence rate of 50 μmol/cm²·s. This fluence rate is higher than the rate sufficient for the maximum photo-reactivation of CO-inhibited cytochrome *c* oxidase at 430 nm in the mitochondria isolated from these species (Fig. 1). In the sperm of sea urchin, echiuroid and starfish, light irradiation at 50 μmol/cm²·s strongly reactivated the CO-inhibited respiration. At this high fluence rate, the action spectra for the photo-reactivation of CO-inhibited respiration in the sperm of sea urchin, echiuroid and starfish were similar to the absorption spectrum of reduced cytochrome *b*, as reported previously (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991). The photo-activation of NADH cytochrome *c* reductase in these sperm probably enhances the rate of respiration at such a high fluence rate, at which the CO-inhibited cytochrome *c* oxidase is completely reactivated at almost all wavelengths. At 10 μmol/cm²·s, a rate at which CO-inhibited cytochrome *c* oxidase was strongly reactivated (Fig. 1), but at which the photo-activation of NADH cytochrome *c* reductase was quite weak, if present at all, in the sea urchin sperm, the CO-exposed sperm of sea urchin exhibited a quite weak photo-reactivation of CO-inhibited respiration with peaks corresponding to those in the absorption spectrum of CO-bound cytochrome *c* oxidase.

AMA inhibited the photo-reactivation of CO-inhibited respiration in the sea urchin sperm completely at 50 μg/ml (Fig. 3C) and weakly at 5 μmol/ml (data not shown). CO was found to inhibit NADH cytochrome *c* reductase as completely as did 50 μg/ml AMA in the mitochondria isolated from the sperm of oyster and abalone, as well as fish, and quite weakly in those from the sperm of sea urchin, echiuroid and starfish. Cytochrome *c* reduction, as in the reactions such as that catalyzed by AMA-sensitive NADH cytochrome *c* reductase, is probably indispensable for the photo-reactivation of CO-inhibited cytochrome *c* oxidase to augment CO-inhibited respiration. The incomplete inhibition of cytochrome *c* reduction by CO probably allows the photo-reactivation of CO-inhibited cytochrome *c* oxidase to cause a weak photo-reactivation of CO-inhibited respiration in the sperm of sea urchin, echiuroid and starfish. The photo-activation of NADH cytochrome *c* reductase, found at the high light fluence rate in the sperm of sea urchin even in the presence of CO, then probably results in another augmentation of respiration in CO-exposed sperm. Additionally, it was found in a preliminary experiment that respiration weakly inhibited by 5 μg/ml AMA was evidently activated by light irra-

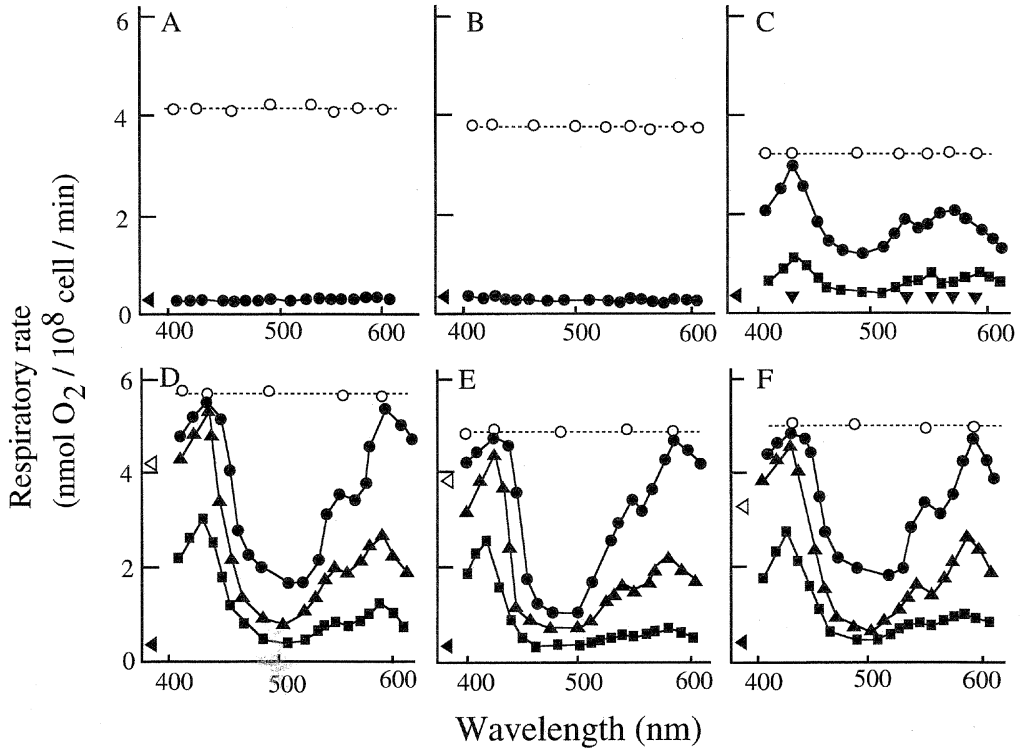

Fig. 3. Action spectra for effects of light irradiation on respiration in sperm of fish (**A**, **D**), oyster (**B**, **E**) and sea urchin (**C**, **F**) in the presence of CO. Experimental procedures are described in Materials and Methods. The respiratory rates in these sperm were also estimated in the presence of 10 μM TMPD, as shown in **D-F**. The respiratory rates estimated under light irradiation at 50 μmol/cm²・s are shown with ● in the presence of CO and ○ in its absence in **A-F**. Respiratory rates in the presence of CO under light irradiation at 10 μmol/cm²・s (■) and at 20 μmol/cm²・s (▲) are also shown in **C-F** and **D-F**. Values under light irradiation at 10 and 20 μmol/cm²・s are omitted in **A-C**, since they are essentially same as under light irradiation at 50 μmol/cm²・s. Values under light irradiation at 20 μmol/cm²・s are also omitted in **C** to simplify figure. Respiratory rates in the presence (**D-F**) and absence of TMPD (**A-C**) in dark are indicated with horizontal dashed lines. Values shown with ◀ in ordinate in **A-F** are respiratory rates in the presence of CO in dark. In **D-F**, respiratory rates in absence of TMPD are shown by in ordinate. Respiratory rate in presence of both CO and TMPD was almost same as in presence of CO. Values marked by ▼ in **C** are respiratory rates in presence of CO and 50 μg/ml AMA under light irradiation at 50 μmol/cm²・s. Respiratory rates in presence of AMA were not shown in **A-B** and **D-F** use they were same as rates in presence of CO under light irradiation. Respiratory rates under light irradiation at above fluence rates were calculated based on relationships between light fluence rate and respiratory rate in presence and absence of CO (data not shown). Action spectra shown in **A-F** are typical of 3 experiments made on different sperm batches of each species.

diation at the high fluence rate even in the absence of CO, in the sperm of abalone, oyster and sea urchin (data not shown). It seems that the photo-activation of NADH cytochrome *c* reductase in these sperm becames apparent as an increase in the respiratory rate, when this complex enzyme is weakly inhibitied.

In the presence of TMPD (tetramethyl paraphynelene-diamine), which is known to mediate non-enzymatical electron transport to cytochrome *c* from cytochrome *b* and ascorbate-like reductants (Lee and Ernster, 1967), the respiratory rate was appreciably enhanced and was made close to zero by CO in the sperm of fish, oyster and sea urchin (Fig. 3), abalone, echiuroids and starfish. The CO-inhibited respiration in the sperm of fish, oysters and sea urchins, as well as those of the other species examined, was strongly activated by light irradiation with peaks corresponding to those in the absorption spectrum of CO-bound cytochrome aa_3 at the fluence rates of 10, 20 and 50 μmol/cm²・s. At the fluence rate of 50 μmol/cm²・s, NADH cytochrome *c* reductase in the sperm of the species examined (other than fish) was strongly activated with peaks corresponding to those in the absoption spectrum of reduced cytochrome *b*. Thus, it is concluded that the photo-reactivation of CO-inhibited respiration in the presence of TMPD resulted solely from the photo-reactivation of CO-inhibited cytochrome *c* oxidase. TMPD-induced artificial cytochrome *c* reduction has been reported to be not affected by light irradiation (Tazawa *et al.*, 1996). It is probable that the TMPD-induced artificial electron transport to cytochrome *c* in the sperm of all of the species examined was at a high enough rate to make the photo-reactivation of CO-inhibited cytochrome *c* oxidase apparent as a strong reactivation of CO-inhibited respiration.

DISCUSSION

In mitochondria isolated from the sperm of abalone, echiuroid, fish, sea urchin and starfish, light irradiation reactivated the CO-inhibited cytochrome *c* oxidase with peaks of photo-

activation at the same wavelengths as those observed in the absorption spectrum of CO-bound cytochrome aa_3 or cytochrome *c* oxidase (Yonetani and Kidder, 1963), in the same manner as in other cell types. In the sperm of sea urchin, echiuroid and starfish, a weak photo-reactivation of CO-inhibited respiration occurred at the light fluence rate sufficient for the strong photo-reactivation of CO-inhibited cytochrome *c* oxidase, with peaks at the same wavelengths as those mentioned above. In the gametes of sea urchin species other than that used in the present study, the photo-reactivation of CO-inhibited respiration is reported to be due to the light-induced reactivation of CO-inhibited cytochrome *c* oxidase, on the basis of difference in the reactivating effect on the CO-inhibited respiration between the wavelengths (Epel, 1963). Activation of CO-inhibited respiration by white light is also found in the eggs of echiuroid (Black *et al.*, 1958). In the sperm of abalone, fish and oyster however, the photo-reactivation of CO-inhibited cytochrome *c* oxidase did not become apparent as an increase in the respiratory rate, though the sperm respiration certainly depended on cytochrome *c* oxidase in the same manner as the sperm of sea urchins starfish and echiuroid.

In the sperm of all species examined, respiration was confirmed to depend on the electron transport in an AMA-sensitive span of the mitochondrial respiratory chain between cytochrome *b* and cytochrome c and on transport to molecular oxygen in the reaction catalyzed by CN^--sensitive cytochrome *c* oxidase. The failure of the photo-reactivation of CO-inhibited cytochrome *c* oxidase to enhance the respiratory rate in the CO-exposed sperm of abalone, fish and oyster is probably the result of the depression of cytochrome *c* reduction, which provides the substrate, reduced cytochrome *c*, for cytochrome *c* oxidase. NADH cytochrome *c* reductase, mediating cytochrome *c* reduction, was inhibited by CO almost completely in the mitochondria isolated from the sperm of abalone, fish and oyster and weakly in those from the sperm of echiuroid, sea urchin and starfish. A weak CO-caused inhibition of cytochrome *c* reduction in the reactions such as those catalyzed by NADH cytochrome *c* reductase in the sperm of echiuroid, sea urchin and starfish is thought to allow the photo-reactivation of CO-inhibited cytochrome *c* oxidase to slightly enhance the respiratory rate in the CO-exposed sperm of these species. The inhibition of cytochrome *c* reduction by AMA blocked the photo-reactivation of CO-inhibited respiration in the sperm of echiuroid, sea urchin and starfish. Non-enzymatical cytochrome *c* reduction by TMPD induced a strong photo-reactivation of CO-inhibited respiration in the sperm of all species examined, with peaks at the same wavelengths as the peaks in the absorption spectrum of CO-bound cytochrome aa_3. These findings indicate that electron transport to cytochrome *c* is indispensable for the photo-reactivation of CO-inhibited cytochrome *c* oxidase to enhance the respiratory rate.

In the mitochondria isolated from the gametes and viscera of abalone, echiuroid, oyster, sea urchin and starfish, the photo-activation of NADH cytochrome *c* reductase and succinate cytochrome *c* reductase occurs with peaks at the same wavelengths as those in the absorption spectrum of reduced cytochrome *b* but does not occur in the mitochondria from fish sperm (Tazawa *et al.*, 1996). In the presence of CO, the photo-activation of NADH cytochrome *c* reductase with the peaks mentioned above was found only in the mitochondria isolated from the sperm of echiuroid, sea urchin and starfish. The light fluence rate for the photo-activation of these complex enzymes in the presence and absence of CO was markedly higher than the rate for photo-reactivation of CO-inhibited cytochrome *c* oxidase. Light irradiation, at the fluence rate high enough to activate these complex enzymes, strongly enhanced the rate of respiration in the presence of CO in the sperm of echiuroid, sea urchin and starfish with peaks of photo-reactivation at wavelengths the same as those in the absorption spectrum of reduced cytochrome *b*. The same finding has been reported in previous papers (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991). The acceleration of cytochrome *c* reduction due to the photo-activation of NADH cytochrome *c* reductase, as well as succinate cytochrome *c* reductase, which occurs in the sperm of echiuroid, sea urchin and starfish even in the presence of CO, probably strongly activates the CO-inhibited respiration at the high fluence rate, at which the maximum photo-reactivation of CO-inhibited cytochrome *c* oxidase was induced at almost all wavelengths.

In the sperm of abalone, echiuroid, oyster, sea urchin and starfish, the mitochondria of which exhibit the photo-activation of NADH cytochrome *c* reductase and succinate cytochrome *c* reductase (Tazawa *et al.*, 1996), light irradiation at the fluence rate high enough to induce the photo-activation of those complex enzymes does not exert any effect on their respiration in the absence of CO (Fujiwara *et al.*, 1991; Yasumasu *et al.*, 1991), in the same manner as in fish sperm, in which these complex enzymes are hardly affected by light irradiation. It is not yet known why the photo-activation of NADH cytochrome *c* reductase does not enhance the respiratory rate in these sperm (which have photo-sensitive complex enzymes) in the absence of CO. We tentatively propose that light irradiation does not cause any increase in the respiratory rate even in sperm with photo-activated NADH cytochrome *c* reductase and succinate cytochrome *c* reductase, unless the reactions such as those catalyzed by NADH cytochrome *c* reductase are rate-limiting in the overall electron transport through whole sperm of the mitochondrial respiratory chain. Indeed, photo-reactivation of respiration occurred in the sperm of echiuroid, oyster and sea urchin, in which the respiration was weakly inhibited by AMA at low concentrations. Under such a weak inhibition of electron transport by a low concentration of AMA, reactions catalyzed by these mitochondrial complex enzymes seem to be made rate-limiting in the mitochondrial respiratory chain.

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