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POSSIBLE INVOLVEMENT OF Fe-S CENTERS AS MAJOR ENDOGENOUS PHOTOSENSITIZERS IN HIGH LIGHT-CAUSED LOSS OF MEMBRANE STRUCTURE AND FUNCTION OF MITOCHONDRIA

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Abstract – Exposure of isolated intact mitochondria to near UV to visible light resulted in not only loss of respiration, the most well-documented phenomenon regarding phototoxic effects in the respiring organelles, but also lipid peroxidation of membranes and mitochondrial swelling; these turned out to be O_2-dependent and thus prevented by anaerobiosis, enhanced by a partial deuteration of the suspension medium, and suppressed by the presence of a singlet oxygen ($^1O_2$) scavenger. Measurements of the spectral dependence of such detrimental effects of light on mitochondrial structure and function revealed that all the resulting spectra bear a significant resemblance to the action spectrum for photogeneration of $^1O_2$ from mitochondrial membranes, which in turn carries the spectral characteristics of light absorption by mitochondrial Fe-S centers. Futhermore, destructing the Fe-S centers by a mercurial treatment of mitochondria brought about a striking reduction of the light-induced membrane peroxidation and swelling of mitochondria. These results are consistent with the suggestion that the impairment of functional, structural integrity of mitochondria caused by strong irradiation is directly related to the production of $^1O_2$ in mitochondria, photosensitized by the Fe-S centers. This paper also presents kinetic data which indicate that, among various membrane-bound protein systems associated with mitochondrial energy metabolism, the respiratory chain is the primary target for photodamage.

INTRODUCTION

Previously, observing that submitochondrial particles (SMP) † prepared from soybean hypocotyls produced singlet oxygen ($^1O_2$, $^1\Delta_2$) upon exposure to near-UV and visible light, our laboratory measured the action spectra for photogeneration of $^1O_2$ from mitochondrial membranes by using both 'intact' SMP and those subjected to the mersalyl acid (MA) treatment; based on these, the Fe-S centers of membrane-bound nonheme iron proteins were suggested as the most important endogenous type II sensitizers in mitochondria.[1] $^1O_2$ such produced appears to be involved not only in photoinactivation processes of the respiratory system which operates in the inner membrane, but also in those of the regulatory enzymes of the citric acid cycle present in the mitochondrial matrix.[1,2]

There is now a copious body of evidence that $^1O_2$ can attack a variety of substrates in biological membranes, resulting in chemically modifing their structures.[3–5] Notably, unsaturated lipids are readily vulnerable to $^1O_2$-mediated peroxidation and many kinds of proteins, which involve $^1O_2$-sensitive amino acid residues such as histidine, cysteine, methionine, tyrosine and tryptophan in their active sites, are very susceptible to oxidative inactivation by this active oxygen species. It would be, therefore, reasonably expected that photoproduction of $^1O_2$ in mitochondrial membranes under high light conditions may be directly related to the light-induced impairment of membrane structure and function of the respiring organelles. This point was scrutinized in the present study, where we particularly focused on the role of the Fe-S centers in the detrimental effects of near UV to visible light on the activities of a number of membrane-bound protein systems, which are associated with mitochondrial energy metabolism, as well as on the alteration of structural integrity of mitochondrial membranes.

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† *Abbreviations:* ADP, adenosine diphosphate; ATP, adenosine 5'-triphosphate; MA, mersalyl acid; Mes, 2-(N-Morpholino)ethanesulfonic acid; NADP, nicotinamide adenine dinucleotide phosphate; Pi, inorganic phosphate; SDS, sodium dodecylsulfate; SMP, submitochondrial particles; $^1O_2$, singlet oxygen; TBARS, thiobarbituric acid-reactive substance; Tris, 2-amino-2-hydroxy-methyl-1,3-propandiol.

This paper presents some results which are pertinent to the concept that the excited Fe-S centers play a crucial role in the functional, structural degradation of mitochondrial membranes by participating in the primary process of photodynamic reactions which apparently proceed *via* the type II mechanism.

MATERIALS AND METHODS

Mitochondrial preparation. Mitochondria from soybean (*Glycine max*) hypocotyls, grown in the dark at *ca.* 25°C for 5-6 days, were isolated according to the procedures of Bonner.[6] Protein was measured by the modified Lowry method.[7]

Photolysis. Mitochondrial suspensions in a long necked glass cuvette (10 mm light path), fitted with a thermostated water jacket (20°C), were irradiated with either white light ($\lambda > 320$ nm), which was passed through a flexiglass plate (6 mm thickness) and water contained in a 500 mL round bottom flask, from a 1 kW Xe-arc lamp (Osram, München, Germany) or monochromatic light obtained from the Xe lamp by the use of a f/3.4 grating monochromator (Applied photophysics, London, UK). During irradiation, samples were bubbled gently with either air or nitrogen.

Measurements of membrane-lipid peroxidation and mitochondrial swelling. The production of thiobarbituric acid-reactive substances (TBARS) from hot acid-digested mitochondria was monitored by absorbance change at 535 nm as an index of the lipid peroxidation, as described in Buege and Aust.[8] The swelling of mitochondria induced by strong irradiation was determined by a change in light scattering at 700 nm, as described by Vercesi.[9]

Determination of mitochondrial respiratory activity. Respiration of mitochondria in a suspension medium (0.3 *M* mannitol, 5 m*M* $MgCl_2$, 10 m*M* KCl and 10 m*M* K-phosphate, adjusted to pH 7.2) was measured by monitoring oxygen uptake, using a polarograph constructed in this laboratory in the presence of 10 m*M* succinate and 1 m*M* ADP, as described previously.[10]

Assay of inorganic phosphate (Pi) carrier. Measurement of optical change at 546 nm (OD_{546}) of mitochondrial samples, which results from organelle shrinking due to water efflux from the mitochondrial matrix in a Ca^{+2}-caused swollen state, accompanied by the Pi uptake, is the basis for assaying the Pi carrier activity. For this, the procedures described by Fonyo *et al.*[11] was followed without any modification, using mitochondria suspended in a medium containing 0.25 *M* sucrose, 5 m*M* Tris-acetate, 2 m*M* Tris-succinate, 4 m*M* $MgCl_2$ and 5 μM rotenone and swollen by the addition of 160 μM $CaCl_2$.

Assay of ADP/ATP translocator. The ATP efflux, that is, the appearance of ATP in the extramitochondrial phase upon the addition of ADP to mitochondrial suspensions, was monitored by the formation of NADPH, which was catalyzed by a coupled enzymatic system comprising hexokinase (1 unit) and glucose-6-phosphate dehydrogenase (0.5 units) in the presence of 2.5 m*M* glucose and 0.2 m*M* $NADP^+$. Except that the build-up NADPH was measured in a spectrophotometer instead of a spectrofluorometer, we followed the procedures described in Atlante *et al.*[12] for the assay.

Assay of Fo-F1 ATPase. Mitochondria were ruptured by ultrasonication so as to expose the inside of the inner membrane along with the ATPase. ATP were added to the ruptured mitochondria in a buffer containing 60 m*M* Tris/Mes (pH 8.5), 50 m*M* KCl and 3 m*M* $MgSO_4$ (3 m*M* ATP for every 30g membrane protein). The hydrolysis reaction of ATP was stopped by the addition of 3 mL of 7% SDS and then Pi was measured as described by Pullman and Penefsky.[13]

Chemicals and instruments. Chemicals were purchased from either Fluka Chemie AG (Buchs, Switzerland), Wako Pure Chemical Ind. (Osaka, Japan) or Sigma Chemical Co. (St. Louis, MO). Enzymes were purchased from Sigma. Throughout the work, spectrophotometric measurements were done with a HP 8452A diode-array spectrophotometer (Hewlett Packard Co. Palo Alto, CA) and oxygen in samples was assayed by using a polarograph constructed in this laboratory. A IL1700 Research Radiometer/Photometer (International Light Inc., Newburyport, MA) was used for the determination of fluence rates of visible light ($\lambda > 400$ nm), while chemical actinometry using ferrioxalate[14] was employed for the fluence rate measurement of near UV ($\lambda > 320$ nm).

RESULTS

Membrane lipid peroxidation

Mitochondrial preparations from soybean hypocotyls were subjected to irradiation with either white light ($\lambda > 320$ nm) or monochromatic light in the ranges from 380 nm to 600 nm and subsequently assayed for lipid peroxidation of mitochondrial membranes. Special care was taken to prevent light-independent autooxidation of membrane lipid; that is, except for the light-treatment, mitochondrial samples were always kept at *ca.* 0°C under a nitrogen atmosphere in the dark.

Measurements of TBARS production from the irradiated miochondria upon hot-acid digestion revealed that membrane lipid peroxidation resulted from the combined action of light and oxygen, which was substantially suppressed by the presence of imidazole in samples and significantly enhanced by a partial deuteration of medium, as summarized in Table 1.

When the dependence of the lipid peroxidation on the irradiating wavelengths was plotted as in Fig. 1, the resulting spectrum, showing an action peak at *ca.* 420 nm, turned out to be very similar to the action spectrum for $^1O_2$ photogeneration from mitochondrial membranes prepared from the same plant source (for this, see the previous paper[1]). Pre-treatment of mitochondria with MA effectively removed the lipid peroxidation effect of light, as clearly demonstrated by a complete disappearance of the 420 nm peak.

Mitochondrial swelling

Mitochondria were suspended in a medium con-

Table 1. Membrane-lipid peroxidation and swelling of soybean hypocotyl mitochondria subjected to various treatments

Treatment*	TBARS production (nmol/mg protein)	Swelling ($\triangle A_{700} \times 10^2$)
Dark+N_2	5.4(0)	0.0
Dark+O_2	5.4(0)	0.0
Light+N_2	5.5(2)	0.4
Light+O_2	8.4(56)	6.8
Light+imidazole+O_2	6.8(26)	2.6
Light+D_2O	9.5(76)	9.4

* Mitochondrial suspensions (0.3 mg protein/mL) were irradiated with white light ($\lambda > 320$ nm and light fluence rate = 500 W/m²) for 20min under either aerobic (O_2) or anaerobic (N_2) conditions at 20°C. [Imidazole] = 10 m*M* and [D_2O] = 50%. Data are averages of duplicate measurements; the relative value of the spread was not greater than 8%. Values in parentheses are the percent of the photostimulation of dark control.

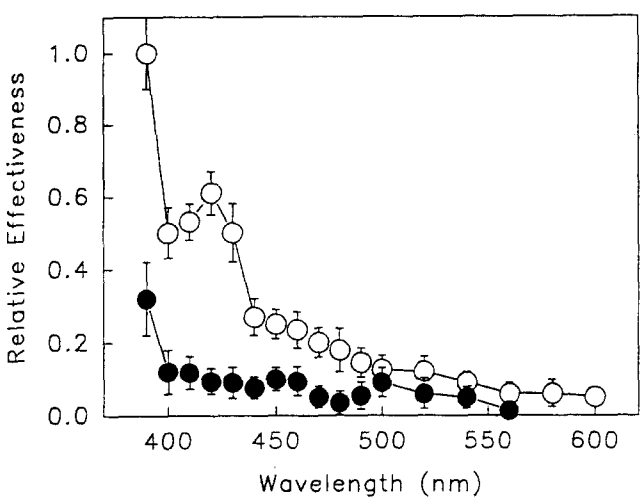

Figure 1. Action spectra for light-induced membrane lipid peroxidation of soybean hypocotyl mitochondria. The effectiveness in TBARS production from intact mitochondria (○), as compared with that from MA-treated mitochondria (●), was determined by dividing the absorbance at 535 nm of TBARS-thiobarbituric acid adduct by the photon fluence rate of the irradiating wavelength. For the treatment of MA, mitochondrial suspensions were diluted into 10 vol of the suspension medium containing MA (1 m*M*), stored for 10 h at 4°C in the dark, and then dialyzed against the suspension medium. Mitochondrial suspensions were aerobically irradiated with monochromatic light at 20°C for 40 min. Data are averages of triplicate measurements.

taining sucrose (0.25 *M*) and irradiated with monochromatic light, and then changes in the extent of light scattering by the organelles were determined at 700 nm. Because the swelling of mitochondria is accompanied by a decrease in their light scattering properties which can be conveniently measured in a spectrometer at a wavelength where no absorption of light by the internal chromophores occurs,[15] the spectral dependence of the decrease in optical density of mitochondrial suspensions upon irradiation should represent the action spectrum for light-induced mitochondrial swelling.

As can be seen in Fig. 2, the action spectrum bears a close resemblance to that for membrane lipid peroxidation by near UV to visible light; further, the effect of the MA-treatment on the light-induced mitochondrial swelling was found out to bear a parallel to that on the lipid peroxidation. Similar to their effects on the peroxidation, imidazole protected mitochondria to a certain extent against the light-induced swelling and D_2O substitution of medium resulted in a substantial increase in the extent of the swelling.

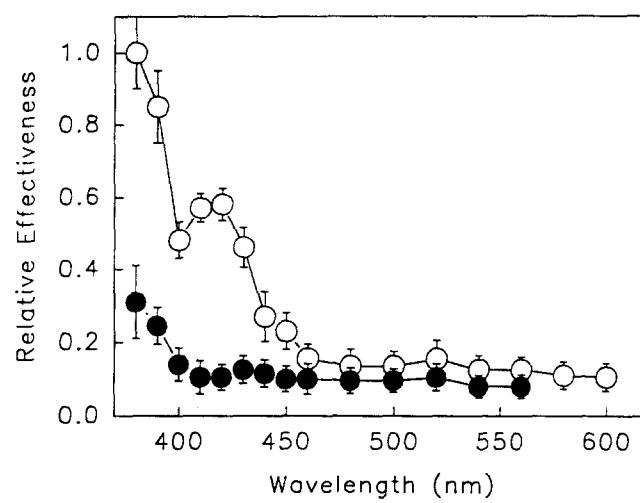

Figure 2. Action spectra for light-induced swelling of soybean hypocotyl mitochondria. The effectiveness of swelling of intact mitochondria (○), as compared with that of MA-treated mitochondria (●), was measured by dividing the decrease in OD at 700 nm of mitochondrial suspensions by the photon fluence rate of the irradiating wavelength. Mitochondria were aerobically irradiated with monochromatic light at 20°C for 60 min. As to the MA treatment, see Fig. 1. Data are averages of triplicate measurements.

Inactivation of membrane proteins involved in mitochondrial energy metabolism

The production of the effective biological energy in cells is largely dependent on the capacity of mitochondria for respiration-coupled phosphorylation and ATP translocation into cytoplasm: the oxidative ATP synthesis is controlled by a number of parameters such as H^+ gradient across the inner membrane, F_0-F_1 ATPase activity and intramitochon-

drial steady state concentration of Pi and ADP; the ADP/ATP exchange activity of the inner membrane seems to determine the cytosolic availability of ATP.[16] In this context, it would be desirable to investigate the light effects on the activities of various membrane-protein systems, which are closely associated with cellular energy production, aiming at confirming the primary target for photodamage to mitochondrial energy metabolism. Among the systems examined, the highest *in situ* susceptibility to photoinactivation was found in the electron carrier chain, as shown in Fig. 3. The transport systems for

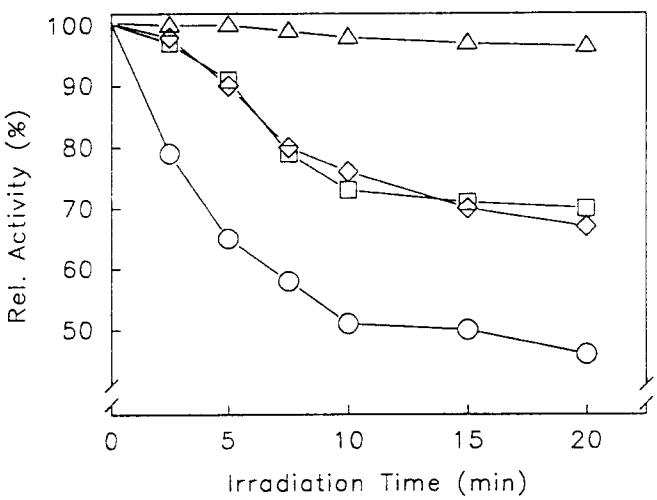

Figure 3. Time courses of photoinactivation of succinate-dependent mitochondiral respiration (-○-), ADP/ATP translocator (-□-), Pi carrier (-◇-), and Fo-F1 ATPase (-△-). Irradiation conditions were same as in Table 1. Data are averages of duplicate measurements.

phosphate compounds, *viz* ADP/ATP antiport and Pi carrier, were also inhibited to some exent, albeit much less compared to the respiratory chain, by strong irradiation. In contrast to these, Fo-F1 ATPase showed the least sensitivity to near-UV and visible light.

Because the respiratory system turned out to be most likely the primary site of the light-induced impairment of mitochondrial function in so far as a decrease in the energy production capacity is concerned, we chose this as a representative of membrane-bound protein systems readily perturbable under high light conditions and measured the spectral dependence of its inactivation. The resulting action spectra, as shown in Fig. 4, again bears a parallel to those for the lipid peroxidation and the swelling of mitochondria (see Figs. 1 and 2). Although the effect of the MA treatment could not be checked because the mercurial *per se* is deadly poisonous for mitochondrial respiration, the results showing the

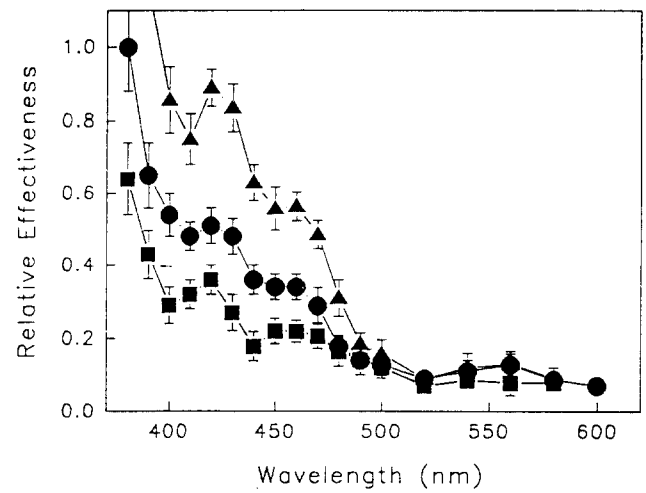

Figure 4. Action spectra for photoinactivation of succinate-dependent respiration of mitochondria from soybean hypocotyls. The effectiveness of photoinactivation of respiration was determined by dividing the decrease in the respiration by the photon fluence rate of the irradiating wavelength. Irradiation conditions were same as in Fig. 1. Data are averages of triplicate measurements. ●: mitochondria were suspended in 'plain' suspension medium. ▲: mitochondria were suspended in the suspension medium containing 50% D_2O. ■: mitochondria were pre-incubated with imidazole (10 m*M*) in 'plain' suspension medium.

effects of imidazole and D_2O on photoinactivation of the respiration were consistent with those observed in relation with the structural alteration of mitochondrial membranes by irradiation.

DISCUSSION

As has long been observed, mitochondria upon exposure to near UV and visible light suffer impairment of their biological functions.[2,17,18] Because this is O_2-dependent and prevented by anaerobiosis, it is generally thought that photodynamic reactions promoted by certain mitochondrial pigments are involved in the damage.

In mitochondria, there exist three classes of visible light-absorbing proteins which are discerned by the types of their prosthetic groups, *viz* flavoproteins, hemoproteins and iron-sulfur proteins. Among these flavoproteins have received special attention from workers who were interested in natural photosensitivity in higher organisms; for some flavins are efficient photosensitizers *in vitro*. Most notably, L. Packer's group[17-19] conducted several elaborative studies on photodamage to the respiring organelles *in vivo* as well as *in vitro*, suggesting flavins as endo-

genous photosensitizers involved, at least, in the inactivation processes of the respiratory chain. Meanwhile, other groups presented some experimental results that imply the possible involvement of mitochondrial hemes as the photosensitizing agents.[20,21] However, our results do not appear to support the importance of either flavins or hemes in connection with the intrinsic light-sensitivity of mitochondria. Then, the only internal chromophores left for experimental scrutiny should be Fe-S centers.

As is established, abundant Fe-S centers (7 centers in animal and 9 centers in plant) are present in mitochondria,[22,23] most of which are associated with complexes I and II of the respiratory chain. In plant mitochondria, three centers denoted as N-1b, N-2 and N-3 are assigned to complex I and other three centers designated as S-1, S-2 and S-3 to complex II; in addition, it seems that there exists the seventh Fe-S center in the alternate NADH pathway of the electron transport.[22] N-1a, another Fe-S center of complex II, and the Rieske center of complex III present in animal mitochondria have not been found in plants.[22,23]

In fact, previously our laboratory provided evidence for the oxidized Fe-S centers as major endogenous sensitizers in mitochondria, in so far as type II processes of photosensitization are concerned.[1] What we have primarily attempted in the present work is therefore to ascertain whether photosensitization of mitochondrial membranes by the internal pigments proceeds *via* the type II (singlet oxygen) mechanism. If so, it would be rather convincing that Fe-S centers are the sensitizing chromophores primarily responsible for photodamage to mitochondria, and hence it would not be unreasonable to expect that the action spectra for such light effects as inactivation of membrane-bound proteins, membrane lipid peroxidation, and mitochondrial swelling show a resemblance to the absorption spectra of mitochondrial Fe-S centers in general shapes as well as in the peak positions, as does the action spectrum for $^1O_2$ photogeneration from mitochondrial membranes.

All spectra presented in this paper conform to this expectation. In addition, the fact that the action bands in blue light region (400-500 nm), which carry the spectral characteristics of light absorption by mitochondrial Fe-S centers,[24] disappeared in the MA-treated samples (Figs. 1 and 2) further supports the concept that these blue light-absorbing chromophores of nonheme iron proteins play a crucial role in photodynamic sensitization of mitochondrial membranes *via* $^1O_2$ mechanism; for mersalyl acid specifically destroys the labile sulfur-containing iron groups.[25,26] The effects of imidazole which scavenges $^1O_2$ and D_2O which prolongs the lifetime of $^1O_2$ on the lipid peroxidation, mitochondrial swelling and the inhibition of respiration in bright light (Table 1) are also in accordance with the type II mechanism for the primary processes of photosensitized reactions occurring in the membranes.

It seems likely that membrane lipid peroxidation, membrane-bound protein inactivation and mitochondrial swelling are interrelated. Lipid peroxidation has been linked to the increased solute permeability of membranes that is directly connected to the swelling and lysis of membrane vesicles, as observed in liposome.[27,28] Non-development of electrochemical potential due, in part, to oxidative modification of membrane proteins can also be attributed to an increase in the permeability of mitochondrial inner membranes for H^+ and other ions.[29] Certain reactive intermediates which are produced during lipid peroxidation processes may affect membrane proteins.[17] The loss of membrane fluidity resulting from lipid peroxidation can also contribute to the decreased enzyme function.[27] Whatever the physico-chemical nature of the interrelations and their mechanistic implications are, it can be said for the moment, based on our own results, that photodynamic action sensitized by the oxidized Fe-S centers is the common cause of the deleterious effects of near-UV and visible light in mitochondria.

In conclusion, our results clearly indicate that light-induced loss of functional and structural integrity of mitochondrial membranes is directly linked to photoproduction of $^1O_2$ by endogenous Fe-S centers in their oxidized states. Taking the results discussed above and those obtained in the previous works from this laboratory done with mitochondrial,[1,2] chloroplasts[30-32] and liposome[33] all together, we consider that Fe-S centers are the most important endogenous chromophores in cells, responsible for natural sensitivity to near UV and blue light that has been observed with many kinds of microorganisms, multicelluar animals and plant cells.

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