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OXIDATION OF SPECIFIC SH PROTEIN OF MITOCHONDRIA BY PHOTODYNAMIC ACTION OF HEMATOPORPHYRIN

RELEVANCE TO UNCOUPLING OF OXIDATIVE PHOSPHORYLATION

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Abstract—Photoexcited hematoporphyrin (Hp) induces the uncoupling of oxidative phosphorylation of mitochondria. The uncoupling was inhibited by pre-incubation of mitochondria with a fluorescent SH reagent, eosin-5-maleimide, which has been shown to react specifically with an essential SH group of the P_i/H^+ symporter [Houstek and Pedersen, *J Biol Chem* **260**: 6288–6295, 1985]. Eosin-5-maleimide labeled 33, 34.5 and 36 kDa proteins in untreated rat liver mitochondria. When eosin-5-maleimide was added after the treatment with Hp plus light, the proteins were not labeled. Singlet oxygen detection by the ESR spin trapping method during photoradiation of Hp was inhibited by amino acids. Cysteine inhibited it more efficiently than histidine, methionine, tryptophan, tyrosine or alanine under the conditions used. HPLC demonstrated that Hp plus light oxidizes cysteine to cystine together with a smaller amount of cysteinesulfinic acid. These results suggest that Hp plus light oxidizes the SH group of mitochondrial protein, probably the P_i/H^+ symporter, with singlet oxygen as a mediator. The possibility of the uncoupling of oxidative phosphorylation through such a modification of the P_i/H^+ symporter is discussed.

Porphyrins have proved useful as sensitizers in cancer photoradiation therapy [1, 2]. Hematoporphyrin derivative (HpD†) or Photofrin® II (a more purified version of HpD) is used clinically. However, the mechanism of photodamage in cancer cells has not yet been fully studied. Mitochondria are a potential target for HpD-induced photosensitization. Ultrastructural studies of cells treated with Hp plus light revealed that the first morphological change in cells occurs within the mitochondria [3]. Fluorescence microscopy demonstrated intracellular binding of HpD to mitochondria [4]. The ATP level in cells exposed to HpD plus light was found to be decreased [5, 6]. Consistently, cell death was found to occur mainly in a single cell following mitochondrial illumination with a laser microbeam [7]. Furthermore, illumination with Hp was found to induce stimulation of mitochondrial respiration in state 4 (i.e. without ADP), which indicates uncoupling of oxidative phosphorylation, in rat liver mitochondria depending on the light intensity and the concentration of Hp [8, 9]. Singh *et al.* [10] showed that dissipation of the electrochemical gradient in mitochondria takes place before cell death. However, the mechanism of uncoupling induced by the photodynamic action of Hp has not yet been elucidated.

The P_i/H^+ symporter is an essential component of the mitochondrial phosphorylating assembly. It contains a SH group which is required for proper functioning. Eosin-5-maleimide was shown to react specifically with the essential SH group of the P_i/H^+ symporter located in the cytosolic surface of the inner mitochondrial membrane [11]. In this paper, we examined the effect of eosin-5-maleimide on the stimulation of state 4 respiration of mitochondria by hematoporphyrin (Hp) plus light to clarify the involvement of the P_i/H^+ symporter.

Previous ESR studies using singlet oxygen ($^1O_2$) traps demonstrated that photoexcited Hp reacts with O_2 to produce $^1O_2$ [12]. In this study, we also measured the relative reactivity of amino acids with $^1O_2$ generated by photoexcited Hp and found that the photochemically generated $^1O_2$ reacts with Cys most effectively.

MATERIALS AND METHODS

Materials. 2,2,6,6-Tetramethyl-4-piperidone hydrochloride and 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy were purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Eosin-5-maleimide was from Molecular Probes, Inc. (Eugene, OR, U.S.A.). *o*-Phthalaldehyde, polyoxyethylene lauryl ether and *N*-acetylcysteine were from the Wako Chemicals Co. (Osaka, Japan). Sodium 1-octanesulfonate was from Nacalai Tesque (Kyoto, Japan). Hp was from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Hp was purified by the method of Granick *et al.* [13], and the purity was checked using HPLC [14].

Illumination of mitochondria in the presence of Hp. Mitochondria were isolated from the livers of Wistar rats by the method of Hogeboom [15]. The functional integrity of the preparations was tested

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† Abbreviations: Hp, hematoporphyrin; HpD, hematoporphyrin derivative; $^1O_2$, singlet oxygen; ESR, electron spin resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

by measuring the respiratory control ratio with ADP using glutamate as the substrate. Mitochondria with an ADP respiratory control ratio greater than 5 were used. The illumination of mitochondria was carried out in a water-jacketed glass vessel with a thermostat (25°), fitted for oxygen measurement with a Clark electrode (Gilson, Paris, France) and containing 1.7 mL of respiratory medium. A 1000 W incandescent light bulb placed at a distance of 43 cm was used as an illumination source. Each medium was stirred magnetically. The power intensity of the light at the level of the vessel was 50 mW/cm^2 as measured by a power energy meter. Glutamate (6 mM) as substrate and Hp (20 μM) were added to the respiratory medium at the beginning of each experiment. About 1 mg of mitochondria protein per mL of respiratory medium was used for each experiment.

Measurement of the respiration rate of mitochondria treated with eosin-5-maleimide. To clarify the involvement of the SH group of P_i/H^+ symporter in the stimulation of mitochondrial respiration by Hp plus light, mitochondria were incubated with 5 μM eosin-5-maleimide in the dark for 5 min. Then, mitochondria were illuminated as described above for a variable duration. The state 4 respiration rate before and after the illumination was measured.

Eosin-5-maleimide labeling of mitochondrial proteins. Mitochondria were illuminated as described above for 20 sec in respiratory medium containing Hp and glutamate. Then 10 μM eosin-5-maleimide was added to the medium. After incubation for 10 min, 50 mM dithiothreitol was added. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 10% polyacrylamide slab gels according to the procedure of Laemmli [16] and the gels were then photographed during excitation with a TF-20L transilluminator (365 nm, 90 W; Vilber Lourmat, Marne la vallée, France).

Measurements of reactivities of $^1O_2$ with amino acids by ESR. The relative yield of $^1O_2$ was measured using 2,2,6,6-tetramethyl-4-piperidone as a $^1O_2$ trapping agent as described previously [14, 17–19]. Typically, samples (100 μL) contained 50 μM Hp, and 100 mM 2,2,6,6-tetramethyl-4-piperidone in 20 mM sodium phosphate buffer at pH 7.4. Where indicated, amino acid (Cys, His, Met, Trp, Tyr and Ala) was added. The samples were illuminated at 0° with the transilluminator (365 nm, 90 W) placed at a distance of 10 cm. For ESR measurements, aliquots of the solutions were taken in calibrated capillaries (50 μL) after illumination. ESR was measured at 25° using a JES-FE-3XG spectrometer (JEOL, Tokyo, Japan) with 100 KHz field modulation. Spectra were recorded with a microwave power of 16 mW and a modulation amplitude of 1.0 G.

HPLC apparatus and conditions. A LC-6A HPLC system (Shimadzu, Kyoto, Japan) equipped with a system controller was used for delivering the mobile phase at a constant flow rate of 0.5 mL/min. A Cosmosil 5C18 Packed Column (4.6 mm i.d. × 150 mm; Nacalai Tesque, Kyoto, Japan) was used for the separation of the products of photooxidation of Cys by Hp. A Shimadzu LC-3A pump was used for delivering the postcolumn reagent at a constant flow rate of 0.3 mL/min. The reagent was prepared by adding 800 mg/L *o*-phthalaldehyde, 1.4% (v/v) ethanol, 1000 mg/L *N*-acetylcystine and 400 mg/L polyoxyethylene lauryl ether to buffer solution (pH 10.0) containing sodium carbonate (0.384 M), boric acid (0.216 M) and potassium sulfate (0.108 M). The separation column and reaction coils were placed in a Shimadzu CTO-2A column oven at 40°. The reaction coil for the fluorescence reaction was a 200 mm long stainless-steel tube (0.5 mm i.d.). The fluorescence intensity of the effluent was measured at the excitation and emission maxima of 348 and 450 nm, respectively, in a Shimadzu RF-500LC spectrofluoromonitor equipped with a xenon discharge lamp. The separation of cysteinesulfinic acid and cysteic acid was carried out by the HPLC method of Stipanuk *et al.* [20]. The amount of the products was calculated by comparing them to the standards.

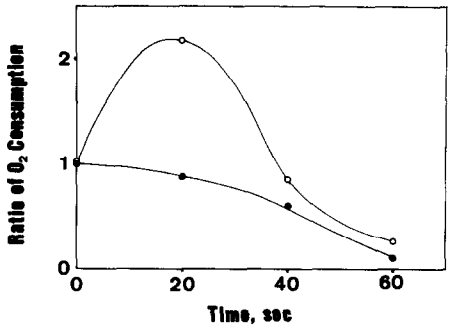

Fig. 1. The effect of eosin-5-maleimide on the stimulation of state 4 respiration mitochondria by Hp plus light. Mitochondria (1 mg/mL) were suspended in the respiratory medium (10 mM $MgCl_2$, 60 mM KCl, 7 mM KH_2PO_4, 87 mM sucrose and 24 mM glycylglycine at pH 7.4) containing 20 μM Hp and 6 mM glutamate as substrate. Then eosin-5-maleimide was added to a final concentration of 5 μM (●) or 0 μM (○). The medium was incubated in the dark for 5 min and illuminated for a variable duration. The ratio of O_2 consumption is the rate of O_2 consumption after illumination divided by that before illumination.

RESULTS

Uncoupling of oxidative phosphorylation by photodynamic action of Hp and the effect of eosin-5-maleimide

Figure 1 shows the effect of eosin-5-maleimide on the stimulation of state 4 respiration by Hp plus light. In the absence of eosin-5-maleimide, mitochondrial state 4 respiration was stimulated and the ratio of O_2 consumption (i.e. the rate of O_2 consumption after illumination divided by that before illumination) was 2.2 for 20 sec of illumination. Further illumination decreased the ratio. When mitochondria were incubated with 5 μM eosin-5-maleimide before illumination, the mitochondrial respiration was not stimulated. Eosin-5-maleimide (5 μM) alone did not affect the respiratory control ratio (data not shown).

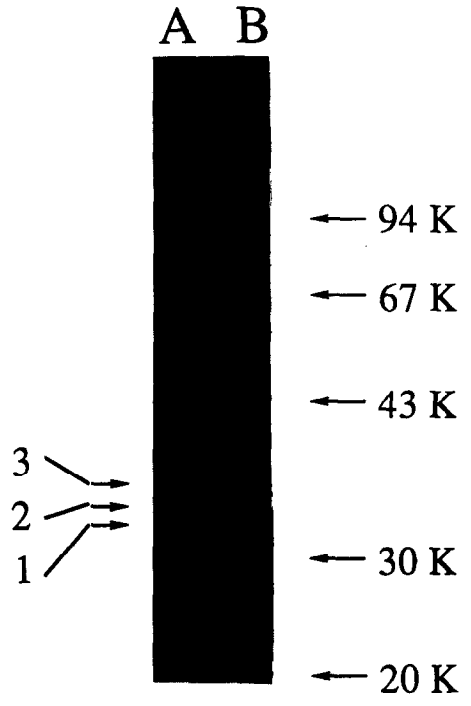

Fig. 2. Eosin-5-maleimide labeling of mitochondrial proteins. Mitochondria (1 mg/mL) were suspended in the respiratory medium (220 mM D-mannitol, 70 mM sucrose, 2.0 mM HEPES, 20 μM Hp and 6 mM glutamate at pH 7.4). (A) The medium was not illuminated; (B) the medium was illuminated for 20 sec. Then, 10 μM eosin-5-maleimide were added and the medium was incubated for 10 min at 25°. After the addition of 50 mM dithiothreitol, SDS–PAGE was performed. Eosin-5-maleimide labeling of separated proteins was visualized by UV light immediately after the electrophoresis. Arrows 1, 2, and 3 indicate the position of bands with molecular masses of 33, 34.5 and 36 kDa, respectively.

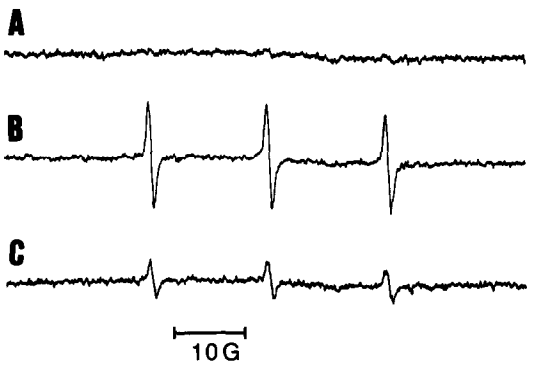

Fig. 3. ESR spectra of nitroxide radical generated during illumination of Hp solution in the presence or absence of Cys. Samples (100 μL) contained 50 μM Hp and 100 mM 2,2,6,6-tetramethyl-4-piperidone in 20 mM sodium phosphate buffer at pH 7.4, with or without 0.2 mM Cys. Samples were illuminated with the transilluminator (365 nm, 90 W) placed at a distance of 10 cm at 0° and ESR spectra were measured. Spectrum A: no Cys, no illumination; spectrum B: no Cys, 15 min of illumination; spectrum C: 0.2 mM Cys, 15 min of illumination.

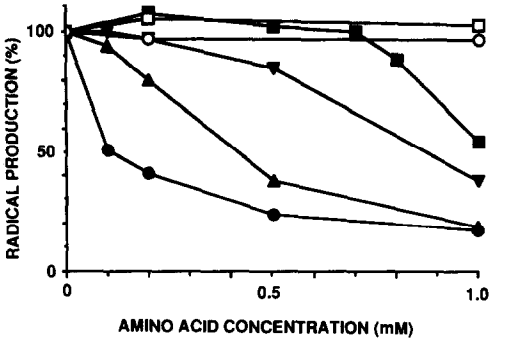

Fig. 4. Effect of amino acids on nitroxide radical production by Hp plus light. Samples (100 μL) contained 50 μM Hp, 100 mM 2,2,6,6-tetramethyl-4-piperidone, and amino acid in 20 mM sodium phosphate buffer at pH 7.4. Samples were illuminated for 10 min and ESR spectra were measured. Cys (●), His (▲), Met (▼), Try (■), Tyr (○) and Ala (□).

Photodynamic effect of Hp on the SH group of mitochondrial protein

Mitochondria were incubated with eosin-5-maleimide and the distribution of protein-bound eosin-5-maleimide on SDS–PAGE was visualized by fluorography. Labeling of the 33, 34.5 and 36 kDa bands was observed in untreated mitochondria (Fig. 2A). When rat liver mitochondria were treated with Hp plus light before the incubation with eosin-5-maleimide, these bands were not observed (Fig. 2B). These results suggest that Hp plus light oxidized the SH group of the P_i/H^+ symporter and prevented the labeling of the P_i/H^+ symporter with eosin-5-maleimide.

Effects of amino acids on nitroxide radical production from 2,2,6,6-tetramethyl-4-piperidone by Hp plus light

Figure 3B shows that the nitroxide radical was produced from 2,2,6,6-tetramethyl-4-piperidone by trapping $^1O_2$ generated during the illumination of Hp. The spectrum of the nitroxide radical was identical with that of 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy. When D_2O was used instead of H_2O, nitroxide radical production was about seven times greater. The addition of 100 mM sodium azide or 10 mM dimethylfuran inhibited completely nitroxide radical production by Hp plus light (data not shown). When Cys was added to the reaction mixture, production of the nitroxide radical was drastically suppressed (Fig. 3C). The effects of different amino acids on nitroxide radical production were compared (Fig. 4) and the order of the inhibitory effect was Cys > His > Met > Trp > Tyr ~ Ala.

HPLC separation and identification of the products of the photoreaction of Hp with Cys

Without the illumination, only peak 2 corresponding to Cys was observed (Fig. 5B). When

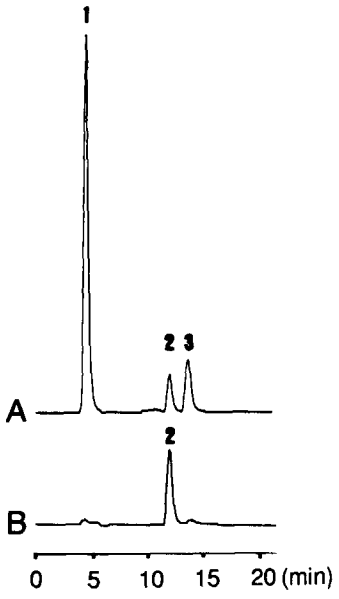

Fig. 5. Ion-pair reversed-phase HPLC of the photooxidation products of Cys. Samples (100 μL) contained 50 μM Hp and 1 mM Cys in 10 mM sodium phosphate buffer at pH 7.4. (A) The sample was illuminated for 10 min as described in the legend to Fig. 3. (B) No illumination. Ten microlitres of the mixtures were injected for HPLC analysis. Mobile phase: 20 mM sodium citrate buffer at pH 3.0/methanol (96/4) containing 300 mg/L sodium octanesulfonate; flow rate, 0.5 mL/min; column temperature, 40°. Peak 1, cysteinesulfinic acid and cysteic acid (not separated); peak 2, Cys; peak 3, cystine.

the reaction mixture of Cys containing Hp was illuminated, peak 2 was decreased from 10 to 4.7 nmol and two other peaks appeared concomitantly (Fig. 5A, peaks 1 and 3). Peak 3 corresponds to 2.2 nmol cystine and peak 1 corresponds to the mixture of cysteinesulfinic acid and cysteic acid, which could not be separated in this system. Further HPLC analysis showed that peak 1 corresponds to the mixture of cysteinesulfinic acid and cysteic acid, which could not be separated in this system. Further HPLC analysis showed that peak 1 is separated into 0.7 nmol cysteinesulfinic acid and less than 0.05 nmol cysteic acid. These results show that Cys is oxidized to cystine (83%), cysteinesulfinic acid (13%) and cysteic acid (<1%). The amount of the oxidation products increased with the duration of illumination.

DISCUSSION

The treatment of rat liver mitochondria with Hp plus light induced uncoupling of oxidative phosphorylation in isolated mitochondria. The result is in accord with the work of Sandberg and Romslo [8]. The uncoupling effect of photoexcited Hp was inhibited by the incubation of mitochondria with eosin-5-maleimide before treatment with Hp plus light, whereas eosin-5-maleimide itself had little effect on the respiratory control ratio at the concentration used. This result leads us to speculate that SH groups located on the cytoplasmic surface on the inner mitochondrial membrane play an important role in the uncoupling of oxidative phosphorylation because eosin-5-maleimide is impermeable to the inner mitochondrial membrane [11].

Noteworthy results were obtained in the experiments involving labeling of mitochondrial proteins with eosin-5-maleimide. After the mitochondria were treated with Hp plus light, eosin-5-maleimide did not label the 33, 34.5 and 36 kDa proteins in mitochondria. The 33 and 34.5 kDa proteins are thought to be the P_i/H^+ symporter because the P_i/H^+ symporter has its essential SH group on the cytoplasmic surface of the inner mitochondrial membrane [11, 21, 22]. Therefore, it is presumed that photoexcited Hp induces the uncoupling of oxidative phosphorylation by modifying the SH group of the P_i/H^+ symporter.

Mitchell [23] suggested that uncouplers are protonophores, i.e. weak acids that increase the membrane proton permeability and collapse the electrochemical gradient by shuttling protons across the membrane. This explanation of uncoupling in mitochondria has been verified in numerous cases for many potent uncouplers [24]. On the other hand, other types of uncouplers have been reported. Takeguchi *et al.* [25] have suggested that some divalent cationic cyanine dyes induce uncoupling by acting on the mitochondrial membrane and forming a leakage-type ion pathway in the bilayer membranes. It has also been suggested that the P_i/H^+ symporter participates in the uncoupling [26].

The photodynamic action of Hp is different from the well-known action of the H^+-conducting uncouplers, protonophores. The ADP/ATP antiporter and the P_i/H^+ symporter are essential components of the mitochondrial phosphorylating assembly. It has been suggested that uncoupling by the $(o\text{-phenanthroline})_2$–Cu complex and by a divalent cationic cyanine dye might be due to their binding with the ADP/ATP antiporter and the P_i/H^+ symporter, respectively [26, 27]. The uncoupling by the photodynamic action of Hp is considered to be related to the P_i/H^+ symporter. The essential SH group of the P_i/H^+ symporter is oriented toward the cytoplasmic surface of the inner membrane [11, 28]. The P_i/H^+ symporter is reactive with eosin-5-maleimide while the ADP/ATP antiporter is not [11]. It is suggested that the SH group forms a disulfide with another neighboring Cys residue when the P_i/H^+ symporter is autoxidized [22]. The SH group is considered to be readily oxidized by the photodynamic action of Hp. Accordingly, our hypothesis is that the oxidation of the SH group of the P_i/H^+ symporter induces the formation of a leakage-type ion pathway causing the uncoupling of oxidative phosphorylation without discrete membrane alterations. Relating to the effects of treatment with Hp plus light on membrane integrity, it was reported that discrete membrane alterations are not the likely cause of the initial loss of pH gradient formation [29].

We have confirmed previously [14, 17, 18, 30] that the ESR method of detecting $^1O_2$ production, based on the reaction of $^1O_2$ with sterically hindered

2,2,6,6-tetramethyl-4-piperidone leading to a stable nitroxide free radical, is useful [31–33]. The ESR experiments reported here demonstrate that the photoexcited Hp reacts with O_2 to generate $^1O_2$. The order of the inhibitory effect on nitroxide radical production was Cys > His > Met > Trp > Tyr ~ Ala. The result suggests that $^1O_2$ reacts most effectively with Cys if type I mechanism (i.e. direct reaction of the excited Hp with amino acid) is negligible. The order was in agreement with that obtained from the rates of oxygen uptake observed on photo-oxidation of amino acids with Rose Bengal [34]. The HPLC analysis reported here showed that $^1O_2$ generated by Hp plus light oxidizes Cys predominantly to cystine and, to a less extent, to cysteinesulfinic acid. The production of cystine, which is the typical product of $^1O_2$ on Cys, is in accord with the work of Cannistraro *et al.* [35]. On the other hand, the participation of type I mechanism is not excluded since cysteinesulfinic acid and cysteic acid were also observed, as minor products [36]. These results raise the possibility that the Cys residue of the P_i/H^+ symporter is oxidized by the photodynamic action of Hp, probably via $^1O_2$, and that oxidation of the Cys residue causes modulated structure of the P_iH^+ symporter, which gives a leakage-type ion pathway causing the uncoupling of oxidative phosphorylation. Pertaining to the high reactivity of Cys with $^1O_2$, it has been suggested that SH-groups may be an important target for the observed photodynamic effects of HpD [37, 38].

Concerning the mitochondrial enzymes, it has been reported that the activities of the proton translocating ATPase, cytochrome *c* oxidase, and succinate dehydrogenase were inhibited by Hp plus light [29, 39, 40]. Atlante *et al.* [41] reported that HpD photosensitization of isolated mitochondria causes impairment of anion translocation. On the other hand, the experiments reported here suggest that the photodynamic action of Hp causes uncoupling of oxidative phosphorylation before it causes significant damage to the electron transport system.

The present study supports the hypothesis that mitochondria are potential targets for HpD photosensitization. Further research is necessary to clarify the mechanism of uncoupling of oxidative phosphorylation by the photodynamic action of HpD *in vivo*. It is especially important to clarify whether or not the photodynamic oxidation of the SH group of the P_i/H^+ symporter induces the formation of a leakage-type ion pathway causing dissipation of the membrane potential.

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