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Photomodulation of Oxidative Metabolism and Electron Chain Enzymes in Rat Liver Mitochondria

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ABSTRACT

Low-level laser irradiation has been applied in a variety of laboratory studies and clinical trials for photobiostimulation over the last three decades. Considerable skepticism exists regarding the concept of photostimulation within the medical community. One of the major difficulties with photoirradiation research is that it lacks experimentally supportable mechanisms for the alleged photobiostimulatory effects. This study was undertaken to determine whether oxidative metabolism and electron chain enzymes in rat liver mitochondria can be modulated by photoirradiation. Oxygen consumption, phosphate potential, and energy charge of rat liver mitochondria were determined following photoirradiation. Activities of mitochondrial enzymes were analyzed to assess the specific enzymes that are directly involved with the photostimulatory process. An argon-dye laser at a wavelength of 660 nm and at a power density of 10 mW/cm^2 was used as a photon source. Photoirradiation significantly increased oxygen consumption (0.6 J/cm^2 and 1.2 J/cm^2, $P < 0.05$), phosphate potential, and the energy charge (1.8 J/cm^2 and 2.4 J/cm^2, $P < 0.05$) of rat liver mitochondria and enhanced the activities of NADH : ubiquinone oxidoreductase, ubiquinol : ferricytochrome C oxidoreductase and ferrocytochrome C : oxygen oxidoreductase (0.6 J/cm^2, 1.2 J/cm^2, 2.4 J/cm^2 and 4.8 J/cm^2, $P < 0.05$). The activities of succinate ubiquinone oxidoreductase, ATPase, and lactate dehydrogenase were not affected by photoirradiation.

INTRODUCTION

Photobiostimulation currently enjoys widespread clinical popularity in Europe, Canada, China and Japan. There is extensive literature on the effects of low-level laser irradiation on cell and bacteria cultures, animal models and clinical studies (1–10). Since Mester *et al.* (1) reported that low-level laser irradiation had a stimulatory effect on wound healing, low-level laser therapy has been successfully used in treating trophic ulcers and indolent wounds of diverse etiologies after traditional treatments had little effect (2,3). Additionally, low-level laser irradiation has been shown to affect the acceleration of bone repair following fracture or radiation-induced necrosis (4–6). Great variations in the type of the experimental animal used, the type and size of the wound treated, the method of assessment of results, the choice of laser wavelengths and fluences and the conditions of the irradiation often produce conflicting results (7–10). The clinical application of laser biostimulation is extremely controversial in the United States. Considerable skepticism exists regarding the concept of photostimulation within the medical community. The efficacy of laser biostimulation has not yet been demonstrated unequivocally with properly controlled experiments and clinical trials. One of the major difficulties with photoirradiation research is that it lacks experimentally supportable mechanisms for the alleged photobiostimulatory effects.

Photosynthesis in both green plants and common living organisms is carried out in cellular membranes where the photochemical reaction center is composed of light-absorbing species (*i.e.* photosensitizer) and electron transfer mediators. This combined redox system serves as an elaborately and completely organized system (11). It is unknown whether mammalian cells have photoactive systems similar to those found in plant cells and common living organisms. Arvanitaki and Chalazonitis compared the action spectrum for *Helix pomatia* myocardium stimulation with the absorption spectrum of isolated mitochondria and demonstrated the ability of mitochondria to act as a subcellular light receptor (12). Chance and Hess (13) have shown that the absorption spectrum of whole cells is qualitatively similar to that of isolated mitochondria. If photoirradiation can induce chemical interactions in mammalian cells, mitochondrial flavins, iron-sulfur centers or hemes should be considered to be potential endogenous photosensitizers that are involved with visible light absorption.

The issue of photosensitivity of mammalian cells was raised in the 1970s when photobiostimulatory effects were being applied to a variety of medical problems. There are many arguments offered regarding the mechanism of photostimulation in mammalian cells. Karu (14) speculates that the mitochondria are the photoreceptors for visible light energy and that the absorption of light by the respiratory chain components may cause a short-term activation of the respiratory chain and oxidation of the NADH pool. This leads to

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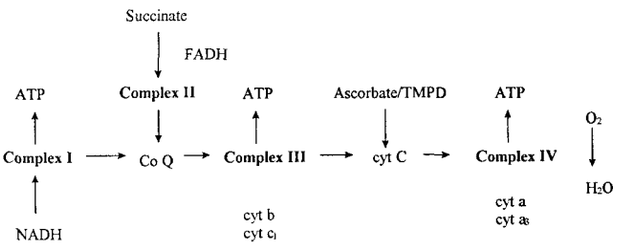

Figure 1. Schema of respiratory chain in hepatic mitochondria.

changes in the redox status of both the mitochondria and the cytoplasm. Activation of the electron-transport chain results in an increase of proton-motive force, an increase in the electrical potential of the mitochondrial membrane and the ATP pool, the alkalization of cytoplasm and finally, the activation of nucleic acid synthesis. However, the lack of detailed supporting evidence for this theory not only makes it difficult to convince most skeptics but also has been the justification for the Food and Drug Administration's withholding of the approval of laser biostimulation devices in the United States (15).

In the last several years, we have investigated photobiostimulatory effects on cellular proliferation, the release of cytokines and growth factors from cells, wound healing, functional preservation of the isolated heart and the immune response in sepsis (16–19). We found that low-level laser irradiation at certain wavelengths and fluences has significant biostimulatory effects on cells and tissues. In this present study, we further investigated the possible mechanism of photomodulation (both stimulatory and inhibitory) using a cell-free system. We assumed that mitochondria in mammalian cells are acting as photoreceptors and that the protein complexes of the mitochondrial respiratory electron chain (Fig. 1) are the photoreaction site. First, we determined whether photoirradiation alters oxygen consumption, phosphate potential and the energy charge of rat liver mitochondria. Then, the activities of mitochondrial enzymes were analyzed to assess the specific enzymes that are directly involved with the photostimulatory process.

MATERIALS AND METHODS

Preparation of mitochondria and inner mitochondrial membranes. Sprague Dawley rats were sacrificed by decapitation. After washing with cold distilled water, the livers were minced in a cold extraction solution (25–30 mL per 10 g tissue) containing 0.25 *M* sucrose, 1 m*M* EDTA and 0.1% bovine serum albumin (BSA).† The tissue was then homogenized using a glass-Teflon Potter-Elvejhem homogenizer. The homogenate was centrifuged at 600 ***g*** for 10 min, and the supernatant fractions were collected and centrifuged again at 15 000 ***g*** for 5 min. The pellets were resuspended in the extraction solution and centrifuged at 15 000 ***g*** for 5 min. Washed mitochondria were suspended in the extraction buffer at a final concentration of 10 mg/mL protein. The mitochondrial suspension was used immediately for oxygen electrode studies and for determination of phosphate potential and energy charge. For enzyme activity analysis, the mitochondrial suspension was stored at −70°C and used within 2 weeks of its initial preparation.

†*Abbreviations:* BCA, bicinchoninic assay; BSA, bovine serum albumin; CoQ_{10}; COX, cytochrome c oxidase; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; TMPD, *N,N,N′,N′*-tetramethyl-phenylene diamine.

Photoirradiation and devices. A Lexel Aurora model 600 argon-dye laser (Lexel, Fremont, CA) was used as the light source for all of our studies. Precise measurement of the laser wavelength was determined *via* a spectrophotometer (RS Instruments, San Jose, CA). The power output was measured using a laser power meter (Molectron Detector Inc., Portland, OR). Photoirradiation was carried out at a wavelength of 660 nm and a power density of 10 mW/cm^2 at room temperature. The requisite experimental fluences were achieved by varying the irradiation time (1, 2, 3, 4 or 8 min) accordingly. The fluences and the wavelength of light utilized in these experiments were chosen based upon our previous investigations and the work of other investigators.

Oxygen electrode studies. Mitochondrial oxygen consumption was analyzed by standard techniques (20) using a Clark-type electrode (Microelectrodes Inc., Londonderry, NH). Mitochondria (5 mg protein) were incubated in a 1 mL custom-designed cuvette chamber equipped with a stirring bar and maintained at 25°C in an incubation medium that consisted of 0.25 *M* sucrose, 2 m*M* $MgCl_2$, 10 m*M* K_2HPO_4, 10 m*M* KCl and 0.2 m*M* EDTA and 4 m*M* HEPES, pH 7.4. Following laser irradiation, oxygen consumption was determined using either succinate at a final concentration of 20 m*M* or ascorbate/*N,N,N′,N′*-tetramethyl-phenylenediamine (TMPD) at final concentrations of 7.2 m*M* and 0.24 m*M*, respectively, as substrates. State 3 oxygen consumption was initiated by the addition of ADP (1 μ*M*) to the incubations. When succinate was used as a substrate, state 3 oxygen consumption was determined in the presence of rotenone at a 4 m*M* final concentration. State 3 respiration using ascorbate/TMPD as substrates was determined in the presence of 0.6 μg/mL antimycin A.

Phosphate potential and energy charge. Phosphate potential and energy charge were determined using mitochondrial incubation techniques as was described by Stucki *et al.* (21). Following laser irradiation, approximately 5 mg of mitochondrial protein was added to a test tube containing 3 mL of the incubation medium (100 m*M* KCl, 5 m*M* L-glutamate and 1 m*M* $MgCl_2$ in 8 m*M* potassium phosphate buffer, pH 7.4) and was incubated at 37°C in a shaker water bath that was shaking at 100 strokes/min. One milliliter from each sample was removed at 7 min and 12 min time intervals and quickly deproteinized by the addition of 1 mL of perchloric acid (0.3 *M*). After neutralization with KOH and centrifugation, the concentrations of ATP, ADP and AMP in the supernatant were determined using enzymatic methods (22). Inorganic phosphorus (Pi) was assayed using a Sigma diagnostic kit. The phosphate potential (G_p) was calculated as follows (21):

$$G_p = [G_{op} + RT \ln(ATP)]/[(ADP) \times (Pi)]$$

where G_{op} = 8.5 kcal, R = gas constant, T = absolute temperature, and ln = natural logarithm. The energy charge was calculated as follows (23):

$$\text{Energy charge} = [(ATP) + 0.5\ (ADP)]/[(ATP) + (ADP) + (AMP)].$$

Assays for mitochondrial protein complexes I, II, II and IV. Assays of mitochondrial protein complexes I, II and III were performed as was described by Ragan *et al.* (24) with the following modifications:

The complex I assay (NADH : ubiquinone oxidoreductase) was modified using a plate reader at 340 nm to measure the oxidization of NADH to NAD^+ at 30°C. Following laser irradiation, 10 μL of mitochondrial protein in 25 m*M* potassium buffer was added to 96 well plates. Each well contained 285 μL of Tris buffer (pH 8.0, 0.05 *M*) and 100 μ*M* NADH. The assay was carried out at 30°C and initiated after the addition of 5 μL coenzyme Q_{10} (CoQ_{10}, 3 m*M*) to each well. The results were expressed as micromoles of NADH oxidized per minute per milligram of mitochondrial protein using a combined NADH-ubiquinone extinction coefficient of 6.81 m*M*/cm^{-1}. The reaction was repeated in the presence of 2.5 μmol/rotenone. The rotenone insensitive activity was subtracted from the total activity, resulting in the rotenone-sensitive or complex I activity.

Complex II (succinate ubiquinone oxidoreductase) was measured by the rate of the secondary reduction of dichlorophenolindophenol by the ubiquinol formed. Following laser irradiation, 10 μL of mitochondrial protein (3 mg/mL) was added to a 1 mL solution containing 10 m*M* succinate, 100 μ*M* EDTA and 75 μ*M* 2,6-dichlorophenolindophenol in 50 m*M* potassium phosphate buffer (pH 7.5)

Table 1. State 3 oxygen consumption of mitochondria following photoirradiation*

	Succinate	Ascorbate/TMPD
Control	155 ± 10	286 ± 37
0.6 J/cm²	212 ± 3†	404 ± 27†
1.2 J/cm²	189 ± 15†	357 ± 79
1.8 J/cm²	186 ± 16†	NA
2.4 J/cm²	166 ± 22	NA

*Oxygen consumption is expressed as natoms/min/mg mitochondrial protein. The data were pooled from three experiments and presented as mean ± SE. N = 8–12.
†$P < 0.05$ *vs* the control.

in a spectrophotometer cell. The reaction began with the addition of 10 μL of CoQ_{10} (100 μ*M*). The decrease in absorbance at 600 nm was used to calculate specific activity (S.A.) using an extinction coefficient of 21/m*M*/cm.

S.A. = Δ absorbency (600 nm)/mg of protein × 21 × Δt.

Complex III (ubiquinol : ferricytochrome C oxidoreductase) was assayed by the rate of reduction of cytochrome c by ubiquinol. A plate reader was used at 550 nm to measure reduced cytochrome c. Following laser irradiation, 5 μL of mitochondrial protein in a sucrose–Tris-HCl buffer was added to a 96 well plate containing 100 μL of 50 m*M* phosphate-buffered saline (PBS) (pH 7.4), 10 μL of 10 m*M* EDTA and 5 μL of 1 m*M* cytochrome c. After equilibration at 30°C, 5 μL of ubiquinol was added to the mixed solution to initiate the reaction. The increase in absorbance at 550 nm was calculated as the S.A. using an extinction coefficient of 18.5/m*M*/cm.

S.A. = Δ absorbency (550)/mg of protein × 18.5 × Δt.

Complex IV (ferrocytochrome C : oxygen oxidoreductase) was assayed by the rate of oxidation of ferrocytochrome c following a decrease in the absorbance at 550 nm (25). Preparation of ferrocytochrome c was as follows: 1.0% cytochrome c was dissolved in 10 m*M* PBS (pH 7.0). The solution was reduced with 2 mg of potassium ascorbate. Excess ascorbate was removed by dialysis in 10 m*M* PBS (pH 7.0) for 24 h with three separate changes of dialysate.

For the assay, 90 μL/well of 7% ferrocytochrome c in PBS (0.01 *M*, pH 7.0) was added to a 96 well plate and equilibrated at 30°C. A blank well was oxidized using 10 μL of 1 *M* potassium ferricyanide. The reaction was initiated by the addition of 10 μL of sample. The decrease in absorbency was measured every 15 s at 550 nm. The activity of cytochrome c oxidase was defined as the first-order velocity constant (k = 2.3 log A [initial]/A[final 1 min] min^{-1}). The specific activity was calculated as:

S.A. = k (concentration cytochrome c)/mg protein.

Activity of ATPase and lactate dehydrogenase (LDH). Mitochondrial ATPase activity was determined in the presence of ouabain using a coupled enzyme reaction according to the method of Scharschmidt *et al.* (26). The activity of LDH (a soluble cytosolic) was measured using a Sigma LDH/LD diagnostic kit.

Protein assay. All protein concentrations in the mitochondrial preparations were determined using the bicinchoninic assay (BCA). Bovine serum albumin (BSA) was used as a standard.

Statistical analysis. Statistical analysis of the differences between control and photoirradiation groups was performed using the Student's *t*-test. A P value of <0.05 was considered statistically significant.

RESULTS

Oxygen consumption of mitochondria following photoirradiation

The results of the oxygen consumption studies are shown in Table 1. The coupling site II of the electron respiratory chain, which is located on the path of electrons from cytochrome b to cytochrome c, was evaluated using succinate as an electron donor and using rotenone to block between NADH and CoQ path. The results show that state 3 oxygen consumption following laser irradiation at fluences of 0.6 J/cm², 1.2 J/cm² and 1.8 J/cm² was significantly increased. When ascorbate/TMDP was used as the substrate, coupling site III of the electron respiratory chain was evaluated following blocking with rotenone and antimycin A. The results imply that photoirradiation alters mitochondrial oxygen consumption. However, the observed changes were significant only at an energy density of 0.6 J/cm².

Phosphate potential and energy charge

Mitochondrial energy metabolism can be characterized by determining the phosphate potential and the energy charge in mitochondrial incubations. Both phosphate potential and energy charge reflect the energy state of a cell or of isolated mitochondria. After a 7 min incubation, both the phosphate potential and energy charge were significantly increased in the laser groups (Table 2). However, there was no significant difference between the control and the laser irradiation groups following a 12 min incubation.

Activity of mitochondrial enzymes

Increased activity of the respiratory chain can be associated with different mitochondrial complexes and enzymes. To assess the specific complexes and enzymes that may respond to photostimulation, the activities of mitochondrial protein complexes I through IV, ATPase and LDH were determined. The enzyme activity results are shown in Tables 3 and 4. Photoirradiation at fluences of 1.2 J/cm² and 2.4 J/cm² increased the activity of complex I ($P < 0.05$). Complex II activity appears not to be affected by photoirradiation. Complex III and complex IV activities were significantly increased following photoirradiation at fluences of 0.6 J/cm², 1.2 J/cm², 2.4 J/cm² and 4.8 J/cm² ($P < 0.05$). Photoirradia-

Table 2. Phosphate potential (Kcal) and energy charge in isolated hepatic mitochondria*

	Control	0.6 J/cm²	1.2 J/cm²	1.8 J/cm²	2.4 J/cm²
Phosphate potential (7 min)	6.62 ± 2.26	10.67 ± 2.58	18.59 ± 5.09†	13.86 ± 5.45†	22.38 ± 6.73†
Phosphate potential (12 min)	17.2 ± 4.58	35.1 ± 18.16	25.5 ± 14.9	15.7 ± 3.21	13.5 ± 2.49
Energy charge (7 min)	0.88 ± 0.04	0.92 ± 0.02	0.93 ± 0.01	0.95 ± 0.02†	0.97 ± 0.01†
Energy charge (12 min)	0.92 ± 0.02	0.95 ± 0.03	0.95 ± 0.01	0.96 ± 0.02	0.92 ± 0.01

*This study was repeated twice. The data were pooled from two experiments and are presented as mean ± SE. N = 8.
†$P < 0.05$ *vs* the control.

Table 3. Activities of mitochondrial electron transport protein complexes*

Group	Complex I (nmol min^{-1} mg $protein^{-1}$)	Complex II (nmol min^{-1} mg $protein^{-1}$)	Complex III (nmol min^{-1} mg $protein^{-1}$)	Complex IV (l min^{-1} mg $protein^{-1}$)
Control	9.33 ± 3.03	9.85 ± 1.44	3.55 ± 0.57	0.514 ± 0.060
0.6 J/cm²	16.47 ± 6.15	8.20 ± 0.49	7.33 ± 1.70†	0.691 ± 0.052†
1.2 J/cm²	33.74 ± 5.96†	9.85 ± 2.34	7.12 ± 1.60†	0.732 ± 0.065†
2.4 J/cm²	45.65 ± 6.90†	7.72 ± 0.23	15.3 ± 3.04†	0.930 ± 0.098†
4.8 J/cm²	N/A	9.45 ± 0.72	7.06 ± 1.49†	0.936 ± 0.070†

*This study was repeated twice. The data were pooled from two experiments and are presented as mean ± SE. N = 6.
†$P < 0.05$ *vs* the control.

tion did not have a statistically significant effect on the activities of LDH and ATPase at any of the fluences studied.

DISCUSSION

We examined mitochondrial oxidative metabolism to determine whether essential mitochondrial functions can be altered following laser irradiation. The oxygen electrode studies showed that laser irradiation at certain fluences significantly increased state 3 oxidation rates when succinate or ascorbate/TMDP were used as substrates. Our results are consistent with other researchers (27,28). Analysis of the phosphate potential and the energy charge has been shown to further characterize mitochondrial function following laser irradiation. Following a 7 min incubation, the mitochondria that received laser irradiation appeared to have a significantly higher phosphate potential and energy charge than mitochondria without photoirradiation. However, we found no difference between control and laser irradiation groups following a 12 min incubation. These data suggest that laser irradiation does alter mitochondrial functions at certain fluences and that photoirradiation may accelerate the redox reaction rate of the electron respiratory chain of mitochondria. However, this stimulatory effect only lasts for a short period. Whether the late-stage interaction, *i.e.* the secondary dark reaction, is related to mitochondrial events following photoirradiation is unclear and warrants further investigation.

Increased activity of mitochondrial electron transport can be associated with a variety of mitochondrial enzyme activities. Photostimulatory effects for a variety of mitochondrial enzymes (*i.e.* protein complexes in respiratory transport chain, ATPase) have been proposed and studied by different researchers (28–31). However, most of the proposed mechanisms are based on oxygen consumption studies and lack of direct experimental support. In this study, we used specific enzyme assays to examine the oxidoreductase activities of the four electron transport protein complexes as well as the activities of ATPase and LDH. Our study demonstrates that photoirradiation can significantly increase the activities of complex I, complex III and complex IV. Photoirradiation did not appear to have a statistically significant effect on the activities of mitochondrial protein complex II, ATPase or LDH.

Based on the findings of our studies, which demonstrate that the electron chain complex I, complex III and complex IV can be activated by photoirradiation, we postulate that the mechanism of photointeraction in mammalian cells is as follows (Fig. 2): metal sites in the protein complexes of the electron respiratory chain play a major role in photomodulation and can directly serve as the principal photoreceptors. When photons from laser light energize the metal sites in these complexes, shaking/vibration of these metals may alter either the enzyme conformation or the redox reaction, and this in turn increases the transfer of electrons throughout the respiratory chain and/or pumping of protons from the membrane. Increased transference of electrons and protons accelerates oxidative metabolism and leads to an increase in ATP synthesis. Increased ATP production may *in turn* promote cellular metabolism, resulting in photobiostimulation. However, we speculate that excessive photoirradiation may produce fully oxidized and fully reduced complexes. These fully reacted complexes will not be associated with electron transfer but will undergo a structural rearrangement (32), thereby resulting in bioinhibitory effects. However, this hypothesis requires specific investigation for confirmation.

Harvard researchers have already used laser light as a tool to shake/knock off binding sites between carbon monoxide and iron in myoglobin (33). In their study, infrared laser light was used to bend carbon monoxide, and an argon laser was used to shake iron from the heme molecule. These experiments provide some insights and seem to support our current theory of laser biostimulation. We used complex IV (cytochrome c and cytochrome c oxidase [COX]) as a model

Table 4. Activities of enzymes in isolated liver mitochondria*

Enzymes	Control	0.6 J/cm²	1.2 J/cm²	1.8 J/cm²	2.4 J/cm²
ATPase†	1.02 ± 0.2	1.15 ± 0.3	1.41 ± 0.51	0.96 ± 0.31	0.72 ± 0.27
LDH‡	9.35 ± 0.28	9.35 ± 0.73	9.56 ± 0.22	9.75 ± 0.32	9.7 ± 0.29

*This study was repeated twice. The data were pooled from two experiments and presented as mean ± SE. N = 8. No statistically significant difference between the control and irradiation groups was demonstrated.
†The ATPase activity is expressed as μmol Pi/mg protein h.
‡The LDH activity is expressed as an international unit/mg protein.

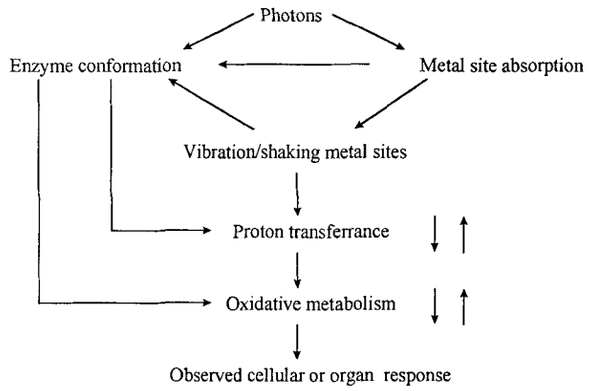

Figure 2. The proposed photostimulatory/inhibitory mechanisms (speculation).

to illustrate our theory in detail because much information is available for the activity of this complex. The main function of COX is energy conservation. This is the terminal enzyme in the respiratory chain. Cytochrome c oxidase is a proton pump situated in the inner mitochondrial membrane of the mammalian cell. This enzyme uses redox chemistry to drive protons from the mitochondrial matrix across the membrane. Electron transfer to cytochrome oxidase is initiated by the binding of cytochrome c to subunit II on the external side of the membrane. This subunit contains a Cu_A center. Most studies of electron transference indicate that a linear sequence of events occurs with the electrons proceeding from cytochrome c to Cu_A then to heme a and then onward to the heme a_3–Cu_B center. Heme a, Heme a_3 and Cu_B are all liganded to residues within subunit I of the oxidase (Fig. 1). Absorbance changes in partly reduced COX have been observed, and the reaction producing the absorbance changes is sensitive to ligands that bind in the a_3–Cu_B site, which suggests that cytochrome a_3 or the immediate environment of this center is associated with the light-induced changes (32). When photons are directly absorbed by the metals in these heme complexes, the shaken vibrated metal groups undergo shaking or vibration that causes increased binding between cytochrome c and COX, and this in turn accelerates electron processing and leads to an accelerated redox reaction.

Our current studies provide some insights into the photostimulatory mechanism in a cell-free system. Whether photobiostimulation involves other mechanisms such as secondary dark reactions and/or enzyme transformation needs further study.

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