# PHOTOINHIBITION OF ISOLATED COMPLEXES I, II, AND III OF BEEF HEART MITOCHONDRIA

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Received 29 October 1973

## 1. Introduction

The inhibitory effect of blue light on exogenous respiration deserves attention because of its possible regulatory influence in cell development and in evolution. Previous investigations have given ample evidence that blue light inhibits respiratory activity in a wide variety of colorless organisms [1–5]. It could be shown that blue light can inhibit respiration by destroying cytochrome $a_3$. In these experiments the inhibitory light was absorbed by the Soret band of the oxidized form of cytochrome $a_3$ [4]. Protection against photodestruction of cytochrome $a_3$ was afforded when cyanide was present or when oxygen was absent. The absorption spectra of irradiated beef heart mitochondria, of yeast and of *Prototheca* cells also indicated that one or more other cytochromes were also destroyed by light but to a smaller extent than the cytochrome $a_3$ [2–4]. One purpose of the work reported here was to determine if the photodestruction of cytochromes other than cytochrome $a_3$ (or cytochrome $a$ and $a_3$ in yeast) resulted in an inhibition of respiratory electron transport. Hatefi et al. [6] showed previously that mitochondria could be separated into four active complexes (complex I through IV) which carried out partial reactions of the electron transport chain, and that the entire electron transport chain could be reconstituted by combining these four complexes in the presence of cytochrome *c*. The positions and activities of these complexes are indicated by scheme 1 taken from Hatefi [7].

The effect of irradiating these complexes individually before reconstruction of the total system has been studied. The present communication reports that a partial inhibition of the reconstituted electron transport chain results from the irradiation of any of the complexes and that the inhibition can be overcome by adding additional non-irradiated complex to the system.

## 2. Materials and methods

Beef heart mitochondria were isolated as described by Crane et al. [8]. Complexes I–III (NADH–cytochrome *c* reductase), I, II, III, and IV were prepared according to published procedures [9–13, resp.]. Respiratory activity of a reconstituted electron transport chain was followed with a Yellow Springs $O_2$-electrode fitted into a 1.0 ml Plexiglass cuvette. NADH-dehydrogenase activity was tested spectrophotometrically by reconstituting dark or irradiated complex I with dark complex III and following the rate of cytochrome *c* reduction in a Beckman DK 2A spectrophotometer. For reconstitution the separated complexes were mixed at high concentration (see [6]), then diluted 1:50 for assay. The systems reconstituted were:

| | |
|---|---|
| a) NADH oxidase | |
| Complex I–III | 4.5 mg Protein |
| Complex IV | 1.3 mg Protein |
| Cytochrome *c* | 0.4 mg Protein |
| b) NADH oxidase | |
| Complex I | 2.2 mg Protein |
| Complex III | 4.5 mg Protein |
| Complex IV | 1.1 mg Protein |
| Cytochrome *c* | 3.5 mg Protein |
| c) Succinate oxidase | |
| Complex II | 1.0 mg Protein |
| Complex III | 2.3 mg Protein |
| Complex IV | 1.1 mg Protein |
| Cytochrome *c* | 0.5 mg Protein |

[6]



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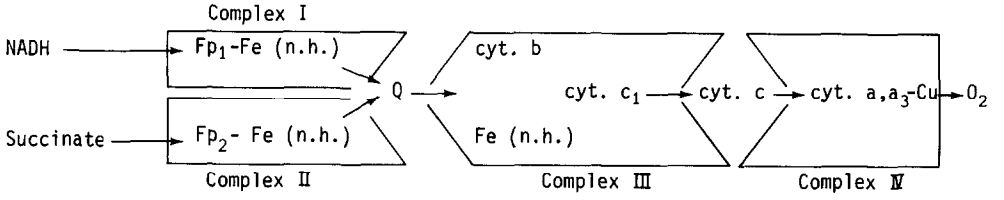
Scheme 1




$10^{-3}$ M NADH (final concn.) or $2.5 \times 10^{-3}$ M succinate were added to the diluted mixture. In some experiments one single complex (see description in the text) was added to the premixed and already diluted set of the other constituents of the respiratory chain. This procedure resulted in maximally the same reconstituted activity as premixing all components, provided the same concentrations were used with both methods. For irradiation of the individual or combined electron transfer complexes, a water-cooled high-pressure mercury lamp, General Electrics AH-6, was used. For activity measurements the complexes were irradiated in high concentration (protein content 30–40 mg/ml), for absorption spectra they were irradiated in a 10-fold dilution with preaerated Tris–sucrose–histidine buffer (0.25 M sucrose, 50 mM Tris–Cl, 1 mM histidine, pH 8.0). The light beam was passed through a glass lens, a blue Corning glass filter no. 5562 and a Corning cut-off filter no. 3-75. During the irradiation period (15–60 min at $5-6 \times 10^5$ or $1-2 \times 10^6$ ergs $cm^{-2} \cdot sec^{-1}$ of blue light) the sample was kept at room temperature (22°C) in a large water-bath.

Difference absorption spectra of non-irradiated and irradiated electron transfer complexes (0.2 ml) were recorded at liquid $N_2$ temperature with a Cary 14 spectrophotometer in line with a PDP/8 computer [14].

## 3. Results and discussion

Irradiation of concentrated mixtures of unresolved complex I–III (NADH – cytochrome *c* reductase) plus complex IV plus cytochrome *c* with $5 \times 10^5$ ergs $cm^{-2} \cdot sec^{-1}$ blue light for 5–30 min led to a decrease of activity, measured as oxygen consumption after NADH had been added as substrate (table 1). A similar loss of activity was observed previously in intact beef heart mitochondria and was ascribed mainly to a destruction of complex IV [4]. The loss of activity could be compensated for by adding back non-irradiated complex IV to the irradiated mixture of complex I–III plus complex IV plus cytochrome *c*, either by premixing or by adding dark complex IV to the diluted irradiated mixture. Both procedures resulted in reconstitutions of similar orders of magnitude. Table 1 also indicates that after a short illumination (5–10 min) respiratory activity can be fully reconstituted to its dark rate by addition of dark complex IV, whereas after a 30 min irradiation only 55–60% of the original

Table 1
Effect of blue light on the electron transport activity of complex I–III and complex IV, and reconstitution of the dark control rate with dark complex IV

| Irradiation | Components | nmoles $O_2$/min |
|---|---|---|
| None | $(I-III+IV+cyt.c)_P$ | 194.5<br>223.0 |
| 5 min | $(I-III+IV+cyt.c)_{i,P}$ | 169.0 |
| 5 min | $((I-III+IV+cyt.c)_i+IV_d)_P$ | 189.0 |
| 10 min | $(I-III+IV+cyt.c)_{i,P}$ | 95.2 |
| 10 min | $((I-III+IV+cyt.c)_i+IV_d)_P$ | 203.0 |
| 10 min | $((I-III+IV+cyt.c)_i+IV_d)_{NP}$ | 203.0 |
| 30 min | $(I-III+IV+cyt.c)_i$ | 66.8 |
| 30 min | $((I-III+IV+cyt.c)_i+IV_d)_P$ | 116.4 |
| 30 min | $((I-III+IV+cyt.c)_i+IV_d)_{NP}$ | 123.5 |

Components premixed according to a) in Materials and methods.
Blue: Corning no. 5562, $5 \times 10^5$ ergs $cm^{-2} \cdot sec^{-1}$. NADH $10^{-3}$ M.
Indices: i = irradiated
d = non-irradiated
P = premixed
NP = not premixed
I–IV = complexes I–IV, resp.
cyt.*c* = cytochrome *c*

activity can be regained by adding back dark complex IV. This observation suggested that components other than cytochrome oxidase might be affected by the longer irradiation periods.

To investigate this hypothesis, complex I–III was irradiated separately before reconstitution with complex IV in the presence of cytochrome *c*. Irradiation with 15–60 min of blue light ($5 \times 10^5$ ergs $cm^{-2} \cdot sec^{-1}$) caused the respiratory activity to decline by 20 to 60%, resp. (table 2A). The activity could be partially reconstituted by adding back either complex I or complex III. Complete or nearly complete reconstitution was achieved by adding back both dark complex I and complex III together (table 2B). (In this experiment dark complex I and/or complex III were added to the other premixed and diluted constituents of the electron transport chain including the irradiation component.)

Irradiation of complex I, II, or III separately before reconstitution into the electron transport resulted also in loss of electron transport activity measured as $O_2$-uptake. Irradiation of complex III with 25 min or 55 min of blue light ($1.2 \times 10^6$ ergs $cm^{-2} \cdot sec^{-1}$) caused a loss of activity of 20% and 40%, respectively (table 3A). Activity was fully restored by adding non-irradiated complex III to the inhibited mixture. Irradiation of complex I for either 30 or 60 min ($1.8 \times 10^6$ ergs $cm^{-2} \cdot sec^{-1}$) caused the activity of the reconstituted system to decrease by 30% (table 3B). The subsequent addition of non-irradiated complex I restored most of the activity. In addition the NADH-dehydrogenase activity of complex I was tested after treatment with 15–120 min of blue light. The enzyme activity was impaired increasingly with prolonged irradiation time, so that after 60 min of light ($6 \times 10^5$ ergs $cm^{-2} \cdot sec^{-1}$) 50% of the dehydrogenase activity was lost (fig. 1).

A 30 min irradiation of complex II with blue light decreased the activity of the reconstituted mixture by about 45% (table 4). Adding back dark complex II by premixing with the inhibited reconstituted electron transport chain restored most of the activity (table 4).

Previous work had shown that irradiation of complex IV resulted in the loss of cytochrome $a_3$. In the present work complex III was examined spectroscopically after various periods of irradiation with blue light. Fig. 2 shows the absorption spectra and fourth derivative spectra [15] of the samples frozen to liquid nitrogen

Table 2

Irradiation of complex I–III and reconstitution of electron transport: A. Effect of irradiation on the respiratory activity. B. Reconstitution of electron transport after irradiation.

| Irradiation | Components | nmoles $O_2$ / min | % Respiration |
|---|---|---|---|
| A. | | | |
| None | $(I–III + IV + cyt.\,c)_{d,\,P}$ | 261.3 | 100.0 |
| 15 min | $(I–III_i + IV_d + cyt.\,c_d)P$ | 205.9 | 78.8 |
| 30 min | $(I–III_i + IV_d + cyt.\,c_d)P$ | 122.1 | 46.7 |
| 60 min | $(I–III_i + IV_d + cyt.\,c_d)P$ | 105.1 | 40.2 |
| B. | | | |
| None | $(I–III + IV + cyt.\,c)_{d,P}$ | 284.0 | 100.0 |
| None | $(I–III + IV + cyt.\,c)_{d,P} + I_{d,NP}$ | 284.0 | 100.0 |
| None | $(I–III + IV + cyt.\,c)_{d,P} + I_{d,NP} + III_{d,NP}$ | 343.6 | 121.0 |
| 60 min | $(I–III_i + IV_d + cyt.\,c_d)P$ | 93.7 | 33.0 |
| 60 min | $(I–III_i + IV_d + cyt.\,c_d)P + III_{d,NP}$ | 133.5 | 47.0 |
| 60 min | $(I–III_i + IV_d + cyt.\,c_d)P + III_{d,NP} + I_{d,NP}$ | 240.0 | 84.5 |
| 60 min | $(I–III_i + IV_d + cyt.\,c_d)P$ | 137.7 | 48.5 |
| 60 min | $(I–III_i + IV_d + cyt.\,c_d)P + I_{d,NP}$ | 204.5 | 72.0 |
| 60 min | $(I–III_i + IV_d + cyt.\,c_d)P + I_{d,NP} + III_{d,NP}$ | 333.7 | 117.5 |

Components premixed according to a) in Materials and methods. $10^{-3}$ M NADH added to each sample. Blue: $5 \times 10^5$ ergs $cm^{-2} \cdot sec^{-1}$. Indices as in table 1.

Table 3
Irradiation of complex I and complex III and reconstitution with dark complex I and complex III, resp.

| Irradiation | Components | nmoles $O_2$ / min |
|---|---|---|
| None | $(I + III + IV + cyt.\ c)_{d,P}$ | 97.3 |
| None | $(I + III + IV + cyt.\ c)_{d,P} + III_{d,NP}$ | 103.1 |
| A. Complex III | | |
| 25 min | $(III_i + (I + IV + cyt.\ c)_d)P$ | 78.0 |
| 25 min | $(III_i + (I + IV + cyt.\ c)_d + III_d)P$ | 99.9 |
| 55 min | $(III_i + (I + IV + cyt.\ c)_d)P$ | 62.5 |
| 55 min | $(III_i + (I + IV + cyt.\ c)_d)P + III_{d,NP}$ | 109.5 |
| B. Complex I | | |
| 30 min | $(I_i + (III + IV + cyt.\ c)_d)P$ | 68.9 |
| 30 min | $(I_i + (III + IV + cyt.\ c)_d)P + I_{d,NP}$ | 99.9 |
| 60 min | $(I_i + (III + IV + cyt.\ c)_d)P$ | 68.3 |
| 60 min | $(I_i + (III + IV + cyt.\ c)_d)P + I_{d,NP}$ | 92.8 |

Components premixed according to b) in Materials and methods. NADH $10^{-3}$ M
Blue: $1.2 \times 10^6$ ergs $cm^{-2} \cdot sec^{-1}$ for complex III, $1.8 \times 10^6$ ergs $cm^{-2} \cdot sec^{-1}$ for complex I.

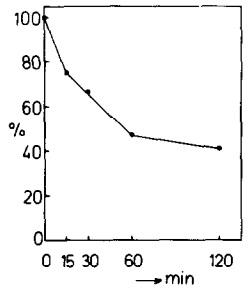



Fig. 1. NADH-dehydrogenase activity of complex I after irradiation with blue light and reconstitution with non-irradiated complex III. Assay: cytochrome *c* reduction. Abscissa: irradiation time (min), ordinate: per cent of enzyme activity. Blue light: $6 \times 10^5$ ergs $cm^{-2} \cdot sec^{-1}$, Corning no. 5562. Protein content of complex I = 76.5 mg/ml, of complex III = 29.7 mg/ml.

* The 60 min sample contained somewhat less material than the other samples.

temperature *. The cytochrome content of complex III was identified previously as cytochrome $b_{562.5}$ (analogous to cytochrome $b_T$) with an $\alpha_1$-band at 562.5 nm at −196°C, an $\alpha_2$-band at 554 nm and a long wavelength $\beta$-band at 535 nm (this $\beta$-band is generally the best diagnostic band for cytochrome $b_{562.5}$ because of its isolation from absorption bands of other cytochromes), cytochrome $b_{560}$ (analogous to cytochrome $b_K$) with a single $\alpha$-band at 559.5 to 560 nm at −196°C and cytochrome $c_1$ with $\alpha_1$- and $\alpha_2$-bands at 553 nm and 549 nm, respectively [16]. In addition a component, probably a cytochrome, with an absorption maximum at 558 nm was noted in the low temperature spectra of complex III. This component was discernible only under certain conditions because of the proximity of the larger band of cytochrome $b_{560}$. It is apparent from the absorption spectra of complex III after various periods of irradiation (fig. 2A) that only cytochrome $b_{560}$ shows any appreciable sensitivity to light.

The fourth derivative spectra (fig. 2B) confirm the increasing loss of cytochrome $b_{560}$ with longer irradiation periods while the other components are little affected. As the peak at 559.5 nm decreases, the fourth derivative band at 557.5 nm becomes more apparent.

Table 4
Effect of blue irradiation on complex II. Reconstitution with dark complex II.

| Irradiation | Components | nmoles $O_2$ / min | % Respiration |
|---|---|---|---|
| None | $(II + III + IV + cyt.\,c)_{d,P}$ | 112.9 | 100.0 |
| 30 min | $((II_i + (III + IV + cyt.\,c)_d)P$ | 62.5 | 55.4 |
| 30 min | $(II_i + (III + IV + cyt.\,c)_d + II_d)P$ | 100.8 | 89.3 |

Components premixed according to c) in Materials and methods. Succinate $10^{-3}$ M.
Blue: $1.8 \times 10^6$ ergs $cm^{-2} \cdot sec^{-1}$.

This band is probably due to the 558 nm component noted previously in complex III [16]. The use of smaller differentiating intervals than were used previously [16] (about 20% smaller) permits the resolution of the 557.5 nm band from the 559.5 nm band and this resolution is increased by the photodestruction of the 559.5 nm band.

Irradiation (30 min, $1.8 \times 10^6$ ergs $cm^{-2} \cdot sec^{-1}$) of complex II does not affect cytochrome $b_{557.5}$ which Davis et al. [16] showed to be present in this complex. In complex I the loss of dehydrogenase and $O_2$-uptake activity after irradiation with blue light could not be correlated with any absorption changes in the 400–500 nm range of the oxidized spectrum of complex I.

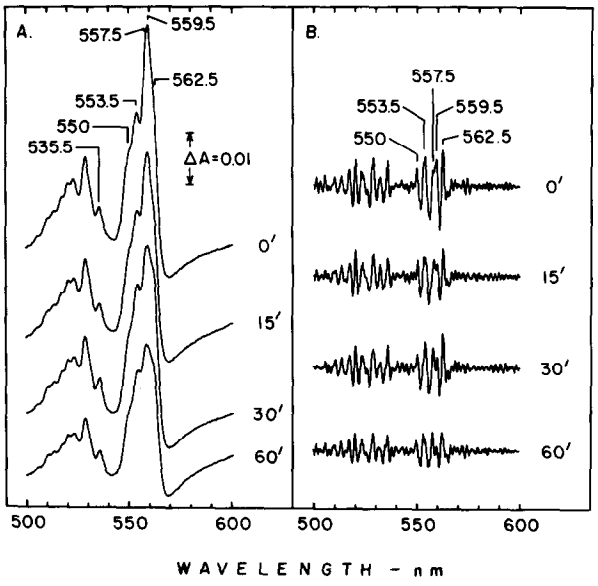



Fig. 2. A. Reduced minus oxidized spectra of complex III after irradiation with blue light for 0, 15, 30 or 60 min, resp.. Blue light: Corning no.5562 + cut-off filter no.3060; $6 \times 10^5$ ergs $cm^{-2} \cdot sec^{-1}$. Complex III (protein = 30 mg/ml) diluted 1:10 with preaerated Tris–sucrose–histidine, pH 8.0. Reduction with dithionite. B. Fourth derivative spectra of the curves under A.

**Acknowledgement**

These investigations were done in Dr. W.L. Butler's laboratory at the University of California San Diego and in Dr. Y. Hatefi's laboratory at Scripps Clinic and Research Foundation in La Jolla. I thank Dr. Butler and Dr. Hatefi for offering me the use of their laboratory facilities and for many helpful discussions. I also gratefully acknowledge the financial support of the Deutsche Forschungsgemeinschaft which made this work possible.

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