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Ronald Waynant
Darrell B. Tata
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Mitochondrial Mechanisms of Laser Phototherapy

Tiina I. Karu

Abstract The terminal enzyme of mitochondrial respiratory chain cytochrome c oxidase is considered as a universal photoacceptor in mammalian cells for visible-to-near IR radiation. Two mechanisms occurring in cytochrome c oxidase under irradiation are investigated experimentally. These are an increase of electron flow inside of cytochrome c oxidase and a relieve of NO block in the catalytic center of cytochrome c oxidase. A novel mitochondrial light-activated cellular signaling pathway (retrograde signaling) has been discovered and investigated. Our results evidence that cytochrome c oxidase can work as a signal generator as well as a signal transducer in irradiated cells.

Keywords: Action and absorption spectra, cytochrome c oxidase, Lorentzian curve fitting, novel light-activated cellular signaling, relieve of NO block, retrograde mitochondrial signaling.

Cytochrome c Oxidase Is a Universal Photoacceptor in Eukaryotic Cells

The action spectra recorded in HeLa cell culture for processes occurring in the cell nucleus (DNA and RNA synthesis rate) and cell membrane (increase in number of cells attached to a glass matrix) in red to near IR region were analyzed by Lorentzian curve fitting [1]. Red-to-near IR part of one of these spectra is presented in Fig. 1. Insofar as the action spectrum resembles the absorption spectrum of the molecule absorbing the light (photoacceptor), the bands in the action spectra were identified by analogy with the metal-ligand systems absorption spectra characteristic of visible-to-near IR spectral range [2]. This analysis allowed us to conclude that all bands in the action spectra (one maximum at 400 nm with the edge of the envelope near 450 nm and two series of doublet bands in the range 620-680 nm and

T.I. Karu
Institute of Laser and Information Technologies of Russian Academy of Sciences, Troitsk, Moscow Region, Russian Federation

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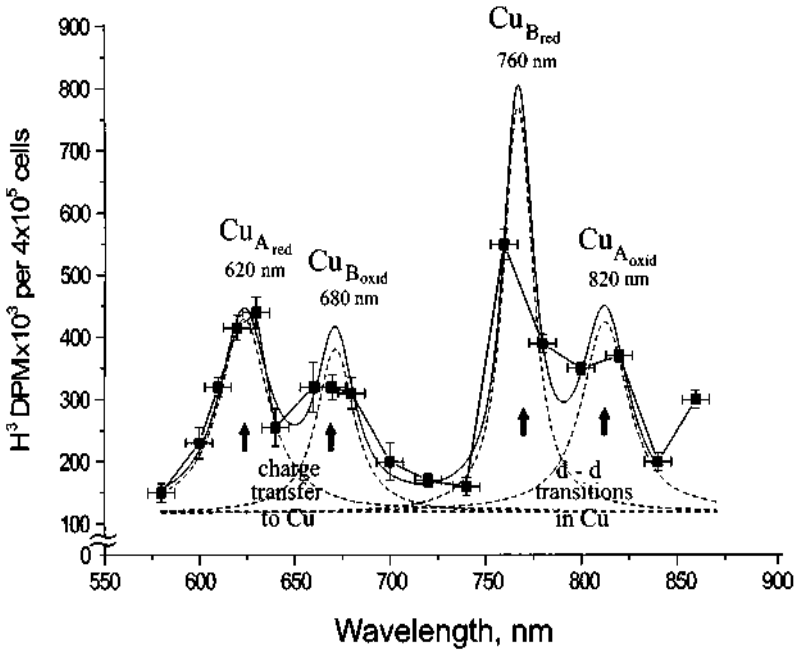

Fig.1. The action spectrum for stimulation of DNA synthesis rate on cellular level. Suggested absorbing chromophores of the photoacceptor, cytochrome c oxidase, are shown (after [2, 3]). Experimental details are described in [1]

760-895 nm with well-pronounced maxima at 620, 680, 760, and 825 nm) may be related to the cytochrome c oxidase. Cytochrome c oxidase is the terminal enzyme of the respiratory chain in eukaryotic cells, which mediates the transfer of electrons from cytochrome c to molecular oxygen. Bands at 404—420, 680 and 825 nm were attributed to a relatively oxidized form of cytochrome c oxidase. The edge of the blue-violet band at 450 nm and the distinct bands at 620 and 760 nm belong to a relatively reduced form of the enzyme [2].

Figure 1 presents only the red-to-near IR part of the action spectrum. It was suggested that the photoacceptor is one of the intermediate forms of cytochrome c oxidase redox cycle. In the red-to-near IR region the 820 nm band is believed belonging mainly to the relatively oxidized Cu_A chromophore of cytochrome c oxidase, the 760 nm band to the relatively reduced Cu_B, the 680 nm band to the relatively oxidized CUB, and the 620 nm band to the relatively reduced Cu_A (Fig. 1).

Comparison of Action and Absorption Spectra: Effect of Irradiation at 830 nm on the Absorption Spectra

Absorption spectra of cellular monolayers were recorded in red-to-near IR region [4] using a sensitive multichannel registration method described in details in [5]. Figure 2a, b present as examples two spectra recorded in (a) enclosed and (b) open

cuvettes. Figure $2a_1$, b_1 present the spectra of the same cells after irradiation at 830 nm. Peak intensity ratios of two bands at 760 and 665 nm (I_{760}/I_{665}) were used to characterize every spectrum quantitatively (see gray vertical lanes in Fig. 2). In the case of equal concentrations of the reduced and oxidized forms of the photoacceptor molecule, the ratio I_{760}/I_{665} should be equal to unity. When the reduced forms prevailed, the ratio I_{760}/I_{665} was greater than unity, and it was less than unity in cases where the oxidized forms dominated [4]. Recall that the internal electron transfer within the cytochrome c oxidase molecule causes the reduction of the molecular oxygen via several transient intermediates of various redox states [6].

The magnitude of the I_{760}/I_{665} criterion was 9.5 for spectrum a (Fig. 2a) and 1.0 for spectrum b (Fig. 2b). By this criterion, irradiation of the cells, whose spectrum is marked by a (I_{760}/I_{665} = 9.5) caused the reduction of the absorbing molecule I_{760}/I_{665} for spectrum a_1 is equal to 16). Irradiation of the cells characterized by spectrum b also caused the reduction of the photoacceptor, as evidenced by the increase of the I_{760}/I_{665} ratio from 1.0 to 2.5 in spectrum bl. In the spectrum of the cells with an initially more reduced photoacceptor (spectrum a), irradiation caused reduction to a lesser extent (16/9.5 = 1.7) than in that of the cells with an initially less reduced photoacceptor (spectrum b). The intensity ratio in this case was 2.5/1 = 2.5.

So, the irradiation at 830 nm caused changes in the initial absorption spectra of the cellular monolayers, which can be interpreted by the I_{760}/I_{665} band intensity ratio criterion as being due to the reduction of the photoacceptor molecule [4].

The $Cu_A \rightarrow$ heme *a* $\rightarrow$ [heme a_3 — Cu_B] $\rightarrow$ O_2 electron transfer within cytochrome c oxidase proceeds rapidly (on a microsecond time scale) between Cu_A and heme *a* and between the catalytic center [heme a_3—Cu_B] and dioxygen. The only rate-limiting stage in the turnover appears to be the internal electron transfer between heme *a* and the [heme a_3— Cu_B] pair. The reduction of the [a_3 — Cu_B] binuclear heme site by the reduced heme *a* occurs on a millisecond time scale [6]. One can speculate that irradiation intensifies exactly this electron transfer stage within the enzyme. It is quite possible that irradiation makes more electrons available for the reduction of dioxygen in the catalytic center of cytochrome *c* oxidase (heme a_3 — Cu_B site). It has long been known that electronic excitation by light stimulates redox processes in organic dyes to intensify electron transfer [7]. This is also true of cytochrome *c* oxidase [8]. The increase of the availability of electrons can be the crucial result of irradiation in situations when all the four electrons are unavailable for the reduction of dioxygen.

Comparison between the absorption (Fig. 2) and action spectra (one example see in Fig. 1) provided evidence that all bands present in the action spectra were present in the absorption spectra as well [3, 4].

A Discovery of a Novel Light-Activated Mitochondrial Cellular Signaling Pathway

The purpose of these experiments was to demonstrate that a signaling pathway exist between the mitochondria (where the suggested photoacceptor cytochrome c oxidase is located) and cellular membrane. As the experimental approach, we used a

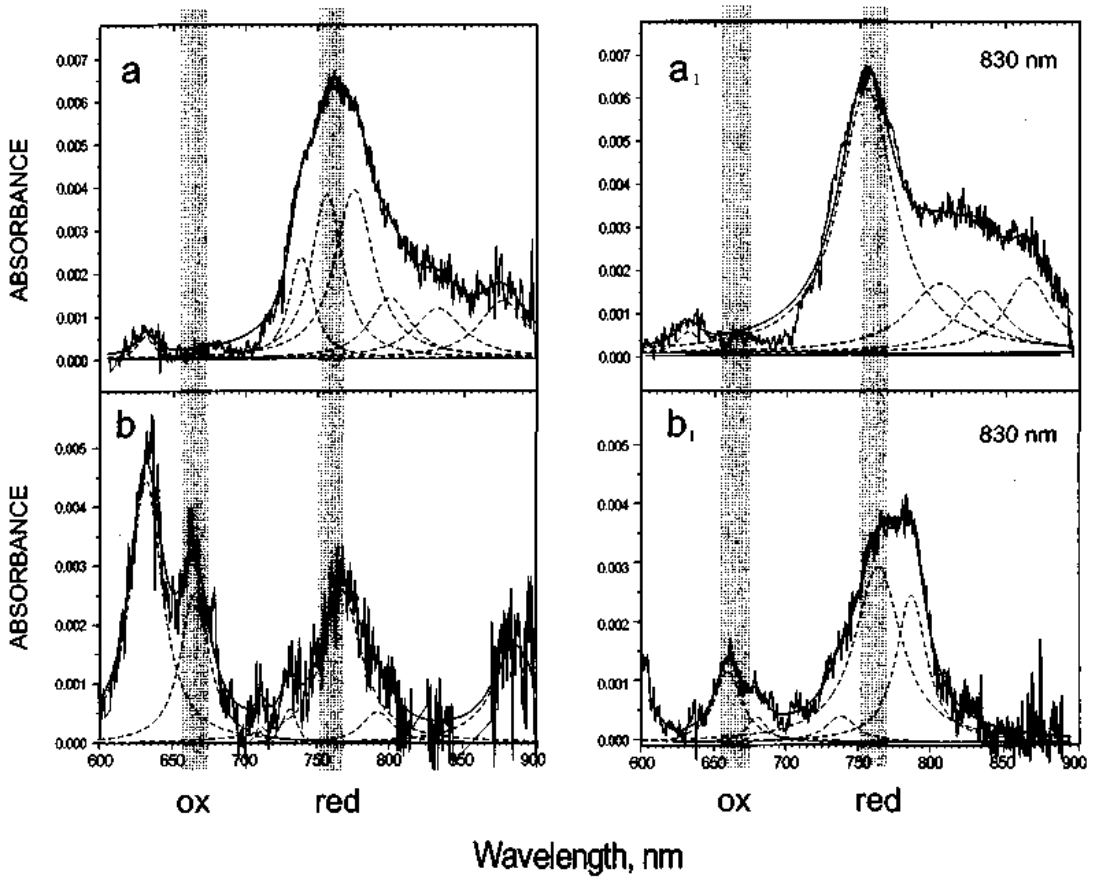

Fig. 2 Absorption spectra of HeLa cell monolayer: (a, b) prior to and (a_1, b_1) after irradiation at 830 nm. a, a_1 – closed cuvette, b, b_1 – open cuvette. Original spectrum, curve fitting (–) and Lorentzian fitting (– – –) are shown as described in [4]. Irradiation procedure is described in [4] in details

modification of the action spectrum associated with the increase of adhesive proper ties of the cell membrane in the range 600-860 nm. NO donor sodium nitroprusside (SNP), sodium azide, which both bond to cytochrome c oxidase catalytic center, as well as ouabain (inhibitor of Na^+, K^+-ATPase in the cell membrane) and amiloride (inhibitor of Na^+/H^+ antiporter in cell the cell membrane) were added to the cellsbefore the irradiation. It is in evidence by comparing these spectra (Fig. 3) that these chemicals have a strong influence on the structure of the intact action spectrum (Fig. 3a) It was suggested that the putative charge transfer complexes to $Cu_{A_{red}}$ $Cu_{B_{oxid}}$ and (see [9] for explanation) are closed for electron transport in the presence of azide. There were practically no changes in electron transport connected with the suggested d-d transitions in Cu_{B_d} chromophores (characterized by doublet bands at 745 and 760 nm), and only a few changes in electron transport occurred, connected with the suggested d-d transitions in $Cu_{A_{oxid}}$ chromophores in near IR region (disappearance of the shoulder at 840 nm (Fig. 3c).

Two charge transfer channels putatively to Cu_{A_d} and Cu_{oxid} as well as two reaction channels putatively connected with d-d transitions in Cu_{d}, and $Cu_{A_{oxid}}$ are reorganized in the presence of NO (Fig. 3b). The action of NO appeared to be quite different from that of azide, which also reacts directly with the binuclear catalytic center of cytochrome c oxidase. Azide bridges the heme of cytochrome a_3 and Cu_B permanently, but NO binds to the catalytic center of cytochrome c oxidase reversibly [10].

The action spectra were recorded also in the presence of two chemicals for which the plasma membrane is impermeable but react with it (amiloride and ouabain, Fig. 3d, e). Ouabain as an inhibitor of Na^+, K^+-adenosine triphosphatase [Na^+, K^+-ATPase] and amiloride as an inhibitor of N^+/H^+ exchanger [NHE], both higher molecular weight substances, cannot react with cytochrome c oxidase (and the a_3-Cu_B center in particular) in the same way as the small ligands N_3 and NO radicals. Our action spectroscopy results (Fig. 3) provide evidence that ouabain as well as amiloride significantly modify the light action spectrum of the increase in the percentage of attached cells [9-11]. The light action spectrum in the presence of ouabain was characterized by a single band at 620 nm and by triplet bands in the near IR region (main peak at 820 nm with shoulders at 800 and 840 nm. Other bands in the red-to-far red region characteristic of the control spectrum fully disappeared in the presence of ouabain. This means that a putative charge transfer channel to Cu_{A_d} (characterized by band at 619 nm) and a channel suggested to be connected with d-d transition in $Cu_{A_{oxid}}$, (band at 820 nm) are working similarly to those of in the control cells, but both channels to Cu_B (the charge transfer channel characterized by the band at 680 nm and a channel due to d-d transition characterized by absorption at 760 nm) are closed in the presence of ouabain.

The light action spectrum in the presence of amiloride has the band only in the near IR region at 831 nm. Noteworthy is the fact that the band at 751 nm in the control spectrum was not only eliminated but amiloride also caused a slight inhibition of cell attachment. This result means that only one reaction channel, namely the channel putatively connected with d-d transition in $Cu_{A_{oxid}}$ chromophore, was working in the

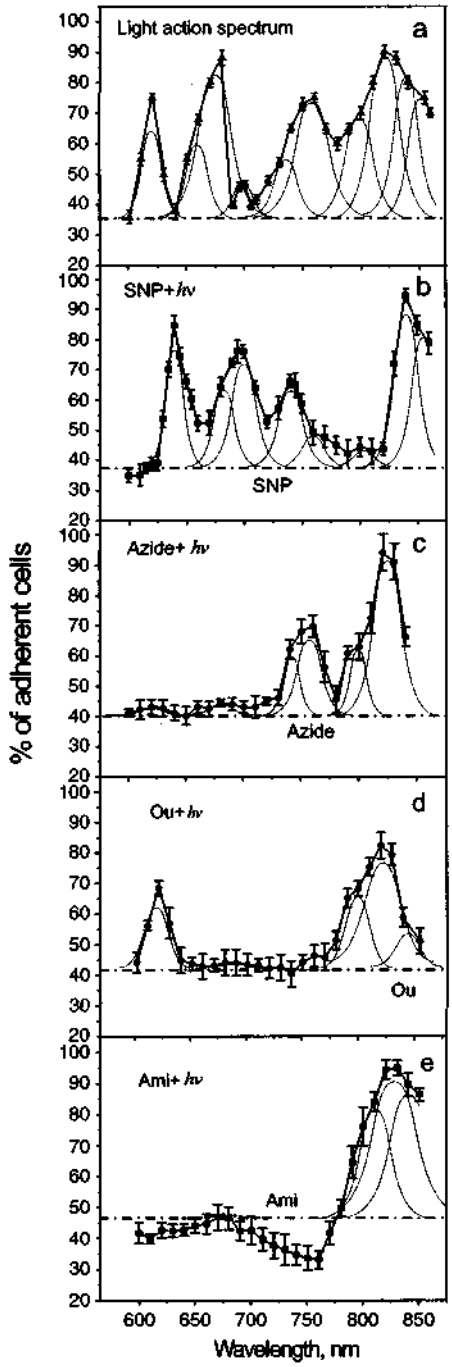

Fig. 3 Action spectra for HeLa cell attachment increase (52 J/m^2, measurements performed 30 min after irradiation): a – without chemicals added or b, c, d, e – sodium nitroprusside, sodium azide, oubain or amiloride added before the irradiation as described in details in [11]

presence of amiloride. Recall that both ouabain and amiloride in the concentrations used ($1x10^{-6}$ M for ouabain and 1.7×10^{-5} M for amiloride) did not statistically significantly influence cell attachment without irradiation.

The novel light-activated mitochondrial cellular signaling pathway could be classified as a mitochondrial retrograde signaling pathway. Mitochondrial retrograde signaling is a pathway of communication from mitochondria to the nucleus under normal and pathophysiological conditions [12]. Recent experimental results confirm the suggestion that cellular responses to light in red-to-near IR region involve retrograde mitochondrial signaling [13].

Conclusions

Our experiments evidence about existence of a light-activated mitochondrial cellular signaling pathway (mitochondrial retrograde signaling). Cytochrome c oxidase acts as a signal generator after the absorption of light quanta as evidenced by light action spectra. But cytochrome c oxidase can act also as a signal transducer as evidenced by results obtained by using the chemicals (Fig. 3).

One can suggest that nitric oxide, a physiological inhibitor of cytochrome c oxidase that binds to its catalytic center dissociates from the catalytic center when the enzyme is reduced by the irradiation. This event could transiently relieve a block in cytochrome c oxidase that causes a reverse of signaling consequences. First, this suggestion may form a basis for explanation of universal effects of various wavelengths in red-to-near IR region phototherapy as well as of various therapeutic uses of this modality.

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