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Absorption measurements of a cell monolayer relevant to phototherapy: Reduction of cytochrome *c* oxidase under near IR radiation

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Abstract

Phototherapy uses monochromatic light in the optical region of 600–1000 nm to treat in a non-destructive and non-thermal fashion various soft-tissue and neurological conditions. This kind of treatment is based on the ability of light red-to-near IR to alter cellular metabolism as a result of its being absorbed by cytochrome *c* oxidase. To further investigate the involvement of cytochrome *c* oxidase as a photoacceptor in the alteration of the cellular metabolism, we have aimed our study at, first, recording the absorption spectra of HeLa-cell monolayers in various oxygenation conditions (using fast multichannel recording), secondly, investigating the changes caused in these absorption spectra by radiation at 830 nm (the radiation wavelength often used in phototherapy), and thirdly, comparing between the absorption and action spectra recorded. The absorption measurements have revealed that the 710- to 790-nm spectral region is characteristic of a relatively reduced photoacceptor, while the 650- to 680-nm one characterizes a relatively oxidized photoacceptor. The ratio between the peak intensities at 760 and 665 nm is used to characterize the redox status of cytochrome *c* oxidase. By this criterion, the irradiation of the cellular monolayers with light at $\lambda = 830$ nm ($D = 6.3 \times 10^3$ J/m^2) causes the reduction of the photoacceptor. A similarity is established between the peak positions at 616, 665, 760, 813, and 830 nm in the absorption spectra of the cellular monolayers and the action spectra of the long-term cellular responses (increase in the DNA synthesis rate and cell adhesion to a matrix).

Keywords: Phototherapy; Photoprotection; Cytochrome *c* oxidase

1. Introduction

Cytochrome *c* oxidase is the enzyme that catalyzes the final step in the mitochondrial respiratory chain: the transfer of electrons from cytochrome *c* to molecular oxygen. Several blocks of experimental data suggest that cytochrome *c* oxidase is sensitive to UV-to-visible light in certain circumstances. First, the flash- or laser-induced reduction of cytochrome *c* oxidase was used as a methodological approach to study the electrogenic and structural changes occurring in the course of the reaction between cytochrome *c* oxidase and dioxygen. This reaction takes advantage of the photolabile bond between CO and the ferrous heme a_3. Laser light with a wavelength of 532 nm [1,2], 514.5 or 488 nm [3], 441.6 nm [4], 413.1 nm [5], or broad-band UV light below 300 nm [6] was used in these studies. Secondly, light in the green-to-red part of the optical region can cause

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physiological responses in cells via activation of cytochrome *c* oxidase. The beat rate of unstained rat heart cells was found to increase for a few minutes following mitochondrial laser microirradiation at 532 nm [7]. Potassium cyanide, a cytochrome *c* oxidase inhibitor, almost completely stopped the photostimulation of the heartbeat rhythm [7]. The action spectrum for stimulating Helix pomatia heart had the same shape as the absorption spectrum of the mitochondria [8]. It was found that following the absorption of light by the respiratory chain, Helix pomatia myocardium behaved like a photosensitive system, showing variations in the period, amplitude, and configuration of the activity potentials [7,8]. State 3 respiration was activated by light at 600 and 420 nm in rat liver and bird brain mitochondria. Uncoupled mitochondria and purified cytochrome *c* oxidase failed to be activated by radiation, suggesting that it was only cytochrome *c* oxidase coupled with oxidative phosphorylation that featured this photosensitivity [9]. The sperm of Urechis unicinctus exhibited photoactivated ($\lambda = 350$–650 nm) CO-insensitive respiration, which was inhibited by antimycin A and potassium cyanide [10]. The authors of [9] suggested that photosensitivity might be a common mitochondrial property in higher animals and could have a physiological significance under certain conditions, e.g., under exposure to orange-red light and high ADP levels. Irradiation of isolated rat liver mitochondria with a He–Ne laser ($\lambda = 632.8$ nm) increased the ATP synthesis, the mitochondrial membrane potential, and the proton gradient [11], cytochrome *c* oxidase being considered to be the photoacceptor involved [12].

Some of the experiments referred to above [11,12] were performed to examine the cellular mechanisms involved in phototherapy (also called low-power laser therapy or photobiomodulation). Phototherapy uses monochromatic light from low-power (below 80 mW) lasers and light emitting diodes (LED's) in the red-to-near infrared (NIR) optical region (~600–1000 nm) to treat in a non-destructive and non-thermal fashion various dermatological, soft-tissue, and neurological conditions [13]. It is believed that this kind of treatment is based on the ability of monochromatic light in the red-to-NIR region to alter the cellular metabolism as a result of its being absorbed by cytochrome *c* oxidase [14–17]. Phototherapy, which is still beyond the mainstream of medicine, has gained attention in the past few years, for this method proved to be useful and to have a great development potential in various fields of medicine and bioengineering. The reduction of the infarct size following a myocardial infraction [18], decrease of methanol-induced retinal toxicity [19], enhancement of the fertilizing capability of sperm cells [20], promotion of the regeneration and functional recovery of tissues with a poor healing potential, such as injured peripheral [21] and optical [22] nerves, improvement of the integration of bony [23] and titanium [24] implants should be mentioned, to name but a few. Light at 670 and 830 nm has recently been shown to upgrade cytochrome *c* oxidase functionally inactivated with potassium cyanide in cultured primary neurons [25].

To further investigate the involvement of cytochrome *c* oxidase as a photoacceptor in the alteration of the cellular metabolism, we aimed our study at, first, recording the absorption spectra of a cellular monolayer in various oxygenation conditions, secondly, investigating the changes caused in these absorption spectra by radiation at 830 nm, and thirdly, comparing between the absorption and action spectra recorded. The 830-nm radiation wavelength is often used for phototherapy purposes [13]. We intend to show that, first, irradiation at $\lambda = 830$ nm causes the reduction of cytochrome *c* oxidase, secondly, the absorption and action spectra are similar in structure in the region of 600–860 nm, and thirdly, the photosensitivity of cytochrome *c* oxidase is not restricted to the UV-to-visible spectral range, but occurs in the far-red and NIR regions as well. With these studies, we also want to draw attention to the as yet unanswered questions as to why such a key enzyme in the cellular metabolism as cytochrome *c* oxidase features photosensitivity and whether this photosensitivity may have a physiological significance. The recent experimental results [26] have demonstrated that the very fast and functional intraprotein electron transfer reactions in bioenergetic systems are not restricted to photosynthetic systems, but occur in cytochrome *c* oxidase as well.

2. Materials and methods

2.1. Cells

The HeLa cells obtained from the Institute of Virology, Moscow, Russia, were cultivated as monolayers in special enclosed Teflon cuvettes provided with quartz windows (described in [27]) at 37 °C in RMPI-1640 (ICN Pharmaceutical, Amsterdam, The Netherlands) with 10% of fetal bovine serum (ICN Pharmaceutical, Amsterdam, The Netherlands) and 100 units/ml of penicillin and streptomycin. The cells were grown in 2 ml of the nutrient medium for 72 h (middle-log phase) when a uniform monolayer covered the entire surface of the cuvette window. The HeLa culture used is characterized by anchorage-dependent growth and forms confluent monolayers. The cells were investigated in the dark or in a dim natural light.

2.2. Optical measurements

The absorption spectra of the cellular monolayers were measured using the setup described in detail in [27]. In short, a parallel light beam from a small incandescent lamp 20 μW in radiant power was directed into a

spectrophotometer with a linear dispersion of 120 Å/mm. The absorption spectra were measured with a multichannel optical analyzer equipped with a Model TCD1301D (Toshiba, Japan) charge-coupled linear photodetector. The total number of the sensitive elements was 3648 per 8 by 200 μm. The spectrophotometer and optical analyzer were constructed by Dr. E. Silkis, Institute of Spectroscopy Russian Academy of Sciences, Troitsk, Russia. The analyzer was coupled to a computer that implemented the signal averaging and data processing procedures. The spectral data were processed (transmission-to-absorption conversion, smoothing, baseline correction, curve fitting, and deconvolution of spectra with the Lorentzian fitting) using the Origin 7.5 (OriginLabs Corp., Northampton, MA, USA) software package. When irradiating the cells and taking measurements, the cuvettes were fixed in the horizontal position, so as to prevent the nutrient medium from covering the windows. The measurements were taken either in enclosed cuvettes or in cuvettes with one of the windows open.

2.3. Irradiation of cellular monolayer

The cells were irradiated with a Model Biotherapy 3ML semiconductor laser (Omega, London, UK). The laser beam parameters were as follows: wavelength 830 nm (3 nm FWHM) output power 18 mW, pulse repetition frequency 700 Hz (duty cycle 80%), fluence 6.3×10^3 J/m^2. The position of the laser beam in the experimental setup for absorbance measurements was similar to that described earlier in [27].

3. Results

The cellular monolayers for all our experiments were grown in enclosed cuvettes. The first series of experiments on the recording of the absorption spectra prior to and after irradiation were conducted with the cuvettes remaining enclosed. A typical spectrum recorded in these conditions prior to irradiation is presented in Fig. 1A, and that recorded after irradiation, in Fig. 1A$_1$. Spectrum A is characterized by strong absorption in the region of 730–850 nm (the bands resolved by the Lorentzian curve fitting method are at 736, 754, 773, and 797 nm, Table 1) and low absorption near 600 nm (a single band at 630 nm) and over 800 nm (the peaks at 830 and 874 nm are resolved, Table 1). This is the spectrum of the cells with the most strongly reduced cytochrome *c* oxidase in our experiments. The irradiation of the cells causes the following changes in the peak positions in this spectrum, as evident from spectrum A$_1$ presented in Fig. 1 and in Table 1. The low-height band is still resolved in the red region at 634 nm. A single strong peak is resolved at 756 nm. The three bands with the peak positions at 807, 834, and 867 nm characterize the region of wavelengths over 800 nm. As a whole, both these spectra, A and A$_1$, have dominating bands in the region of 750–770 nm.

A typical spectrum of the cells with the most strongly oxidized cytochrome *c* oxidase in our experiments is presented in Fig. 1C. In this case, the cuvettes were opened for 30 min to let the cellular monolayer to dry in the air. The red band in this spectrum is resolved at 616 nm, and the far-red bands, at 665 and 681 nm. The NIR region is characterized by weak bands at 712, 730 and 762 nm and by two strong bands at 813 and 872 nm (Table 1). The irradiation of this monolayer caused no changes in the absorption spectrum, as evidenced by Fig. 1 (spectrum C$_1$) and Table 1. This is not an unexpected finding because the respiratory chains do not function in dry cells. This experiment was needed to record the spectrum of the cells with the certainly oxidized cytochrome *c* oxidase. As a whole, spectrum C in Fig. 1 is characterized by strong absorption bands in the red region, as well as in the NIR region of wavelengths over 800 nm, and extremely weak bands in the region of 750–770 nm. Comparison between spectra A and C allows us to conclude that the band at 750–770 nm is characteristic of relatively reduced photoacceptor molecules, and the band at 650–680 nm, as well as that at 800–870 nm, of oxidized ones.

In the third series of experiments, the cuvettes were held open for 5 min prior to taking measurements. The absorption spectra of the cells in these conditions are typically characterized by a band at 633 nm in the red region and the bands with the peak position at 666, 711, 730, 767, 791, and 880 nm in the far-red and NIR regions (Fig. 1B, Table 1). The irradiation of these cells causes significant changes in the absorption spectra in all spectral regions (spectrum B$_1$ in Fig. 1). The band at 750–800 nm becomes dominant in the spectra of the irradiated cells. There are no bands resolved at wavelengths over 800 nm, as well as in the region of 600–630 nm. Spectrum B$_1$ features bands with the peak positions at 661, 681, 739, 765, and 788 nm (Table 1).

For quantitative characterization purposes, as well as for comparison between the recorded absorption spectra, we decided to use intensity ratios between certain absorption bands. The use of spectral band intensity ratios to analyze various spectra is not a new issue for spectroscopy in general [28], but to our best knowledge, it has not been used in the absorption spectroscopy of isolated mitochondria or living cells. Calculation of the intensity ratios was proved to provide benefits for exact comparison of spectra in the IR region [28]. We used the band present in all absorption spectra near 760 nm (exactly at 754, 756, 767, 765, and 762 nm) (Table 1) as a characteristic band for the relatively reduced photoacceptor. The band used by us to characterize the relatively oxidized photoacceptor was the one near 665 nm

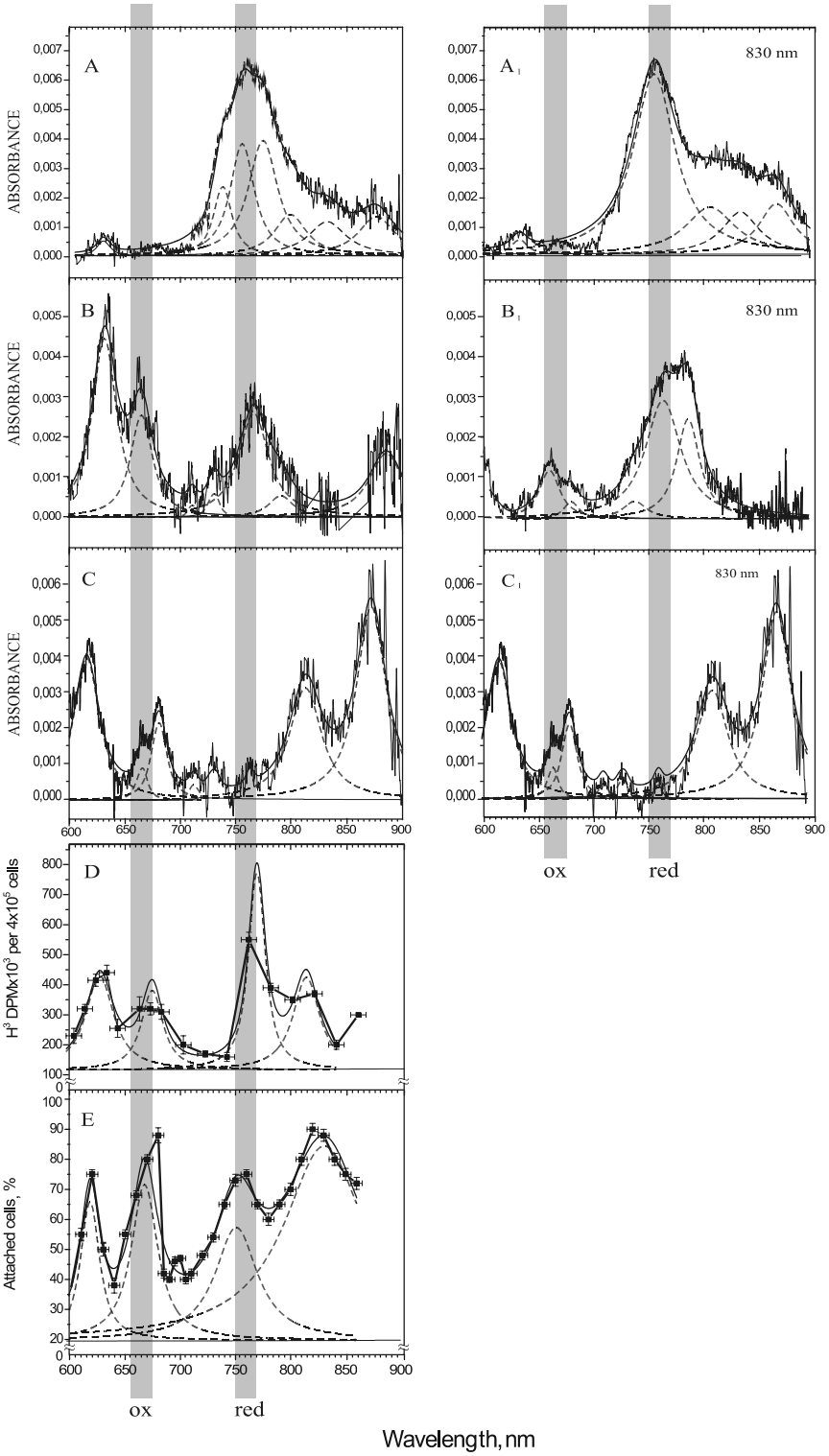

Fig. 1. Absorption spectra of HeLa cell monolayer: (A–C) prior to and (A_1–C_1) after – irradiation at 830 nm. A, A_1 – enclosed cuvette, B, B_1 – open cuvette, C, C_1 – air-dry monolayer. Original spectrum, curve fitting (–) and Lorentzian fitting (–––) are shown. Action spectra for (D) stimulation of DNA synthesis and (E) stimulation of cell adhesion to a glass matrix, measured respectively 1.5 h after irradiation of HeLa cell monolayer ($D = 100$ J/m^2, $t = 10$ s, $I = 10$ W/m^2) and 30 min after irradiation of HeLa cell suspension ($D = 52$ J/m^2, $t = 0$ s, $I = 1.3$ W/m^2). Experimental curves (-■-■-, adapted from [34,35]), curve fitting (–) and Lorentzian fitting (–––) are shown.

(exactly at 666, 661, and 665 nm) in spectra B, B_1, C, C_1 (Table 1). This band is so weak it could not be resolved by the Lorentzian fitting method in spectra A and A_1 belonging to the most strongly reduced photoacceptor in our experiments. For this reason, we used in our intensity calculations for spectra A and A_1 absorption

Table 1
Peak positions in absorption and action spectra of HeLa cells in red-to-NIR region as resolved by Lorentzian fitting

Absorption spectra						Action spectra		Characterization	
A ($R^2 = 0.99$)	A_1 ($R^2 = 0.98$)	B ($R^2 = 0.95$)	B_1 ($R^2 = 0.98$)	C ($R^2 = 0.95$)	C_1 ($R^2 = 0.95$)	D DNA synthesis ($R^2 = 0.97$)	E Adhesion ($R^2 = 0.91$)		
–	–	–	–	616	616	–	618		Oxidized photoacceptor
(630)	(634)	633	–	–	–	624	–		Reduced photoacceptor
–	–	666	661	665	665	672	668	Gray line in Fig. 1	Oxidized photoacceptor
–	–	–	681	681	681	–	–		
–	–	(711)	–	(712)	(712)	–	–	Gray line in Fig. 1	Reduced photoacceptor
736	–	(730)	739	(730)	(730)	–	–		
754	756	767	765	(762)	(762)	767	751		
773	–	–	–	–	–	–	–		
797		(791)	788	–	–	–	–		
–	807	–	–	813	813	813	–		Oxidized photoacceptor
830	834	–	–	–	–	–	831		
874	867	880	–	872	872	Not measured	Not measured		

A, B, C – absorption spectra before, and A_1, B_1, C_1 – after irradiation at 830 nm. R^2 – mean-square deviation of fitting. Weak bands are marked with brackets.

on the curve fitting level at 665 nm. The gray vertical lines in Fig. 1 mark the bands chosen. The intensity ratio I_{760}/I_{665} was calculated to characterize every spectrum. In these simple calculations, we used only the peak intensities (peak heights) and not the integral intensities (peak areas) that are certainly needed for further developments. 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 prevail, the ratio I_{760}/I_{665} is greater than unity, and it is less than unity in cases where the oxidized forms dominate. 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 [37,45].

The magnitude of the I_{760}/I_{665} criterion is 9.5 for spectrum A, 1.0 for spectrum B, and 0.36 for spectrum C. By this criterion, irradiation of the cells, whose spectrum is marked by A ($I_{760}/I_{665} = 9.5$) causes 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 causes 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 B_1. In the spectrum of the cells with initially more reduced photoacceptor (spectrum A), irradiation causes reduction to a lesser extent ($16/9.5 = 1.7$) than in that of the cells with initially less reduced photoacceptor (spectrum B). The intensity ratio in this case is $2.5/1 = 2.5$).

Fig. 1 also presents two action spectra, one for the stimulation of the DNA synthesis in our HeLa cells (D) and the other for the stimulation of the attachment of the cells to a glass matrix (E). Recall that the action spectrum is a plot of the relative effectiveness of light differing in wavelength in causing a particular biological response. Under ideal conditions, the action spectrum should mimic the absorption spectrum of the light-absorbing molecule whose photochemical alteration causes the effect [29]. The action spectra of Fig. 1 were recorded earlier [30,31], but now they have been processed by the curve fitting and Lorentzian deconvolution method, as shown in Fig. 1C and D and Table 1. The results of the analysis of five action spectra recorded for various cellular responses, together with the experimental results on the dichromatic irradiation of the cells and on the modification of the action spectra with chemicals, allowed us earlier to suggest that the photoacceptor mirrored in these action spectra is a component of the respiratory chain [14] and, quite probably, one of the intermediates of the cytochrome *c* oxidase turnover [15]. However, no exact comparison between the absorption and action spectra in the red-to-NIR region was made because of the lack of the absorption spectra of living cells in this region. The absorption spectra of individual living cells were recorded at wavelengths of up to 650 nm years ago with the aim to identify the respiratory chain enzymes. The absorption spectrum of whole cells in the visible region was found to be qualitatively similar to that of isolated mitochondria [32]. The extension of optical measurements from the visible spectral range to the far-red and NIR regions (650–1000 nm) was undertaken late in the seventies for the purpose of monitoring the redox behavior of cytochrome *c* oxidase in vivo [33]. These studies led to the discovery of a "NIR window" into the body and the development of NIR spectroscopy for monitoring tissue oxygenation [34,35].

The two action spectra presented in Fig. 1D and E are characterized by four bands whose peak positions are situated close to each other, namely, 624 and 618 nm, 672 and 668 nm, and 767 and 751 nm, respectively (Table 1). There is a significant difference in the peak positions of spectra D and E at wavelengths over 800 nm (813 and 831 nm, Table 1). However, the peak positions at 813 and 831 nm are resolved by deconvolution in absorption spectra A, A_1, and C, C_1, Table 1). Comparison between the absorption and action spectra presented in Fig. 1 evidences that all the bands present in action spectra D and E are present in the absorption spectra as well (Table 1). However, there are more peaks resolved by the Lorentzian fitting method in the absorption spectra than in the action spectra. This controversy can be explained by the definition of the action spectrum that mirrors the absorption spectrum of the primary photoacceptor. This is an advantage and a specificity of the action-spectrum spectroscopy as compared to other types of spectroscopy. It is well known that the transient species of the cytochrome *c* oxidase turnover are extremely difficult to confidently identify by optical means in physiological conditions. The primary photoacceptor is believed to be one of the turnover intermediates of cytochrome *c* oxidase that has not as yet been identified [15].

The I_{760}/I_{665} intensity ratio is 2.4 for spectrum D and 0.74 for spectrum E, which means that the redox state of the photoacceptor molecule differs between these two spectra, it being more reduced in spectrum D. As far as the I_{760}/I_{665} intensity ratio is concerned, spectrum D is close to absorption spectrum B_1. The two photoresponses whose action spectra are presented in Fig. 1D and E belong to reactions occurring in different parts of the cell, namely, in the nucleus and in the plasma membrane, respectively. It means that the cellular signaling cascades from the photoacceptor [14] can differ as well. It cannot also be ruled out that it is different intermediates of the cytochrome *c* oxidase turnover that play the role of the photoacceptor for these two cellular responses.

4. Discussion

A growing body of evidence suggests that cytochrome *c* oxidase is the photoacceptor in the red-to-NIR spectral range; its activation by light initiates intracellular signaling cascades, resulting in various cellular responses. Several recent experimental results show a connection between the up-regulation of cytochrome *c* oxidase by light and protection of intoxicated retina [19] or neurons [25].

It was found in the present work that the spectral region at 710–790 nm in the recorded absorption spectra of cellular monolayers was characteristic of a relatively reduced photoacceptor, while the region at 650–680 nm characterized the relatively oxidized state of the photoacceptor. The finding that the bands in the region 710–790 nm are characteristic of a relatively reduced cytochrome *c* oxidase is supported by the data presented in [36–39]. The band at ~785 nm is present in the spectrum of the fully reduced unliganded five-coordinated ferrous heme a_3^{2+} [36,37]. The epr-undetectable copper (Cu_B) is believed to display an absorption band in the NIR region, centered at ~740 nm [38], that appears as a distinct peak in one of the low-temperature trapped intermediates [39]. The reduced enzyme and its fully reduced CO-bound form also feature an absorption band at ~710 nm that was assigned to the low-spin ferrous heme *a* [37]. The peak at 711–712 nm is very weak in our absorption spectra, but nevertheless it was resolved by the Lorentzian fitting method (Table 1). Our earlier suggestion was that the peak appearing in the action spectra near 760 nm could be assigned to the reduced Cu_B [15], but the participation of the heme a_3 was not taken into account in this analysis.

So far no strong absorption bands of cytochrome *c* oxidase or its turnover intermediates were recorded in the 650–680-nm wavelength range of the far-red spectral region. The fully developed absorption band at 655 nm, as well as the 800-nm band attributed to the oxidized Cu components, was recorded at a low temperature [40]. A small absorption band assigned to an intermediate (compound A) was recorded at ~660 nm [37]. The appearance of the 655-nm absorption band suggests that Cu_B is oxidized and participates in a spin-coupled state [41,42]. It is believed that the 655-nm feature can arise from a charge transfer band of the high-spin ferric heme a_3 that is modulated by the redox state of Cu_B. As the binuclear center is reduced, the 655-nm band disappears [41,42]. It was suggested in the earlier analysis [15] that the spectral bands in this region were of charge-transfer nature and characteristic of the oxidized form of Cu_B. The literature data [39,41,42] do not exclude the possibility that the 650–680-nm bands in the absorption spectra are characteristic of a oxidized form of cytochrome *c* oxidase. It is also not ruled out that the I_{760}/I_{665} band intensity ratio can roughly characterize the redox status of the catalytic center in cytochrome *c* oxidase, the [heme a_3-Cu_B].

The study of the far-red to NIR absorption spectra of the membrane-bound cytochrome *c* oxidase at low temperatures shows that there are overlapping traces covering the entire wavelengths range from 680 to 870 nm [40,41]. It is generally agreed that the broad absorption band at wavelengths over 800 nm, centered at 830 nm, belongs to the oxidized Cu_A center of cytochrome *c* oxidase. This dimeric copper complex with four ligands is responsible for 77% of the absorbance at 810–820 nm, while the contributions of heme *a* and [heme a_3-Cu_B] are 18 and 5%, respectively [37]. The strong absorption

of Cu_A in this region being dominant, the weak underlying lines in the absorption spectrum of cytochrome *c* oxidase are masked [36]. In our absorption spectra, three bands appear in this region, with peak positions at 807–813, 830–834, and 867–880 nm (Table 1). The two bands at 820 and 870 nm were resolved by the Lorentzian fitting of the large band at wavelengths over 800 nm [34].

It has long been known that the reduced form of cytochrome *c* oxidase has a peak at 605 nm [32]. For the membrane-bound cytochrome *c* oxidase, this peak can be red-shifted for ~10 nm as compared to that for solubilized enzyme [43]. Some of its isospectral peroxy turnover intermediates have a peak at ~607 nm in their absorption spectra [44]. 75% of absorption in this region is accounted for by the low-spin heme *a*, and 25%, by the high-spin heme a_3. Some contribution from Cu_A at ~615 nm was also suggested [38]. Our experimental absorption spectra recording conditions allow us to suggest that the peak at 616 nm (spectra C, C_1, Table 1) can be characteristic of the relatively oxidized photoacceptor, and the weak absorption bands at 630–634 nm, of the relatively reduced photoacceptor (Table 1).

The second finding of the present work is that irradiation at 830 nm causes 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.

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 [45].

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 [46]. This is also true of cytochrome *c* oxidase [47]. 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. It is well known that it is only in the ideal case where the supply of electrons from cytochrome *c* is unlimited that electrons are always present to reduce oxygen. However, this appears to be not always the case with the cytochrome *c* oxidase turnover, provided that the electron supply to its catalytic center becomes a rate-limiting factor [45]. Indeed, the electron transfer activity of an isolated cytochrome *c* oxidase molecule, inhibited by low concentrations of potassium cyanide, intensified under exposure to visible light [48]. Irradiation also increased the reduction rate of the heme groups of cytochrome *c* oxidase in the presence of sodium dithionite. Based on these results, the authors of the above work [48] suggested that the photostimulation of the intramolecular electron transport occurred at the interheme transfer stage. The variability of the electron transfer rate at the interheme point is believed to be a controlling factor in the turnover of cytochrome *c* oxidase [45].

The possibly photosensitive respiratory chain enzymes have never gained researchers' attention as functional photoacceptors (such as chlorophyll and rhodopsin). However, the fragmentary knowledge gathered so far forces one to ask whether the photosensitivity of these enzymes may have a physiological significance in spite of a full adaptation of living systems to photons as a natural external factor. Menez and coworkers [49] suggested that preirradiation with IR light (e.g., during sunrise) is a natural process whereby cells are protected against the solar UV radiation that was acquired and preserved through evolutional selection and plays an important role in life support. They found that visible-to-near-IR radiation at 400–2000 nm protected human dermal fibroblasts from UVA and UVB cytotoxicity [49]. It was found in [49] that this protection was independent of the protein neosynthesis, there being no dependence on the induction of heat-shock proteins. Red-to-NIR radiation is nowadays being used in therapeutical devices for the treatment of a variety of injuries [13]. Recall that light therapy was already known in the ancient times and that red light was used in medicine long before the advent of the laser [50]. Visible-to-NIR monochromatic (laser) light is also being used to protect cells against the harmful effect of ionizing [51–53] or UV [54–56] radiation. This type of protection can be considered as a functional upgrading or activation of the pertinent photoacceptor [51]. A similar phenomenon also occurs in the case of chemically intoxicated cells [16,17,19,25]. This type of photoprotection is not restricted to eukaryotic cells alone, but occurs, for example, in *Escherichia coli* as well [53,54]. The terminal enzymes of the respiratory chain of *E. coli* can be activated by red-to-NIR radiation [50].

The recent experimental measurements of the gene expression in human fibroblasts by the cDNA microarray analysis technique evidence that 111 genes in 10 functional categories, some of them related to the mitochondrial energy metabolism, are regulated by radiation at 670 nm [57]. The evolutional, natural-selection explanation of the development of "useful" photosensitivity to red-to-NIR radiation is consistent with the data about the "optical window" of organisms for radiation in this region of the spectrum [34].

5. Conclusion

Our experimental results evidence, first, that there is a correspondence between the absorption spectra of the cellular monolayers and the action spectra for various cellular responses in the red-to-NIR region. Secondly, irradiation at $\lambda = 830$ nm causes reduction of the photoacceptor, as judged by the I_{760}/I_{665} band intensity ratio criterion. The transient photosensitive species (the photoacceptor) has not as yet been identified. The question as to whether this is the $[a_3^{2+} \cdot O_2]$ photosensitive cytochrome *c* oxidase turnover species whose formation and disappearance have been observed experimentally [58], or something else, remains open.

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