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A Novel Mitochondrial Signaling Pathway Activated by Visible-to-near Infrared Radiation¶

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ABSTRACT

The number of cells attached to glass substratum increases if HeLa cell suspension is irradiated with monochromatic visible-to-near infrared radiation before plating (the action spectrum with maxima at 619, 657, 675, 700, 740, 760, 800, 820, 840 and 860 nm). Treating of cell suspension with sodium azide (2×10^{-5} *M*), sodium nitroprusside (5×10^{-5} *M*), ouabain (1×10^{-6} *M*) or amiloride (1.7×10^{-5} *M*) before irradiation significantly modifies the spectrum of cell attachment enhancement. A light-induced mitochondrial signaling pathway can be regulated by small ligands directly binding to the catalytic center of cytochrome *c* oxidase (N_3, NO) as well as by chemicals specifically binding to plasma membrane enzymes (ouabain, amiloride). The comparative analysis of action spectra allows the conclusions that first, Cu_A and Cu_B chromophores of cytochrome *c* oxidase could be involved as photoacceptors and second, various signaling pathways (reaction channels) between cytochrome *c* oxidase and cell attachment regulation are at work.

INTRODUCTION

Research into the mechanisms of cell–cell and cell–matrix interactions is motivated by the involvement of the cell surface and its adhesive interactions in the regulation of such processes as growth, differentiation, morphogenesis, wound repair, formation of metastases, to name but a few. The cells from freshly resuspended monolayer require energy to initiate and maintain their attachment to glass (1) as well as to collagen-coated surfaces (2). It was found in these studies that conditions inhibiting adenosine triphosphate (ATP) synthesis (treatment of cells with inhibitors of respiratory chain) reduce the ability of cells to attach to substrata. Vice versa, cell attachment can be increased in conditions where ATP extrasynthesis occurs, *e.g.* by the activation of the respiratory chain by irradiation of cells with monochromatic light in the visible–to-near infrared (NIR) spectral region (3).

In this study the experimental approach of using light action spectra instead of absorption spectra provides two benefits. Recording of absorption spectra of living cells in the far red-to-NIR region in physiologic conditions as well as interpretation of these spectra is limited because of the technical difficulties associated with broad overlapping and weak absorption bands of redox carriers in this region and extremely fast electron transfer during redox cycles. Recall that any graph representing a biological response as a function of wavelength is called an action spectrum; the action spectrum coincides (some limits exist) with the absorption spectrum of the photoacceptor molecule (4). A light action spectrum not only provides information about the primary photoacceptor but also allows one to make suggestions about the reaction channels between the photoacceptor molecule and cellular response measured experimentally.

Comparative analysis of three light action spectra recorded in the wavelength range 330–860 nm (DNA and RNA synthesis rates, cell attachment) and the available spectroscopic data for biomolecules absorbing in the visible-to-NIR region led to the suggestion that the photoacceptor in mammalian cells is a redox intermediate of the terminal enzyme of the respiratory chain, cytochrome *c* oxidase (5). Coincident action spectra for processes in the nucleus (DNA and RNA synthesis) and in the plasma membrane (attachment) suggest that the photoacceptor is the same and imply the existence of signaling pathways between the photoacceptor located in mitochondria and the plasma membrane as well as between the mitochondria and nucleus (5,6). Direct action of radiation in the visible-to-NIR region on cell attachment cannot be considered because cell–matrix and cell–cell attachment molecules isolated and characterized so far belong to the large family of glycoproteins, which are not capable of absorbing light in this optical region (7,8).

In this study we analyze comparatively five light action spectra (the control spectrum and the spectra when ouabain, amiloride, sodium nitroprusside [SNP] or sodium azide was added before irradiation to the cell suspension) with the aim to characterize changes in the photoacceptor molecule leading to cell attachment modification. Nitric oxide released from SNP as well as $-N_3$ from sodium azide primarily bind to heme a_3–Cu_B center of cytochrome *c* oxidase and thereby regulate its catalytic activity (10–12). Two other chemicals (inhibitor of Na^+,K^+–adenosine triphosphatase [ATPase] ouabain and inhibitor of N^+/H^+ exchanger [NHE] amiloride), as higher molecular weight substances, cannot react

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Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; FWHM, full wide at half maximum; NHE, N^+/H^+ exchanger; NIR, near infrared; SNP, sodium nitroprusside.

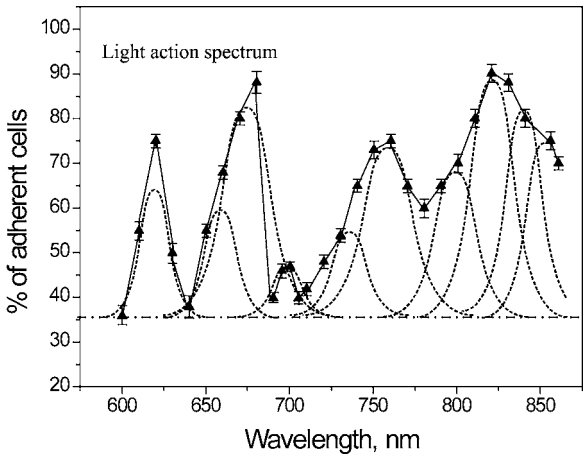

Figure 1. Dependence of cell attachment on wavelength (light action spectrum) used for irradiation of cell suspensions (CW light, fluence 52 J/m^2, intensity 1.3 W/m^2, irradiation time 40 s, adhesion measurements performed 30 min after irradiation). The dotted line indicates the corrected baseline. The dashed lines indicate Lorentzian fitting.

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 radical. It was suggested that signals produced by such chemicals are transduced to mitochondria via protein–protein interactions by binding the chemicals to the cell membrane (13). Xie and Askari (13) believe that the activation of this regulation pathway is a common feature of the signal transducing function of Na^+,K^+–ATPase in most cells, including HeLa. Literature data evidences that ouabain (14,15) and amiloride (16) can modulate cell attachment and ouabain can regulate the activity of cellular respiration as well (17).

Early research into the photobiology of action of monochromatic visible light on cells was initiated by the successful use of lasers in medicine, mainly for the improvement of tissue repair (18). Recent research results evidence that improving cell metabolism by irradiation with monochromatic (laser) light provides benefits in the treatment of infraction (19) and carpal tunnel syndrome pain (20), the repair of spinal cord nerves (21), wound healing in diabetic mice (22), promotion of cell survival (23) and increase of proliferation (24,25).

The aim of this work was to study the model of cell attachment cellular signaling pathways that include mitochondria and cytochrome *c* oxidase in particular and can be regulated by visible-to-NIR radiation. It will be shown that various signaling pathways that include cytochrome *c* oxidase can modulate cell attachment.

MATERIALS AND METHODS

Cells. HeLa cells, obtained from the Institute of Virology, Moscow, Russia, were cultivated as a monolayer in closed Carrell vials (diameter 45 mm), at 37°C in 5 mL of RMPI-1640 (Flow Laboratories, London, UK), with 10% fetal bovine serum (Flow Laboratories), and 100 U/mL of penicillin and streptomycin (ICN Pharmaceutical, Amsterdam, The Netherlands). A total of 1.5×10^6 cells were plated per vial and grown for 72 h (middle–log phase). The HeLa culture used is characterized by anchorage-dependent growth and forms a confluent monolayer. Cells were harvested using 0.02% Versene solution (37°C) (ICN Pharmaceutical), and suspension for irradiation was prepared in medium RMPI-1640 (Roswell Memorial Park Institute) containing 10% bovine embryo serum. Cell culture processing was performed in dark or under dim natural light. Extraneous illumination (sunshine or artificial light) was avoided.

Irradiation. Monochromatic radiation was obtained by means of a monochromator designed by Dr. A. Lifshits at the Institute of Spectroscopy, Russian Academy of Sciences, Troitsk, to have bandwidths of 400–700 nm and 540–1050 nm. The monochromator was operated in accordance with an autocollimation scheme. A single slit, 1.2 mm wide, was used to direct radiation both into and out of the monochromator. Dispersion amounted to 8 nm/mm. Thus, the exit radiation bandwidths came to 10 nm (full wide at half maximum [FWHM]). The dispersive element used was a diffraction grating of 1200 lines per millimeter. To obtain radiation in region 600–860 nm, the second order of the grating, a thermal filter and a 600 nm cut-off glass filter, placed between the radiation source (a stabilized 250 W high-pressure xenon arc lamp) and the monochromator, was used. The source radiation was focused with a positive lens and delivered by a fiber light guide to the upper part of the slit. After diffraction of the beam, the monochromatic radiation was exited from the lower part of the slit and transmitted by another light guide. In the range of wavelength 600–860 nm, the output power of the monochromator and the power measured after the sample were constant. A Coherent Fieldmaster power meter suitable for measuring powers in the microwatt range was used for the measurements. Light intensity in experiments was 1.3 W/m^2; dose, 52 J/m^2; irradiation time, 40 s. The light spot fully covered the vial with cell suspension (0.38 cm^2).

The samples of cellular suspension were irradiated in special glass sample cells. These cuvettes were made as follows. Two glass rings with ground-in edges (inner diameter 0.7 cm, height 0.4 cm, volume 130 μL) were stuck on a microscope slide 5 cm distance apart. Both wells were filled with cell suspension (85 000 cells per vial). Always, one of the vials was irradiated and the other was used as control. Optimal irradiation conditions (shape and dimensions of the vial, number of cells per vial) were developed in a special series of experiments. Irradiation was performed at room temperature in the dark. The control well was specially protected from light during irradiation experiment. A new pair of sample cells was used for every measurement.

Measurement of cell–glass adhesion. The criterion by which changes in the adhesion properties of the cell membrane were judged was the number of cells that attached themselves to the bottom of the cuvette during the course of the 30 min at 37°C. This time was chosen according to experiments describing the time course of attachment of HeLa cells to the glass substratum under our experimental condition (3). In control culture, 42.5 ± 2.5% of cells adhered to the surface after 30 min incubation. Thus, in these conditions, the stimulatory and inhibitory effects of the irradiation and chemicals could be measured under the same conditions. After incubation, the nutrient medium was removed, and the cuvettes were washed with warm (37°C) Hanks' solution (ICN Pharmaceutical) to remove nonattached cells. The attached cells were trypsinized, and their number was counted with a hemocytometer. Each data point represents the mean of at least 10 independent measurements.

Chemicals. Amiloride, ouabain, sodium azide and SNP were from Sigma Chemical Co. (St. Louis, MO). Ten microliters of freshly prepared solution of a chemical in noncolored Hanks' solution (37°C) was added to each cuvette; 10 μL of Hanks' solution without the chemical was added to the control cuvettes. Stock solutions of the chemicals were made up freshly before the experiments and protected from light. The toxicity of the chemicals was assessed using the trypan blue exclusion test: the concentrations used were found to be nontoxic under our experimental conditions (viability of cells >95%). The solutions of inhibitors did not show any absorption bands in the optical region under study (checked by recording the absorption spectra).

Statistical analysis. The results obtained were statistically processed by means of GraphPad Prism 4.0 (San Diego, CA) program packages and expressed in terms of the mean ± SEM from 10 measurements. $P < 0.05$ was considered significant using analysis of variance and Student's *t*-test.

Deconvolution of action spectra with Lorentzian fitting was made using the Origin 7.5 (Northampton, MA) software program.

RESULTS

Light action spectrum in red–NIR region

The percentage of cell attachment to the glass surface is increased on irradiating the cell suspension samples with certain wavelengths. Figure 1 shows the measured light action spectrum as well as the deconvolution of this spectrum with Lorentzian fitting. Table 1 shows the peak positions of the Lorentzian fitting. The light action spectrum of cell adherence to glass (Fig. 1) is characterized by a single peak at 619 nm; triplet peaks at 657 nm, 675 nm (main

Table 1. Comparison of peak positions in control light action spectrum of increase of cell adherence with peak positions in light action spectra when azide, SNP, ouabain or amiloride are added before irradiation of cell suspension. Peak positions are calculated from Lorentz fit data; changes <5 nm are considered nonsignificant. Main peaks are marked bold*

Control spectrum		Azide, 2×10^{-5} *M*		SNP, 5×10^{-5} *M*		Ouabain, 1×10^{-6} *M*		Amiloride, 1.7×10^{-5} *M*	
Band structure, chromophore putatively responsible for absorption in main band (27)	Peak positions (nm)	Peak positions (nm)	Changes in peak positions in comparison with control spectrum	Peak positions (nm)	Changes in peak positions in comparison with control spectrum	Peak positions (nm)	Changes in peak positions in comparison with control spectrum	Peak positions (nm)	Changes in peak positions in comparison with control spectrum
Single									
$Cu_{A_{red}}$	**619**	—	Disappeared	—	Disappeared	**620**	No changes	—	Disappeared
				642	New band				
Triplet	657	—	Disappeared	—	Disappeared	—	Disappeared	—	Disappeared
$Cu_{B_{oxid}}$	**675**	(~680 v.w.sh.)	Disappeared	685	Shift +10 nm	(~680 v.w.sh.)	Disappeared	(~670 v.w.sh.)	Disappeared
	700 w.sh.	—	Disappeared	**700**	New band	—	Disappeared	—	Disappeared
Doublet	740	745	No changes	**742**	New distinct band	—	Disappeared	—	Disappeared
$Cu_{B_{red}}$	**760**	**760**	No changes	(~760 v.w.sh.)	Disappeared	(~760 v.w.sh.)	Disappeared	—	Disappeared
Quartet	800 s.sh.	800 w.sh.	No changes	(~805 v.w.sh.)	Disappeared	800 s.sh.	No changes	811 s.sh.	Shift +11 nm
$Cu_{A_{oxid}}$	**820**	**825**	No changes	—	Disappeared	**820**	No changes	**831**	Shift +11 nm
	840 s.sh.	—	Disappeared	**842**	No changes	840 w.sh.	No changes	842	No changes
	860 s.sh.	Not measured		856 s.sh.	No changes	—	Disappeared	Not measured	

*w.sh., weak shoulder; s.sh., strong shoulder; v.w.sh., very weak shoulder (statistically not significant).

Figure 2. Dependence of cell attachment on wavelength used for irradiation of cell suspension (CW light, fluence 52 J/m^2, intensity 1.3 W/m^2, irradiation time 40 s, adhesion measurements performed 30 min after irradiation) with sodium azide (2×10^{-5} *M*) added before irradiation. The dotted line indicates the attachment of cells in the presence of azide without irradiation. The dashed lines indicate Lorentzian fitting.

peak) and 700 nm (a weak shoulder); doublet peaks at 740 and 760 nm (main peak) as well as by quartet peaks at 800, 820, 840 and 860 nm (Table 1). In this quartet, the main peak is at 820 nm, and peaks at 800, 840 and 860 nm are resolved as shoulders.

The bands similar to the action spectrum represented in Fig. 1 light action spectra were identified by analogy with the absorption spectra of the metal–ligand systems ([26], review [5]). On the basis of this work, the main contribution to the 619 nm band is believed to arise from the reduced Cu_A, to the 675 nm band from the oxidized Cu_B, to the 760 nm band from the reduced Cu_B and to the 820 nm band from the oxidized Cu_A chromophores of cytochrome *c* oxidase. The bands at 619 and 675 nm are mainly due to charge transfer from ligands to copper atoms; the bands at 760 and 820 nm are due to d–d transitions inside the copper atoms (26). On the basis of the study of Karu and Afanasyeva (26), it was suggested that the photoacceptor is a yet not identified intermediate of redox cycle of cytochrome *c* oxidase that appears in turnover (5).

The study of Karu and Afanasyeva (26) was performed before the three-dimensional crystallographic structure of cytochrome *c* oxidase was solved in 1995 and many details about its catalytic mechanism were not known (10). The exact contributions of absorption of every redox intermediate to the broad and overlapping absorption bands are still not known. Recent experimental absorption studies in the red–NIR region (27–29) do not contradict the suggestions made by Karu and Afanasyeva (26) (review [5]). The present interpretation of modification of light action spectra with chemicals is based on our earlier work on the interpretation of action spectra (26).

Modification of the light action spectrum with inhibitors of respiratory chain

Figure 2 shows the light action spectrum when azide was added to cell suspension before the irradiation. Concentration of azide used in this experiment, 2×10^{-5} *M,* does not influence cell attachment without irradiation (40.8 ± 2.5%, marked by dotted line in Fig. 2, *vs* 42.5 ± 2.5% in control). Doublet bands at 745 and 760 nm and doublet bands at 800 and 825 nm (Table 1) characterize the action spectrum shown in Fig. 2. Comparison of this action spectrum (Fig.

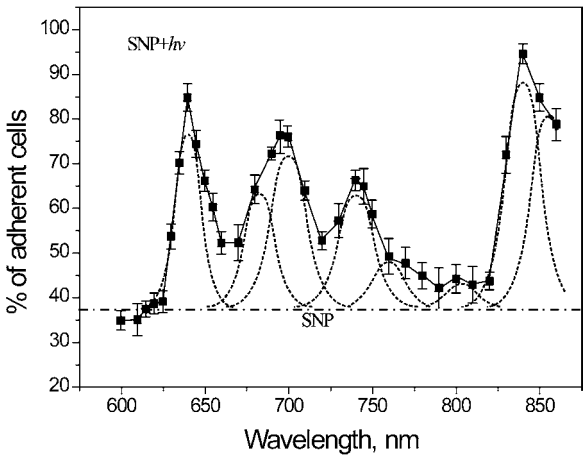

Figure 3. Dependence of cell attachment on wavelength used for irradiation of cell suspensions (CW light, fluence 52 J/m^2, intensity 1.3 W/m^2, irradiation time 40 s, adhesion measurements performed 30 min after irradiation) with SNP (5×10^{-5} *M*) added before irradiation. The dotted line indicates the attachment of cells in the presence of SNP without irradiation. The dashed lines indicate Lorentzian fitting.

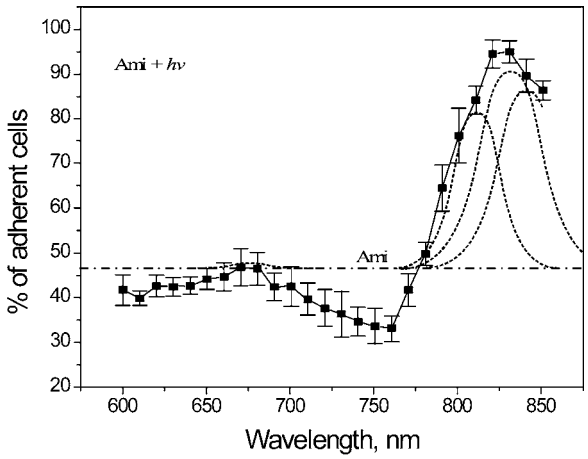

Figure 5. Dependence of cell attachment on wavelength used for irradiation of cell suspensions (CW light, fluence 52 J/m^2, intensity 1.3 W/m^2, irradiation time 40 s, adhesion measurements performed 30 min after irradiation) with amiloride (1.7×10^{-5} *M*) added before irradiation. The dotted line indicates the attachment of cells in the presence of amiloride without irradiation. The dashed lines indicate Lorentzian fitting.

2) with the control spectrum (Fig. 1) shows that the most dramatic changes occur in the red region (peak at 619 nm is fully eliminated) as well as in the far-red region (the triplet bands with main peak at 675 nm and with shoulders at 657 and 700 nm in the control spectrum are fully eliminated, Table 1). This finding may mean that the putative charge transfer complexes to $Cu_{A_{red}}$ and $Cu_{B_{oxid}}$ are closed for electron transport in the presence of azide. There are practically no changes in electron transport connected with suggested d–d transitions in $Cu_{B_{red}}$ chromophore (characterized by doublet bands at 745 and 760 nm), and only few changes in electron transport connected with suggested d–d transitions in $Cu_{A_{oxid}}$ chromophore in NIR region occur (disappearance of the shoulder at 840 nm, Table 1).

Figure 3 shows the measured action spectrum as well as Lorentz fit when SNP (5×10^{-5} *M*) is added to cell suspension samples before irradiation. SNP at a concentration of 5×10^{-5} *M* causes a statistically nonsignificant inhibition of cell attachment without irradiation (37.9 ± 2.1% *vs* 42.5 ± 2.5% in control). The new action spectrum is characterized by a single band at 642 nm, doublet bands at 685 and 700 nm (main peak), single band at 742 nm (with a weak shoulder at 760 nm) and doublet bands at 842 nm (main peak) and 856 nm (Table 1).

On the whole, the comparison of data in Table 1 allows one to state that two charge transfer channels putatively to $Cu_{A_{red}}$ and $Cu_{B_{oxid}}$ as well as two reaction channels putatively connected with d–d transitions in $Cu_{B_{red}}$ and $Cu_{A_{oxid}}$ are reorganized in the presence of NO (Table 1). The action of NO appears 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 (10), but NO can bind to cytochrome a_3, Cu_B or both (11,12).

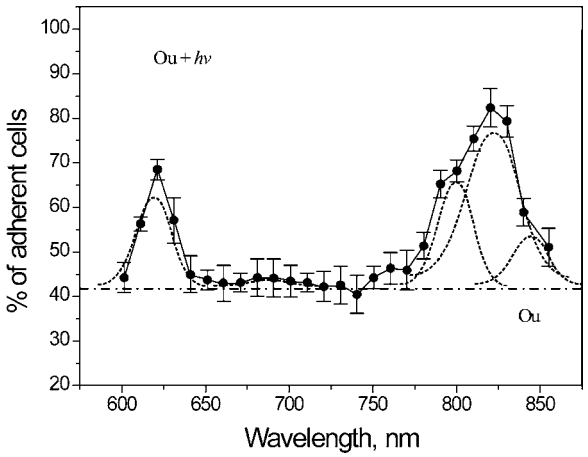

Figure 4. Dependence of cell attachment on wavelength used for irradiation of cell suspensions (CW light, fluence 52 J/m^2, intensity 1.3 W/m^2, irradiation time 40 s, adhesion measurements performed 30 min after irradiation) with ouabain (1×10^{-6} *M*) added before irradiation. The dotted line indicates the attachment of cells in the presence of ouabain without irradiation. The dashed lines indicate Lorentzian fitting.

Modification of the light action spectrum with molecules acting via interaction with cell membrane

Our results of action spectroscopy provide evidence that ouabain (Fig. 4) as well as amiloride (Fig. 5) significantly modify the light action spectrum of increase of percentage of attached cells increase (Fig. 1). The light action spectrum in the presence of ouabain is characterized by a single band at 620 nm and by triplet bands in NIR region (main peak at 820 nm with shoulders at 800 and 840 nm, Table 1). Other bands the in red-to-far red region characteristic of the control spectrum (Fig. 1) fully disappeared in the presence of ouabain (Fig. 4). It means that putative charge transfer channel to $Cu_{A_{red}}$ (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 these in the control cells, but both channels to Cu_B (charge transfer channel characterized by band 680 nm and a channel due to d–d transition characterized by absorption at 760 nm) are closed in the presence of ouabain.

Figure 5 shows the light action spectrum in the presence of amiloride. This action spectrum has the bands only in NIR region, which are characterized by +11 nm shifts of strong shoulder at 800 nm and main peak at 820 nm as compared with those in the control

spectrum (Table 1). Noteworthy is the fact that the band at 760 nm in the control spectrum is not only eliminated but amiloride also causes a slight inhibition of cell attachment below the control level. This result means that only one channel, namely the one putatively connected with d–d transition in $Cu_{A_{oxid}}$ chromophore, is working in the presence of amiloride. Recall that both ouabain and amiloride in concentrations used (1×10^{-6} *M* for ouabain and 1.7×10^{-5} *M* for amiloride) do not statistically significantly influence cell attachment without irradiation (42.1 ± 3.2% and 47.1 ± 2.1%, respectively, *vs* that in control, 42.5 ± 2.5%).

These results evidence that despite not binding directly to the catalytic center of cytochrome *c* oxidase, ouabain and amiloride actively influence cellular respiration in the irradiated cells via inhibition of NHE and K^+,Na^+–ATPase.

DISCUSSION

The number of HeLa cells attached to the glass substrate increases after irradiation of cell suspension with certain wavelengths in the visible-to-NIR optical region (the action spectrum in Fig. 1). This finding evidences, first, that a new subpopulation of cells enters an adhesive state because of the irradiation and second, a photoacceptor governs this process. In other words, the irradiation opens some new reaction channel(s) for attachment increase via activation of the photoacceptor. The result that the chemicals added before irradiation strongly change the light action spectrum shows that reaction channels activated by light are either closed, as evidenced by full disappearance of bands in action spectra in Figs. 2–5, or modified, as evidenced by appearance of new peaks (Fig. 3) or shifted peak positions (Fig. 5). Recall that chemicals in concentrations used do not influence cell attachment without irradiation.

The bands in the light action spectrum (Fig. 1) are believed to belong to a not yet identified redox intermediate of cytochrome *c* oxidase turnover (5,26). Redox absorbance changes in living cells recorded in the spectral range 530–890 nm (30) as well as changes in optical activity of absorbing chromophores at 250–780 nm (31) occurring under laser irradiation support this suggestion.

Let us make two remarks before the analysis of possible absorbing chromophores in recorded action spectra. First, the photosensitivity of mitochondria (32) and cytochrome *c* oxidase in particular (33–38) is known for a long time. Purified cytochrome *c* oxidase and the enzyme in isolated mitochondria were photoreduced by green light (33) as well as by UV radiation ($\lambda < 300$ nm) (35). Pulsed laser light at 532 nm caused redox absorbance changes and electrogenic events in partly reduced cytochrome *c* oxidase, indicating increased electron transfer from Cu_B to $Fe^{3+}_{a_3}$ (35). In the experiments described by Hallen *et al.* (35), fully oxidized cytochrome *c* oxidase did not display any measurable absorbance changes. He–Ne laser radiation (632.8 nm) caused a remarkable increase in electron transfer in cytochrome *c* oxidase both in soluble preparation as well as in isolated mitochondria (38). Taken together, measurements of light-induced electrogenic events and absorbance changes indicate that irradiation can induce structural changes in cytochrome *c* oxidase when working with purified enzyme (33–38), isolated mitochondria (38–40) or living cells (5,30–32,41).

A possible photomodulation of other mitochondrial enzymes (42,43) is not considered here because as a rule they do not contain known chromophores absorbing at 700–900 nm. One can suggest that measured changes in glutamate dehydrogenase properties under irradiation at 632.8 nm (43) are due to absorbance of a semiquinone form of this flavoprotein.

Second, our interest in mitochondrial photosensitivity and their ability to regulate cellular metabolism is connected with molecular mechanisms of low-power laser therapy, a modality used in clinical practice for various purposes (18–25). It was suggested that the mechanism of low-power laser therapy at the cellular level is based on the absorption of monochromatic visible and NIR radiation by components of the mitochondrial respiratory chain causing changes in their redox properties and acceleration of electron transfer (primary reactions), which are followed by secondary (dark) biochemical reactions of cellular signaling (6). The model of cell attachment together with the methodical approach of action spectroscopy used in this study allow a complex study of both primary and secondary cellular responses.

The result that azide and nitric oxide, which are known as inhibitors of cytochrome *c* oxidase, modify the action spectrum of cell attachment increase supports our suggestion (5,26) that cytochrome *c* oxidase could be the photoacceptor. This result also corroborates a recent hypothesis (44) that the mitochondrial NO signaling pathway could be an alternative function of cytochrome *c* oxidase. An unexpected result is that ouabain and amiloride, which do not react directly with cytochrome *c* oxidase, modify significantly the absorption of the photoacceptor (mirrored by the action spectrum in our case). This finding is consistent with a view (13) that the signal-transducing role played by Na^+,K^+–ATPase from plasma membrane to mitochondria via protein–protein interactions could be one of its standard functions. The inhibition of cellular respiration by ouabain is measured experimentally (17). It is not clear now how the signal from NHE, which is inhibited by amiloride, is transduced to mitochondria. It is known that amiloride induces fast intracellular acidification (45). An intracellular signaling pathway is believed to mediate the effects of cell adhesion and receptors of pH_i (16).

Suggested participation of copper chromophores of cytochrome *c* oxidase in various redox states ($Cu_{A_{red}}$, $Cu_{A_{oxid}}$, $Cu_{B_{oxid}}$, $Cu_{B_{red}}$) (5,26) allows to propose that at least four reaction channels from cytochrome *c* oxidase, which are regulated by light, regulate cell attachment as well. These chromophores are indicated in the first row of Table 1. Percentage of the newly attached subpopulation under our experimental conditions is from ~47% at irradiation wavelength of 820 nm and ~40% at 680 nm to ~33% at 760 nm and ~26% at 620 nm (Fig. 1). Not a simple structure of main bands (Fig. 1) resolved by Lorentzian fitting (Table 1) shows not only a complex nature of the reaction channels but also a feature of these channels for subtle adjustment. Changes in peak positions as well disappearance or appearance of bands in the light action spectrum in the presence of chemicals evidence that we are dealing with a dynamic system of photoacceptor(s) fast reacting to changing conditions both in the vicinity of absorbing chromophores as well as in the plasma membrane.

An analysis of data in Table 1 allows one to arrive at the following conclusions. The only band fully disappearing (in the presence of azide, ouabain, and or amiloride) or strongly modified (by NO) in the light action spectrum is the triplet with main peak at 675 nm and shoulders at 657 and 700 nm. This finding may point to increase in reduction of Cu_B chromophore in our experimental conditions (insofar as these peaks are thought to belong to $Cu_{B_{oxid}}$). A 655 nm band has long been considered to be characteristic of fully oxidized cytochrome *c* oxidase and disappears by reduction (46). This peak at 655 nm (putatively at 657 nm in our spectrum,

Fig. 1) is believed to belong to $a_3^{2+} - Cu_B^{2+}$ binuclear center and disappears by the formation of peroxy and ferryl forms of the enzyme in turnover (47). There is some evidence that the properties of this transition are affected by the oxidation state of Cu_B (48). Beinert *et al*. (47) also recorded an absorption near 680 nm in mixed-valence CO-bound cytochrome *c* oxidase.

In the study of Szundi *et al*. (27), a shoulder at ~700 nm in the ferryl difference spectrum was observed. Similar weak shoulder resolved in our light action spectrum (Fig. 1) has disappeared in the presence of all chemicals except SNP. Szundi *et al*. (27) found that absorption near 710 nm was increasing by reduction of cytochrome *c* oxidase. Appearance of a new peak at 700 together with redshift and decrease in intensity of the peak at 675 nm (Table 1) may indicate that a new charge transfer channel is open in the presence of NO. One has to emphasize that a very weak absorption of Cu_B chromophore (46) can be strongly amplified in the action spectra.

Comparison of spectral peak positions in the presence of NO and azide shows that in the first case distance between peaks putatively belonging to $Cu_{B_{red}}$ (760 nm in control) and $Cu_{A_{oxid}}$ (820 nm in control) increases, which may be connected with the opening of a new channel at 642 nm. At the same time, there is a shortening of the distance between peaks putatively characteristic of $Cu_{A_{red}}$ (619 nm in control) and $Cu_{B_{oxid}}$ (680 nm in control, Table 1). In the presence of azide, the situation is quite opposite. The distance between peaks at 760 and 820 nm shortens, and two peaks at 619 and 680 nm disappear at all (Table 1). This finding could be consistent with differences in the binding of these two ligands to the catalytic center of cytochrome *c* oxidase (10–12).

In the presence of chemicals, which do not bind to cytochrome *c* oxidase (amiloride, ouabain), the doublet bands at 740 and 760 nm putatively characteristic of $Cu_{B_{oxid}}$ (Table 1) fully disappeared. The chemicals, which directly bind to the catalytic center of cytochrome *c* oxidase, do not influence this doublet (azide) or only modify it (nitric oxide). An absorption at 740 nm, which appears at low-temperature measurements by the formation of one of the intermediates (compound C), and an absorption at 760 nm (together with absorption at 796 nm), which appears by partial oxidation of cytochrome *c* oxidase (compound B), have been recorded (49). Compound B is a peroxy intermediate with ferric peroxide–cupric Cu complex. Compound C is also a peroxy intermediate of turnover but different from compound B (50). The presence of two peaks at 740 and 760 nm in our control action spectrum may evidence that the photosensitive intermediate is a peroxy compound. The data presented in the study (34) supports this suggestion. The appearance and disappearance of a photosensitive intermediate, which could be a $a_3^{2+} - Cu_B^{1+}$ intermediate, was shown in this study. Recent studies evidence that the peroxy intermediate is really a mixture of several peroxy intermediates (51,52).

Two bands in our control action spectrum are believed to be connected with the Cu_A chromophore. These are the single band at 619 nm and a compound band in the NIR region with main peak at 820 nm and shoulders at 800, 840 and 860 nm (Table 1). There is a consensus in the literature data that a large absorption band centered at 820–830 nm is characteristic of the $Cu_{A_{oxid}}$ chromophore, which gives ~80% of absorption in this region (27). Cu_A is also suggested to make a small contribution around 615 nm, which is obscured in part by intense and overlapping transitions from cytochromes *a* and a_3 (53). The red absorption band of isolated mitochondria in steady-state–coupled turnover is a broadband from ~590 nm to ~630 nm centered near 610 nm (54). Clore *et al*. (55) trapped at low-temperature measurements an intermediate absorbing at 610 nm and noticed that for membrane-bound oxidase there is a redshift of this band as compared with soluble oxidase and also the FWHM of this band is broader in this case. They suggested that this is a charge transfer transition. The suggestion that the band at 619 nm in our light action spectrum (Table 1, Fig. 1) belongs to $Cu_{A_{red}}$ is also supported by opposite time course of absorption changes at this region $(Cu_{A_{red}})$ and in the NIR region at 830 nm $(Cu_{A_{oxid}})$ (48).

We have found in this study that treating of cell suspension with sodium azide, SNP, ouabain or amiloride before irradiation significantly modifies the spectrum of cell attachment enhancement. The light-induced mitochondrial signaling pathway can be regulated by small ligands directly binding to the catalytic center of cytochrome *c* oxidase (N_3, NO) as well as by chemicals specifically binding to plasma membrane enzymes (ouabain, amiloride).

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