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Thermal Lens Determination of Cytochrome *c* and Its NO Complex

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Abstract—Spectrophotometric and thermal lens measurements showed that cw laser beam (450–530 nm, up to 100 mW) does not affect the absorption band of cytochrome *c*. Therefore, thermal lensing is used for determining cytochrome *c* (III) ($c_{min} = 1 \times 10^{-7}$ mol/L at $\lambda = 488.0$ nm; $c_{min} = 3 \times 10^{-8}$ mol/L at $\lambda = 514.5$ nm) and its active form, cytochrome *c* (II) ($c_{min} = 1 \times 10^{-8}$ mol/L at $\lambda = 514.5$ nm). The enhancement of the sensitivity of determination of these species as compared with conventional spectrophotometry is more than two orders of magnitude. The optimal conditions for the formation of the NO complex of cytochrome *c* for its photometric determination were selected: the molar ratio of dodecyl sulfate (a modifying agent) to cytochrome *c* is 1 : 30 at a working wavelength of 560 nm. When exposed to laser radiation, the nitrosyl complex of cytochrome *c* dissociates to form cytochrome *c* (III). The decomposition of this complex can be monitored by thermal lensing (514.5 nm) down to a level of 1×10^{-7} mol/L.

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Much recent attention has been focused on the problem of apoptosis, programmed cell death, which is one of the main reasons for cell death in various diseases [1]. Cytochrome *c* plays a central role in the initiation of apoptosis [2–8]. It is known that a critical event in the initiation of apoptosis is cytochrome *c* release from mitochondria into the intercellular space; however, the mechanism of this release remains unclear. Currently, it is believed that a key role in this process is played by the peroxidase activity of cytochrome *c* [1, 2]. Study of cytochrome *c* release from mitochondria is of importance for the elucidation of the mechanism of apoptosis development and the action of corresponding drugs. Therefore, routine and rapid quantification of cytochrome *c* (both the total amount and the content of active forms) is important for medical and biological purposes.

Nitric oxide (NO) is a key physiological regulator. Of special interest is the interaction of NO with heme proteins, including cytochrome *c* [9], to form nitrosyl complexes. This interaction affects the peroxidase activity of cytochrome *c* [10], which can be used for regulating apoptosis.

This work deals with the choice of conditions for quantification of cytochrome *c* forms and its nitrosyl complex by molecular spectroscopy methods. As such methods, we used spectrophotometry as a basic method and thermal lensing as a laser-based method of molecular absorption spectroscopy, which ensures high sensitivity of the determination of various substances [11, 12].

EXPERIMENTAL

Instrumentation. On the basis of the available thermal lens spectrometer setup [13], we created a setup for thermal lensing of biological samples. A thermal lens was excited in a cell (an optical path length of 1 cm) by an Innova 90-6 argon ion laser (Coherent, United States; TEM_{00} mode, $\lambda_e = 488.0$ and 514.5 nm). An SP 106-1 He–Ne laser (Spectra Physics, United States) with $\lambda_p = 632.8$ nm (TEM_{00} mode, 10 mW) was used as a probe laser. Setup parameters are summarized in Table 1. The setup allows one to change the geometry of the optical scheme, the excitation beam power in the range 1–200 mW, and the irradiation duration in the range 0.01–10 s. For spectrophotometric measurements, a Shimadzu UV1240 scanning spectrophotometer (Japan) with quartz cells (an optical path length of 1 cm) was used.

Data processing. Thermal lens measurements represent a sequence of on and off cycles of the excitation

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Table 1. Parameters of the configuration of a dual-beam thermal lens spectrometer with a single-channel signal detection system

Excitation laser	Wavelength λ_e, nm	514.5	488.0
	Confocal distance z_{ce}, mm	6.4	6.7
	Power range in a cell, mW	1–50	1–50
	Cross sectional radius of the laser beam waist ω_{0e}, μm	55 ± 5	
	Focal length of a focusing lens f_e, mm	300	
Probe laser	Wavelength λ_p, nm	632.8	
	Focal length of a focusing lens f_p, mm	185	
	Confocal distance z_{cp}, mm	3.1	
	Laser power in a cell P_p, mW	3	
	Cross sectional radius of the laser beam waist ω_{0p}, μm	25.0	
Optical scheme parameters	Optical path length l, mm	10.0	
	Cell–detector distance, cm	180	
	Geometric parameter B	0.72	
	Chopper frequency φ, Hz	0.5–4	

laser (thermal lens formation/dissipation). Is a result, a series of signals θ can be obtained [12]:

$$\theta = \frac{1}{B}\left(1 - \sqrt{\frac{I_{off} - I_{on}}{I_{on}} + 1}\right), \quad (1)$$

where I_{off} and I_{on} are the intensities at the center of the probe beam in the absence of a thermal lens and when the lens is completely developed, respectively, and B is a geometric parameter.

The signal θ is recalculated into absorbance by the equation

$$A = \frac{\theta}{2.303 E_0 P_e}, \quad (2)$$

where P_e is the power of the laser beam with the wavelength λ_e inducing the thermal lens [12] and E_0 is the sensitivity factor of thermal lens measurements (an increase in sensitivity as compared with spectrophotometry for a excitation beam power of 1 mW),

$$E_0 = \frac{dn/dT}{k\lambda_p}, \quad (3)$$

where dn/dT is the temperature gradient of the refractive index, k is the thermal conductivity of a medium, and λ_p is the probe beam wavelength.

Reagents and solvents. Cytochrome *c* from horse heart 99% (Sigma, M = 12 383) for biochemical studies, sodium dodecyl sulfate (SDS, 99% Fluka, M = 288.4), $NaH_2PO_4 \cdot 2H_2O$ (pure for analysis, Khimmed), NaOH (aqueous solution, pure for analysis), 1 M KCl (pure for analysis, Khimmed) and ascorbic acid (reagent grade) were used.

Solutions of cytochrome *c* (1 mmol/L) and SDS were prepared by dissolving a weighed sample of a reagent in distilled water. A phosphate buffer solution with pH 7.4 and a concentration of 20 mmol/L was prepared by dissolving a weighed sample of $NaH_2PO_4 \cdot 2H_2O$ in distilled water. The required pH 7.4 was adjusted by adding NaOH while monitoring the acidity with a pH meter. The required ionic strength was created by adding a KCl solution. All prepared solutions were kept in the dark at 4°C. Ascorbic acid was used for preparing ascorbate, required for the reduction of cytochrome *c*. A solution of 5 mg of ascorbic acid in 100 mL of water was titrated with an NaOH (pH 12) solution to pH 7. The resulting solution was diluted with water (1 : 5) and used for reducing cytochrome *c*.

Gaseous nitric oxide was obtained by a dry method from KNO_2, KNO_3, Cr_2O_3, and Fe_2O_3 [14, 15]: $3KNO_2 + KNO_3 + Cr_2O_3 \longrightarrow 2K_2CrO_4 + 4NO$ (iron(III) oxide is required for the agglomeration of the chromate). The evolved NO was bubbled through the four traps with NaOH (20%) to remove higher nitrogen oxides and then through an absorbing trap (two compartments were filled with 10 M NaOH and the third one, with distilled water [16]). A quartz tube filled with a mixture of the reagents with argon was heated at 300°C until gas evolution ceased. The resulting gas was used for saturating degassed water obtained by bubbling an argon flow thorough distilled water for 15 min.

Preparation of nitrosyl complexes of cytochrome *c*. The water saturated with NO was added to a mixture cytochrome *c* (III) (7×10^{-6} M) and SDS (0.22 mM) in the presence of 4×10^{-6} M ascorbate in the phosphate buffer solution (20 mM, pH 7.4), the total mixture volume being 2 mL. The concentration of the resulting NO solution was unknown; therefore, the ratio of cytochrome *c* to NO was selected before each experiment.

Reduction of cytochrome *c* by ascorbate. A 9-mg sample of cytochrome *c* was dissolved in 100 mL of water. Then, 1.25 mL of the resulting solution was sampled. A 5-mg portion of SDS was dissolved in 24 mL of

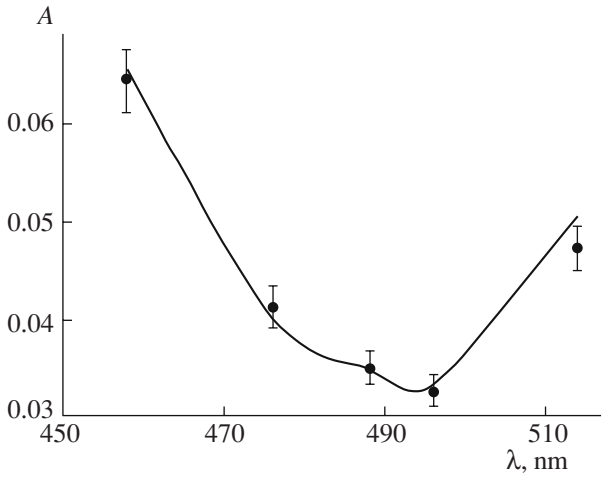

Fig. 1. Comparison of the absorption spectra of 5.7 μM cytochrome *c* (III) obtained by spectrophotometry and thermal lensing. The solid line shows the absorbance obtained from spectrophotometric measurements, and data points correspond to the absorbance recalculated from the thermal lens signal.

water. A 0.4-mL portion of this solution was sampled. Then, 1.25 mL of 100 nM phosphate buffer solution was added. To the resulting solution, 0.2 mL of the prepared ascorbate solution was added. The spectra of the reaction products were recorded on a spectrophotometer immediately after preparation of the solution.

Determination of the nitrosyl complex of cytochrome *c*. The solutions of the nitrosyl complex of cytochrome *c* were exposed to a laser beam (514.5 nm, 40 mW) for 10, 70, or 370 s. In the case of thermal lens measurements, a thermal lens signal was recorded during these periods; in the case of spectrophotometric measurements, the absorption spectra of the irradiated mixture were recorded after these periods of time.

RESULTS AND DISCUSSION

Study of the laser beam effect on cytochrome *c*. Comparison of the absorbance of cytochrome *c* solutions determined by spectrophotometry and calculated from the thermal lens signal at wavelengths of its generation showed that the beam has no effect on cytochrome *c* (Fig. 1). Hence, cytochrome *c* and its active forms can be studied by thermal lens spectrometry, which is more sensitive than spectrophotometry.

Performance parameters of the quantification of cytochrome *c*. We constructed calibration curves for cytochrome *c* (III) and cytochrome *c* (II) for spectrophotometric measurements (in the maxima of absorption bands) and for thermal lens measurements. The calibration curves for cytochrome *c* (III) were constructed at two wavelengths, 488.0 and 514.5 nm, and for cytochrome *c* (II), only at 514.5 nm (at 488.0 nm, the molar absorptivity of cytochrome *c* (II) is small). The results are summarized in Table 2. The resulting detection limits correlate with the available data [17]. The reproducibility of determination of cytochrome *c* at a level of 10^{-7} M is no worse than that of the spectrophotometric determination. The increase in sensitivity for both cytochrome *c* forms is more than 200-fold, which is in agreement with the expected increase in the sensitivity of thermal lensing.

Synthesis of the NO complex of cytochrome *c* (III). Among the conditions of synthesis of nitrosyl complexes of cytochrome *c*, the SDS–to–cytochrome *c* and cytochrome *c*–to–NO ratios were selected. SDS acts as an agent facilitating the access of the ligand to the heme [10]. We found that the optimal ratio of SDS to the protein is in the range from 1 : 30 to 1 : 50. However, since

Table 2. Equations of calibration curves and characteristics of the sensitivity of the spectrophotometric and thermal lens determination of cytochrome *c*

Method	Cytochrome *c* (III)	Cytochrome *c* (II)
Spectrophotometry	$A_{530} = (1.1 \pm 0.1)c + 1.1 \times 10^{-2}$ ($n = 12$, $P = 0.95$; $r = 0.9989$) $c_{min} = 7 \times 10^{-6}$ M $s_r(10^{-5}$–10^{-4} M) = 0.02–0.05	$A_{550} = (1.7 \pm 0.2)c + 1.1 \times 10^{-2}$ ($n = 12$, $P = 0.95$; $r = 0.9979$) $c_{min} = 2 \times 10^{-6}$ M $s_r(10^{-5}$–10^{-4} M) = 0.03–0.05
Thermal lens spectrometry (40 mW)	$\vartheta_{488} = (0.220 \pm 0.002)c + 1.1 \times 10^{-2}$ ($n = 12$, $P = 0.95$; $r = 0.9994$) $c_{min} = 1 \times 10^{-7}$ M $s_r(10^{-7}$–10^{-6} M) = 0.01–0.04	$\vartheta_{514.5} = (0.558 \pm 0.003)c + 3.9 \times 10^{-2}$ ($n = 12$, $P = 0.95$; $r = 0.9994$) $c_{min} = 1 \times 10^{-8}$ M $s_r(10^{-8}$–10^{-6} M) = 0.02–0.06
	$\vartheta_{514.5} = (0.396 \pm 0.004)c + 2.2 \times 10^{-2}$ ($n = 12$, $P = 0.95$; $r = 0.9997$) $c_{min} = 3 \times 10^{-8}$ M $s_r(10^{-8}$–10^{-6} M) = 0.02–0.05	

Note: For the calibration curves, concentration are in mmol/L (spectrophotometry) and μmol/L (thermal lensing).

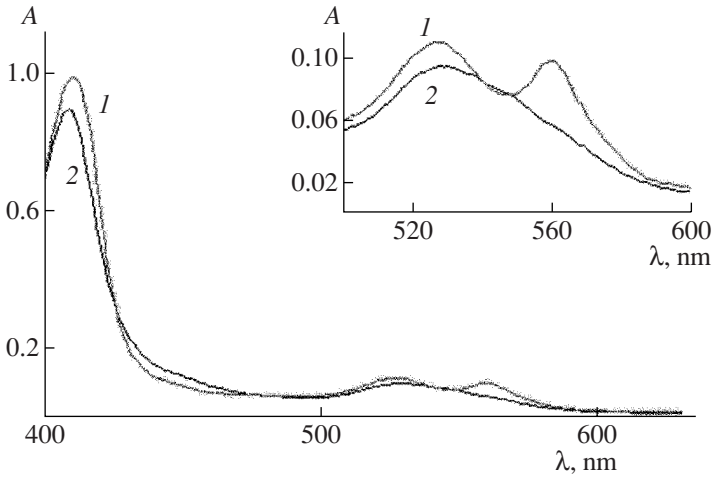

Fig. 2. Absorption spectrum of cytochrome *c* (III) (*1*) with and (*2*) without NO.

SDS is a surfactant and tends to form colloid solutions, we chosen the minimal value of this ratio (1 : 30).

Inasmuch as proteins denature at an interface, the formation of an interface when a gas is bubbled through a solution is undesirable. Therefore, to obtain the NO complex of cytochrome *c*, gaseous nitric oxide was not passed through the solution of the protein with SDS; rather, the reaction was carried out in aqueous solutions preliminarily saturated with NO. Solutions of NO were added 5 min after the other reagents. The products of the reaction of cytochrome *c* with NO were identified from changes in the absorption spectrum as compared with the same amount of a solution of all components except NO. A buildup of the absorption at 560 nm (Fig. 2), which is evidence of the presence of the NO complex of cytochrome *c* [10], was observed when 100–300 μL of the NO solution was added; a further increase in the NO volume did not lead to further changes in the absorption spectrum of cytochrome *c*.

Study of the laser beam effect on the NO complex of cytochrome *c*. The complex was exposed to laser radiation, which led to rather rapid decomposition of the NO complex of cytochrome *c* (Fig. 3). The complex decomposed (the peak at 560 nm decreased) and the spectrum of cytochrome *c* was restored in 10 s. In the absence of laser radiation, the complex is stable and does not decompose over time; therefore, the decomposition of the complex is only caused by the action of the laser beam. Figure 3 shows that the complex decomposes to produce cytochrome *c* (III), but a fraction of nascent cytochrome *c* again reacts with NO present in the system. Thermal lens determination of the nitrosyl complex of cytochrome *c* is possible at the level of concentrations achieved for cytochrome *c* (II, III), i.e., 10^{-7} M, and gives the same gain in sensitivity as compared with spectrophotometry and in the case of determination of cytochrome *c* (III), i.e., about a 200-fold increase.

We selected the conditions of the spectrophotometric and thermal lens determination of cytochrome *c*, its active form, and its complex with NO. We found that a laser beam does not lead to changes in the absorption spectrum of cytochrome *c* of cytochrome *c*. This observation makes it possible to use high-sensitivity thermal lens spectrometry for determining cytochrome *c* (II) and cytochrome *c* (III) at a level of 10^{-8} mol/L, which cannot be achieved by most other methods of determination of cytochrome *c*. The conditions found can be used for studying inhibition and reactivation of peroxidase activity of cytochrome *c* under the action of NO [10] by thermal lens spectrometry, which was beyond the scope of the present paper. In addition, laser-regulated and -controlled photodissociation of the NO complex of cytochrome *c* can be used in laser therapy.

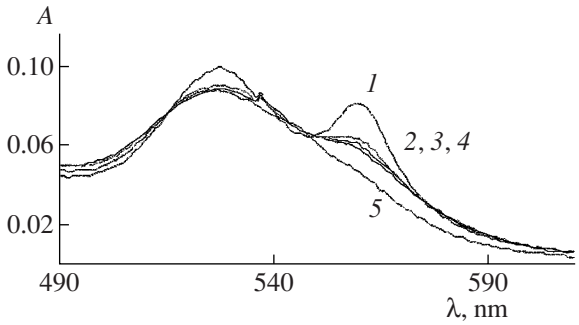

Fig. 3. Decomposition of the NO complex of cytochrome *c* induced by laser beam: the absorption spectra of (*1*) the intact cytochrome *c*–NO complex and (*2–4*) after exposure to a laser radiation for (*2*) 10, (*3*) 70, and (*4*) 370 s; (*5*) the absorption spectrum of cytochrome *c* (III) is shown for comparison. The complex was obtained by mixing 20 μL of a 0.89 mM solution of cytochrome *c*, 110 μL of a 5.5 mM solution of SDS, 1470 μL of a 20 mM solution of phosphate buffer (pH 7.4, and 400 μL of degassed water saturated with NO.

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