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Biochimica et Biophysica Acta 1321 (1997) 221–228

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Intramolecular oxidation of cytochrome *c* by covalently attached sulfoaromatic molecules

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Received 27 March 1997; revised 25 June 1997; accepted 30 June 1997

Abstract

Two photosensitive molecules, 1-maleimidopyrene-3,6,8-trisulfonate (MPTS) and *N*-acetylaminoethyl-1-aminonaphthalene-5-sulfonate (AEDANS), are employed to drive the intramolecular oxidation of the heme residue in cytochrome *c*. Intense pulse illumination (60–120 MW cm^{-2}) of MPTS and AEDANS in the aqueous solution by the third harmonic frequency of Nd–Yag laser drives a two successive-photon process of the dyes. The oxidized products originating from the dyes react with variety of electron donors. MPTS and AEDANS residues were covalently linked the *Saccharomyces cerevisiae iso*-1-cytochrome *c* by labeling of its single sulfhydryl group. When pulsed by intensive laser beam the heme of the labeled ferrocytochrome *c* undergoes fast oxidation. Transient absorption spectroscopy was used to directly measure the rate constants for the photoinduced electron-transfer reaction from the ferros heme group to the oxidized dyes. The rate constant was found to be $(3.6 \pm 0.4) \times 10^4$ s^{-1} for MPTS derivative. The rate of the heme oxidation in AEDANS derivative was faster than response time of our detection system (20 ns). Rapid photooxidation of cytochrome *c* makes it a useful tool for fast initiation of electron transfer in oxidized direction within complexes of cytochrome *c* with the other redox proteins. © 1997 Elsevier Science B.V.

Keywords: Photooxidation; Cytochrome *c*; Time-resolved spectroscopy; Electron transfer

1. Introduction

Biological electron transfer reactions between redox groups in proteins are key steps in photosynthesis, respiration and many other processes. However, the measurement of the actual electron transfer between redox carriers remains a difficult problem. While the electron transfer between the redox carriers can readily be measured by steady state kinetics, time resolved measurements have until recently been limited to photosynthetic systems, where photochemical activation initiates electron transfer allowing the resolution of the nanosecond and microsecond electron transfer dynamics [1,2]. The inability of non-photosynthetic systems to interact with radiation can be circumvented by using external photosensitive elements as the electron donor. Current experimental

Abbreviations: MPTS, 1-maleimidopyrene-3,6,8-trisulfonate; AEDANS, *N*-acetylaminoethyl-1-aminonaphthalene-5-sulfonate; IAEDANS, *N*-iodoacetylaminoethyl-1-aminonaphthalene-5-sulfonate; $AEDANS_{ox}$ and $MPTS_{ox}$ are oxidized radicals of AEDANS and MPTS, correspondingly

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PII S0005-2728(97)00058-3

methods employ excited flavines [3,4], ruthenium complexes [5,6] or zinc-substituted heme [7,8] as light-driven photo-reductants. Of these, the ruthenium complexes have proven to be the most practical. Pulse irradiation of Ru(II) complexes results in the formation of a low potential excited state ($E^0 = -0.72$ V), capable of reducing compounds of chemical and biological nature. Upon relaxation of the oxidized metal ion to the ground state, Ru(III) acts as an oxidant ($E^0 = 1.31$ V) and becomes re-reduced. This method has been utilized to study electron transfer in cytochrome *c* [6,9] and within complexes of cytochrome *c* with other proteins [10–15].

We have recently introduced [16] a new method, capable of delivery an intensive oxidative pulse. The method is based on pulse excitation of water soluble sulfoaromatic molecules. Intensive illumination of pyranine (8-hydroxypyrene-1,3,6-trisulfonate) by the third harmonic frequency of an Nd–Yag laser drives a two successive-photon oxidative process. The first photon excites the dye to its first electronic singlet state. The second photon interacts with excited molecule, the process which yields solvated electron and oxidized pyranine radical. The oxidized compound of pyranine has a well defined absorption in the visible range of the spectrum and, under conditions where the solvated electrons are quenched, it is a stable compound that reacts as a one electron acceptor with a variety of reductants. This methodology has been implemented to study oxidation of cytochrome *c* in solution by pyranine [17]. Yet, as the reaction proceeds by two reactants, free in solution, the measured kinetics is a superposition of the diffusion controlled encounter plus the dynamics of the intramolecular electron transfer. In addition, no information is available concerning the encounter complex during electron transfer for bimolecular reactions. The way to overcome the disadvantages of bimolecular systems is to attach the photoactive molecules to the particular site of protein by covalent modification of one of its amino acid residues. In the present paper, the pyrene–trisulfonate and aminonaphtalene–sulfonate residues were attached to yeast cytochrome *c* molecule via its single cysteine residue. The photoinduced intramolecular oxidation of the heme by both labels was measured directly by transient absorption spectroscopy and the rate constants of electron transfer were estimated.

2. Materials and methods

2.1. Materials

MPTS was made by Lambda Fluorescence (Austria). IAEDANS was purchased from Molecular Probes (USA). $FeSO_4$ and $K_4[Fe(CN)_6]$ AR were Merck products. All other chemicals were obtained from Sigma (USA).

2.2. Transient absorption measurements

The sample (1.5 ml), contained 5 mM HEPES (pH 7.0), dyes (20 μM) or labeled cytochrome *c* (30 μM) together with 200 μM ascorbate was placed in four face 1 cm quartz cuvette. The oxygen dissolved in the solution was removed by continuous bubbling of Ar 30 ml min^{-1} through the cuvette for 15 min and throughout the experiment as well. Excitation of the dyes was initiated by the third harmonic frequency of a Nd–Yag laser (355 nm, 2 ns FWHM, 17 mJ $pulse^{-1}$), which was focused on the side of cuvette over a spot having a surface of 0.3 cm^2. Continuous monitoring of the redox state of MPTS and AEDANS dyes was carried out at 458 and 560 nm, respectively. The state of the heme group was monitored at 550–556 nm (isosbestic point of cytochrome *c*). The probing beam, either the 458 nm band of a CW Argon laser or the output of 'Coherent' CR-599 Dye laser with Rhodamine-110 dye at 550, 556 and 560 nm, was crossing the pulse irradiated face of the cuvette perpendicular to the excitation beam. The probing beam was directed to monochromator photomultiplier assembly and transients were stored and averaged by a Tektronix TDS 520A digital oscilloscope as previously described [16]. The response time of the detection system is equal to 20 ns. The transients are the average of 20 pulses collected at a frequency of 0.02 Hz. Photoexcitation causes no irreversible damage of the dyes and results in no change of their steady-state absorption spectra. Oxidation of cytochrome was quantitated by the absorbance coefficient difference of $\epsilon_{550-556} = 19\,000$ M^{-1} cm^{-1}.

Transient spectra were measured in the Biophysical Institute in Szeged using an Excimer laser and a monitoring system consisting of a dispersing element,

gated image intensifier and a multichannel analyzer [18].

2.3. Preparation of N-acetylcysteine derivatives of AEDANS and MPTS

N-Acetylcysteine derivative of AEDANS was prepared as previously described [19]. *N*-Acetylcysteine–MPTS was prepared by incubation of 5 mM *N*-acetylcysteine with 5 mM MPTS in 50 mM HEPES (pH 7.6) at room temperature for 2 h.

2.4. Preparation of AEDANS–cytochrome c derivative

Cytochrome *c* (*iso*-1) from *Saccharomyces cerevisiae* (1 mM) in 100 mM HEPES (pH 6.5), containing 2 mM ascorbate was mixed with freshly prepared IAEDANS (2 mM) and incubated for 4 h at 38°C. The labeled protein was separated from non-bound dye on 1×20 cm G-25 Sephadex 'coarse' column. The protein eluted in a void volume was collected and chromatographed on 1.6×7 cm CM-Sephadex column in 20 mM potassium phosphate buffer (pH 6.5), containing 1 mM dithioerythritol, using a linear gradient of KCl (0–500 mM). This procedure allow to separate the labeled cytochrome from the unlabeled one. The main peak, eluted from the column in $\sim$ 250 mM KCl, contains 1/1 labeled cytochrome *c*. The AEDANS content in the protein fractions was quantitated on the basis of the absorbance coefficient of $\epsilon_{250} = 18\,000\ M^{-1}\ cm^{-1}$ [19] and cytochrome *c* content on the bases of absorbance coefficient of $\epsilon_{550} = 28\,000\ M^{-1}\ cm^{-1}$. Analysis of free SH-groups with Ellman reagent [20] clearly showed that the labeled protein contains no free cysteines and thus the label is linked to a single cysteine residue of cytochrome *c*.

2.5. Preparation of MPTS–cytochrome c derivative

Yeast cytochrome *c* (0.5 mM) in 50 mM HEPES (pH 7.5), containing 2 mM ascorbate was mixed with freshly prepared MPTS (0.5 mM) and incubated for 2 h at room temperature. The labeled protein was separated from non-bound dye on 1×20 cm G-25 Sephadex 'coarse' column. The modified cytochrome *c* was separated from non-modified one and purified to homogeneity by ion-exchange chromatography. The protein eluted in void volume was applied on 1.6×7 cm CM-Sephadex column in 20 mM potassium phosphate buffer (pH 8.0), containing 1 mM dithioerythritol and eluted from the column by linear gradient of KCl (0–500 mM). The main peak, eluted from the column in $\sim$ 250 mM KCl, containing 1/1 labeled cytochrome *c*. The MPTS content in the protein fractions was quantitated on the basis of the absorbance coefficient of $\epsilon_{372} = 30\,000\ M^{-1}\ cm^{-1}$. Analysis of free SH-groups with Ellman reagent showed no free cysteines in the fraction.

3. Results

3.1. Redox perturbation of aqueous solution, induced by MPTS

The photochemistry of pyranine in water solutions was studied in details in our previous work [16]. Since the aim of the present study was to initiate the intramolecular electron transfer from the reduced heme to photooxidant, covalently attached to cytochrome *c*, we selected the MPTS molecule as a photoreactive protein label. We assumed that being the homologue of pyranine, MPTS will drive the photochemical redox reactions similar to that of pyranine. Indeed, intensive pulse illumination of aqueous solution of *N*-acetylcysteinil derivative of MPTS ejects an electron from the excited molecule and a pair of reactants, a solvated electron and an oxidized aromatic moiety are formed. As in the case of pyranine (see [16]) oxidized photoproduct, $MPTS_{ox}$ is characterized by an intensive absorbance at 458 nm, a wavelength where the ground state compound has a negligible absorbance. This allows us to monitor the process of formation and relaxation of the oxidized dye at 458 nm. In the absence of added reductant the absorption decay is rather slow (hundreds of microseconds), but if reductants are added the bleaching of the dye is accelerated. The kinetics of $MPTS_{ox}$ reduction by Fe^{2+} ions is shown on Fig. 1. The rate of the reaction is increased linearly with concentration as evident from Fig. 1.

The crucial point for application of MPTS as an intraprotein redox perturbant is the ability of the dye to oxidize the heme group of ferrocytochrome *c*. Time resolved kinetics of photosensitized oxidation of 20 μM cytochrome *c* in the aqueous solution in

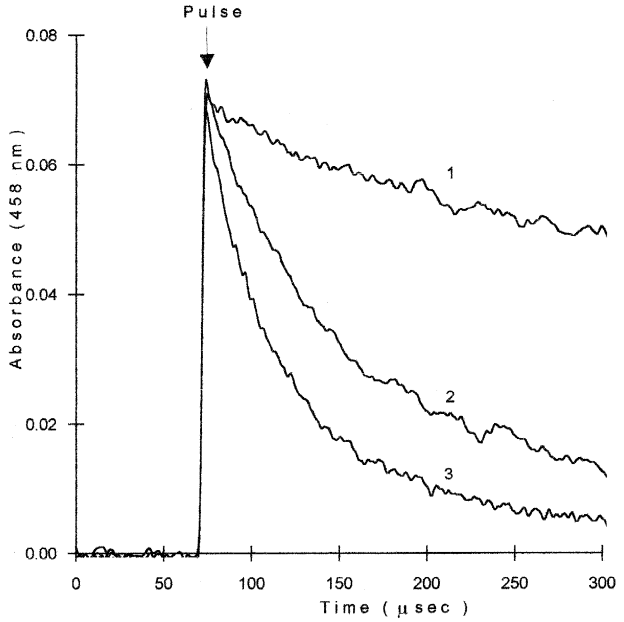

Fig. 1. The dynamics of $MPTS_{ox}$ reduction by Fe^{2+} ions. *N*-Acetylcysteine–MPTS solution (5 μM) in 0.1 mM HCl (curve 1) in the presence of 100 (curve 2) and 200 μM $FeSO_4$ (curve 3) was intensively (50 ml min^{-1}) purged with Ar for 15 min and pulsed by the third harmonic frequency of a Nd–Yag laser (355 nm, 17 mJ $pulse^{-1}$) as described in Section 2. The absorbencies of $MPTS_{ox}$ were measured over time at 458 nm.

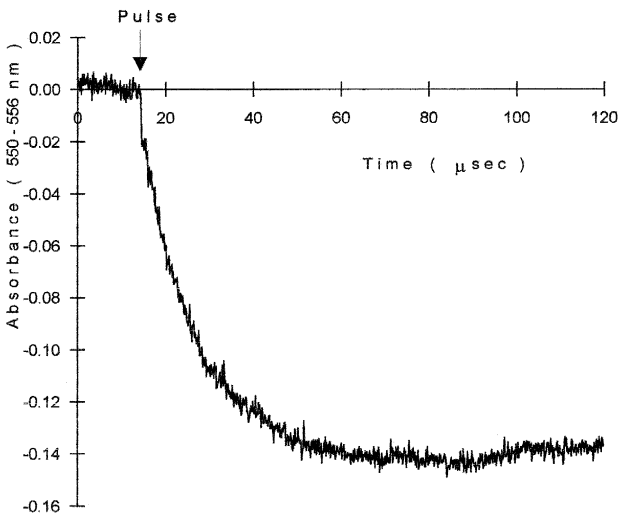

Fig. 2. Transient kinetics for electron transfer in a solution, containing 20 μM cytochrome *c*, 80 μM MPTS, 100 μM ascorbate, 5 mM HEPES, pH 7.0. The transient at 550 nm minus 556 nm, the cytochrome *c* isosbestic, is measured as described in Section 2. The transient is fit by a single exponential decay with having a rate constant $k_{obs} = (7.1 \pm 0.3) \times 10^4$ s^{-1}.

the presence of the 80 μM concentration of the dye is shown on Fig. 2. The transient as seen in the figure is monophasic, with a rate constant of $(7.1 \pm 0.3) \times 10^4$ s^{-1}. As in the case of reaction of cytochrome *c* with pyranine [17] the rate is compatible with that for diffusion controlled reactions. Thus the electron transfer that follows the complex formation is not a rate limiting step.

3.2. Intramolecular heme oxidation by covalently bound MPTS residue

To make the redox perturbation strictly directed to the cytochrome heme, MPTS residue was covalently

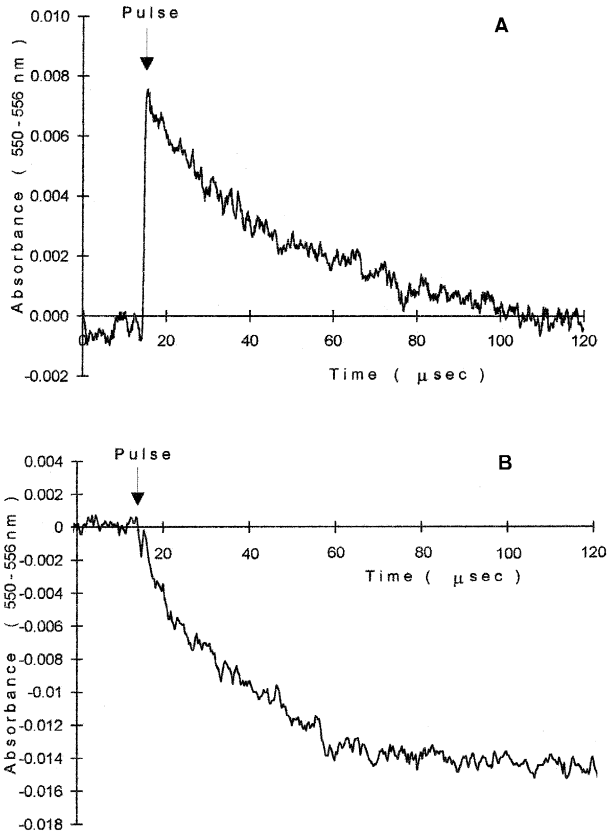

Fig. 3. Intramolecular reduction (A) and oxidation (B) of cytochrome *c*–MPTS derivative. The cytochrome *c* (cys102)–MPTS adduct (30 μM) in 5 mM HEPES buffer, pH 7.0, containing either 200 μM ferricyanide to keep the cytochrome *c* in its oxidized form (A) and 200 μM ascorbic acid to keep the heme reduced (B), was subjected to 20 laser pulses to obtain high signal to noise ratio. Before and during the excitation argon was bubbled into the cuvettes to maintain anaerobiosis. The changes in the heme redox states were monitored at 550–556 nm as described Section 2.

attached to the yeast cytochrome *c* molecule. Pulse illumination of the reduced labeled protein results in its heme oxidation by the oxidized MPTS radical (see Fig. 3B). The rate constant was equal to $(3.6 \pm 0.4) \times 10^4$ s^{-1} and the rate of the heme oxidation was independent on concentration of the cytochrome–MPTS. This kinetic behavior is consistent with intracomplex electron transfer mechanism. When the oxidized protein was subjected to the laser pulse excitation, instantaneous (within the resolution of our system) reduction of the heme by the electron, released from the excited dye molecule, took place. Subsequent re-oxidation of the heme by the oxidized dye molecule brought the system to the initial prepulsed state (Fig. 3A). The rate of the heme re-oxidation is equal to that of oxidation of the initially reduced cytochrome *c* (see Fig. 3B). The rather slow rate of the heme oxidation may reflect low driving force of the reaction. The ΔG^0 value for the reaction of heme oxidation by the oxidized label is equal to 0.62 V, the value which is based on $E^0 = 0.88$ V for the label [16] and $E^0 = 0.26$ V for cytochrome *c*. To increase the driving force of the oxidative process reaction we have substituted the MPTS label for AEDANS.

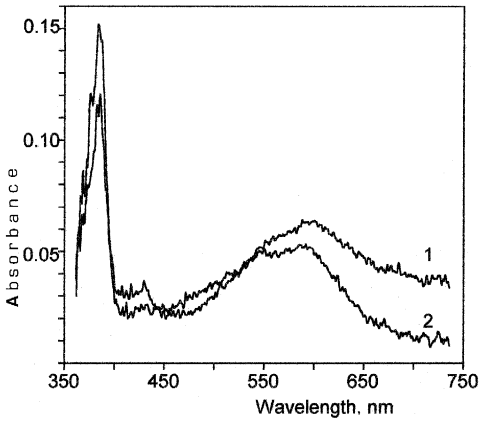

Fig. 4. The absorption spectrum of photoproduct generated by pulse excitation of 100 μM solution of *N*-acetylcysteine derivative of AEDANS in 10 mM HEPES, pH 6.0. The solution was bubbled with Ar for 15 min to remove oxygen and pulsed by the Eximer laser (309 nm, 5 mJ $pulse^{-1}$). The changes in the absorption were measured by an instrumental setup consisting of dispersion element, gated image intensifier and multichannel analyzer. The difference spectrum, corrected for the decremental absorbance of ground state of *N*-acetylcysteine derivative of AEDANS, was measured 0.4 (curve 1) and 2.5 μs (curve 2) after the excitation pulse. The base line drift did not exceed 0.002 optical units.

3.3. Photochemistry of AEDANS

When pulsed with an intensive laser beam the AEDANS linked to *N*-acetylcysteine residue undergoes fast photooxidation. As in the case of pyranine [16] a successive two photon reaction ejects an electron from the molecule and an oxidized AEDANS ($AEDANS_{ox}$) is formed. Transient spectra recorded within the first microsecond revealed a broad absorption band at the wavelength longer than 600 nm (see Fig. 4), together with a spectral band from 550 to 600 nm. The former is attributed to solvated electrons, and the later to the oxidized AEDANS radical. Transient spectra recorded several microseconds after the pulse is attributed only to the oxidized radical, as hydrated electrons have a short life-time ($\tau_{1/2} \sim 1$ μs). The oxidized radical of AEDANS absorbs light at much longer wavelength as the ground state form of AEDANS does. This allowed us to monitor the process of $AEDANS_{ox}$ formation and relaxation at the wavelengths from 550 to 600 nm. The spontaneous relaxation of the oxidized radical is accelerated in the presence of reductants. The dynamics of reduction of the oxidized radical was measured with several electron donors: Fe^{2+}, Mn^{2+}, $K_4[Fe(CN)_6]$, Co^{2+} and ascorbic acid. The rate of reaction with Co^{2+} was surprisingly fast and submilimolar concentrations of the reductant reduce $AEDANS_{ox}$ in less than microsecond. Fig. 5 depicts the kinetics of $AEDANS_{ox}$ reduction by Co^{2+} ions. A second-order rate constant of $(7.0 \pm 0.2) \times 10^9$ M^{-1} s^{-1}, calculated from the linear dependence of the pseudofirst-order rate constant on Co^{2+} concentration (not shown), is compatible with that for diffusion controlled reactions. The second-order rates of $AEDANS_{ox}$ reduction by Fe^{2+} and Mn^{2+} ions are at least two orders of magnitude lower than diffusion controlled rate. The redox potential of Co^{2+}/Co^{3+} couple (1.81 V) is higher then the potentials of Fe^{2+}/Fe^{3+} (0.77 V) and Mn^{2+}/Mn^{3+} (1.51 V) couples. Thus the ΔG^0 for the reduction of $AEDANS_{ox}$ by Co^{2+} is smaller than that of the radical reduction by the alternative ions. According to Markus theory [21,22] in the 'normal' region ($\Delta G^0 < \lambda$) the rate constant of electron transfer increases with increase of driving force. When $-\Delta G^0$ is equal to λ the

reaction is barrierless. If the driving force is increased further ($\Delta G^0 > \lambda$) the rate constant decreases with increasing driving force, the 'inverted' region. Reduction of $AEDANS_{ox}$ by the metal ions fits well with the model: The reduction by Co^{2+} is almost barrierless and reduction by Fe^{2+} and Mn^{2+} are in the 'inverted' region. This analysis enables us to make a crude estimation of the redox potential of $AEDANS_{ox}$ radical. It should be higher than that of Co^{2+}/Co^{3+} couple and lying in the range of 2 V. High redox potential of the dye makes possible to drive the oxidation of aromatic amino acid residues. The dynamics of the reaction was followed by looking at the relaxation of the oxidized radical. As seen in Fig. 6, the spontaneous relaxation of $AEDANS_{ox}$ radical is accelerated in the presence of millimolar concentration of histidine. The effect of phenylalanine, tryptophan, and tyrosine was similar to that of histidine; Non-aromatic amino acids even at 10 mM concentration had no effect on the relaxation rate.

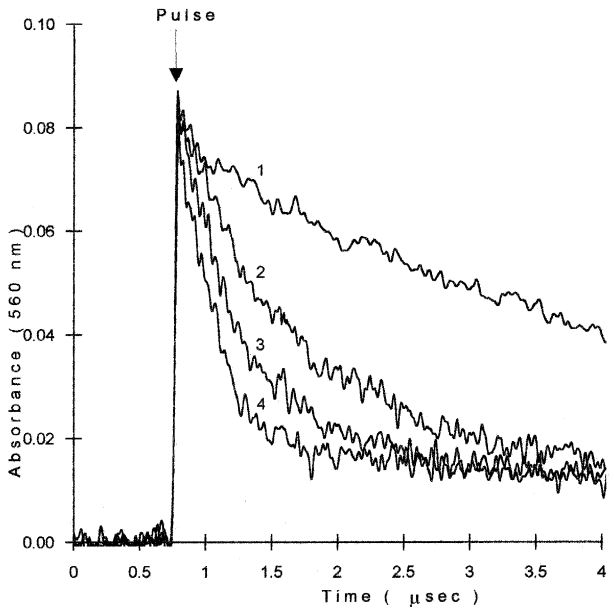

Fig. 5. Reduction of the oxidized *N*-acetylcysteine–AEDANS radical by Co^{2+}. 20 μM of *N*-acetylcysteine–AEDANS derivative in 1 mM HEPES, pH 7.0, was pulsed by the third harmonic frequency of a Nd–Yag laser (355 nm, 2 ns FWHM, 17 mJ $pulse^{-1}$). Formation of $AEDANS_{ox}$ and its relaxation in the absence (curve 1) and in the presence of 100 (curve 2), 200 (curve 3) and 400 μM (curve 4) Co^{2+} was monitored by 'Coherent' CR-599 Dye laser with Rhodamine-110 dye at 560 nm (see Section 2).

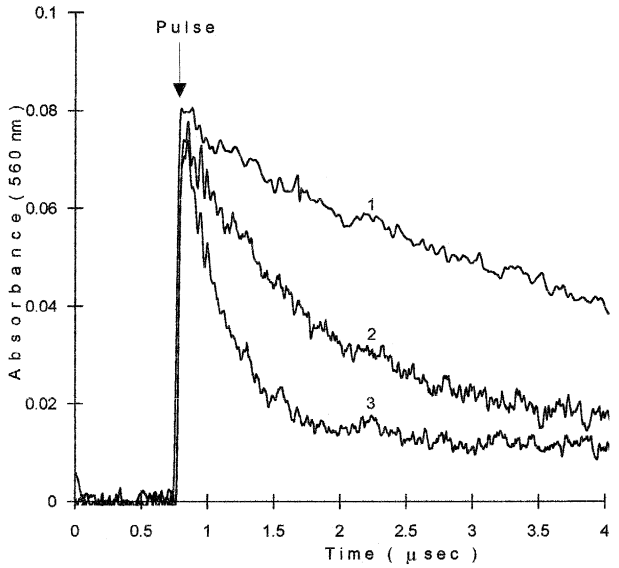

Fig. 6. Reduction of the oxidized *N*-acetylcysteine–AEDANS radical by histidine. *N*-Acetylcysteine–AEDANS solution (20 μM) in 1 mM HEPES, pH 7.0, (curve 1) in the presence of 0.5 (curve 2) and 2 mM histidine (curve 3) was pulsed and measured as described in Section 2.

3.4. Intramolecular electron transfer in cytochrome c–AEDANS complex

Preincubation of yeast cytochrome *c* with stoichiometrical amount of IAEDANS results in covalent modification of a single cysteine 102 residue of the protein. As the compound can modify methionine and histidine residues in addition to cysteines, the labeling of the cytochrome was performed with stoichiometrical concentration of IAEDANS at neutral pH in order to increase the selectivity of the label. Under these conditions almost 1/1 labeling of the yeast protein has been achieved within 2 h. When bovine heart cytochrome *c*, which lack cysteine, was preincubated in the same conditions only 10% of the label was bound to the protein. This result strongly indicates that cysteine is the primary target of IAEDANS attack. Analysis of free SH-groups in the modified yeast cytochrome *c* content with Ellman reagent [20] also clearly showed that the labeled protein contains no free cysteines.

When pulsed by an intensive laser beam the heme group of the labeled ferrocytochrome *c* undergoes rapid intramolecular oxidation by $AEDANS_{ox}$ radical (Fig. 7). The oxidation is much faster ($\tau_{1/2} < 20$ ns)

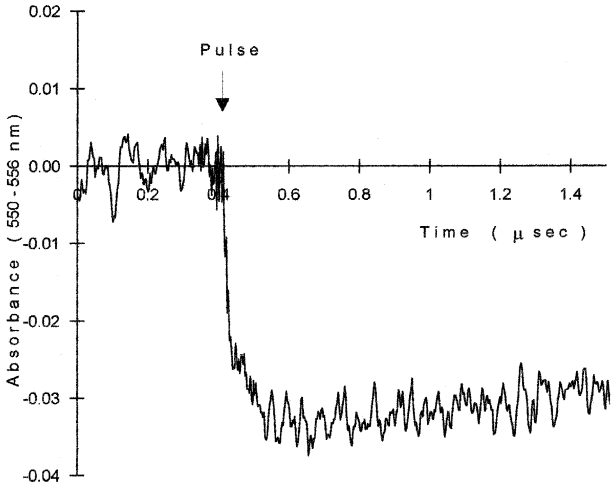

Fig. 7. Light-induced oxidation of the modified cytochrome *c*. The solution of 30 μM cytochrome *c*–AEDANS in 5 mM HEPES, pH 7.0, was pulsed in the presence of 200 μM ascorbate by the third harmonic frequency of a Nd–Yag laser (355 nm, 2 ns FWHM, 17 mJ pulse^{-1}). The transients were recorded at 550 and 556 nm as described in Section 2.

than that by MPTS radical (see Fig. 3B) and proceeds within the time resolution of our measuring system.

4. Discussion

The inability of non-photosynthetic systems to interact with radiation had been circumference by introduction of external photosensitive elements to the assay. The experimental methods which are currently in use employ excited flavines [3,4], Ruthenium complexes [5,6] or zinc substituted heme [7,8] as light driven photoreductants. In this respect, the oxidative pulse described in the paper expand the research possibilities as oxidative dynamics can be studied too.

We have demonstrated that under high photon flux (50–100 MW cm^{-2}) the sulfoaromatic compounds undergo a fast photooxidation: A successive two photon reaction ejects an electron from the molecule and a pair of reactants, solvated electron and oxidized aromatic moiety are formed. Under conditions where the solvated electrons are quenched, the oxidized compounds originating from pyrene–trisulfonate and aminonaphthalene–sulfonate residues can react as one electron acceptors with variety of reductants. The redox potential of the AEDANS residue is sufficiently high ($\sim$ 2 V) to accept electrons from Co^{2+} and free aromatic amino acids. In contrast to AEDANS, the potential of MPTS oxidized radical was insufficient to react with high-potential electron donors.

Binding of these labels to the single cysteine residue (cys102) of the yeast cytochrome *c*, yielded a covalently linked photoproducts which can oxidize the heme group (see Figs. 3 and 7). The rate of electron transfer from the heme iron to the label is strongly different for MPTS and AEDANS residues. The former is oxidizing the heme at least three orders in magnitude slower than the later one. According to Marcus theory the rate of non-adiabatic outer shell electron transfer between two reactants depends on the distance between electron donor and acceptor as well as on the thermodynamic factor, i.e., the balance between driving force and nuclear reorganization energy. As both photoactive labels employed in this study are attached to the same site of cytochrome *c*, the main factors determine reactivity of the labels are driving force and nuclear reorganization energy. In case of the heme oxidation by $AEDANS_{ox}$ radical, the absolute value of free energy ($\sim$ 2 V) is probably strongly exceed the reorganization energy, which seems to vary within a range of 0.5–1 V for most reactions. In this case, strong difference between the electron transfer rates found for AEDANS and MPTS can hardly be explained by difference in free energies for both reactions. An alternative explanation is based on direct participation of amino acid residues as the electron carriers in the electron pathway from the heme to AEDANS label. Indeed, the ability of $AEDANS_{ox}$ to oxidize free amino acids in solution has been demonstrated in the present work (see Fig. 6). The oxidized amino acid radical, formed as a result of redox reaction with $AEDANS_{ox}$, can then conduct the oxidative perturbation to the heme directly or via oxidation of alternative amino acid residues. Possible oxidation of amino acids by photoinduced high-potential oxidants should be taking into account when drawing conclusions about the mechanism of electron transfer in proteins. This is true, in particular, for the oxidation of redox active groups in proteins by the covalently bound Ruthenium complexes, the experimental approach providing information about distance dependence of the

electron transfer rates in proteins [9,23–26]. As the redox potential of Ru^{2+}/Ru^{3+} pair in bis(bipiridine) dicarboxypyridine complex is equal to 1.3 V the oxidation of aromatic amino acid residues by Ru^{3+} seems feasible.

The employment of substituted cytochrome *c* derivatives (like cytochrome *c*–AEDANS adduct), where oxidation of the heme is completed within ~ 20 ns, provides us with a direct tool for 'extracting' one electron from cytochrome *c* being bound to the other redox proteins and measuring the resulting electron redistribution with the same level of time resolution.

Acknowledgements

This research is supported by the Israel Academy of Sciences and Humanities (538/95). We thank Prof. M. Gutman for helpful discussions and Dr. L. Zimanyi for the measurement of transient absorption spectra.

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