ELSEVIER

Biochimica et Biophysica Acta 1228 (1995) 87–90

BBA Biochimica et Biophysica Acta

Rapid Report

Light-induced oxidation of cytochrome *c*

Alexander B. Kotlyar *, Natalia Borovok

Laser Laboratory for Fast Reactions in Biology, Department of Biochemistry, Tel Aviv University, Ramat Aviv 69978, Israel

Received 18 October 1994; accepted 27 October 1994

Abstract

Photooxidation of pyranine (8-hydroxypyrene-1,3,6-trisulfonate) by an intensive UV laser pulse (20–60 MW/cm^2) leads to an instantaneous formation of the oxidized form of the dye. The redox reaction between the oxidized dye and ferrocytochrome *c* was followed by transient absorption spectroscopy looking either at re-reduction of oxidized pyranine or the oxidation of the cytochrome. At high ionic strength (100 mM Hepes and 20 mM KCl, pH 6.5), second-order kinetics of ferrocytochrome-*c* oxidation was observed with a rate constant of $(3.2 \pm 0.3) \cdot 10^9$ M^{-1}s^{-1}. At lower ionic strength a 1:1 complex was formed between the cytochrome and pyranine. The rate for intracomplex electron transfer was found to be $(3.6 \pm 0.2) \cdot 10^6$ s^{-1}. This rapid photooxidation of cytochrome *c* makes it a useful tool for fast initiation of a synchronized electron flow within redox proteins.

Keywords: Pyranine; Cytochrome *c*; Photooxidation; Electron transfer

Cytochrome *c* is water-soluble, low molecular weight hemoprotein component of the mitochondrial and bacterial respiratory chain. While associated with the inner mitochondrial membrane it transfers electrons between *b*-c_1 complex and cytochrome oxidase. Cytochrome *c* is strongly cationic protein with p*I* value greater than 10 and the net charge of +6 at pH 7.0. The positively charged residues are predominantly lysines. Numerous studies have revealed that lysines, especially those located around the heme crevice, play an important role in the interactions of the cytochrome with many proteins like: cytochrome oxidase, *b*-c_1 complex [1–5], cytochrome b_5 [6–9], cytochrome-*c* peroxidase [10,11] and others. The electrostatically stabilized complex formed between cytochrome *c* and alternative proteins represent one of the simplest systems for the study of interprotein electron transfer. Several techniques are available to initiate the electron flow within such complexes. These include photosensitized reduction of cytochrome *c* in the presence of flavins and EDTA [12–15], flash photolysis of zinc-substituted heme proteins [16–19] or photoexcitation of covalently attached ruthenium complexes [20–24]. All these techniques can deliver the photoreductive pulse to isolated cytochromes or cytochrome complexes.

We have recently introduced a new method capable of delivering an intensive oxidative pulse (unpublished results). The reaction is based on excitation of pyranine (Pyr, 8-hydroxypyrene-1,3,6-trisulfonate) molecule to its second electronic singlet state by intensive laser pulse. The second singlet state of pyranine anion ejects an electron and relaxes to a ground state oxidized radical (Pyr_{ox}). The solvated electron released from the excited pyranine reacts preferentially with H^+. The reaction of Pyr_{ox} with hydrogen is rather slow and the oxidized product decays with a rate constant of 0.1–0.2 min^{-1}. Its redox potential is sufficiently high E_m (Pyr/Pyr_{ox}) $\approx$ 1 V to oxidize a wide variety of electron donors of chemical and biological nature.

In the present paper we report the use of photooxidative pulse for rapid oxidation of reduced cytochrome *c*. The electron transfer from the ferro heme group of the cytochrome to Pyr_{ox} has been followed in a μs time frame and the kinetic parameters of the reaction were measured.

Cytochrome *c* from horse heart was obtained from Sigma. Pyranine was made by Eastman Kodak. The sample (1 ml), contained Hepes (1 or 100 mM) cytochrome *c* (50–200 μM) and 150 μM ascorbate (pH 6.5) was placed in four-phase quartz semimicrocuvette with front face 1 cm and depth 0.4 cm. The anaerobic conditions was reached by continuous bubbling of argon at 30 ml/min through the cuvette for 15 min prior to and during the experiment. Electron transfer was initiated by an excitation

* Corresponding author. E-mail: sasha@hemi.tau.ac.il. Fax: +972 3 6415053.

SSDI 0005-2728(94)00165-0

pulse, third harmonic frequency of Nd-YAG laser (355 nm, 2 ns FWHM, 17 mJ/pulse), which was focused on the 0.4 cm side of cuvette over a spot having a surface of 0.15 cm^2. Continuous monitoring of the redox state of pyranine and cytochrome *c* was carried out respectively at 458 nm and 550 minus 556 nm (the reference 556 nm wavelength is at an isosbestic point of cytochrome *c*). The probing beam, either the 458 nm band of a CW Ar laser (458 nm) or the output of 'Coherent' CR-599 Dye laser with Rhodamine-110 dye (550 and 556 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 2430A digital oscilloscope as previously described [25]. The response time of the detection system is 20 ns. The transients are the average of 128 pulses collected at a frequency of 0.02 Hz. Oxidation of cytochrome and reduction of Pyr_{ox} was quantified on the basis of the absorbance coefficient difference of $\epsilon_{550-556} = 19000\ M^{-1}cm^{-1}$ and absorbance coefficient $\epsilon_{458} = 27000\ M^{-1}cm^{-1}$, respectively.

Excitation of pyranine with intensive UV laser pulse resulted in its photooxidation. The oxidized product — Pyr_{ox} — produced is characterized by an intensive absorbance ($\epsilon = 27000\ M^{-1}cm^{-1}$) at 458 nm, a wavelength

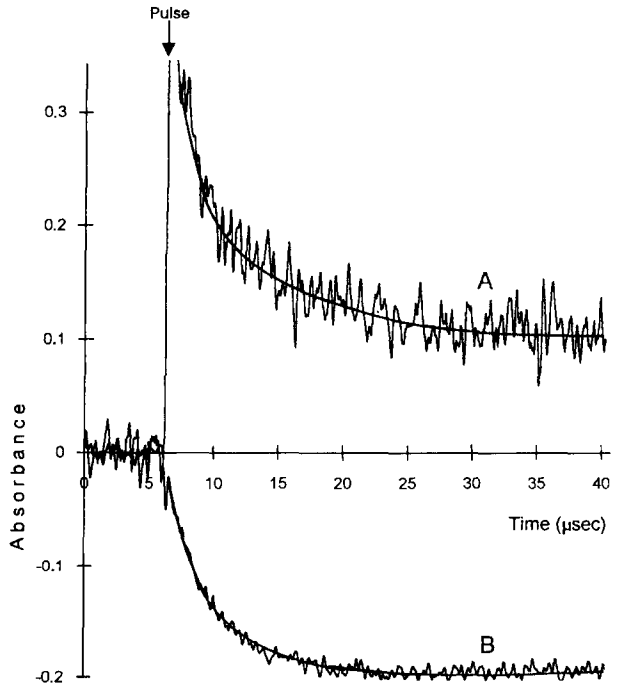

Fig. 1. Transient kinetics for electron transfer in a solution containing 100 μM cytochrome *c*, 300 μM pyranine, 150 μM ascorbate, 20 mM KCl, 0.1 M Hepes (pH 6.5). (A) The transient at 458 nm was due to the formation and decay of Pyr_{ox}. (B) The transient at 550 nm minus 556 nm, the cytochrome-*c* isosbestic. Both transients were fit by a single exponential decay (solid line) having a (first-order) rate constant $k_{obs} = (3.2 \pm 0.2) \cdot 10^5\ s^{-1}$.

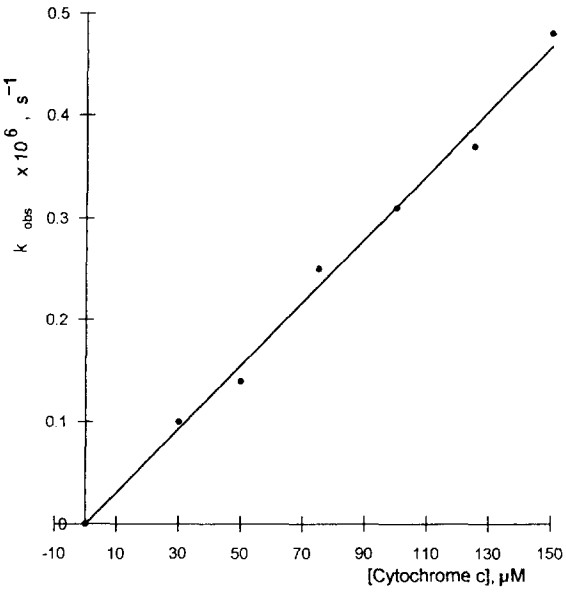

Fig. 2. Second-order plot for oxidation of reduced cytochrome *c* by Pyr_{ox}. The details are as shown in Fig. 1. The second-order rate constant calculated from the slope was $k = (3.2 \pm 0.3) \cdot 10^9\ M^{-1}s^{-1}$.

where the protonated ground state compound has a negligible absorbance. Fig. 1A shows Pyr_{ox} has an unresolved rise time followed by a few μs decay. The decay corresponds with re-reduction of Pyr_{ox} by ferrocytochrome *c* back to the ground-state form. The residual absorbance of the transient is due to the difference in the extinction coefficients of ferri- and ferrocytochrome *c* at 458 nm ($\Delta\epsilon = 9500\ M^{-1}cm^{-1}$). The electron transfer from cytochrome *c* to Pyr_{ox} is shown in Fig. 1B. The amount of cytochrome *c* which was oxidized during the transient ($\approx 25\ \mu M$) is approximately equal to that of Pyr_{ox}, indicating a preferential re-reduction of the oxidized dye by the ferrocytochrome. The transients at 458 and 550–556 nm, as seen in Fig. 1 are monophasic, with a rate constant of $(3.2 \pm 0.2) \cdot 10^5\ s^{-1}$. The rate constant ($k_{obs}$) increases linearly with cytochrome-*c* concentration from 50 to 150 μM as evident from Fig. 2. A second-order rate constant of $(3.2 \pm 0.3) \cdot 10^9\ M^{-1}s^{-1}$, calculated from the slope of the line in Fig. 2, is compatible with that for diffusion controlled reactions. Thus the electron transfer that follows the complex formation is not a rate limiting step.

At low ionic strength addition of cytochrome *c* shifts the p*K* of pyranine from 7.9 to 7.2 (not shown). The observed shift is equated with the enhanced binding of deprotonated form of the dye with the hemoprotein [26]. The probable site for the electrostatically stabilized complex is the positively charged polylysine clusters located on the cytochrome surface [27].

The pyranine-cytochrome complex is not a very stable one ($K_d \approx 30\ \mu M$) and rather high concentration ($> 50\ \mu M$) of cytochrome *c* is required to measure the p*K*-shift. Under these conditions the oxidation of cytochrome *c*

corresponds with an electron transfer within the cytochrome-pyranine complex (see Fig. 3). The rate constant of $(3.6 \pm 0.2) \cdot 10^6$ s^{-1} estimated from the data of Fig. 3 was independent on the concentration of the complex, while the total amplitude of the transient increases with cytochrome-pyranine concentration from 50 to 200 μM. The fact that the rate constant was independent of concentration is indicative of an intramolecular electron transfer mechanism.

Under conditions where we have both the free pyranine and the 1:1 complex the reaction reveals two well-distinguished phases (see Fig. 4). At first we observe a fast redox event having a time constant typical of intracomplex reaction (see Fig. 3). The rest of the reaction is significantly slower corresponding with the collision controlled reaction between Pyr_{ox} and cytochrome *c*. The fast-phase rate constant $(4.0 \pm 1.0) \cdot 10^6$ s^{-1} was independent of cytochrome-*c* concentration. In contrast, the rate constant of the second one $(2.7 \pm 0.2) \cdot 10^5$ s^{-1} increases with the concentration of cytochrome *c*. This kinetic behavior is consistent with combination of intracomplex and intermolecular electron transfer mechanisms.

Previous studies employed the photoexcitation of photosensitive compounds to reduce cytochromes and even cytochrome complexes [12–24]. The oxidative pulse method, described in this paper, is capable of delivering a microsecond oxidative pulse to cytochrome *c*. Being a strong oxidant ($E_m \approx 1$ V) Pyr_{ox} may be employed for study of both the low and high potential section of the respiratory chain of mitochondria and bacteria.

This research is supported by the US Navy Office of Naval Research (Grant No. N00014-89-J1622) and the United States–Israel Binational Science Foundation (91-00226). The authors are grateful to Dr. M. Gutman for his assistance throughout this study.

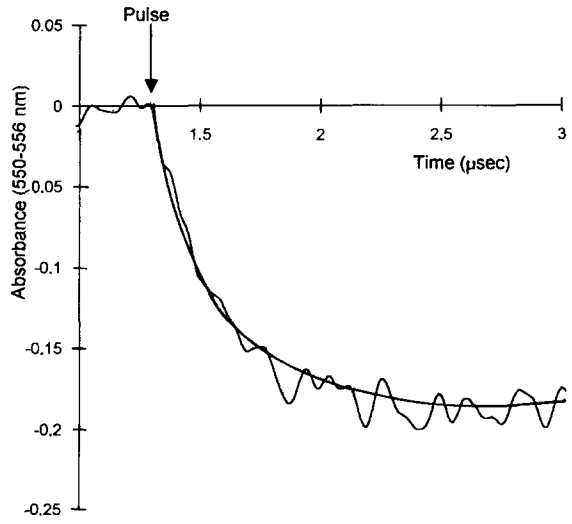

Fig. 3. Cytochrome *c*-Pyr_{ox} intracomplex electron transfer. The solution contained 100 μM cytochrome *c*, 100 μM pyranine, 150 μM ascorbate, 1 mM Hepes (pH 6.5). The transient at 550–556 nm was fit an exponential (solid line) with $k_{obs} = (3.6 \pm 0.2) \cdot 10^6$ $M^{-1}s^{-1}$.

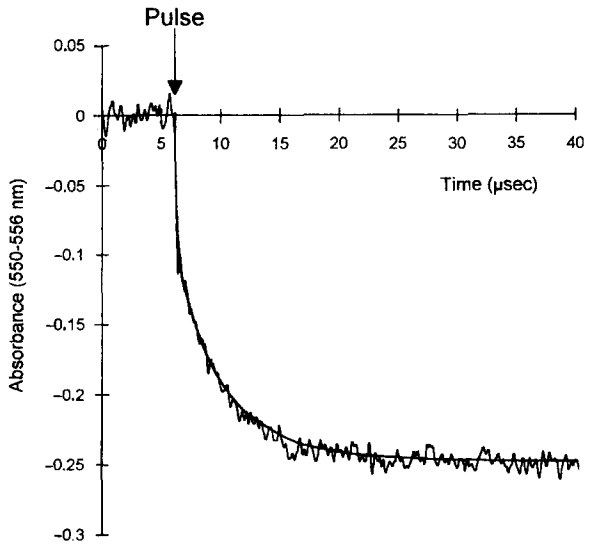

Fig. 4. Biphasic absorption transient following a laser pulse at low ionic strength. The solution contained 100 μM cytochrome *c*, 300 μM pyranine, 150 μM ascorbate, 1 mM Hepes (pH 6.5). The solid line is the theoretical fit assuming two exponential reactions measured at 550–556 nm. The rate constants estimated for the fast and slow phases were $(4.0 \pm 1.0) \cdot 10^6$ s^{-1} and $(2.7 \pm 0.2) \cdot 10^5$ s^{-1}, respectively.

References

[1] Staudenmayer, N., Ng, S., Smith, M.B. and Millett, F. (1977) Biochemistry 16, 600–705.
[2] Smith, H.T., Staudenmayer, N. and Millett, F. (1977) Biochemistry 16, 4971–4974.
[3] Ahmed, A.J., Smith, H.T., Smith, M.B. and Millett, F. (1978) Biochemistry 17, 2479–2483.
[4] Speck, S.H., Ferguson-Miller, S., Osheroff, N. and Margoliash, E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 155–159.
[5] Rieder, R and Bosshard, H.R. (1978) J. Biol. Chem. 253, 6045–6053.
[6] Salemme, F.R. (1976) J. Mol. Biol. 102, 563–568.
[7] Ng, S., Smith, M.B., Smith, H.T. and Millett, F. (1977) Biochemistry 16, 4975–4978.
[8] Stonehuerner, J., Williams, J.B. and Millett, F. (1979) Biochemistry 18, 5422–5427.
[9] Eley, C.G.S. and Moore, G.R.(1983) Biochem. J. 215, 11–21.
[10] Leonard, J.J. and Yonetani, T (1974) Biochemistry 13, 1465–1468.
[11] Erman, J.E. and Vitello, L.B. (1980) J. Biol. Chem. 255, 6224–6227.
[12] Hazzard, J.T., Poulos, T. and Tollin, G. (1987) Biochemistry 26, 2836–2848.
[13] Hazzard, J.T., McLendon, G., Cusanovich, M.A. and Tollin, G. (1988) Biochem. Biophys. Res. Commun. 151, 429–434.
[14] Eltis, L., Mauk, A.G., Hazzard J.T., Cusanovich, M.A. and Tollin, G.(1988) Biochemistry 27, 5455–5460.
[15] Meyer, T.E., Zhao, Z.G., Cusanovich, M.A. and Tollin,G. (1993) Biochemistry 32, 4552–4559.
[16] McLendon, G., Winkler, J., Nocera, D., Mauk, A.G. and Gray, H.B. (1985) J. Am. Chem. Soc. 107, 739–740.
[17] McLendon, G. and Miller, J.R. (1985) J. Am. Chem. Soc. 107, 7811–7816.
[18] Liang, N., Mauk, A.G., Pielak, G.J., Johnson J.A., Smith, M. and Hoffman, B.M. (1988) Science 240, 311–313.

[19] Zhou, J.S. and Kostic, N.M. (1992) Biochemistry 31, 7543–7550.
[20] Pan, L.P., Frame, M., Durham, B., Davis, D. and Millett, F. (1990) Biochemistry 29, 3231–3236.
[21] Geren, L., Hahm, S, Durham, B. and Millett, F. (1991) Biochemistry 30, 9450–9457.
[22] Harm, S., Durham, B. and Millett, F. (1992) Biochemistry 31, 3472–3477.
[23] Willie, A., Stayton, P.S., Sligar, S.G., Durham, B. and Millett, F. (1992) Biochemistry 31, 7237–7242.
[24] Heacock, D.H., Lin, R.-Q., Yu, C.-A., Yu, L., Durham, B. and Millett, F. (1993) J. Biol. Chem. 268, 27171–27175.
[25] Gutman, M., Kotlyar, A.B., Borovok, N. and Nachliel, E. (1993) Biochemistry 32, 2942–2946.
[26] Tong L.K.J. and Glesmann M.C. (1957) J. Am. Chem. Soc. 79, 4305–4309.
[27] Smith, H.T., Ahmed, A.J. and Millett, F. (1981) J. Biol. Chem. 256, 4984–4990.