IDENTIFICATION AND ASSAY OF SYNCHROTRON RADIATION-INDUCED ALTERATIONS ON METALLOENZYMES AND PROTEINS

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1. Introduction

In pulsed radiolysis studies, 4 components of the respiratory chain have been demonstrated to accept electrons from the products of the interaction of ionizing radiation and water molecules: cytochrome *c*, a heme protein electron-transfer component [1]; ubiquinone, an electron-transfer component [2]; riboflavin (and presumably FAD and FMN), an electron-acceptor from substrates [3]; and NAD, the principal redox agent of the citric acid cycle in the mitochondrial respiratory chain [4]. In the last 3 cases, the 2 e^- redox mediators are reduced to the semiquinone, while in cytochrome *c*, Fe^{3+} is reduced to Fe^{2+}. The reactions are extremely rapid: rate constants approaching the diffusion-limited values of 10^{10} M^{-1} s^{-1} are obtained at physiological pH values in the case of cytochrome *c*. In fact, hydrated electrons will radicalize nearly any organic substance, which in turn can react rapidly with cytochrome *c*, causing its reduction [1]. In pulse radiolysis studies of cytochrome oxidase, 2.45 μM hydrated electrons are produced of which ~50% or 1.15 μM react with 1.15 μM cytochrome oxidase in a half-time of 3.7 μs [5]. In synchrotron radiation studies, usual beam intensities at the sample (~6×10^{11} photons/s) are calculated to give ~2.5 μM hydrated electrons/s. Thus, a similar, or even more efficient, reaction may occur with the 1 mM cytochrome oxidase in the 0.5–1 h X-irradiation required for adequate accuracy of data collection. Since a variety of metalloproteins have been employed in wide ranging studies [6–9], with similar reactions possibly occurring, it seems useful to report here our studies in which the redox state of the metalloproteins under irradiation could be monitored continuously by reflectance spectrophotometry. Such irradiation can result in:

(i) Permanent alterations leading to stable reaction products;
(ii) Formation of transient intermediates that are sufficiently long-lived so that they may be observed or trapped;
(iii) Formation of transient species that exist at substantial concentration only during irradiation.

This contribution deals with methods of detection of radiation-induced alterations in these 3 categories by on-line optical and EPR monitoring of the sample redox state.

Direct tests of radiation-induced damage are best made by using the sample itself as its own dosimeter, since this avoids extrapolation from measurements of radical generation to calculations of possible damage. The absorption bands in cytochrome oxidase and in the copper protein stellacyanin, due to their oxidized copper components, have been observed to disappear rapidly during X-irradiation at room temperature at ~6×10^{11} photons/s [8]. On the other hand, significant stability to irradiation has been observed at −20°C and −100°C, suggesting that a highly diffusable species such as hydrated electrons is the agent causing the damage [9].

2. Materials and methods

2.1. *Spectrophotometer*

The split beam spectrophotometer in these studies used circuitry developed together with C. C. Yang [10,11] and consists of a 250 mm focus Bausch and Lomb grating monochromator (600 lines/mm) in

which the second order overlap was minimized using a Wratten 15 gelatin filter which blocked below 500 nm. The scan range was 959–390 nm, but the long light guide curtailed this to 800–400 nm. The chopping frequency and other details are mentioned in [11]. Baseline corrections are read into a computer memory, and the spectra represent the differences with respect to the baseline.

The temperature at which the sample is irradiated has been found to be the most important of the variables. In the case of cytochrome oxidase, samples have been studied at −100°C and −20°C. These temperatures were obtained by flowing thermostatically regulated nitrogen gas through a liquid nitrogen heat exchanger, and then through an electrically controlled reheater. These gasses flow through a styrofoam cryostat holding the reference and measured cytochrome oxidase samples plus the fibre optics probes.

2.2. *'On-line' monitoring of sample damage by reflectance spectrophotometry*

A light guide coupling of a 'split-beam' spectrophotometer directly to the cytochrome oxidase sample under irradiation is afforded by 6 ft of crofon fibre optics. This light guide couples the 2 separate beams from the spectrophotometer directly to the reference and measured samples as housed in a plexiglas cryostat. Light reflected from the samples is collected separately, joined together in a single light guide, and conducted to a photomultiplier located conveniently within the protective steel enclosure (hutch).

The wavelength scanning range available through this length of fibre optics is ~800–400 nm, although the wavelength interval used is ~959–390 nm. The reflectance changes obtained from the ~1 mM cytochrome oxidase sample were fairly large due to the effective optical coupling obtained with the fibre optics. Characteristic absorbance changes (ΔA) of 0.05 and 0.5 at the copper and heme α bands, respectively, were observed. Approximations to absolute spectra were obtained using for reference material a variety of substances, but usually frozen stopcock grease, which seemed most effective over the whole wavelength range. Lack of proportionality between the ΔA in the visible and Soret regions was also characteristic of the reflectance method: the apparent changes for the Soret band were no greater than those at the α band due to pathlength changes with wavelength. Due to the large excess of electron acceptors (e.g., cytochrome oxidase) over that of the hydrated electron concentration, no ΔA_{650} were detected in the restricted period of the experiment [5].

2.3. *EPR monitoring*

The samples were monitored by electron paramagnetic resonance (EPR) immediately (<1 min) before and after irradiation. Spectra were obtained from a Varian E-4 EPR spectrophotometer equipped with a Scanlon wide bore (8 mm i.d.) low temperature quartz dewar in conjunction with an Air Products low-temperature helium-flow system. This dewar accepted the sample holders used for synchrotron studies so that no transfer of fluids or warming occurred. Temperatures were measured with calibrated carbon resistors and were constant at ~10 K. Measurement of the EPR signals varied because of the nature of the derivative spectrum for each paramagnetic center. Due to free radical generation at $g = 2.0$, relative changes in the Cu signals were measured using peak height of the $g = 2.18$ 'absorption-like' part of the EPR detectable Cu_a signal (table 1). The low spin heme was measured in a similar manner using the peak height of the $g = 3.05$ 'absorption-like' part of the heme powder spectrum. Relative changes in the high spin heme were determined using a peak-to-trough measurement of the derivative $g = 6$ signal. All spectra were obtained at 10 mW with a modulation amplitude of 1.0 mT (10 G) and modulation frequency of 100 kHz.

2.4. *Synchrotron intensities*

In this series of monitorings, the synchrotron was operated in a 'dedicated' mode with intensities of 2.6 GeV and currents of 50–60 mA corresponding to $\sim 6 \times 10^{11}$ photons/s [8,9,12] (II-3 FOCUSED EXAFS). The beam was focused on an elliptical spot 2 × 4 mm. The scans were at the Fe K edge between 7050 and 7850 keV. The $1/e$ penetration of the radiation for these samples was 1 mm [13].

2.5. *Optics*

Reflectance spectrophotometry and the X-irradiation occurred on opposite sides of the sample. The optical penetration varied considerably with wavelength: the $1/e$ distance for the infrared region was approximately the sample thickness, but for the visible and near ultraviolet regions it was fractions of a millimeter. The EPR studies averaged the total sample. Possible errors due to this asymmetry were

controlled by optical monitoring external to the synchrotron 'hutch' on either side to check for large differences. None was found in the infrared region. In order, however, to obtain a uniform optical reading independent of, e.g., frost accumulation, on the sample itself, reflectance spectra were recorded through the lucite back of the sample. Thus, the only possibility of underestimating sample changes in the on-line condition would be in the α and γ band regions of the heme.

2.6. *Sample holder and sample preparations*

Samples were prepared in the plexiglas holders, containing an elliptical space, from which all optical, EPR, and EXAFS studies were made. The holders were filled with cytochrome oxidase, usually in a paste form. The oxidized state was verified by ferricyanide addition. The redox state was optically and EPR monitored prior to irradiation. In the case of fig.2, identical samples [15,25] were prepared from aliquots of the same preparation. No. 15 served as room temperature control, while 25 was exposed to X-rays in fig.1. Optical transmission and reflectance were measured in these controls. All samples were maintained at low temperatures throughout their studies, except for that of fig.1.

The cytochrome oxidase preparations were made by modification of the procedure of T. Yonetani by Y. Ching (personal communication) [14].

3. Results

3.1. *On-line evaluation of radiation damage at room temperature*

In a special allocation of 1.5 h beam time, the temperature of the cryostat, containing a cytochrome oxidase sample sealed with mylar tape, was raised to room temperature. The on-line spectrophotometer permitted recording every 4 min. Fig.1 plots the kinetics of ΔA_{780} with respect to ΔA_{690}; an absorbance decrease is plotted as a downward deflection (sample 25 was recorded differentially with respect to identical sample 15). The traces start at t = 9:56, at the start of irradiation, and show a small absorbance difference between the reference and measure samples at the two wavelengths measured. Eight minutes after the completion of-1 scan, there is a highly significant loss of A_{780} in the irradiated sample. This change reaches a slightly higher amplitude after the second

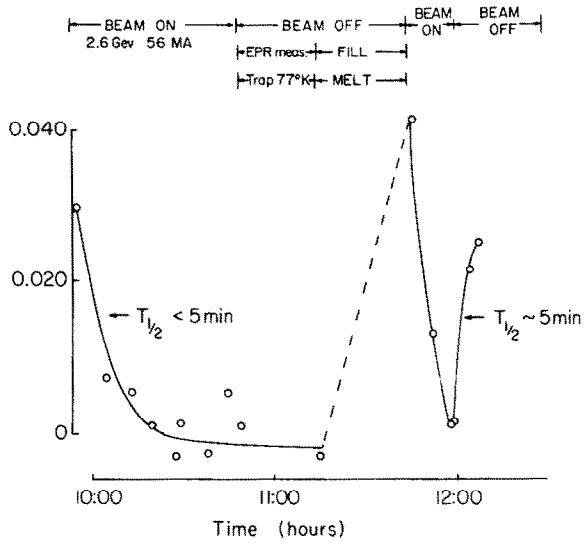

Fig.1. On-line monitoring of radiation induced changes: near infrared ΔA at room temperature under conditions of synchrotron irradiation, 2.6 GeV, 56 mA (6×10^{11} photons/s, sample 25 ~1 mM cytochrome oxidase). A_{780} decrease with respect to A_{690} decrease is indicated as a downward deflection ($-\Delta A$).

scan (t = 10:14). A maximum absorption was reached in 30 min, $t_{1/2}$ being somewhat <5 min. The change is >50% complete within the single 7 min scan.

Interruption of the synchrotron function terminated the recording after ~1 h. The sample was removed from the cryostat and immediately chilled to liquid nitrogen temperature to trap any unstable species. The EPR spectra show normal g = 3, 4.3, and 6 signals: the fraction of the spin concentration in the high spin state, as estimated by the formulation in [15,16], is <10% with >90% in the low spin state prior to irradiation. After irradiation, the EPR spectra showed that 80% remained in the low spin state and 20% in the high spin state, as illustrated by fig.2. The sample was then returned to +23°C and observed by optical data to have retained the ΔA during the melting phase. During the next 0.5 h no irradiation occurred and at the end of that interval the alteration had completely disappeared.

The synchrotron then was allowed to irradiate the sample for 15 min at the end of which the allocated time was abruptly terminated and the decay of the ΔA was followed over the 20 min available prior to the required dismantling of the apparatus. A half-time of decay at room temperature of 5 min was observed.

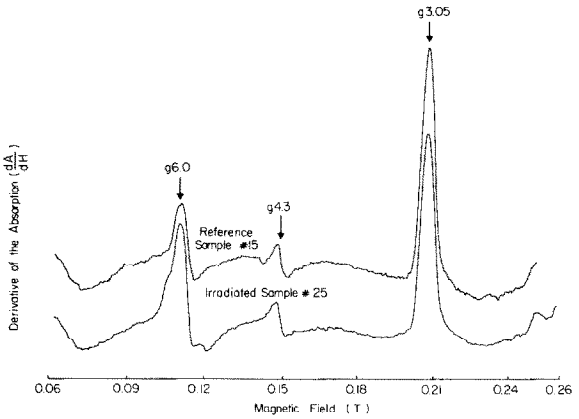

Fig.2. EPR spectra of the identical samples, no. 15 irradiated control, and 25 irradiated as in fig.1, at 2.6 GeV, 56 mA. Samples were run at point marked in fig.1 EPR meas at 10 K, at 10 mW and modulation amplitude of 0.01 mT (10 G).

4. Discussion

This paper quantitates the extent of room temperature alterations on the sample state. Optically a $t_{1/2} < 5$ min under X-irradiation and a recovery time of $t_{1/2} \sim 5$ min without irradiation are observed. This we interpret as due to a steady state of reduction of the infrared absorbing copper components, Cu_a (and Cu_{a3}) [7].

EPR scans show reduction of the heme as well, and show an intensification of the mixed valence signal at $g = 6$, as is characteristic of the EPR signals in cytochrome oxidase [17].

Simultaneous Fe EXAFS spectra obtained on this and other samples after 20 min X-ray exposure at room temperature clearly show that the first shell contribution of the form initially identified as oxidized (fig.2) is similar (± 0.02 Å) to that of the form initially identified as reduced + CO ($Na_2S_2O_4$ + CO). No further change in either of these first shell contributions occurs during exposures up to 60 min. At room temperatures, all the EXAFS contributions of the chemically reduced form ($Na_2S_2O_4$ + CO) differed from those obtained for X-irradiation of similar samples at low temperature. The higher shell contributions of the room temperature-exposed, initially-oxidized form are similar, nevertheless, to those of the low temperature-exposed, oxidized form. (The oxidized and the reduced forms showed small or insignificant optical and EPR changes at low temperature (table 1).) This indicates that X-ray exposure at room temperature produced structural forms unlike those of the states studied at low temperature.

It seems therefore impossible to obtain EXAFS of authentic oxidized cytochrome oxidase at room temperature at $\sim 6 \times 10^{11}$ photons/s without producing a major redox change and an altered structural form.

The mechanism of the reaction is presumably due to formation of radical species by the excitation of water molecules and the formation of e^-_{aq} [1] which serves as a reductant for the oxidase. Electron transport is activated and reduction to a steady state level ensues. Recovery to the oxidized state may require diffusion of oxygen into the heavily irradiated portion of the sample ($t_{1/2} \sim 5$ min) [9].

At −20°C and −100°C, the very large decrease in

Table 1
Optical and EPR monitoring of cytochrome oxidase alteration by 7 keV photons

(6×10^{11} photons/s, 2.6 GeV, 50–70 mA, temp. ≃ −100°C, expt. 4007)

Sample no.	State of enzyme	Scans (~10 min)	Optical change		EPR change		
			'Cu' 800–730 nm	'Heme' 605–630 nm	$g = 6$	$g = 3$	$g = 2.18$
24	Oxidized	12	<5		+15	<5	+12
21	Oxidized	7	<5				
2	Oxidized	10	<5				
0	Oxidized	10			+ 9	−7	−13
8	Reduced	6		−2			
12	Reduced	15		−4			

Percentage changes are measured with respect to non-irradiated control; EPR changes are measured from peak amplitudes

the diffusion of H_2O and e^-_{aq} in frozen solids ($\sim 10^6$- and $\sim 10^9$-fold, respectively [18]) would decrease the rate of sample alteration [16]. Such effects are demonstrated in table 1 when the temperature of the cryostat was lowered to $-100^\circ C$; the samples were monitored optically and by EPR immediately before and after (<1 min) the interval of ~10 scans (~ 100 min X-irradiation). The optical data appear regular and precise; some scatter of EPR data is characteristic of sample placement problems in the large EPR dewar. Thus low temperatures or flow systems seem essential for synchrotron X-ray studies of authentic redox states of metalloenzymes, proteins, and related model systems.

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References

[1] Land, E. J. and Swallow, A. J. (1971) Arch. Biochem. Biophys. 145, 365–372.
[2] Land, E. J., Simic, M. and Swallow, A. J. (1971) Biochim. Biophys. Acta 226, 239–240.
[3] Land, E. J. and Swallow, A. J. (1969) Biochemistry 8, 2117–2125.
[4] Simic, M., Taub, I. A., Tocci, J. and Hurwitz, P. A. (1975) Biochem. Biophys. Res. Commun. 62, 161–167.
[5] Van Buuren, K. J. H., Van Gelder, B. F., Wilting, J. and Braams, R. (1974) Biochim. Biophys. Acta 333, 421–429.
[6] Stanford Synchrotron Radiation Laboratory Users Group Meetings (1976–79) SSRL reports no. 76/11, 77/11, 78/09, 79/05.
[7] Powers, L., Blumberg, W. E., Chance, B., Barlow, C. H., Leigh, J. S., jr, Smith, J., Yonetani, T., Vik, S. and Peisach, J. (1979) Biochim. Biophys. Acta 546, 520–538.
[8] SSRL report no. 79/05 (1979) pp. 1–20.
[9] Chance, B. (1979) SSRL report no. 79/05, p. 28.
[10] Chance, B., Williams, F. C., Yang, C. C., Busser, J. and Higgins, J. (1951) Rev. Sci. Instrum. 22, 683–689.
[11] Chance, B. (1957) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. D. eds) pp. 273–328, Academic Press, New York.
[12] Doniach, S., Landau, I., Spicer, W. E. and Winick, H. (1975) SSRL report no. 75/09.
[13] Burr, A. F. (1977–78) in: CRC Handbook of Chemistry and Physics (58th edn) p. E139, CRC Pub. Co., W. Palm Beach, FL.
[14] Yonetani, T. (1961) J. Biol. Chem. 236, 1680–88.
[15] Aasa, R. and Vanngård, T. (1975) J. Mag. Res. 19, 308–315.
[16] Hartzell, C. R. and Beinert, H. (1976) Biochim. Biophys. Acta 423, 323–338.
[17] Aasa, R., Albracht, S. P. J., Falk, K., Lanne, B. and Vanngård, T. (1976) Biochim. Biophys. Acta 542, 260–272.
[18] Fletcher, N. H. (1970) Chemical Physics of Ice, p. 160, Academic Press, New York.