Changes in the Spin State and Reactivity of Cytochrome c Induced by Photochemically Generated Singlet Oxygen and Free Radicals*

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This work compares the effect of photogenerated singlet oxygen (O2(1Δg)) (type II mechanism) and free radicals (type I mechanism) on cytochrome c structure and reactivity. Both reactive species were obtained by photoca- tion of methylene blue (MB·) in the monomer and dimer forms, respectively. The monomer is predominant at low dye concentrations (up to 8 μM) or in the presence of an excess of SDS micelles, while dimers are predominant at 0.7 mM SDS. Over a pH range in which cytochrome c is in the native form, O2(1Δg) and free radicals induced a Soret band blue shift (from 409 to 405 nm), predominantly. EPR measurements revealed that the blue shift of the Soret band was compatible with conversion of the heme iron from its native low spin state to a high spin state with axial symmetry (g ~ 6.0). Soret band bleaching, due to direct attack on the heme group, was only detected under conditions that favored free radical production (MB· dimer in SDS micelles) or in the presence of a less structured form of the protein (above pH 9.3). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry of the heme group and the polypeptide chain of cytochrome c with Soret band at 405 nm (cyte-405) revealed no alterations in the mass of the cyto-405 heme group but oxidative modifications on methionine (Met65 and Met80) and tyrosine (Tyr74) residues. Damage of cyto-405 tyrosine residue impaired its reduction by diphenylacetaldehyde, but not by β-mercaptoethanol, which was able to reduce cyto-405, generating cytochrome c Fe(II) in the high spin state (spin 2).

The association of cytochrome c with lipid bilayers is modu- lated by the heme iron redox states (1). This association changes the cytochrome c spin state (1, 2) and influences its reactivity with aldehydes, ketones (3), and peroxides (4). These reactions produce free radicals and excited states, both able to attack cytochrome c amino acid side chains and heme group. In this regard, the reaction of cytochrome c with t-butyl-hydroperoxide leads to bleaching of the Soret band, with conversion of the heme protein from its low spin form with rhombic symmetry to a high spin form with axial and rhombic symmetry (3). In this case, the damage in the heme group was promoted by free radicals, but similar effects are also expected from excited species. Among the excited species, singlet oxygen O2(1Δg) is of particular interest because of its high electrophilicity and relatively long lifetime (2–4 μs in H2O and ~700 μs in CCl4) (5). This excited species, which can be generated by an energy transfer mechanism (6, 7), exhibits high reactivity and can be produced in biological systems (8, 9). In mammalian cells, O2(1Δg) can be generated during oxidative stress, and it is able to attack DNA, protein thiol groups, and membrane lipids (11–20).

Proteins are important targets for the pro-oxidant action of O2(1Δg). In the case of heme proteins, this excited species can damage both the apoproteins and the prosthetic group (21–23). Because the vital biological function of these proteins is dependent on the integrity of the heme group, it is to be expected that the heme group is particularly resistant to modifications by reactive species, including O2(1Δg). In fact, when hemoglobin and cytochrome c are exposed to O2(1Δg), chemical modifications of the chromophore constitute only a minor reaction pathway. However, although data reported in the literature have demonstrated O2(1Δg)-induced modifications in the heme group of Neurospora crassa catalase, the chemical nature of the modifications is still under investigation (24–26). In the apoprotein structures, amino acids are one of the main sites of attack by O2(1Δg) (27, 28).

Singlet oxygen mediates the photodynamic action of some drugs. The process known as photodynamic therapy (PDT) involves in situ generation of reactive species promoting tumor regression (29). The mechanism of tumoral destruction involves the direct oxidation (type I mechanism) of biological targets (membranes, proteins, and DNA), as well as oxidation mediated by O2(1Δg) (type II mechanism) (30). In the type II mechanism, O2(1Δg) is generated through energy transferred from excited triplet species to molecular oxygen (30). The use of sensitizers that accumulate in proliferating cells to treat tumors and other diseases is currently widespread. In this regard, methylene blue (MB·) is a sensitizer used in vitro for a variety of applications, including energy conversion and photodynamic therapy (31).

Cytochrome c is a mitochondrial protein fundamental to the cell’s respiratory process. The fact that the apoptosis cascade

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§ The abbreviations used are: MB, methylene blue; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; EPR, electron spin resonance; cyte-405, cytochrome c with Soret band at 405 nm; DPAA, diphenylacetaldehyde.
involves cytochrome c-promoted caspase activation (32, 33) demonstrates that, in the cells, cytochrome c plays a broader role than the transport of electrons in the respiratory chain. Therefore, \( \text{O}_2(1\Delta_g) \)-mediated alterations in cytochrome c structure and reactivity can influence activation of caspases in the apoptosome. On the other hand, photodynamic generation of photodynamic products containing cytochrome c may be accompanied by free radical production (34), and discriminating the effect of each reactive species on the cytochrome c structure still poses a challenge.

**MATERIALS AND METHODS**

*Chemicals—* Cytochrome c (horse heart, type III) and SDS were acquired from Sigma. MB ³⁻ was purchased from Aldrich and double recrystallized from ethanol. Diphenylacetalddehyde was purchased from Aldrich. Ile⁷-angiotensin III (AngIII), human adrenocorticotropic hormone fragments 18–36 (hACTH-(18–36)), α-cyano-4-hydroxycinnamic acid, sinapinic acid, and trifluoroacetic acid were obtained from Amersham Biosciences (Upsala, Sweden). Sequencing grade trypsin was purchased from Promega (Madison, WI). Water was bidistilled from an ultrapure water system (Prominence, Shimadzu Scientific Instruments Inc., Columbia, MD).

*Cytochrome c Oxidation Experiments—* The measurements were performed on freshly prepared cytochrome c solutions (200 μM) in a 1-cm light path quartz cuvette. Absorbance spectra were obtained after subtraction of MB ³⁻ spectral contribution in this region.

**RESULTS**

Effect of pH on \( \text{O}_2(1\Delta_g) \)-induced Oxidative Modifications on Cytochrome c—Cytochrome c was exposed to \( \text{O}_2(1\Delta_g) \) generated by irradiation of MB ³⁻ (methylene blue), over a pH range of 3.0 to 12.0. Fig. 1 and its inset show alterations in the cytochrome c spectra promoted by \( \text{O}_2(1\Delta_g) \) at pH 7.4. The inset shows spectral changes in the cytochrome c Q bands obtained after subtraction of the spectral contribution of MB ³⁻ in the visible range. The spectrum of cytochrome c in the presence of MB ³⁻, obtained before irradiation (time 0), was typical of native cytochrome c Fe(III) low spin state (thick solid line). This result suggests that MB ³⁻ did not induce any significant alteration in the cytochrome c structure and heme iron spin states. Upon irradiation, two phenomena occurred concomitantly: the reduction of cytochrome c by excited MB ³⁻ and irreversible conversion of cytochrome c Fe(II) to Fe(III) mediated by \( \text{O}_2(1\Delta_g) \) associated with bleaching and blue shift of the Soret band (left panel and inset). In the early stages of irradiation, the reduction of cytochrome c was predominant, and the spectrum corresponded to the partial conversion of cytochrome c from ferric to ferrous form (dotted line, left panel and inset). At this time, the bleaching of the Soret band was already evident. As the irradiation progressed, irreversible heme iron oxidation took place (thin solid line, left panel and inset). Progressive bleaching of MB ³⁻ 664 nm occurred throughout the irradiation (Fig. 1, right panel). Similar results were obtained in a phosphate-buffered solution of \( \text{D}_2\text{O} \) at pH 7.8 (not shown), but the rate of bleaching was much slower than that observed in water.

**Matrix-assisted Laser Desorption Ionization (MALDI) Time-of-flight (TOF) Mass Spectrometry—** The MALDI-TOF MS analyses were performed using an Ettan MALDI-TOF Pro system equipped with a quadratic-field reflectron and a timed ion gate. Protein identification was conducted in reflectron mode with positive ionization at 20 kV. The sample was, in this case, mixed with an equal volume of 50% acetonitrile, and 0.5% trifluoroacetic acid saturated with α-cyano-4-hydroxycinnamic acid. A 0.5 μl of the mixture (containing 8 pmol of protein) was loaded onto the stainless steel MALDI slides for analysis. The external calibration was performed before protein identification with AngIII and hACTH-(18–36). The data were analyzed using the Ettan MALDI-ToF Pro software system.

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2. **Fig. 1. Changes in the cytochrome c and MB³⁻ spectra promoted by dye irradiation.** Samples containing 4 μM cytochrome c and 8 μM MB³⁻ were irradiated in 5 mM phosphate buffer, at 35 °C, in a glass chamber with a 500 watt halogen light bulb placed at a distance of 30 cm. Cytochrome c and MB³⁻ spectra were obtained before irradiation (thick solid line) and after 3 and 15 min of irradiation (dotted and thin solid line, respectively). The spectra shown in the inset correspond to the spectral changes in the visible region obtained after subtraction of MB³⁻ spectral contribution in this region.
the irreversible heme iron oxidation was 6.5-fold ($k_{obs}$ in water = 0.81 ± 0.08 ms⁻¹ and $k_{obs}$ in D₂O = 5.4 ± 0.75 ms⁻¹). The rate of cytochrome $c$ reduction and oxidation was measured over a pH range of 3.0 to 9.0 in which cytochrome $c$ remains in its native conformation and spin state. Within this range of pH, different rates of cytochrome $c$ reduction promoted by MB⁺ and the irreversible heme iron re-oxidation produced by O₂(1Δg) were obtained. Fig. 2A shows the kinetic curves for cytochrome $c$ reduction and subsequent re-oxidation obtained in carbonate buffer, pH 6.1, 7.2, and 9.1, according to changes in the intensity of Q bands (insets). Fig. 2B shows the titration curves for the two processes compared with the pH curve for the O₂(1Δg) quantum yield resulting from the energy transfer from 3MB* to O₂ in the triplet ground state O₂(1Δg) and obtained from Ref. 37. In this work, the authors demonstrated that the quantum yield of 3MB* produced by irradiation and the generation of O₂(1Δg) by energy transfer both obey the same pH curve, since the former gives rise to the latter. According to Fig. 2B, only the pH curve for the irreversible oxidation ($pK_a = 6.3$) remained close to the pH curve for the O₂(1Δg) quantum yield previously determined, which displays a $pK_a$ value = 7.0. Complete cytochrome $c$ reduction, without subsequent blue shift of the Soret band, was detected during irradiation of MB⁺, when the O₂(1Δg) yield was drastically decreased by N₂ purging of the medium or by chemical and physical trapping by histidine or urate added to the solution (not shown). These results suggested that the oxidative damage in the cytochrome $c$ structure was dependent on the triplet MB⁺ and O₂(1Δg) quantum yields, but the limiting-rate factor for the photochemical reduction of cytochrome $c$ heme iron did not depend on the quantum yield of triplet species.

At highly alkaline pH values (11.0–12.0), where cytochrome $c$ is not in the native conformation, the irradiation of the protein in the presence of MB⁺ with visible light led to a progressive bleaching of the Soret band, without significant alterations in the maximal peak wavelength (not shown). Similar results were observed when the reaction was performed in D₂O phosphate buffer, but bleaching was considerably faster (not shown). The rate constant ($k_{obs}$) for the O₂(1Δg)-mediated Soret band bleaching was calculated at 0.21 ms⁻¹ in buffered water and at 1.4 ms⁻¹ in buffered D₂O. Therefore, in this condition, the quantum yield of O₂(1Δg) was also the limiting-rate factor for the oxidative damage of the protein. The intense bleaching observed at high pH values (above cytochrome $c$ pI) suggested damage in the protein’s prosthetic group.

In the Presence of SDS Micelles It Was Possible to Discriminate the Effect of Free Radicals and O₂(1Δg) on Cytochrome $c$ Structure—In aqueous aerated solution, photosensitized MB⁺ can give rise to both type I and II mechanisms. In order to clarify the specific contribution of the oxidative species, O₂(1Δg) and free radicals, cytochrome $c$ was exposed to conditions that favored either type I or II mechanisms (38). Fig. 3A shows that, at pH 7.4, in the presence of excess SDS micelles, type II mechanism predominated, and the irradiation of cytochrome $c$ led to a blue shift of the Soret band without previous detection of heme iron reduction. In this condition, after the blue shift, only discrete bleaching of the Soret band was detected. At the same pH value, when type I mechanism was predominant, only bleaching of the Soret band (Fig. 3B) was detected. Bleaching of the cytochrome $c$ Soret band has already been observed upon exposure of cytochrome $c$ to free radicals generated in the course of the reaction between cytochrome $c$ heme iron and t-butyl hydroperoxide (4).

These alterations in cytochrome $c$ Soret band were associated to different cytochrome $c$ spin states detectable by direct EPR of the heme iron. Concentration of cytochrome $c$ samples was a limiting factor to detect heme iron EPR signal. Therefore, all EPR data were obtained in high protein (higher than 100 μM) and dye concentrations and were accompanied by simultaneous UV-vis spectra (not shown) in order to ensure the presence of either MB⁺ dimer or monomer forms. In this condition, a longer irradiation time (50 min) was required to obtain the same alterations in the spectrum of cytochrome $c$ obtained in shorter irradiation times in low protein and dye concentrations. This may be attributed to the effect of an internal filter produced by the high concentration of the dye and the protein. In a homogeneous medium, under the experimental conditions required to acquire EPR spectra, MB⁺ was predominantly in the dimer form (not shown) and therefore, under irradiation, generated predominantly free radicals.

The EPR spectra were analyzed considering the different proportions of cytochrome $c$ high spin states obtained in 50 min of irradiation, with $g = 6.0$, assigned to protein with amino acid
residues oxidized and with $g = 4.3$, assigned to protein with damage in both amino acid residues and prosthetic group.

Fig. 4A, spectrum $a$, shows the EPR spectrum (X-band) of 100 $\mu$M cytochrome $c$ in 5 mM phosphate buffer, pH 7.4, at 11 K, a condition in which cytochrome $c$ is in the native form. Spectrum $a$ corresponds to the well-known Fe(III) low-spin form with a rhombic symmetry that displays signals at $g_y = 3.07$ and $g_z = 2.23$. After 30 s of irradiation, the EPR signal of Fe(III) low spin form was drastically decreased (spectrum $b$), which was coherent with cytochrome $c$ reduction previously observed in the early stages of irradiation (Fig. 1, dashed line, left panel and inset). In the subsequent spectra, obtained at different MB$^+$ irradiation times, it was possible to see the appearance and increase of two forms of heme iron spin states represented by spectrum $c$. Spectrum $c$ indicates the presence of the high spin Fe(III) form with axial symmetry ($g = 6.0$) and a small amount of the high spin Fe(III) form with rhombic symmetry ($g = 4.3$). The intensity of the signal of each high spin state was quantified and the proportion of the high spin states expressed as the ratio of the maximal intensity obtained for each spectra ($g = 6.0g_y = 4.3$ ratio). After 50 min of irradiation in aerated medium, the $g = 6.0/g_y = 4.3$ ratio was $\sim 4.0$. In this sample, a change of cytochrome $c$ low spin species to high spin species was accompanied by a blue shift with discrete bleaching of the Soret band (not shown). According to data in the literature (3), these results suggested the loss of the heme iron sixth coordination due to oxidation of the Met$^{80}$ residue by free radicals. In $N_2$ saturated buffer, the EPR spectrum obtained after 30 s of irradiation showed significant loss of the heme iron low spin state signal, which was compatible with the conversion of cytochrome $c$ from ferric to ferrous form (spectrum $d$). After 50 s of irradiation, a small amount of $g = 6.0$ and 4.3 species was detected (spectrum $e$) and the $g = 6.0/g_y = 4.3$ ratio was $\sim 2.0$. In this case, the impairment of heme iron oxidation by molecular oxygen prevented the detection of the EPR signal from native and damaged protein. During irradiation in the high field region, the appearance of a free radical spectrum was evident (solid line). A computational simulation revealed $g_z = 1/3 (g_1 + g_2 + g_3)$ equal to 2.0079, where $g_1 = 2.0100, g_2 = 2.0088,$ and $g_3 = 2.0050$. This free radical signal was also evident when the experiments were performed at pH 12.0 (not shown).

EPR spectra of cytochrome $c$ heme iron obtained during
Oxidative Changes in Cytochrome c Structure and Reactivity

TABLE I

Identifiable tryptic fragments of cytochrome c in the presence of MB⁺ before and after irradiation, as detected by MALDI TOF/TOF Mass spectrometry.

Data were obtained as described under “Materials and Methods.” Residue number is based on the sequence of the mature protein.

| Meas. mass | Ave isotopic | Comp. mass | Error | Res. start | Res. end | M. cut | Meas. mass | Meas. mass | Peptide sequence |
|-----------|-------------|------------|-------|------------|----------|--------|------------|------------|-----------------|
| 1305.725 | Mono        | 1305.693   | 0.0326 | 89         | 99       | 1      | 1305.693   | 1305.759   | GEREELJAYLK    |
| 1632.637 | Mono        | 1632.812   | -0.1742| 9          | 22       | 1      | 1632.637   | 1632.649   | IFVYKACQICTVEK |
| 1583.751 | Mono        | 1583.758   | -0.0065| 39         | 53       | 1      | 1583.791   | 1583.792   | KTGQAPPSYTDANK|
| 1167.685 | Mono        | 1167.615   | 0.0706 | 28         | 38       | 1      | 1167.685   | 1167.662   | TGPNLHGLGRR   |
| 1295.897 | Mono        | 1295.710   | 0.9698 | 28         | 38       | 1      | 1295.710   | 1295.755   | TGPNLHGLGRR   |
| 1433.013 | Mono        | 1432.789   | 0.0369 | 26         | 38       | 1      | 1432.806   | 1432.806   | HKTGNLHGLGRR  |
| 1445.669 | Mono        | 1445.663   | 0.0080 | 50         | 53       | 1      | 1455.685   | 1455.684   | TYQAPPSYTDANK |
| 1457.750 | Mono        | 1457.805   | -0.0533| 74         | 86       | 1      | 1457.750   | 1457.810   | YIPGRTMIFAGIK |
| 2136.960 | Mono        | 2137.040   | -0.0799| 56         | 73       | 1      | 2153.275   | 2153.275   | GWTGWEETLMLEYLPKK |
| 778.404 | Mono        | 778.441    | -0.0367| 80         | 86       | 1      | 778.565    | 794.511    | MIFAGIK       |
| 906.762 | Mono        | 906.536    | 0.1660 | 80         | 87       | 1      | 906.702    | 922.692    | MIFAGIK       |
| 677.175 | Mono        | 677.375    | -0.1997| 74         | 79       | 1      | 693.102    | 693.102    | YIPGRTK       |
| 2080.420 | Mono        | 2080.019   | 0.2187 | 56         | 72       | 1      | 2096.174   | 2096.174   | GWTGWEETLMLEYLPKK |
| 1494.853 | Mono        | 1494.691   | 0.1621 | 61         | 72       | 1      | 1510.642   | 1510.642   | EETLMLEYLPKK  |

* Increase in the expected mass of the peptide fragment compatible with oxidation of Met65.
* Mass of the peptide fragment indicates that Met65 was not oxidized.
* Increase in the expected mass of the peptide fragment compatible with oxidation of Met80.
* Increase in the expected mass of the peptide fragments indicates oxidation of Tyr40 and Tyr73.

irradiation at pH 12 exhibited a higher yield of the species, with g = 4.3, since the g = 6.0/4.3 ratio decreased to ~0.9 (spectra not shown). This result was coherent with a major exposure of the heme group to the attack by free radicals. Similarly to what was observed at pH = 7.4, the yield of g = 6.0 was impaired when irradiation occurred in an N₂ atmosphere. In this condition, the yield of g = 4.3 species remained the same and the g = 6.0/4.3 ratio decreased to 0.35 (spectra not shown). Considering the less compact cytochrome c structure at pH 12.0, this result suggested that a free radical-mediated attack on the heme group would be improbable without concomitant re-oxidation of the heme iron.

In the experimental conditions for EPR measurements, the exclusive monomer form of MB⁺ and predominant generation of O₂(1Δg), can only be achieved in the presence of high SDS concentrations (above 15 mM). In this condition, g = 6.0/4.3 ratio increased to 20.0 due to a high yield of the high spin Fe(III) form with axial symmetry (g = 6.0) (Fig. 4B). In the micellar medium, the absence of significant amounts of the g = 4.3 species characterized of oxidized porphyrin (4) indicated that, in the presence of SDS micelles, O₂(1Δg) attacks the cytochrome c heme group less efficiently than free radicals.

Photochemically generated O₂(1Δg) Attacks Cytochrome c Side Chain Amino Acids and Changes the Reactivity of the Hemeprotein—Two mechanisms may account for the O₂(1Δg) -mediated cytochrome c conversion from low to high spin state with g = 6.0 and Soret peak at 405 nm, herein named ctc405: attack on the heme group or attack on amino acid side chains leading to changes in the protein structure. The main amino acid residues that are targets for O₂(1Δg) are present in the cytochrome c structure, but with differing degrees of accessibility. Cytochrome c structure exhibits two methionine residues (65 and 80), four tyrosine residues (48, 67, 74, and 98), one tryptophan residue (59), and three histidine residues (18, 26, and 33). The MALDI-TOF mass spectrometry analysis of cytochrome c fragments obtained by digestion with trypsin, exposed for different lengths of time to photochemically generated O₂(1Δg), revealed, after 30 s of irradiation, a mass increase of 16 units in the fragment containing amino acid residues from 56 to 73 (Table I). In this fragment, both Met65 and Tyr40 are candidate targets for the pro-oxidant species. However, it is more probable that the oxidation process occurred in Met65 since it is more accessible in cytochrome c structure. In this case, the mass increase of 16 units was compatible with the formation of sulfoxide derivative (Table I). Besides the Met65 oxidation, the protein fragments containing oxidized Met80 and Tyr74 (increase of 16 units) were also observed after trypsin digestion of samples subjected to irradiation for 5 and 120 min (Table I). The MALDI-TOF analysis of the heme group extracted from the protein before and after 40 min of exposure to O₂(1Δg) and free radicals, at pH 8.0, revealed no significant modification in the prosthetic group as compared with native cytochrome c. The MALDI-TOF spectra of the sample irradiated at pH 8 for 40 min revealed 617.2 and 635.4 Da mass peaks compatible with free heme [M + H]⁺ and [M + H]⁺ + H₂O, respectively (not shown). The same result was obtained for the control sample (t = 0, not shown). The MALDI-TOF analysis and SDS-PAGE of non-digested ctc405 (not shown) discarded the possibility of protein fragmentation.

The attack on cytochrome c amino acid residues pointed to the possibility of changes in the protein reactivity, according to previous results that will be described herein. Diphenylacetaldehyde (DPAA) reduces cytochrome c via a pH-dependent mechanism that involves interaction of DPAA phenyl groups with the side chain of two tyrosine groups (probably Tyr40 and Tyr72) (39). On the other hand, reduction of cytochrome c by thiol groups was pH-independent and did not involve interaction of the reducing agent with the tyrosine side chains of cytochrome c. Fig. 5A shows that native cytochrome c was completely reduced by DPAA 2 min after addition of the aldehyde, at pH 10.5 (the optimal pH for the reaction). Under the same conditions, ctc405 was not reduced, even after a long incubation time. Similarly, ctc405 was not exposed to irradiation (not shown), suggesting that MB⁺-promoted cytochrome c reduction was also dependent on interactions with cytochrome c amino acid side chains. However, ctc405 was promptly reduced by β-mercaptoethanol and exhibited spectral alterations assigned to cytochrome c Fe(II) in the high spin form (spin 2) (1, 40), as shown in Fig. 5B. The red shift of the Soret band without an increase of the Q bands is similar to what occurs when ferrous oxyhemoglobin is converted to its deoxy form that exhibits Fe(II) in the high state because of the loss of Q₉ as the sixth ligand (40).

DISCUSSION

Effect of Type I and II Mechanisms on the Cytochrome c Spin States—The irradiation of cytochrome c and MB⁺ in a homogeneous medium (phosphate or carbonate buffer) led to the
exposure of the protein to both O$_2$(1$^\Delta_g$) (type II mechanism) and dye-derived free radicals (type I mechanism). Three pro-oxidant species possibly mediate direct oxidation of cytochrome c (type I mechanism): excited MB$^+$ ($^3$MB$^+$) and its radical forms MB$^\text{•}$ and MB$^\text{2•}$, according to Equations 1–6.

In the presence of low MB$^+$ concentrations, although both type I and type II mechanisms occurred, the monomer form of MB$^+$ was prevalent and a predominant contribution from O$_2$(1$^\Delta_g$) to both events (blue shift and bleaching) was evident, since D$_2$O buffered media enhanced the rate of the oxidative reactions (see $k_{\text{obs}}$ values for cytochrome c re-oxidation, at pH 7.4 and for Soret band bleaching, at pH 11.0, in water and D$_2$O buffered media). The role of O$_2$(1$^\Delta_g$) as the chief mediator for the conversion of native cytochrome c to cyt-405 was also confirmed by the similarity displayed by the titration curve of cytochrome c re-oxidation and that of O$_2$(1$^\Delta_g$) quantum yield (36) (Fig. 2B). However, in a homogeneous medium, another event was detected during irradiation of cytochrome c in the presence of MB$^+$: cytochrome c reduction (Figs. 1 and 2A). This implies that MB$^+$, which was formed as shown in Equation 7, was able to transfer one electron to the cytochrome c heme iron (10).

The titration curve obtained for MB$^+$-mediated cytochrome c

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**Equations**

**Equation 1**

\[
\text{MB}^+ \xrightarrow{hv} \text{MB}^\text{•} + \text{RH}
\]

**Equation 2**

\[
\text{MB}^+ + \text{O}_2 \rightarrow \text{MB}^\text{•} + \text{O}_2^\text{•} + \text{R} + H^+
\]

**Equation 3**

\[
^1\text{O}_2 + \text{cytcFelli} \rightarrow \text{cytc405Felli} + \text{O}_2^\text{•} -
\]

**Equation 4**

\[
\text{MB}^+ + \text{RH} \rightarrow \text{MB}^\text{•} + \text{R} + H^+
\]

**Equation 5**

\[
\text{MB}^\text{•} + \text{RH} \rightarrow \text{MB}^\text{2•} + \text{R} + H^+
\]

**Equation 6**

\[
\text{MB}^+ + \text{cytcFelli} \rightarrow \text{cytcFell} + \text{MB}^+
\]

**Equation 7**

\[
\text{MB}^+ + \text{cytcFelli} \rightarrow \text{cytcFell} + \text{MB}^+
\]
reduction (Fig. 2B) suggested that the quantum yield of $^{3}\text{MB}^{\bullet\bullet}$ was not the limiting step in this process. In this case, the electron transfer from MB to the cytochrome c heme iron was likely dependent on the protonation of tyrosine residues, as previously observed in the process mediated DPAA (38), which, as expected, was unable to reduce cytc405 (Fig. 5A).

The results obtained with SDS micelles, under conditions selectively favoring either type I or II mechanisms, suggested that the blue shift of the Soret band was promoted by $O_2$($^1\Delta_g$) and bleaching by free radicals. However, the increase observed in the Soret band bleaching ($k_{obs}$) rate in D$_2$O phosphate buffer at pH 11.0 (not shown) indicated that bleaching could also be produced by the attack of $O_2$($^1\Delta_g$). Therefore, predominance of either bleaching or blue shift of the Soret band seemed dependent on protein structure. This assumption was clarified through EPR experiments.

EPR measurements were carried out in the presence of a high MB$^+$ dimer/monomer ratio, a condition in which a high yield of free radicals was expected. During the first few seconds of irradiation in a homogeneous medium, at pH 8.0, cytochrome c was reduced by MB$^+$ (Equation 7), generating Fe(II) cytochrome c which was EPR silent (Fig. 4A, traces a and d). During subsequent irradiation, both cytochrome c Fe(II) and Fe(III) displayed susceptibility to the attack of $O_2$($^1\Delta_g$) and free radicals. The predominance of the signal $g = 6.0$ (Fig. 4A, trace c) suggested that oxidative damage occurred predominantly in the polypeptide chain. Fe(II) cytochrome c damaged by pro-oxidant species can be re-oxidized to Fe(III)cytc405, but the opposite cannot occur since cytc405 is impaired, which prevents it from being reduced by MB$^+$ (not shown). In the absence of O$_2$, the decrease in the $g = 6.0$ signal observed in samples irradiated at pH 8.0 was attributed to the fact that a smaller amount of Fe(III)cytc405 was generated due to impaired $O_2$($^1\Delta_g$) production and because Fe(II)cytc405 was not re-oxidized by O$_2$ and remained EPR silent (compare traces c and e in Fig. 4A).

At highly alkaline pH values, due to the easier access of the heme group to attack by free radicals and $O_2$($^1\Delta_g$), the prothionic group and the polypeptide chain of both Fe(III) and Fe(II) cytochrome c were both susceptible to the attack of the pro-oxidant species, increasing the yield of 4.3 signal (not shown). Scheme I illustrates the above discussion.

In the presence of SDS micelles (Fig. 4B), a condition that favored the monomer form of MB$^+$ and, hence, the generation of $O_2$($^1\Delta_g$) (type II mechanism), formation of the high spin form with axial symmetry ($g = 6.0$) was almost exclusive, suggesting that cytochrome c amino acid side chains were the main target for $O_2$($^1\Delta_g$). We have already reported that SDS micelles induce changes in the cytochrome c spin state from the native low spin form to the alternative low spin form with less rhombic symmetry (2). Therefore, in this condition, where the type II mechanism acts exclusively, the almost total absence of signal at 4.3, assigned to chemical damage in the heme group, suggests a poor ability of $O_2$($^1\Delta_g$) to oxidize the heme group of cytochrome c associated to SDS micelles. According to Fig. 3B, the same did not hold true for free radicals.

MB$^+$ dimers in SDS micelles (Fig. 3B) could not be obtained under the experimental conditions applied for the EPR experiments (high cytochrome c and MB$^+$ concentrations), so it was impossible to complement the results shown in Fig. 3B with EPR measurements.

An additional important piece of information was extracted from EPR measurements: the appearance of an EPR signal characteristic of a small molecule-free radical. This free radical signal was detected only in conditions in which the MB$^+$ dimer was present (Fig. 4A) but was absent in the presence of high concentrations of SDS, where $O_2$($^1\Delta_g$) was the only pro-oxidant species formed (Fig. 4B). This result suggests that this free radical signal may originate from an MB$^+$-derived radical or from a radical of the protein produced by an MB$^+$ radical-directed reaction.

**Singlet Oxygen Mediated Chemical Modifications in the Cytochrome c Amino Acid Residues**—At this point, an important question emerged: was cytc405 a consequence of a modification in the heme group or in amino acid side chains of native cytochrome c? The answer was provided by the results obtained from mass spectrometry of the heme group extracted from cytochrome c structure after irradiation in the presence of MB$^+$ at pH 8.0. The same mass values were found for native cytochrome c and cytc405. The peptide digestion and fragment analysis of cytochrome c irradiated for 30 s confirmed oxidation of an amino acid residue in the fragment encompassing the amino acid residues 56–73. In this fragment Trp, Met, and Tyr were susceptible to pro-oxidant attack. However, the increase of 16 units of mass in this fragment was compatible with both Met$^{45}$ and Tyr$^{47}$ oxidation (30), although Met$^{45}$ was the more probable candidate for oxidation due to its greater accessibility. The analysis of samples subjected to longer irradiation times indicated oxidation of Met$^{45}$, coincident with the occurrence of Soret band blue shift. The oxidation of Tyr$^{47}$ was also concomitant with the oxidation of Met$^{45}$ and...
confirms the presumed lack of reactivity of cytochrome c with DPAA and MB.

The Reactivity of cyt c405—In previous studies we reported that changes in the spin states of cytochrome c mediated by charged interfaces can strongly influence its reactivity (3, 4). The results obtained with cyt c405 indicate that alterations in reactivity also occur in this high spin form of cytochrome c. However, while cytochrome c associated with DCP vesicles exhibits high reactivity with DPAA, cyt c405 failed to be reduced by this aldehyde. These findings reinforce the assumption that this high spin form of cytochrome c derives from chemical modifications in its amino acid residues, including Tyr74, a putative essential residue of the reduction promoted by DPAA (38). Otherwise, thiol-promoted reduction of native cytochrome c did not exhibit pH dependence (not shown) and, as expected, cyt c405 was promptly reduced by /H9252-mercaptoethanol, generating a reduced high spin form of heme iron: cytochrome cFe(II) spin 2 (1, 40). The results obtained with the model systems (cytochrome c and MB /H11001, in solution and micelles) raises important questions about the apoptotic process triggered by photodynamic therapy.

Conclusions—Based on the results described herein, it can be concluded that the cytochrome c structure, influenced by the pH of the medium and its association with interfaces, is crucial to determine the type of damage produced by free radicals and O₂(1Δg). In the presence of SDS micelles, a clear difference was detected when O₂(1Δg) or free radicals were the exclusive prooxidant species. This difference is particularly interesting because the reactions occurred in a heterogeneous medium, which mimics, at least partially, the biological conditions. In this regard, the effect of O₂(1Δg) on cytochrome c bound to mitochondrial mimetic membranes is under investigation in our laboratory.

Photodynamic therapy leads to tumoral cell death through intense oxidative stress, with generation of singlet oxygen in high yield, inducing cytochrome c detachment from the inner mitochondrial membrane and consequently triggering apoptosis in cytosol. What has yet to be ascertained, however, is whether the oxidative stress produced by photodynamic therapy also affects cytochrome c and whether the latter, after being oxidized, is still able to cause apoptosis, or if only non-oxidized cytochrome c molecules trigger apoptosis in this condition. These topics are also currently under investigation in our laboratory.

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Changes in the Spin State and Reactivity of Cytochrome c Induced by Photochemically Generated Singlet Oxygen and Free Radicals

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