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Research paper

Marked difference in cytochrome *c* oxidation mediated by $HO^{\cdot}$ and/or $O_2^{\cdot -}$ free radicals *in vitro*

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Abstract

Cytochrome *c* (cyt *c*) is an electron carrier involved in the mitochondrial respiratory chain and a critical protein in apoptosis. The oxidation of cytochrome *c* can therefore be relevant biologically. We studied whether cytochrome *c* underwent the attack of reactive oxygen species (ROS) during ionizing irradiation-induced oxidative stress. ROS were generated via water radiolysis under ionizing radiation (IR) *in vitro*. Characterization of oxidation was performed by mass spectrometry, after tryptic digestion, and UV-visible spectrophotometry. When both hydroxyl and superoxide free radicals were generated during water radiolysis, only five tryptic peptides of cyt *c* were reproducibly identified as oxidized according to a relation that was dependent of the dose of ionizing radiation. The same behavior was observed when hydroxyl free radicals were specifically generated (N_2O-saturated solutions). Specific oxidation of cyt *c* by superoxide free radicals was performed and has shown that only one oxidized peptide (MIFAGIK + 16), corresponding to the oxidation of Met80 into methionine sulfoxide, exhibited a radiation dose-dependent formation. In addition, the enzymatic site of cytochrome *c* was sensitive to the attack of both superoxide and hydroxyl radicals as observed through the reduction of Fe^{3+}, the degradation of the protoporphyrin IX and the oxidative disruption of the Met80–Fe^{3+} bond. Noteworthy, the latter has been involved in the conversion of cyt *c* to a peroxidase. Finally, Met80 appears as the most sensitive residue towards hydroxyl but also superoxide free radicals mediated oxidation.

Keywords: Cytochrome *c*; Oxidation; Mass spectrometry; Reactive oxygen species; Ionizing radiation

1. Introduction

Cytochrome *c* (cyt *c*) is a mitochondrial electron carrier protein that has been highly conserved during evolution [1]. It plays a dual role in triggering apoptosis and mitochondrial oxidative phosphorylations. Under normal physiological conditions, it functions as an electron carrier in the respiratory chain between complexes III and IV. Mitochondria are the main intracellular source of reactive oxygen species (ROS), generated as side products during mitochondrial respiration. As a result of increased ROS production and/or deficient ROS scavenging within the mitochondria, cyt *c* is a key sensor that helps to maintain cell homeostasis. Cytochrome *c* is a 12 kDa peripheral mitochondrial protein located on the outer surface of the inner mitochondrial membrane (IMM). It has a single polypeptide chain of 104 amino acid residues with a heme group (protoporphyrin IX and Fe^{3+}) covalently linked with two cysteines (Cys-14, Cys-17) and surrounded by tightly packed hydrophobic side chains. There is a hydrophobic channel from the protein surface where basic residues facilitate the binding to several macromolecules (oxidases, lipids) to the heme crevice. This particular structure is critical for cyt *c* redox properties and biological functions.

In the past decades, evidence has accumulated supporting the concept that upon ionizing radiation (IR) exposure, signals originated from the plasma membrane to activate pathways

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that culminated in apoptosis. Within seconds of irradiation, sphingomyelinases are activated and catalyze sphingomyelin into ceramide. Ceramide facilitates apoptosis through the release of cyt *c* from the mitochondria independently from p53 [2,3]. IR-induced lipid-protein interactions occur at the level of the plasma membrane and influence cellular function. Mechanistically, neither IR-induced oxidative modifications of proteins at the mitochondrial level nor IR-induced lipid-protein interactions have been studied to better understand IR-induced apoptosis. The oxidation of small peptide models by specific ROS showed differences between aerobic and anaerobic conditions: decreases in isoelectric point and fragmentation predominating after aerobic irradiation, and aggregation occurring under anaerobic conditions. Specific amino-acid modifications in small peptides were identified [4,5]. With the development of mass spectrometry, it has also become possible to identify specific residue sites in proteins that are modified by ROS [6,7]. Protein oxidation is involved in a large number of disease processes, with mitochondria as a major source of ROS. Aging and cancer have a broad range of abnormal proteins and oxidative processes. Noteworthy, translational research focusing on cancer and apoptosis are incrementally using mass spectrometry applied to proteomics for the understanding of biologic processes.

We tested whether we could identify any IR-induced modifications of the molecule of cyt *c*. We hypothesized that cyt *c* could be oxidized in a non-random manner. If so, these modifications of structure might change its functions and interactions *in vivo*. To identify IR-induced oxidative modifications, cyt *c* was irradiated *in vitro* under water radiolysis conditions. All putative sites of oxidations on amino-acid residues were analyzed by mass spectrometry. In particular, mass spectrometry elucidated the mechanism of methionine sulfoxide formation by various oxidants. *In vitro*, oxidative stress mostly affects C-terminal methionine residues in contrast to what is seen *in vivo*. The goal of this study was to determine which residues of cyt *c* would be modified by the presence of hydroxyl and/or superoxide radicals generated by IR-induced water radiolysis.

2. Materials and methods

2.1. Materials

Horse heart cytochrome *c* (96% pure) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. Trypsin (sequencing grade) was purchased from Roche Diagnostics (Indianapolis, IN, USA). All solvent were of HPLC grade. Ultra pure water (resistivity = 18.2 MΩ, Maxima Purelab Ultra, Elga) was used throughout.

2.2. Oxidation of cyt c via water radiolysis

Gamma radiolysis of water is a powerful way to simulate oxidative stress conditions *in vitro*. Reactive oxygen species (ROS) are homogeneously generated in solutions. Substrates directly undergo ROS' attack, as occurs *in vivo*. Gamma irradiations were performed with an IBL 637 irradiator (CIS Biointernational, Gif-Sur-Yvette, France), using a Cesium 137 γ-ray source of 222 TBq (6000 Ci) activity. Under gamma radiolysis, the total amount of ROS can be increased or decreased by increasing or decreasing the radiation dose (expressed in Gy, 1 Gy = 1 J kg^{-1}), that is by selecting the time the sample is exposed to the ^{137}Cs gamma ray source: the longer the exposure, the higher the radiation dose. For solutions in low concentrations, γ-ray interactions with substrates occur indirectly. Radiolytic effects are only due to radical species produced by water radiolysis (i.e. $HO^{\cdot}$, e^{-}_{aq} and $H^{\cdot}$) [8]. The dosimetry was determined according to the Fricke method [8,9]. The dose rate was 8 Gy min^{-1} in our experiments.

In the presence of oxygen, i.e. in aerated solutions, water radiolysis leads to the generation of hydroxyl ($HO^{\cdot}$) and superoxide ($O_2^{\cdot -}$, produced from scavenged e^{-}_{aq} and $H^{\cdot}$ by O_2) free radicals, with formation yields of 2.8×10^{-7} and 3.4×10^{-7} mol J^{-1} [8], respectively (the radiolytic yield is the number of free radical produced per unit of energy absorbed, i.e. per joule). Hydroxyl radical $HO^{\cdot}$ is the most reactive species. It has a high one-electron oxidation potential of $E^{\circ\prime}(HO^{\cdot}/H_2O) = 2.34$ V at pH 7.0 [8] and reacts with numerous biomolecules with high rate constants (usually 10^9–10^{10} L mol^{-1} s^{-1} [10]). Conversely, superoxide anion $O_2^{\cdot -}$ reacts poorly with biological substrates [11].

Selection of generated free radicals during water radiolysis was performed as following: in the absence of oxygen, i.e. in N_2O-saturated aqueous solutions, hydroxyl free radicals ($HO^{\cdot}$) were specifically generated with a production yield of 5.6×10^{-7} mol J^{-1} [8]. In the presence of sodium formate 10 mM, selective generation of superoxide radicals ($O_2^{\cdot -}$) was achieved with a production yield of 6.2×10^{-7} mol J^{-1}.

Cytochrome *c* is a hydrophilic protein easily solubilized in water. All aqueous solutions of cyt *c* were prepared at a concentration of 50 μM in Na-phosphate buffer (20 mM) at pH 7.0. Volumes of 5 ml of aqueous solutions were introduced into a test-tube and irradiated.

In deaerated experiments, volumes of 5 ml of aqueous solutions were introduced into special test-tubes closed at the top and equipped with two thin glass tubes for deaeration and saturation with N_2O gas. Molecular oxygen was completely removed from solutions. The action of hydroxyl free radicals on cyt *c* could therefore be specifically assessed. Aqueous solutions were bubbled for 1 h at a flow-rate of approximately 1 ml min^{-1}. Test-tubes were then hermetically closed until analysis to avoid contact between solutions and molecular oxygen present in the air before or after irradiation. To study superoxide free radical specific action, cyt *c* was prepared in sodium formate aerated aqueous solution (10 mM). Prior to each set of experiments, glassware was carefully washed with TFD4 soap (Franklab, France), rinsed with ultra-pure water and finally heated at 400 °C for 4 h to avoid pollution by any remaining organic compounds.

2.3. Tryptic digestion of cyt c

Trypsin was used for cyt *c* digestion prior to mass spectrometric sequencing. Irradiated aqueous solutions of cyt *c* were