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Biochemical properties of cytochrome c nitrated by peroxynitrite

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Abstract

Nitration of tyrosine residues is taken as evidence for intracellular formation of peroxynitrite. Cytochrome c (cyt c) can be nitrated by peroxynitrite and nitrated cyt c has been observed in cells and tissues under stress conditions. Here we studied the biochemical properties of nitrated cyt c in order to understand its potential roles in nitrative stress. Nitration of cyt c resulted in disruption of the heme-methionine bond and rapid binding to cyanide. Equilibrium unfolding by guanidine hydrochloride showed that cyt c was slightly destabilized upon nitration but the unfolding transition of nitrated cyt c was highly cooperative indicating that the overall folding was largely preserved. Nitrated cyt c could not be reduced by superoxide and did not support electron transfer between ascorbate and cyt c oxidase. Nitration of cyt c resulted in a tremendous increase in peroxidase activity so that nitrated cyt c rapidly oxidized dihydrodichlorofluorescein even in the presence of a high concentration of glutathione. Enhanced peroxidase activity of nitrated cyt c was responsible for H_2O_2-induced oxidation of phospholipid membranes and H_2O_2/NO_2^--mediated nitration of other proteins. These results suggest that nitration of cyt c by peroxynitrite may exacerbate oxidative damage to mitochondrial proteins and membranes.

Keywords: Cytochrome c; Peroxynitrite; Nitrotyrosine; Peroxidase

1. Introduction

Superoxide and nitric oxide are primary reactive oxygen/nitrogen species produced in cells. They react with each other at a diffusion-controlled rate ($k \sim 10^{10}$ $M^{-1}s^{-1}$) to form peroxynitrite. Being a strong oxidant, peroxynitrite modifies a variety of intracellular molecules including proteins, nucleic acids, and lipids (reviewed in [1]). Formation of 3-nitrotyrosine is considered a footprint of intracellular production of peroxynitrite.

Producing both superoxide and nitric oxide, mitochondria are a primary site for the formation of peroxynitrite. Evidence for the mitochondrial formation and reactions of peroxynitrite is summarized in a recent review by Radi et al. [2]. As a protein confined in the mitochondrial intermembrane space, cyt c may react with peroxynitrite as well. Indeed the formation of nitrotyrosine in cyt c has been observed in cells and in vivo including macrophages treated with NO donors [3], cancer cells treated with peroxynitrite [4], neurons deprived of oxygen and glucose [5], chronic allograft nephropathy [6], and renal cells undergoing ischemia/reperfusion [7].

According to Cassina et al. [8], treatment of cyt c with peroxynitrite results in nitration of tyrosine-67, disruption of the heme-methionine bond, inactivation of electron transport activity, and enhanced oxidation of 2,2′-azino-bis-[3-ethylbenzothiazoline]-6-sulfonate (ABTS) in the presence of H_2O_2. Nakagawa et al. [9] reported that nitrated cyt c does not support mitochondrial membrane potential.

Extending previous work by others [8,9], we studied the biochemical properties of nitrated cyt c in greater detail in order to understand its potential roles in nitrative stress. Cyanide binding and equilibrium unfolding of nitrated cyt c were analyzed. ABTS was replaced by 2',7'-dichlorodihydrofluorescein ($DCFH_2$), a widely used fluorescent probe for intracellular ROS, and the peroxidase activity of nitrated cyt c was measured under various conditions. We also examined other properties of nitrated cyt c relevant to oxidative stress, including reducibility by superoxide, peroxidatic oxidation of phospholipid membrane, and peroxidatic nitration of other proteins.

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2. Materials and methods

Horse heart cytochrome c, bovine milk xanthine oxidase, bovine liver catalase, bovine erythrocyte Cu,Zn-superoxide dismutase (CuZnSOD), and bovine serum albumin were from Sigma (St. Louis, MO, USA). Anti-nitrotyrosine antibody and anti-rabbit IgG were from Upstate Biochemicals (Lake Placid, NY, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. Peroxynitrite was prepared by the reaction of H_2O_2 and isoamyl nitrite [10]. Concentration of peroxynitrite was measured spectrophotometrically by using $\epsilon_{302} = 1670\ M^{-1}cm^{-1}$. $DCFH_2$ was obtained via hydrolysis of 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR, USA) in 1 mM NaOH. Buffer solutions were treated with Chelex-100 and 0.1 mM diethylenetriaminepentaacetic acid (DTPA) to remove trace metal ions. All measurements were carried out at 25 °C.

Nitrated cyt c was prepared by treating cyt c (500 μM) in 0.2 M KPi (pH 7.2) with aliquots (~300 μM each) of peroxynitrite until the 690 nm band disappeared. Normally the final concentration of peroxynitrite reached ~20 mM. Nitrated cyt c was dialyzed overnight to remove the decomposition products of peroxynitrite.

In order to obtain the equilibrium unfolding profiles, 5 μM of native or nitrated cyt c in 0.2 M KPi (pH 7.2) was incubated with 0-5 M guanidine hydrochloride for 4 h and the tryptophan fluorescence at 355 nm was measured using a spectrofluorometer (Perkin-Elmer LS-55, USA). The excitation wavelength was 290 nm.

Peroxidase activity of cyt c was measured with $DCFH_2$ as a substrate. To 10 μM $DCFH_2$ and 250 μM H_2O_2 in 0.1 M KPi (pH 7.2) was added 500 nM of native or nitrated cyt c. Increase in the fluorescence of DCF due to peroxidatic oxidation of $DCFH_2$ was measured at 520 nm with an excitation at 480 nm.

For the measurement of peroxidatic oxidation of phospholipid membranes, heart cardiolipin and plant phosphatidylcholine were mixed in chloroform at a ratio of 3:7. The resulting solution was dried with a stream of nitrogen and suspended in 20 mM HEPES (pH 7.4). Liposomes were prepared by a membrane (pore size 20 μm) extrusion technique. In order to measure the lipid hydroperoxide formation, liposomes (7.4 mg/ml) were incubated with 1 mM H_2O_2. Peroxidatic oxidation was initiated by adding 5 μM cyt c and the oxygen consumption was measured by using a Clark type oxygen electrode (Rank Brothers, Cambridge, UK).

Nitrotyrosine in proteins was detected by Western blotting. To CuZnSOD or albumin (20 μM) was added 1 mM H_2O_2, 0.5 mM nitrite, and 20 μM cyt c. The reaction was allowed to proceed for 10 min. The samples were subjected to 15% SDS-PAGE and nitrotyrosine was detected by using anti-nitrotyrosine antibody.

3. Results

Cassina et al. [8] reported that addition of peroxynitrite to native cyt c resulted in modified cyt c, in which tyrosine-67 was nitrated and the heme-methionine bond was cleaved. We treated cyt c with aliquots of peroxynitrite to ensure complete bleaching of the absorption band at 690 nm, an indicative of the heme-methionine bond. The Soret band at 410 nm was also blue-shifted to 405 nm (Fig. 1, inset). Since the sixth ligand of the heme, methionine-80, is disrupted in peroxynitrite-treated cyt c, nitrated cyt c is expected to bind strong field ligands such as cyanide and azide much more efficiently than the native protein. Addition of cyanide to the nitrated cyt c resulted in a red-shift of the Soret band to 412 nm (Fig. 1, inset), which we attribute to a six-coordinate heme-cyanide complex. Cyanide binding to native cyt c was extremely slow in agreement with previous work [11].

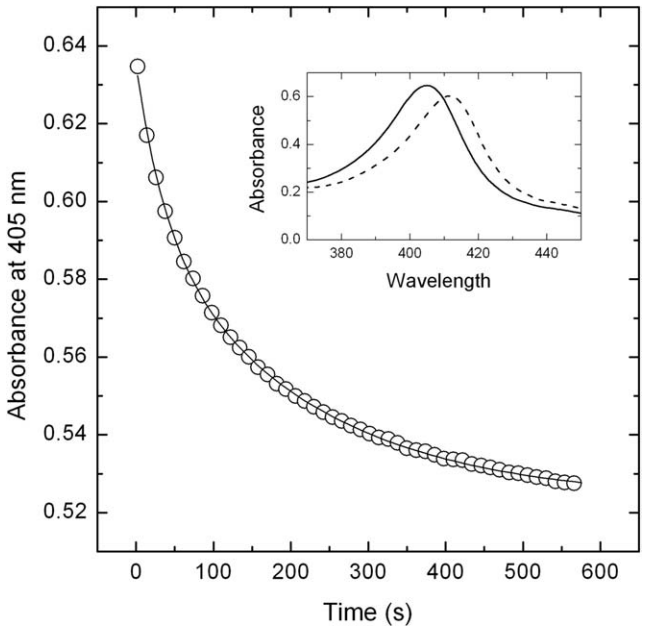

Fig. 1. Binding of cyanide to nitrated cyt c. Cyanide (1 mM) was added to nitrated cyt c (5 μM) in 0.1 M potassium phosphate (pH 7.2) and the absorbance at 405 nm was followed. The data were fitted by two exponentials (solid line). Inset: The Soret band of nitrated cyt c before (solid) and after (dotted) addition of cyanide.

The time-dependent decrease in the 405 nm absorption yielded the kinetic parameters of the cyanide binding. As shown in Fig. 1, the data was fitted by two exponentials, $A(t)=0.039\exp(-0.028t)+0.073\exp(-0.0048t)+0.52$, which consisted of 35% fast phase and 65% slow phase. Assuming a second order reaction (i.e. rate = k[cyt c][CN^-]) and using [CN^-] = 1 mM and [cyt c] = 5 μM, we obtained the rate constants k for the fast and slow phases to be 28 and 4.8 $M^{-1}s^{-1}$, respectively. The biphasic kinetics may be due to a mixed population of singly and doubly nitrated cyt c. Multiply nitrated cyt c has been demonstrated by Cassina et al. [8].

Equilibrium unfolding of cyt c has been studied extensively due to availability of various spectroscopic techniques that can probe the structure of cyt c [12]. We obtained unfolding profiles of native and nitrated cyt c to examine their stability toward chaotropic agents. Cyt c has a unique tryptophan residue at position 59 which moves away from the heme when cyt c is unfolded by a chaotropic agent like guani-