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Structural and functional characterization of phosphomimetic mutants of cytochrome *c* at threonine 28 and serine 47

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ABSTRACT

Protein function is frequently modulated by post-translational modifications of specific residues. Cytochrome *c*, in particular, is phosphorylated *in vivo* at threonine 28 and serine 47. However, the effect of such modifications on the physiological functions of cytochrome *c* – namely, the transfer of electrons in the respiratory electron transport chain and the triggering of programmed cell death – is still unknown. Here we replace each of these two residues by aspartate, in order to mimic phosphorylation, and report the structural and functional changes in the resulting cytochrome *c* variants. We find that the T28D mutant causes a 30-mV decrease on the midpoint redox potential and lowers the affinity for the distal site of *Arabidopsis thaliana* cytochrome c_1 in complex III. Both the T28D and S47D variants display a higher efficiency as electron donors for the cytochrome *c* oxidase activity of complex IV. In both protein mutants, the peroxidase activity is significantly higher, which is related to the ability of cytochrome *c* to leave the mitochondria and reach the cytoplasm. We also find that both mutations at serine 47 (S47D and S47A) impair the ability of cytoplasmic cytochrome *c* to activate the caspases cascade, which is essential for triggering programmed cell death.

1. Introduction

Post-translational modifications of proteins are relevant regulatory mechanisms to control an ample set of cell metabolic processes. One of the most usual modifications is phosphorylation. Besides the concomitant change in electrostatics, phosphorylation may alter the structural features of proteins [1] and affect the interactions with their partners [2]. Phosphorylation is modulated by kinases and phosphatases, which are regulated by redox signaling [3–5]. The latter is important in the context of mitochondria, which constitute the main source of reactive oxygen/nitrogen species (RNOS) in the cell [6].

Cytochrome *c* (Cyt *c*) is a small soluble heme-protein localized in the mitochondrial intermembrane space under homeostatic conditions [7]. It takes part in the electron transport chain (ETC), carrying electrons from the cytochrome bc_1 complex (Cyt bc_1) to cytochrome *c* oxidase (COX). Recently, nuclear magnetic resonance (NMR) and computational data revealed that cytochrome c_1 (Cyt c_1), a subunit of Cbc_1, has two binding sites for Cyt *c*. The first site – the so-called proximal site – is located near the heme and is compatible with electron transfer. The second site – the so-called distal site – lays far from the heme and it has been proposed to be a local energy minimum in the restrained Cyt *c* diffusion pathway towards COX [8,9]. Notably, Cyt *c* also interacts with two binding sites on COX [9]. However, the surface regions on Cyt *c* responsible for binding to Cyt c_1 [8,9] and COX [10] are different. Under severe oxidative stress conditions, Cyt *c* exits mitochondria and acts as a programmed cell death (PCD) inductor by interacting with several proteins in the cytoplasm and the nucleus [11–15]. The first reported cytoplasmic interaction of Cyt *c* was with the apoptosis protease activation factor-1 (Apaf-1), which is responsible for the apoptosome assembly and subsequent activation of caspases [16,17]. The apoptosis-inducing form of Cyt *c* has been suggested to be membrane-bound [18–21]. Indeed, a substantial population of Cyt *c* is bound to cardiolipin (CL) in the mitochondrial inner membrane [22].

Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; Apaf-1, apoptosis protease activation factor-1; Cyt bc_1, cytochrome bc_1 complex; Cyt *c*, cytochrome *c*; Cyt c_1, cytochrome c_1; COX, cytochrome *c* oxidase; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CL, cardiolipin; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DSC, differential scanning calorimetry; E'_0, midpoint reduction potential; EMSA, electrophoretic mobility shift assay; ETC, electron transfer chain; H_2DCF, dichlorofluorescein diacetate; HEK, human embryonic kidney; ITC, isothermal titration calorimetry; MD, molecular dynamics; NMR, nuclear magnetic resonance; RMSD, root mean square deviation; T_m, midpoint melting temperature; PCD, programmed cell death; TOCL, 1,1′2,2′-tetraoleoylcardiolipin.

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Binding to CL results in a conformational change in the protein [23–25] allowing the entry of a hydrogen peroxide molecule [26]. This enhances the peroxidase activity of Cyt *c* [19,27], so favoring CL oxidation and the release of Cyt *c* to the cytosol [28,29]. Both functions are regulated by post-translational modifications, such as nitration [30–32] or phosphorylation [33–35].

Phosphorylation of Cyt *c* accompanies several pathological situations, including ischemia or cancer [36,37]. *In vivo*, Cyt *c* is phosphorylated in Tyr48 and Tyr97 [33,35]. Tyrosine phosphorylations of Cyt *c* have been classically studied by Tyr to Glu mutation [38,39]. However, this change causes a substantial decrease in the residue volume and surface area. To solve this problem, a new approach based in the Evolved tRNA Synthetase Technique has been lately published [40]. Recently, two additional phosphorylation sites at Thr28 and Ser47 have been reported in the human skeletal muscle [41] (Fig. S1A). Nonetheless, how these modifications affect the structure and the function of Cyt *c* remains unclear. The difficulty of the analysis is that the specific Cyt *c*-phosphorylating kinase remains unknown and isolated Cyt *c* does not preserve the *in vivo* phosphorylation at these positions.

In this work, we analyze two phosphomimetic mutants of human Cyt *c* in which Thr28 or Ser47 are replaced by aspartate (T28D and S47D, respectively). For comparative purposes, two other mutants at the same positions (T28A and S47A) were analyzed to differentiate the effects due to the presence of a negatively charged residue. We show that none of these amino acid substitutions affect the overall structure of Cyt *c*. However, the T28D mutation alters the ability of Cyt *c* to bind to the distal site of *Arabidopsis thaliana* Cyt c_1 and diminishes its redox potential, whereas its oxidation *in vitro* by COX becomes more efficient. In addition, mutations on Ser47 affect the functionality of Cyt *c* in terms of peroxidase activity and ability for caspase activation.

2. Materials and methods

2.1. Site-directed mutagenesis, protein expression and purification of recombinant proteins

Genetic manipulations were performed on a pBTR-1 plasmid (pCyt*c*WT) comprising the CYCS and CYC3 genes, coding for human Cyt *c* and the yeast Cyt *c* heme lyase, respectively. The last enzyme is required for the proper cytoplasmic maturation of human Cyt *c*. Endogenous *c*-type cytochromes in *Escherichia coli* are usually exported to the periplasm space. However, the sequences of the above recombinant gene lacked topogenic sequences, thereby avoiding the export of the gene products to periplasm. Then, the resulting proteins were located in the bacterial cytoplasm. The selectable marker of pCytcWT was a cassette that conferred ampicillin resistance to cells containing this plasmid [42]. pCyt*c*WT was mutated by replacing the ACC triplet coding Thr28 and GGC triplet coding Ser47 in CYCS gene – coding Cyt *c* – with GAT to obtain T28D and S47D, and with GCG to T28A and S47A mutants, respectively. For this purpose, the one-step mutagenic PCR with Accusure™ DNA Polymerase (Bioline) was used following manufacturer's instructions. *E. coli* DH5α was used as a host in all cloning procedures. Plasmid DNA was transferred to *E. coli* strain following the standard heat-shock transformation method. In all cloning procedures involving PCR amplification, the sequences of the amplified fragments were checked with the aid of a commercial sequencing service (StabVida, Caparica, Portugal). The new plasmids, containing the mutated sequences, were called, pCyt*c*T28D, pCyt*c*S47D, pCyt*c*T28A and pCyt*c*S47A. The primers for mutagenic PCR were the following ones:

5-AAACACAAAGATGGTCCCAAC-3 (pCyt*c*T28D forward);
5-GTTGGGACCATCTTTGTGTTT-3 (pCyt*c*T28D reverse);
5-AAACACAAAGCGGGTCCCAAC-3 (pCyt*c*T28A forward);
5-GTTGGGACCCGCTTTGTGTTT-3 (pCyt*c*T28A reverse);
5-CCGGGCTACGATTACACGGCG-3 (pCyt*c*S47D forward);
5-CGCCGTGTAATCGTAGCCCGG-3 (pCyt*c*S47D reverse);
5-CCGGGCTACGCGTACACGGCG-3 (pCyt*c*S47A forward);
5-CGCCGTGTACGCGTAGCCCGG-3 (pCyt*c*S47A reverse).

In order to obtain the four human Cyt *c* mutants T28D, S47D, T28A and S47A, *E. coli* BL-21 (DE3) cells were transformed with the plasmids pCyt*c*T28D, pCyt*c*S47D, pCyt*c*T28A and pCyt*c*S47A, respectively. Cells were cultured for 24 h at 30 °C and 150 rpm in LB rich media supplemented with ampicillin and δ-aminolevulinic acid at final concentrations of 0.1 mg mL^{-1} and 0.1 mM, respectively. Cells were collected by centrifugation (9,000*g* for 10 min), and then resuspended in 1.5 mM borate buffer pH 8.5 (10 mL of buffer per 1 L of culture), supplemented with 1 mM PMSF, 0.02 mg mL^{-1} DNase and 0.2 mg mL^{-1} lysozyme. The cytoplasmic fraction was obtained by sonication on a BRANSON Digital Sonifier® cell disrupter. The cells were intermittently sonicated on ice for 30 s with 60 s allowed for cooling and a 40% vibration amplitude. The total sonication time was 4 min. The resulting suspension was centrifuged at 20,000*g* for 15 min. Then, the supernatant was collected and loaded onto a carboxymethyl cellulose ion exchange column (Sigma® C-4146) pre-equilibrated with 1.5 mM borate buffer pH 8.5. The different mutants of Cyt *c* were eluted from the column along a 36–360 mM NaCl gradient and tested by UV–Vis spectrophotometry in a Jasco V-650 apparatus. The A_{280}/A_{550} ratio of the resulting Cyt *c* preparations in the reduced state was *ca.* 1.0, as previously described [30]. Tryptic digestion and MALDI-TOF analysis (Brüker Daltonics, Germany) confirmed the molecular mass and the different substitutions for each mutant. Protein concentrations were determined by visible spectrophotometry, using an extinction coefficient of 29 mM^{-1} cm^{-1} at 550 nm wavelength for the reduced Cyt *c* species.

2.2. Circular dichroism spectroscopy

All circular dichroism (CD) spectra were recorded using a Jasco J-815 spectropolarimeter equipped with a Peltier temperature-control system. CD intensities are presented in terms of molar ellipticity [θ_{molar}] using molar protein concentration [43]. The secondary structure analyses were carried out by recording far-UV CD spectra (185–250 nm) at 25 °C in a 1-mm quartz cuvette. Samples contained 3 μM protein in 10 mM sodium phosphate pH 6.5, supplemented with 10 μM potassium ferricyanide. For each sample, 20 scans were averaged and analyzed using the CDpro software package [44,45] with the SMP50 and SP37A reference set, as well as with the CLSTR option to compare with a set of proteins with similar folds.

The coordination of the heme iron atom to the S_δ atom of Met80 – the sixth axial ligand of heme group – was analyzed by visible (B-band) recording visible CD spectra (300–600 nm) at 25 °C in a 10-mm quartz cuvette, as previously reported [46]. Samples contained 30 μM protein in 10 mM sodium phosphate pH 6.5, supplemented with 100 μM potassium ferricyanide.

Thermal unfolding was monitored between 20 and 105 °C (with a heating rate of 1 °C/min) by recording the CD signal at far-UV in a 10-mm quartz cuvette. For these assays, the oxidized Cyt *c* species were at 3 μM final concentration in 10 mM sodium phosphate pH 6.5 supplemented with 10 μM potassium ferricyanide. Changes in the tertiary structure of the protein upon increasing the temperature were simultaneously monitored by measuring the changes in total fluorescence at 270 nm excitation wavelength, which are attributed to the exposure of Trp59 to the solvent [47]. The sample mixture contained 30 μM Cyt *c* in 10 mM sodium phosphate pH 6.5 and 100 μM potassium ferricyanide. The experimental data were fitted to a two state native-denatured equilibrium model.