Nitration of tyrosine 74 prevents human cytochrome c to play a key role in apoptosis signaling by blocking caspase-9 activation

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Abstract: Tyrosine nitration is one of the most common post-transcriptional modifications of proteins, so affecting their structure and function. Human cytochrome c, with five tyrosine residues, is an excellent case study as it is a well-known protein playing a double physiological role in different cell compartments. On one hand, it acts as electron carrier within the mitochondrial respiratory electron transport chain, and on the other hand, it serves as a cytoplasmic apoptosis-triggering agent. In a previous paper, we reported the effect of nitration on physicochemical and kinetic features of monotyrosine cytochrome c mutants. Here, we analyse the nitration-induced changes in secondary structure, thermal stability, haem environment, alkaline transition and molecular dynamics of three of such monotyrosine mutants – the so-called h-Y67, h-Y74 and h-Y97 – which have four tyrosines replaced by phenylalanines and just keep the tyrosine residue giving its number to the mutant. The resulting data, along with the functional analyses of the three mutants, indicate that it is the specific nitration of solvent-exposed Tyr74 which enhances the peroxidase activity and blocks the ability of Cc to activate caspase-9, thereby preventing the apoptosis signaling pathway.

Keywords: Alkaline transition; Apoptosis; Cytochrome c; Peroxidase activity; Post-translational modification; Tyrosine nitration.

Abbreviations: Ac-LEHD-AFC, N-acetyl-Leu-Glu-His-Asp-(7-amino-4-trifluoromethyl coumarin); Apaf-1-xl, apoptosis protease-activating factor-1-x-long; BSA, bovine serum albumin; Cc, cytochrome c; CD, circular dichroism; CLSTR, CLuSTeR CDPro database; DSF, differential scanning fluorimetry; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; H2DCF, reduced 2′7′- dichlorofluorescein; HEPES, N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid; MD, molecular dynamics; PC9, pro-caspase 9; PMSF, phenylmethanesulphonylfluoride; RMSD, root mean square deviation; RMSF, root mean square fluctuations; RNS, reactive nitrogen/oxygen species; Tm, midpoint melting temperature.

1. Introduction

Protein tyrosine nitration is a post-translational modification that occurs under the action of a nitrating agent and results in the addition of an NO2 group at the ortho position of the phenolic hydroxyl group. Although more extensively studied in animals, it seems to be a common process in all living organisms, ranging from bacteria to yeasts, plants and animals. The fact that tyrosine nitration is a low yield process in vivo [1] makes it difficult for analytical methods to detect such covalent modification in complex proteomes [2]. However, it exhibits an apparent high selectivity and specificity for the modified proteins and is a more stable post-translational modification than tyrosine phosphorylation. Actually, the cumulative protein tyrosine nitration might be responsible for alterations in protein function, turnover and localisation [3–5], with the concomitant implication in the pathogenesis of some diseases related to nitrosative stress [3, 6, 7]. Initially, the generation of nitrotyrosine was considered an irreversible process, but recent reports indicate that the accumulation of nitrated proteins results from the increased generation
of reactive nitrogen/oxygen species (RNOS), as well as from the inhibition of the proteasome [8] and/or the denitration system [7].

Mitochondrial matrix appears to be the primary locus for protein tyrosine modification because the peroxynitrite anion – the in vivo nitrating agent formed by the reaction between superoxide anion and nitric oxide – can diffuse from extramitochondrial compartments into mitochondria or can be formed intramitochondrally through the generation of RNOS because of the activity of complexes I–III of the respiratory chain [9,10]. Even though RNOS are mainly neutralised in cells by specific mechanisms or enzymes, such as manganese superoxide dismutase, under physiological conditions [11,12], the excessive RNOS production under nitrooxidative stress leads to the oxidation and nitrination of many mitochondrial proteins, such as the referred superoxide dismutase, whose inactivation involves a positive feedback cycle for nitrination.

Respiratory cytochrome c (Cc) is the main target for RNOS in mitochondria, where it is both nitrated and nitrosylated in vivo [10,13–15]. In human Cc, there are five tyrosine residues, at positions 46, 48, 67, 74, and 97 (Fig. 1A). Four of them are widely conserved, whereas Tyr46 is present in humans and plants but not in horse (Fig. 1C). Previous studies show that the most surface-exposed tyrosines in horse Cc – Tyr74 and Tyr97 – are available for nitrination [16], although in vivo assays also suggest modifications on partially buried Tyr67 [17], in which the catalysis of nitrination is favoured by the proximity of the haem-group. The effects of tyrosine nitrination on protein function have been previously analysed using horse [4,14,16,18,19] and bovine [20] Cc. Among the changes induced by nitrination, the spectral changes due to the loss of haem coordination by Met80, which acts as the sixth ligand of the iron atom, should be highlighted. The behaviour followed by the haem coordination along pH titration starts in a conformational state III at neutral pH [21,22] and ends in an alkaline state IV at basic pH. Although keeping the low-spin state of iron, this involves a significant change in the haem axial ligands because of displacement of Met80 and substitution by Lys73 or Lys79 [22,23]. Replacement of Met80 by alanine disturbs the protein localisation [24], and the Cc alkaline transition between states III and IV shifts towards more physiological conditions upon nitrination [4,18,25]. Nitrated Cc, whose redox potential is drastically changed, is unable to react with the complexes cytochrome bc1 and cytochrome c oxidase, so failing in sustaining the respiratory electron flow [19,26].

In addition to the well-established role of Cc in energy metabolism, the haem protein triggers the apoptosis signaling pathway. In fact, it seems that nitrination impairs the ability of Cc to activate caspases [17,20,26] during programmed cell death. Moreover, the posttranscriptionally modified Cc undergoes changes in its haem moiety and enhances its peroxidatic activity [1,18,19]. Also, the catalytic properties of Cc have been extensively studied in the apoptosissubduing Cc–cardiolipin complex [27–30].

The experiments herein presented are focussed on the mononitrosylated mutants of human Cc h-Y67, h-Y74 and h-Y97 previously designed in our laboratory [26], in which all its tyrosine residues except one – just that at the indicated position – have been substituted by phenylalanine. The h-Y46 and h-Y48 mutants are not studies here because a comparison between the diamagnetic 1H-NMR spectra of the nitrated and non-nitrated species of the five mononitrosylated Cc mutants reveals no signal dispersion of amide and methyl protons in the nitrated species of h-Y46 and h-Y48 (the so-called h-Y46:N and h-Y48:N), thus suggesting that protein unfolding and/or aggregation events could take place upon specific tyrosine nitrination at these two positions (Fig. 1B). The wild-type (h-WT) and null (h-Null) Cc species – the latter, with all the five tyrosines replaced by phenylalanines – are used as controls. Based on this set of haem proteins, we have analysed their physicochemical properties in terms of secondary structure, thermal stability, haem environment and dynamic behaviour. Also, the functional studies reveal that the specific nitrination of solvent-exposed Tyr74 enhances the peroxidase activity and blocks the caspases activation ability of Cc, thereby making this protein a master key in the apoptosis signaling pathway.

2. Materials and methods

2.1. Protein expression and purification

Recombinant human Cc, either the WT species or the mononitrosylated mutants, were expressed in E. coli strains and further purified by ionic exchange chromatography, as previously described [26]. The synthesis of peroxynitrite and the nitrination of the different Cc samples was as reported in [18,26] with minor modifications. Both Fe3+-EDTA concentration and number of additions of peroxynitrite were increased up to 1.5 mM and 10 bolus additions, respectively. The nitrination reaction was performed in acidic conditions (pH 5.0). Afterwards, nitrated Cc species were intensively washed with 10 mM potassium phosphate (pH 6.0) and purified as in ref. [26]. The tryptic digestion and MALDI-TOF (Bruker-Daltonics, Germany) analyses confirmed not only that the nitrated Cc preparations were purified to 95% homogeneity but also that the molecular mass and the specifically nitrated tyrosine of each mutant
were right. Moreover, Western Blotting Solution (Amersham) using antibodies anti-nitrotyrosine (Biotem) corroborated the presence of -NO₂ groups in the Cc samples undergoing the nitration protocol. Samples were concentrated to 0.2–2.0 nM in 5 mM sodium phosphate buffer (pH 6.0). The extinction coefficients of monothioline mutants – nitrated and non-nitrated forms – used to determine protein concentration by spectrophotometric methods are summarised in Supplementary Data [31].

Recombinant human Apaf-1-XL was expressed and purified as described in [32,33]. Apaf-1-XL was stored until use at -80 °C, at 2–10 μM final concentration, in 20 mM HEPES-KOH buffer (pH 8.0), supplemented with 10 mM MgCl₂, 1 mM dithiothreitol (DTT) and 20% glycerol (v/v).

Pro-caspase 9 (PC9) was subcloned into a pET23b vector with a C-terminal 6-His-tag. The expression plasmid was transformed into BL21(DE3)pLys S Codon plus E. coli strain. Cultured cells were growing for 3 h at 30 °C before harvesting upon induction with 0.2 mM IPTG at OD₆₀₀=0.6. PC9 was purified by using a 5 mL affinity column His-Trap HP (GE Healthcare), according to the manufacturer’s recommendations. Further purification was performed by ion exchange using a 5 mL column HiTrap Q HP.

2.2. Circular dichroism spectroscopy

All circular dichroism (CD) spectra were recorded on a Jasco J-815 spectropolarimeter, equipped with a Peltier temperature-control system and a 1-mm quartz cuvette. CD intensities are presented in terms of molar ellipticity [θ]molar using molar protein concentration and a 1-mm path-length. The analyses of the secondary structure of oxidised Cc samples – nitrated and non-nitrated – were carried out by recording their far-UV CD spectra (185–250 nm) at 25 °C. Protein concentration was 3 μM, and the buffer was 5 mM sodium phosphate (pH 6.0), supplemented with 10 μM potassium ferricyanide. For each sample, 20 scans were averaged and analysed using the CDpro software package [34] with the SMP50 reference set, as well as with the CLSTR option to compare with a set of proteins with similar folds.

Thermal unfolding was monitored between 30 and 105 °C. Temperature was increased at a rate of 1 °C per min, and protein unfolding was monitored by recording the CD signal at 220 nm. No reversibility of protein unfolding was observed for any of the samples. For all these assays, the oxidised Cc species at 3 μMfinal concentration were solved into 5 mM sodium phosphate buffer (pH 6.0) with 10 μM potassium ferricyanide. The experimental data were fitted to a two-state native-denatured model [35].

2.3. NMR spectroscopy

The oxidised Cc samples at ranging 0.4–0.6 mM concentration were prepared in H₂O(90%)/D₂O(10%) solutions of 5 mM sodium phosphate buffer (pH 6.0) with 0.2 mM potassium ferricyanide. NMR spectra were recorded at 25 °C on a Bruker Avance DRX spectrometer operating at 500 MHz ¹H frequency. The spectra were processed and analysed with the Bruker TOPSPIN 2.0 software (Bruker BioSpin 2006). Standard 1D ¹H NMR spectra were recorded using a spectral width of 20 ppm. Water suppression was achieved by pre-saturation pulses [36]. Spectral processing required a sine-square window function, as well as a polynomial baseline correction, which was made automatically.

For pH titration of oxidised Cc species, the pH of the samples was adjusted at the desired value by adding 0.1–0.5 M NaOH or 0.1–0.5 M HCl, and the 1D ¹H spectra were recorded at 25 °C by using the superWEFT pulse sequence (RD-P180-r-P90-AQ) to observe the fast relaxing signals [37]. In the superWEFT experiments, the spectral window was 100 ppm, the acquisition plus the relaxation delays were 32 ms and the inter-pulse delay, τ, was typically around 40 ms. An exponential window function combined with a polynomial baseline correction was used for spectral processing. The pK₅ values were determined by fitting the volume of haem methyl 8, haem methyl 3 and Met80 methyl signals of the Cc species as a function of pH to the Henderson-Hasselbalch equation.

2.4. EPR spectroscopy

EPR spectra were collected in a Bruker EMX spectrometer, equipped with an Oxford Instruments ESR-900 continuous-flow helium cryostat. Spectra were recorded at -258 °C, using a microwave attenuation of 20 dB (2 mW) and modulation amplitude of 1 mT. Four scans were averaged for each spectrum. Samples contained 100 μM Cc in 10 mM phosphate buffer at varying pH.

2.5. Electronic absorption spectroscopy

Electronic absorption spectra were recorded in the 600–750 nm range to analyse the band centred at 699 nm, indicative of the haem–Met-80 coordination [38], using a Beckman DU® 650 spectrophotometer in a 1-mL quartz cuvette of 10-mm path-length. The assays were performed with oxidised nitrated or non-nitrated Cc in 5 mM sodium phosphate buffer, supplemented with 0.2 mM ferricyanide, at a protein
concentration as high as 0.6 mM because of the relatively low intensity of the absorption band ($\epsilon_{280}=865 \text{ M}^{-1} \text{ cm}^{-1}$ in the 4.5–7.0 pH range) [25,39]. For pH titration studies, the optical spectra were collected at varying pH, which was adjusted at the desired value by adding 0.1–0.5 M NaOH or 0.1–0.5 M HCl. The pH values were determined by fitting the absorbance at 699 nm of the Cc species as a function of pH to the Henderson-Hasselbalch equation.

2.6. Molecular dynamics simulations

The Molecular Dynamics (MD) calculations were run using both a homology model and the most representative NMR structure of human Cc (PDB access code 1J3S) [40]. The MD simulations using the NMR structure showed large fluctuations and partial unfolding of the loop between residues 20 and 28, despite several equilibration protocols were assayed. The homology model was calculated with MODELLER [41] using the X-ray diffraction structures of Cc from horse heart (1HRC) [42], yeast (1YCC) [43], yeast iso-2 (1YEA) [44], and rice (1CCR) [45]. The maximum backbone RMSD between the templates and the nine best models resulting from annealing simulations was 0.74 Å.

MD simulations were carried out using AMBER 9.0 [46] under the AMBER 96 force field [47] in a Dell PowerEdge cluster. All calculations (minimum distance between protein and cell faces, initially set to 10 Å) cell geometry and PME electrostatics with an Ewald summation cutoff of 9 Å [48] force field parameters, corresponding to the oxidised haem moiety. The charges and force field parameters of nitrated tyrosine residues were derived from ab initio calculations in GAMESS [49] using the 631G* Basis set (unpublished data). Chloride counterions were added to neutralise charges. All systems were solvated with TIP3P water molecules [50] using the TLEAP module of AMBER. Protein side-chains were then energy-minimised (250 steepest descent and 750 conjugate gradient steps) down to a RMS energy gradient of 0.0125 kJ mol$^{-1}$ Å$^{-1}$, using the SANDER module of AMBER. Afterwards, solvent and counter-ions were subjected to 500 steps of energy minimisation and then submitted to NPT-MD using isotropic molecule position scaling and a pressure relaxation time of 2 ps at 25 °C. Temperature was regulated with Berendsen’s heat bath algorithm [51] using a coupling time constant equal to 0.5 ps. After 100 ps simulation, the density of the system reached a plateau. Then, for each protein, the whole system was energy-minimised and submitted to NVT-MD at 25 °C, using 1.6 fs integration time steps. Snapshots were saved every 4 ps. The SHAKE algorithm [52] was used to constrain bonds involving hydrogen atoms. Coordinate files were processed using the PTRAJ module of AMBER.

2.7. Peroxidase activity assays

The peroxidase activity of the different Cc species was determined as previously described [28], with minor modifications, by following the fluorescence increase of reduced 2′,7′-dichlorofluorescein (H$_2$DCF) upon oxidation by hydrogen peroxide. The fluorescence emission of Cc samples in a 1.0 mL quartz cuvette, with an excitation wavelength of 502 nm, were measured at 522 nm in a Cary Eclipse (Varian) fluorescence spectrophotometer with optical slits of 1.5 nm. Each sample contained 1 μM Cc and 500 nM H$_2$DCF in 50 mM sodium phosphate buffer, at a pH value ranging between 5.0 and 9.0. Upon addition of 100 μM H$_2$O$_2$, the peroxidase activity was estimated by monitoring the fluorescence increase at 522 nm. Bovine serum albumin (BSA) was used as a control. Each experimental data were the average of at least eight independent measurements.

2.8. Apaf-1/Cc cross-linking and light scattering

For cross-linking experiments, 100 μg of Jurkat cell extracts, obtained and treated as previously described [26], were incubated for 15 min at 37 °C with 200 μM ATP, 200 μMDTT, 20 mM KCl, 2.5 μM Cc and 4 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in a final volume of 25 μL. To stop the reaction by unfolding the proteins, 10 μL of Laemmli buffer was added. The resulting sample was first loaded onto a 6% polyacrylamide electrophoretic gel, and after transferred to a nitrocellulose membrane (Trans-Blot, BioRad), which was incubated overnight with polyclonal antibodies against Cc. Labelled proteins, after being incubated with a second antibody anti-rabbit IgG-HRP conjugate (Sigma), were detected by exposition to an X-ray film upon addition of the ECL Western Blotting Solution (Amersham). As a control of the cross-linking experiments, antibodies against Apaf-1 (Santa Cruz Biotechnologies) were used. 100 μL of Jurkat cells extract, previously obtained as described above, was incubated with 2 μg of anti-Apaf-1, which interacts with protein A in a Sepharose matrix (GE Healthcare). After centrifugation, the precipitated matrix was dissolved in Laemmli buffer, loaded onto a polyacrylamide gel and detected as explained above.

Light scattering was followed in a Jasco FP-6500 spectrophotometer, using a 0.1 mL quartz cuvette (light path, 10 mm) with an excitation and emission wavelength of 500 nm. Light dispersion was successively recorded, first with the buffer alone (20 mMHEPES-KOH, pH 7.5, containing 10 mM KCl, 1.5 mM
MgCl2, 1 mM EDTA and 1 mM EGTA), for 1 min, upon addition of Apaf-1-xl at 1 μM final concentration for one more minute, and finally upon addition of Cc at 10 μM final concentration.

2.9. Caspase-9 activation

In vitro assay of caspase-9 activation was as described in [33]. Nitrated and non-nitrated Cc species, at 20–40 nM final concentration, were incubated with 100 nM Apaf-1 and 100 μM dATP in a total volume of 195 μL of 20 mM HEPES-KOH buffer (pH 7.5) containing 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.1 mM PMSF. After 1 h incubation at 30 °C, 5 μL of 4 μM PC9 were added to each sample up to reach a final concentration of 100 nM. After incubation for another 10 min at 30 °C, the caspase-9 substrate Ac-LEHD-AFC (Sigma) was added to 50 μM final concentration. The increase in fluorescence resulting from Ac-LEHD-AFC cleavage was determined in a Victor fluorescent detector (Perkin Elmer, USA), using an excitation wavelength of 400 nm and an emission wavelength of 508 nm. Each experimental data were the average of at least three independent measurements.

3. Results

3.1. Changes in secondary structure

Far-UV CD spectra reveal that the tyrosine-by-phenylalanine mutations significantly favour the α-helix formation at the expense of turns (see Supplementary Data). This is as expected because of the larger tendency of phenylalanines and tyrosines to be in helices and turns, respectively [53]. However, the α-helical content of monotypyrine Cc mutants is larger than that of h-Null, a finding that can be specifically ascribed to the particular re-arrangement of the H-bond network (see MD data below). Moreover, the CD spectra (Fig. 2) show no substantial changes in the global secondary structure content of the nitrated and non-nitrated forms of h-Y67, h-Y74 and h-Y97.

Protein oxidation could take place during the peroxynitrite treatment. Hence, to test the effect that the nitration reaction could have on Cc folding, the h-Null Cc mutant – with all its five tyrosine residues replaced by phenylalanines – was subjected to the same nitration protocol as the monotypyrine mutants. The far-UV CD spectrum of the resulting protein, the so-called h-Null:N, is identical to that of the non-treated h-Null (see Supplementary Data), thus suggesting that the differences in secondary structure mentioned above are specifically due to nitrated tyrosines.

3.2. Thermal stability of cc mutants

Given that nitration itself hardly alters the global folding of Cc (Fig. 2), we tested whether the chemical treatment of Cc withONOO−, a highly reactive molecule, or addition of a −NO2 group to tyrosine residues could affect the thermal stability of the metalloprotein. 220-nm CD spectroscopy showed that the loss of the phenolic −OH group because of the tyrosine-by-phenylalanine mutation has no consequences on haem protein stability (Table 1). Actually, h-WT and h-Null show similar values for the midpoint melting temperature (Tm), namely 85.9 °C and 85.3 °C, respectively (see Supplementary Data). Moreover, the h-Null:N mutant, which was subjected to nitration, behaves as h-Null in terms of stability (Table 1).

The change in the Tm value (ΔTm) of the monotypyrine Cc mutants upon addition of a ∼NO2 group to the remaining tyrosine residue depends on the position of the modified residue. Whereas the thermal unfolding curves of h-Y67 and h-Y67:N are almost identical, the addition of a ∼NO2 group to Tyr74 or Tyr97 destabilises the proteins by ca. 4 or 2.5 °C, respectively (Fig. 3 and Table 1). These changes in stability between nitrated and non-nitrated forms are confirmed by differential scanning fluorimetry (DSF) using the fluorescent SYPRO Orange dye, although ΔTm is a little higher with some mutants (Table 1). As the paramagnetic ∼NO2 group added at positions Tyr74 and Tyr97 is quite close to the π-orbital electrons of two aromatic residues (Trp59 and Phe10, respectively), the resulting repulsive forces between negative charges could compromise protein stability. Actually, the repulsion Tyr74-NO2−Trp59 is substantially more intense, so explaining the lower Tm value of this monotypyrine mutant and its greater instability upon nitration. Essentially, Trp59 is placed in between Tyr67 and Tyr74. Unlike Tyr74-NO2−, Tyr67-NO2 has no effect on Tm because its ∼NO2 group does not lie on the π-electron cloud of Trp59.

3.3. Alkaline transition

In the pH range between 1 and 12, oxidised Cc shows at least five different conformations because of changes in haem axial ligands and/or protein folding [38,54,55]. Previous work from Radi’s group [25] shows that the addition of a ∼NO2 to Tyr74 of horse Cc shifts the pKs value of the alkaline transition towards neutral pH. We thus investigated the effect of nitration on the pH dependence of monotypyrine mutants of human Cc in which only one of the residues Tyr67, Tyr74 or Tyr97 is chemically modified. First, 1H NMR analyses, along with visible absorption and EPR measurements, were performed to link
the presence of Tyr-NO₂ with the pKₐ value of the well-described alkaline transition in cytochromes. Second, the pH dependence of each nitrated monooxygine Cc mutant was correlated with the measurements of its peroxidase activity.

The haem group and its axial iron ligands in cytochromes have been extensively characterised by ¹H NMR spectroscopy [56,57]. Actually, the superWEFT pulse sequence has been used in pH titration studies to observe the fast relaxing signals located close to the Fe(III) ion of Cc species [37]. In our superWEFT experiments, the spectral window was 100 ppm to display the resonances corresponding to haem methyl 8, haem methyl 3 and that of the haem axial ligand Met80 (Fig. 4A). Actually, the ¹H NMR spectra of Cc mutants showed that the resonances corresponding to the neutral and alkaline species are identical to those observed with WT. Interestingly, the multiple set of haem methyl-8 signals with nitrated Cc mutants reveals that such a group adopts several conformations because of an increased in the dynamics of the Cc Ω-loop upon nitration (see below). For all Cc samples, the down- and up-field signals underwent substantial line broadening upon pH titration. However, the changes affecting the haem methyl resonances revealed an overall perturbation of the haem environment, whereas the loss of the intense up-field signal at ca. –20 ppm, corresponding to the ε-CH₃ group of Met80, unequivocally evidenced the detachment of Met80 from the haem iron.

Moreover, the broad resonance located at ca. –9 ppm can be only identified in the alkaline forms of h-WT, h-Y67 and h-Y67:N. It closely resembles a signal assigned to Lys79, which could serve as the axial ligand instead of Met80. Even though such signal is not detected in h-Y74:N, the EPR measurements (see below) confirm not only that the low-spin character at the Fe(III) centre is conserved for all these samples, but also that the metal site structure in the alkaline form is independent of the Cc post-translational modification by nitration.

The pH titrations allow us to determine a pKₐ value for every nitrated and non-nitrated forms of the three monooxygine Cc mutants (Fig. 4A), along with those of the h-WT and h-Null species. The chemical process itself, as well as methionine oxidation derived from the nitration reaction, has no effect on either the neutral or the alkaline forms of Cc, as can be seen in the h-Null:N species used as a control. Alkaline transition was checked to be a reversible process for all Cc samples, recovering the ¹H line-width at pH ca. 6.5 (see Supplementary Data).

In Fig. 4A, the pH titrations of nitrated and non-nitrated forms of h-Y67, h-Y74 and h-Y97 followed by ¹H NMR spectroscopy are shown. In all cases, raising the pH resulted in similar spectral perturbations although the pKₐ value of the alkaline transition is highly dependent on the mutant and on its nitration state. Interestingly, the tyrosine-biphénylalanine mutations in h-Y74, h-Y97 (Fig. 4A) and h-Null increase the pKa values by more than one pH unit compared with h-WT (pKₐ of 9.3) (see Supplementary Data). Nonetheless, in the case of h-Y67 (Fig. 4), the ¹H NMR spectroscopy displays some perturbations compared with the spectrum of h-WT, although the pKa value of h-Y67 is lowered by 0.8 pH units (pKa of 8.5, Table 2). This finding demonstrates, on one hand, that the H-bonding network in which Tyr-OH is involved modulates the transition of Cc from state III to “alternative low-spin conformations,” which in most cases correspond to state IV. On the other hand, the deprotonation of tyrosine in the h-Y67 mutant specifically triggers a conformational change in the protein coupled to the shift of alkaline transition towards near-to-neutral pH, as described by Radi and co-workers [25]. Although this is in agreement with the proximity of Tyr67 to the haem pocket (Fig. 1A), nitration of such residue has little effect on the pKₐ value (Table 2).

Nitration of the most solvent-exposed tyrosine residues – Tyr74 and Tyr97 – makes the respective h-Y74 and h-Y97 mutants behave in a different way, a finding that is in marked contrast to the previously reported data by Abrira et al. [25] but that can be ascribed to the fact that the chemical procedure to nitrate Tyr97 also nitrates Tyr74 when it is present in the protein. Here, the alkaline transition for the h-Y97: N mutant is identical to that of h-Y97, whereas the nitration of Tyr74 (in the so-called h-Y74:N mutant) lowers the pKₐ to 7.8 (Table 2). It thus seems that both Tyr67-OH and Tyr74-NO₂ collaborate in shifting the alkaline transition of human Cc to physiological conditions, which becomes a phenomenon of biological relevance.

Fig. 4B shows the EPR spectra of nitrated and non-nitrated forms of h-Y67, h-Y74 and h-Y97 at different pH values. At pH 6.0, all the samples show a mixture of high- and low-spin states. Actually, the signal with a g-value of 5.7 is typical of a high-spin Fe(III) atom in a tetragonal ligand field, consistent with a penta-coordinated iron atom. Such assignment is compatible with the existence of a 625 nm band in the visible absorption spectra (Fig. 5). In addition, two signals with g-values of 3.03 and 2.22 reveal a major population of hexa-coordinated low-spin iron, in agreement with NMR data. Finally, a small signal with a g-value of 4.30 indicates the presence of a high-spin ferric species with rhombic symmetry. This is
normally attributed to a population displaying damage at the metal site. Other signals are not easily identified at first glance, but they suggest that other low-spin states might also be populated at this pH value.

The shapes of the EPR spectra of h-Y67, h-Y67:N and h-Y74:N change significantly with increasing pH, as expected from other spectroscopic data herein presented, and only the signal at g equal to 4.30 remains unchanged. Both the signal corresponding to pentacoordinated high-spin iron and that matching the low-spin species fade along pH titration. Simultaneously, new low-spin signals appear. The h-Y67 mutant exhibits a main signal with $g_s=3.4$ and $g_a=2.2$. The h-Y67:N and h-Y74:N forms show, among others, signals at $g$-values of 3.2 and 2.1. Such changes appear, at least partially, at pH 8.0, in agreement with the alkaline transition $pK_a$ values calculated from NMR and UV/Vis spectra. In contrast, the spectra of h-Y97, h-Y97:N and h-Y74 do not change within the assayed pH range, in agreement with their high $pK_a$ value.

It is worth noting that the high-spin contributions are much larger in the case of h-Y67:N and h-Y74:N. Actually, their intensities are high enough as to make it feasible to detect a signal with a $g$-value equal to 2.0, which could be assigned to the weak $g$ of this species. Upon nitration, the two spin-state populations vary according to changes in both the relative energy and dynamics of the molecule. The absence of the high-spin state at high pH is consistent with a larger covalence (ligand field) in the haem moiety, as expected from a change of methionine by lysine.

The Fe-S$_8$(Met80) bond can also be studied by UV/Vis spectrometry by following the charge transfer band at 699 nm at varying pH [58]. The detailed spectrophotometric pH titration of h-Y67 and h-Y67:N shows that their 699 nm band is lost at pH values higher than 8.0 ($pK_a$ of 8.8 and 8.3, respectively; Table 2), thereby suggesting that only the Tyr67-OH group – and not Tyr67-NO$_2$ – is responsible for the alkaline transition to occur ‘earlier’ than in h-WT (Fig. 5).

In addition, the intensity of the 699 nm band in h-Y74:N does also significantly decrease when the spectrum is recorded at pH 8.0, shifting the $pK_a$ value by 1.5 units compared to h-WT ($pK_a$ of 7.8, Table 2). This behaviour retains the $pK_a$ drop of the phenolic –OH group from ca. 10 to 7.5 upon tyrosine nitration [59]. However, only Tyr74 nitration is capable of triggering the alkaline transition because no differences were observed between the nitrated and non-nitrated forms of h-Y67 and h-Y97 (Fig. 5 and Table 2). Also, the increase in the alkaline transition $pK_a$ upon tyrosine-by-phenylalanine replacement occurs in the h-Y74 and h-Y97 mutants (Fig. 5), as well as in the h-Null species, in good agreement with our NMR and EPR data (Table 2).

3.4. Molecular dynamics calculations

To test putative structural changes of Cc upon mutation and further nitration, we performed MD calculations on oxidised h-Y67 and h-Y74 as compared with the oxidised h-WT species. The h-Y97 forms were not calculated because none of them (either non-nitrated or nitrated) showed substantial changes in secondary structure and alkaline transition $pK_a$. Oxidised forms of single tyrosine mutants were simulated in both non-nitrated and nitrated states. The trajectory lengths were 10 ns, except those of the h-WT and h-Y74: N forms, which were 15.8 and 18.4 ns. All the trajectories reached a plateau in the backbone root mean square deviation (RMSD) with respect to the starting structure after a period ranging between 400 ps and 1 ns (Fig. 6). The maximum backbone RMSD average (at the plateau) along the calculations ranged from 0.91 to 1.53 Å, with the highest value corresponding to the h-Y74:N simulation.

The analysis of atomic fluctuations (Fig. 6) along the trajectories shows that all Cc species have a very flexible region between residues 20 and 29. In fact, the conformation of such a loop in the NMR structure of reduced human Cc (PDB code 1J3S) [40] is very different compared with other cytochromes, like those herein used as templates in the homology model (see Materials and methods). Calculations starting from the NMR structure were unstable, with average RMSD values larger than 4, because of unfolding of the 20–29 loop and surrounding regions. Another flexible region corresponds to the residues 42–47, which are near the above mentioned region.

Nitrated Tyr67 or Tyr74 affects the dynamics of the Ω-loop (comprising residues from Asn70 to Ile85), which contains the sixth Fe ligand (Met80) and nearby regions. The comparisons between fluctuations in the non-nitrated and nitrated forms of the h-Y67 and h-Y74 mutants are thus shown in Fig. 6. In addition to the two hotspots displayed in all simulations, h-Y67:N exhibits an enhanced motion in the sequence stretch between residues 71 and 77, as well as between residues 82 and 83. A minimum in this region corresponds to Met80, whose bond to the haem iron atom cannot be broken along simulations and imposes a restraint on the dynamics. In addition, the force field parameterisation [48] of this bond corresponds to a single state of the haem moiety. Hence, such an enhancement of the dynamics in the Ω-
loop is probably underestimated in the simulations. The fluctuations of the stretch involving residues 86–91, at one end of the \(\Omega\)-loop, are larger in h-Y74:N than in h-Y74.

In agreement with our CD data, h-Null and the monotyrosine Cc mutants hardly differ in their secondary structure content. However, the nitration of h-Y67 and h-Y74 slightly destabilises their \(\alpha\)-helices (see Supplementary Data). Actually, helices 2, 3 and 4 – the latter helix is located next to the \(\Omega\)-loop – are somewhat perturbed in h-Y67:N and h-Y74:N; the latter mutant even shows a destabilisation of the N-terminus of helix 5, at the other end of the \(\Omega\)-loop. Moreover, residues 35–37 shift their secondary structure from turns to 310-elices in the two nitrated mutants.

It has been recently proposed [25] that a H-bond between Tyr67-OH and the \(S_\delta\)(Met80) atom is responsible for the change induced by nitration in the alkaline transition \(pK_a\) of horse Cc. However, our analysis of the H-bonds involving residue side-chains along the MD trajectories (see Supplementary Data) reveals that no H-bond involving the \(S_\delta\)(Met80) atom can be detected in any calculation, but a weak H-bond is established by the \(O_γ\)(Thr78) atom with Tyr67-OH.

There are no differences in the highest occupancy (N85\%) H-bonds between the different Cc forms (see Supplementary Data). The largest differences in the H-bonding patterns between h-WT and the mutant species are located in the surroundings of the carboxyl group of the ring A propionate. Most of these changes are, however, small shifts on the occupancies of native bonds rather than the complete loss of any given bond. Nevertheless, Tyr48-OH binds to ring A propionate in h-WT but is missing in h-Null and all monoyrosine mutants. The occupancy of this bond is 76\% in h-WT, and its absence could explain the particular behaviour of h-Y67: The energy cost of burying the H-bonds surrounding Tyr67 cannot be compensated by such a neighbouring stabilising interaction. Hence, alkaline transition is favoured since it takes the Tyr67 ring outside, as inferred from the NMR structure of the alkaline yeast iso-1-ferricytochrome (PDB code 1LMS) [22]. This is not the case for h-Y74 because the hydroxyl group of Tyr74 is already exposed to solvent at neutral pH.

In summary, no differences in H-bonding can explain the different behaviours of the Cc species as regards their alkaline transition and peroxidase activity (see below).

### 3.5. Peroxidase activity of cc mutants

Like most haem proteins, Cc exhibits peroxidase activity. Lawrence and co-workers [60] attributed this peroxidase activity to the formation of a compound I-type intermediate when Cc reacts with \(H_2O_2\). However, there are controversial data concerning the relationship between any disruption of the Fe-\(S_\delta\)(Met80) bond of Cc and its peroxidase activity. Some authors attributed the observed increase in peroxidase activity upon alkaline transition [18,19,25] to the breakage of this bond. Others [28,61] propose that the protein matrix controls the catalytic activity of Cc by impairing the access of \(H_2O_2\) molecules to the haem crevice.

If the breakage of the Fe-\(S_\delta\)(Met80) bond is important for the peroxidase activity, this might be higher at pH values below the alkaline transition \(pK_a\) because of the exchange to penta-coordinated state, as inferred from our EPR data. Moreover, the alkaline form shows a stronger ligand field and, hence, a lower rate of ligand exchange and a more stable low-spin (hexa-coordinated) species. In contrast, the haem group shows a large solvent accessibility in the alkaline form of yeast iso-1-ferricytochrome [22], which is more susceptible to the effects of \(H_2O_2\). Therefore, we studied the pH dependency of the peroxidase activity of Cc.

Fig. 7 shows the typical kinetic traces of peroxidase activity assays. After a short time lag, the final reaction product accumulates and becomes detectable by fluorimetry. When comparing the data obtained with different Cc species, the slopes of the kinetic traces are similar at a given pH value but increase substantially from pH 7.5 to 8.0, no matter if the alkaline transition \(pK_a\) is around 11, as in h-Y97. Like in other systems [28], the peroxidase activity of h-Y97 increases with increasing pH, although the Fe-\(S_\delta\)(Met80) bond remains “intact”. Assuming a pH-dependent increase in the accessibility of the haem group [62], the differences in activity between h-Y67, h-Y74, h-Y97 and h-Null are related rather to the lag time than to the kinetic slopes (Fig. 7). The low peroxidase activity of h-WT could be ascribed to its tighter H-bonding network, which makes the protein structure more compact and hinders the access of \(H_2O_2\) to the haem group (see Supplementary Data). In agreement with our MD calculations (see above), Tyr48 may play a critical role since its replacement by phenylalanine impairs the formation of the stable H-bond with ring A propionate (see above) in h-WT but not in any of the mutants.

Among the nitrated mutants, only h-Y74:N shows a peroxidase activity significantly greater than its respective non-nitrated form (Fig. 7). Thus, the nitration of such particular tyrosine could be mainly
responsible for the change in enzymatic reaction. This seems to be due to a shortening of the lag phase rather than to an increase in the kinetic slope.

It is well-documented that the existence of nitrated tyrosines enhances the gain of peroxidatic activity of the Cc protein [18,19]. This phenomenon correlates well with a shift of the alkaline transition pKₐ towards near-neutral pH values [25], which are much closer to physiological conditions. In agreement with this, the h-Y74:N mutant experiences an “early” alkaline transition. These results provide insight into the role of Cc nitration, which switches from an electron transport carrier [26] into a peroxidase. So post-translationally modified Cc by nitration of Tyr74 could trigger the oxidation of mitochondrial phospholipid membranes, crucial for the further release of proapoptotic factors, including Cc [27], and caspases activation during the cell death pathway.

3.6. Cc-dependent activation of caspases

In a previous work [26], we studied the activation of caspases 3 and 7 upon the addition of non-nitrated or nitrated Cc forms to Jurkat cell extracts. The major drawback of such assays was that human Cc not only interacted with Apaf-1 and Cc, the cross-linking and light scattering measurements demonstrate that the protein complex is formed independently of whichever tyrosine residue is nitrated (Fig. 8). Actually, the increase in light scattering of the Apaf-1 samples upon addition of either nonnitrated or nitrated Cc mutants reveals that the complex is being formed. Therefore, the low ability of most nitrated Cc mutants to activate caspase-9 (Fig. 8) suggests that the chemically modified proteins are not fully functional in the apoptosome assembly. Actually, non-functional aggregates of the apoptosome have previously been observed when the exchange between Apaf-1-bound dADP and exogenous dATP fails [64].

As shown in Fig. 8, all non-nitrated mutants conserve most of their ability to activate caspase-9 at pH 7.5, thus suggesting that the tyrosine residues themselves are not essential for triggering apoptosis. Interestingly, h-Y97 and h-Null exhibit a decrease of ca. 20% in the caspase-9 activity compared to h-WT. However, nitration alters the ability of monotyrosine Cc mutants to activate caspases depending on the position of the tyrosine nitrated (Fig. 8). Actually, h-Y97:N shows activity levels similar to h-Y97, but the h-Y67:N and h-Y74:N mutants yield much lower values than their respective non-nitrated forms. At high protein concentration, h-Y67:N maintains ca. 45% of the h-Y67 activity and h-Y74:N only retains ca. 15% of the unmodified protein. Even more interesting is the finding that h-WT:N possesses an activity similar to h-Y74:N. Therefore, the inhibitory effect on caspase-9 activation observed with the polynitrated h-WT species could be ascribed to the specific nitration of Tyr74 rather than to any cooperative effect among different Tyr-NO₂ groups, contrary to the previously suggested mechanisms [26].

The identical behaviour of h-Null and h-Null:N assures that the nitration-dependent decrease in the ability of Cc to activate caspase-9 is not due to unspecific protein modifications arising from the treatment with peroxynitrite. All these data indicate that the interaction of Cc with Apaf-1 takes place mainly through the Ω-loop and, in particular, through the residues Lys72, Lys73 and Tyr74 [26,65,66]. Upon Tyr74 nitration, the alkaline transition shifts towards near-to-neutral pH values (pKₐ of 7.8), which induces the replacement of Met80 by Lys73 or Lys79 as haem axial ligand. Such conformational changes in the Ω-loop drastically affect the electrostatic surface properties of Cc, thereby contributing to the assembly of a non-functional apoptosome, which is unable to activate caspases and to drive cells to apoptosis.

4. Discussion

Post-translational covalent modifications – namely, phosphorylation, glycosylation and nitration; although the latter could rather be considered as a co-translational change [67] – are critical to coordinate the compartmentalised metabolism and destiny of proteins inside the cells, so defining the diversity of physiological roles played by these proteins. Actually, the RNOS produced in mitochondria are responsible for oxidation of protein tyrosine to 3-nitrotyrosine, thereby causing the loss or gain in protein functions.

Respiratory Cc is an excellent case study in which the specific addition of a –NO₂ group to Tyr74 inside the mitochondria has opposite effects on the energy metabolism and on the first steps of apoptotic signaling. On the basis of the lower redox potential values observed in nitrated Cc forms, our group has
recently reported that h-Y74:N is unable to react with cytochrome c oxidase, the well-known complex IV of the mitochondrial respiratory chain [26]. The data herein presented likewise indicate that the specific nitration of Tyr74 elicits a peroxidase activity in Cc at alkaline pH values. Investigation is currently in progress to check whether such activity could be further involved in the oxidation of membrane lipids, such as cardiolipin, and/or in the release of proapoptotic mitochondrial factors to the cytoplasm. It is well-described that non-nitrated Cc leaves to the cytoplasm after mitochondrial membrane permeation and continues the development of the apoptotic program by forming a functional apoptosome, upon binding to Apaf-1, in the cytosol.

Therefore, the post-translationally modified Cc upon nitration, mainly associated to the mitochondrial phospholipid membrane, seems to be essential in triggering the “early” apoptotic signaling events, whereas the cytoplasmic non-nitrated Cc amplifies the programmed cell death signal by caspases cascade activation. Interestingly, the two extra functional aspects of nitrated Cc – peroxidase activity and inactivation of apoptosome – are due to the specific nitration of Tyr74 and do not respond to any cooperative effect of the –NO$_2$ group added to any other tyrosine residue. The cell compartmentalisation of the apoptosis process, with nitrated proteins mainly in the mitochondria but not in the cytosol, would be partially lost by the presence of cytoplasmic nitrated Cc, which specifically competes with non-nitrated Cc by the Apaf-1 binding site and blocks the PC9 activation by forming a non-functional apoptosome.

An even higher level of complexity is attained when considering the oxidation of phosphatidylserine, a phospholipid allocated in the inner leaflet of plasma membrane, because of the strengthened catalytic activity of nitrated Cc. It has been proposed that the externalisation of oxidised phosphatidylserine on the surface of the plasma membrane is one of the hallmarks essential for the clearance of apoptotic cells via phagocytic pathways [61].

However, the low overall yield of in vivo protein nitration arises a number of questions about the physiological relevance of nitrated Cc. Protein nitration is a relatively widespread modification observed in vivo in a large number of proteins, both under normal and disease conditions, but it has been reported that only 1–5 residues over 10,000 tyrosines are nitrated in stressed tissues [4]. Taking also into account that no more than 10% of Cc is normally bound to the mitochondrial inner membrane [68], it is tempting to say that most of the Tyr74-nitrated Cc is closely associated to mitochondrial cardiolipin and, therefore, is unlikely that it can be involved in blocking the apoptosome formation in the cell cytoplasm.

The behaviour of h-Y74:N herein described in the apoptotic context must respond to drastic molecular changes experienced upon nitration in terms of haem environment, secondary structure content, thermal stability and alkaline transition of Cc. Actually, the addition of a –NO$_2$ group to Tyr74 makes the protein increase its content in turns but keep its overall folding.

Both Asn52 and Trp59 interact with the haem propionate groups (see Supplementary Data). Asn52 is a buried residue – with large effects on the global stability of the 16-residue Ω-loop [69] – that interacts with Tyr67, which is located in the 60's α-helix [70], through a water-mediated H-bond. It has recently been reported that the Hbonding network surrounding Tyr67 is essential for maintaining protein stability and increasing peroxidase activity [71]. Our pH titrations and MD studies reveal certain rearrangements in one of these interactions involving Thr78 and Tyr67, whose phenolic group is crucial in shifting the alkaline transition pK$_\text{a}$ towards neutral pH. It is worth mentioning that, contrary to previous assumptions [25], Tyr67 and Met80 are not close enough as to form a H-bond in any of the 20 conformations of the human Cc NMR structure [40]. Even though no direct contacts could be detected between Tyr67 and Met80, they are both connected through Ω-loop Thr78. Thus, Tyr74 nitration may perturb its interaction with Trp59, which would be tuned towards Asn52 to disrupt a water-mediated H-bond with Tyr67. Then, the Tyr67-Thr78 H-bond is impaired and the whole Ω-loop is shifted, as shown in Fig. 9. An alternative pathway has been proposed in nitrated horse Cc [25], in which the addition of a –NO$_2$ group to Tyr74 creates a steric hindrance with Glu66. However, Glu66 only contributes to the Ω-loop stability by means of a salt bridge with Lys73 [73], whose role in the Cc alkaline transition has been extensively discussed [74]. It thus seems that nitration of Tyr74 destabilises the Ω-loop and makes it much more flexible, as shown in our MD calculations. As a result, the alkaline transition shifts towards physiological pH values. Actually, the alkaline transition experienced by Cc matches the Ω-loop unfolding [74].

The peroxidase activity of the haem group depends on the rate of exchange of the Fe axial ligand by H$_2$O$_2$. In its turn, such a rate relies on the strength of the metal coordination and haem moiety accessibility. In addition, the protein dynamics modulates both terms. When Tyr74 is nitrated, the Ω-loop increases its mobility even at neutral pH, thereby weakening the bond between the S$_\text{ε}$(Met80) and Fe atoms, as the appraisal of the high-spin species indicates (Fig. 4). Above physiological pH, the S$_\text{ε}$(Met80) atom is replaced by the nearby Lys79 ε-amino group [72], which may form a stronger bond with the
metal, with the concomitant decrease in high-spin haem Fe population (Fig. 4). It is remarkable that the haem group is highly exposed to solvent in the alkaline structure of Cc [22]. Hence, the tighter binding of lysine to Fe might lead to a decrease in the intrinsic ligand exchange rate (with H2O2), but the larger accessibility for H2O2 yields a net increase in the peroxidase activity of Cc. In the alkaline structure, the conformational changes of theΩ-loop – which contains several residues, namely Lys72 and Lys73, that are involved in binding to Apaf-1 and cardiolipin – impact both on the assembly of a non-functional apoptosome unable to activate caspases [66] and on the electrostatic/hydrophobic interactions with anionic phospholipids [28,75]. In fact, the loss of tertiary structure caused by the interaction of Cc with mitochondrial membrane [75] favours the extended lipid anchorage to the haem crevice. Nonetheless, further studies concerning the interaction between mononitrotyrosine Cc species and anionic phospholipids embedded in micelles or liposomes are required to delve into the early stages of apoptosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

References


