Nitration of Solvent-Exposed Tyrosine-74 on Cytochrome c Triggers Heme Iron-Methionine-80 Bond Disruption: Nuclear Magnetic Resonance and Optical Spectroscopy Studies

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Summary

Cytochrome c, a mitochondrial electron transfer protein containing an hexacoordinated heme, is involved in other physiologically-relevant events, such as the triggering of apoptosis, and the activation of a peroxidatic activity. The latter occurs secondary to interactions with cardiolipin and/or post-translational modifications, including tyrosine nitration by peroxynitrite and other nitric oxide-derived oxidants. The gain-of-peroxidatic activity in nitrated cytochrome c has been related to a heme site transition in the physiological pH region, which normally occurs at alkaline pH in the native protein. Herein, we report a spectroscopic characterization of two nitrated variants of horse heart cytochrome c by using optical spectoscopies and NMR. Highly pure nitrated cytochrome c species modified at solvent exposed Tyr-74 or Tyr-97 were generated after treatment with a flux of peroxynitrite, separated and purified by preparative HPLC and characterized by mass spectrometry-based peptide mapping. It is shown that nitration of Tyr-74 elicits an early alkaline transition with a $pK_a = 7.2$, resulting in the displacement of the sixth and axial iron ligand Met-80 and replacement by a weaker Lys ligand to yield an alternative low spin conformation. Based on the study of site-specific Tyr to Phe mutants in the four conserved Tyr residues, we also show that this transition is not due to deprotonation of nitro-Tyr-74, but instead we propose a destabilizing steric effect of the nitro group in the mobile Ω-loop of cytochrome c, which is transmitted to the iron center via the nearby Tyr-67. The key role of Tyr-67 in promoting the transition through interactions with Met-80 was further substantiated in the Y67F mutant. These results therefore provide new insights about how a remote post-translational modification in cytochrome c such as tyrosine nitration triggers profound structural changes in the heme ligation and microenvironment and impacts in protein function.
Introduction

Cytochrome c is a small globular heme protein present at > 1 mM concentration in the intermembrane space of mitochondria, where it participates as a redox partner in the mitochondrial electron transport chain (1-4). In addition, when released to the cytosol it is involved in the apoptotic pathway (5,6). The iron ion at the heme moiety is hexacoordinated with His-18 and Met-80 as fifth and sixth heme ligands, respectively. (7) Cytochrome c can switch between the ferric and ferrous redox forms, with a characteristically high \(E^\circ\) of +260 mV (8,9). Spectroscopic studies have indicated that cytochrome c\(^{3+}\) (ferricytochrome c) can exist in at least five distinct conformations in the pH range 1-12, resulting from changes in heme axial coordination and/or in protein folding (10-12). Conformational state III is the dominant species at near neutral and physiological pH, and its structure has been studied extensively at near-atomic resolution (13-17). The transformation of the state III species (the “neutral” species from now on) into state IV species (the “alkaline” species) upon pH increase is called the alkaline transition (which occurs with a \(pK_a\) around 9.3) (13,18-24). This conformational change is present in all known isoforms of type-c cytochrome and implies the displacement of Met-80 from the heme and its substitution by another ligand, Lys-72 or Lys-7 (13,18,24,25), keeping the iron in a low spin state.

While initially not appreciated as a phenomenon of biological relevance, recent work has established that a transition from state III to alternative states can be triggered on a pH-independent fashion under biologically-relevant conditions and plays a role in mitochondrial physiology and apoptotic signaling (26). In this regard, cytochrome c association to a pool of the mitochondrial phospholipid cardiolipin-containing fatty acids with a high degree of unsaturation (27-29) and post-translational modifications such as methionine oxidation and tyrosine nitration (30,31) promote the transition of cytochrome c to “alternative low spin conformations” (32), which in some cases correspond to state IV.

Indeed, when the neutral species converts into the alkaline form, a band in the visible spectrum centered at 695 nm, which is indicative of the iron-sulfur bond established between the heme iron and Met-80 weakens in intensity and is eventually lost (10), and data obtained by magnetic circular dichroism and low temperature EPR have clearly shown that the heme is kept as low spin (27,32). As an alternative to these spectroscopic methods, changes in heme electronic structure in cytochrome c\(^{3+}\) can be monitored by looking at the paramagnetically-shifted resonances in \(^1\)H NMR spectra (33-35), as both species are low-spin and give sharp, sensitive signals, with different chemical shifts under a slow exchange regime (13,19). NMR allows not only the characterization of the different conformations, but it also assists in the determination of the \(pK_a\) for the observed transition, which can be followed at the residue level (19,35). Moreover, cytochrome c\(^{3+}\) - cardiolipin interactions have been preliminarily evaluated by \(^1\)H-NMR (36).

Among the chemical modifications that seem to alter the conformational state of cytochrome c\(^{3+}\) and, in particular the heme microenvironment, is the nitration of tyrosine residues. Indeed, mammalian cytochromes c contain four highly conserved tyrosines, two solvent-exposed (Tyr-74 and Tyr-97) and two buried internally (Tyr-67 and Tyr-48). While the functional impact of the nitration of cytochrome c by tetranitromethane was studied by seminal early work (37-39), a renewed interest appeared recently (40,41) due to the fact that tyrosine nitration has been established as a post-translational modification occurring \textit{in vivo} due to the reactions of nitric oxide
(•NO)-derived oxidants (31) and that can influence protein structure and function (for a recent review see) (42). In this regard, we should note that addition of a nitro group (-NO2) to tyrosine residues, reduces the pKa of the phenolic –OH group by 2 or 3 units and adds a bulky moiety of 45 Da. Nitrated cytochrome c has been detected in cellular and animal models of disease associated to nitroxidative stress (43-45).

We have established that cytochrome c tyrosine nitration can occur via a variety of biologically-relevant reaction mechanisms that include peroxynitrite (ONOO–/ONOOH; ) (40,41), nitrite (NO2–) plus hydrogen peroxide (H2O2) (46) and •NO plus H2O2 (47) dependent reactions (for a review see) (48). Peptide mapping studies have revealed that during peroxynitrite-mediated cytochrome c nitration, the first nitrated tyrosine residues are either Tyr-74 or Tyr-97, consistent with the fact that peroxynitrite-derived radicals (either via homolytic cleavage to yield hydroxyl (•OH) and nitrogen dioxide (•NO2) radicals or secondary to the reaction with carbon dioxide that yield carbonate radicals (CO3•‾) and •NO2) will preferentially react with solvent-exposed tyrosine residues1 (40). Other biochemical pathways of cytochrome c nitration have been reported (46,47), which may also involve the nitration of Tyr-67 (48). Nonetheless, we have observed that the nitro-Tyr-97 and nitro-Tyr-74 cytochrome c species undergo important functional changes that include the induction of a peroxidatic activity (i.e. reduction of a peroxide such as H2O2 with the concomitant oxidation of a second substrate via a heme-dependent catalytic cycle) (40,41), the loss of electron transport capabilities (40,41) and the inability to induce apoptosome activation (48).

While the functional effect, from none to either gain- or loss-of-function (31,42), of tyrosine nitration on a variety of proteins has been under active investigation over the last decade, structural studies are almost lacking. In particular, one crucial unanswered issue is how the site-specific nitration of one or more tyrosine residues in cytochrome c is able to decrease the pKa of the alkaline transition towards the neutral region and result on a conformational switch to render peroxidatic activity; the latter has been related to cardiolipin oxidation (29,40,56) and initiation of mitochondrial apoptotic signaling (26).

In this paper, we have explored the effect of nitration at the solvent-exposed tyrosine residues (Tyr-97 and Tyr-74) of horse heart cytochrome c on the pKa of its alkaline transition and the changes in the heme ligation and protein conformation by a combination of NMR and optical spectroscopies. This work allows us to provide a structural rationale for the remote effect of Tyr-74 nitration onto the alkaline transition, which results in the gain-of-peroxidatic activity upon nitric oxide-derived oxidants reactions. Moreover, we propose a mechanism by which tyrosine nitration is conformationally transmitted within the protein framework. These data provide novel insights on the structural changes triggered by tyrosine nitration on cytochrome c and highlights the utility of NMR of paramagnetic systems for assessing the effects of post-translational modifications and interactions with protein and phospholipids ligands on the heme ligation and conformational states of cytochrome c3+.

1 Peroxynitrite is the product of the diffusion-controlled reaction (k = 1.6 x 1010 M–1s–1; (49)) between superoxide (O2•‾) and •NO radicals. Cytochrome c3+ reacts at moderate rates with O2•‾ (k = 3 x 105 M–1s–1; (50)) and very slowly with •NO (k = 7.2 x 102 M–1s–1, (51,52)), and therefore can inhibit peroxynitrite formation (53) and decrease nitration yields. Nonetheless, cytochrome c3+ can still be nitrated by fluxes of O2•‾ and •NO (unpublished data), underscoring the concept that is not possible to completely block peroxynitrite formation, even by strong radical scavenging systems (31,54,55).
Experimental Procedures

Chemicals. Horse heart cytochrome c (C-7752) was obtained from Sigma. This cytochrome c preparation was selected because it is purified without the use of trichloroacetic acid, which otherwise leads to substantial amounts of deamidated and polymeric forms (57). The purity of the cytochrome c preparation used herein was >95% as determined by chromatographic, spectroscopic, electrophoretic, and mass spectrometric criteria. Sequencing grade modified trypsin was obtained from Promega. Hydrogen peroxide was purchased from Baker. D2O (99.8 % ²H purity) was purchased from Sigma. All other reagents were analytical-grade. HPLC solvents were of the highest available quality. Peroxynitrite was synthesized, quantitated, and handled as previously described (58) and contained less than 20 % of residual nitrite.

Nitration and purification of modified cytochrome c species

A large scale nitration and purification procedure was adapted from previously described methods (40,48) to obtain the amounts of mononitrated cytochrome c species required for the optical and NMR spectroscopy studies performed herein. Peroxynitrite was added as a continuous flow of 0.134 mM min⁻¹ during 30 min to cytochrome c³⁺ (200 µM) by using a motor-driven multi syringe system (SAGE Instrument Boston, USA) making three vials of 0.5 ml each time, under vigorous shaking condition in 200 mM potassium phosphate with 25 mM NaHCO₃ and 100 µM DTPA at pH 7.0 and 25°C. The final pH was always controlled and kept below 7.4. After reaction, salts were removed by a chromatography step in a HiTrap Desalting column (Amersham Biosciences) and the mixture of nitrated cytochrome c forms was passed through a cation exchange sulfopropyl-TSK preparative column (21.5 mm(ID) x 15.0 cm (L), Tosoh Biosep) in order to purify the mononitrated species. The flow rate was 3 ml/min and the column was equilibrated with 10 mM ammonium acetate buffer (pH 9.0), kept during 20 min in this buffer and then peaks were eluted using a linear gradient from 10 mM to 400 mM ammonium acetate (pH 9.0) from 20 to 80 min and kept until 90 min 400 mM ammonium acetate. Peaks “2” and “3” from the elution (Fig. 1A), containing nitrated cytochrome c (Note: peak “1” corresponds to unmodified cytochrome c³⁺) were re-purified in the same column (Fig. 1B) using the same solvent gradient, to avoid any cross-contamination. High purity of different modified cytochrome c species was confirmed by mass spectrometry analysis of the whole protein and monitoring the nitration of the tyrosine-containing peptides as described in (40,48). Mass spectrometry analysis was performed with a MALDI TOF/TOF Spectrometer (Applied Biosystems model 4800 Analyzer, Framingham, E.U.A.) on the Analytical Biochemistry and Proteomics Core Facility of the Institut Pasteur de Montevideo (www.pasteur.edu.uy).

HPLC peak “2” contained only the nitrated peptide 92EDLIAY(NO₂)LK99 of 1009.2 Da corresponding to the nitration of Tyr-97, and peak “3” contained only the nitrated peptide 74Y(NO₂)IPGTK79 of 723.3 Da corresponding to the nitration of Tyr-74 (40,48).

Site-directed mutagenesis, expression and purification of recombinant cytochrome c mutants

The plasmid pJRhrsN2 (kindly provided by Dr. Jon Rumbley, Chemistry Department University of Minnesota Duluth) was employed to produce wild type recombinant cytochrome c and the Y97F, Y74F, Y67F and Y48F mutants. Plasmid pJRhrsN2 codes for horse heart cytochrome c described in (59), carrying the two
substitutions H26N and H33N. The four single Y to F mutants were obtained using QuikChange II site-directed mutagenesis kit (Stratagene, CA, USA). The resultant pJRhrsN2 derived plasmids were transformed into *E. coli* strain BL21 Star (DE3) (Invitrogen) for protein expression.

Recombinant proteins were expressed and purified according to (59) with minor modifications. Twenty-five ml of starter culture grown in LB medium and 100 μg/ml ampicillin was inoculated in 1 L of Terrific broth with the antibiotic. The culture was grown with vigorous shaking until A550nm = 0.8. Expression of cytochrome *c* Y to F mutants was induced with 0.8 mM IPTG for 50 hours at 303 K. The protein was purified using a CM-Sepharose fast flow column (Amersham Pharmacia Biotechnology). Fractions with a 410/280 nm absorbance ratio > 4.0 were considered pure, as supported by SDS-PAGE analysis.

**1H NMR spectroscopy**

Lyophilized samples were dissolved in 100 mM phosphate buffer prepared in D$_2$O at pH 7. NMR spectra were acquired with a Bruker Avance II spectrometer operating at 600.13 MHz (1H frequency), with presaturation of the water signal during the relaxation delay. A spectral window of 70 ppm was used, and the total recycle time was 300 ms.

For pH titration studies, the pH was adjusted close to a desired value by addition of 0.1 – 1 M NaOH or 0.1 – 1 M HCl dissolved in D$_2$O, and a 1H spectrum was recorded at 298 and 318 K. After recording each spectrum, the pH was measured again and the mean value (before and after the experiment) was reported. The reported pH values were corrected for the deuterium isotope effect (60). The pKa values were determined by fitting the intensity of heme methyl 8, heme methyl 3, and Met80 methyl signals of the neutral species as a function of pH to a Henderson-Hasselbach equation.

**Absorption and Circular Dichroism spectroscopies**

Electronic absorption spectra were obtained in the 650 – 750 nm region to evaluate the band centered at 695 nm indicative of the heme iron-Met-80 coordination (10) using a Shimadzu UV-2401 PC spectrophotometer in a 0.1-ml quartz cuvette of 1-cm path. Due to the relatively weak absorption coefficient of this band ($\varepsilon_{695} = 865$ M$^{-1}$cm$^{-1}$ for control cytochrome $c^{3+}$ in the pH range 4.5-7.0; this work, see also (61)), assays were performed with 0.5 mM cytochrome $c^{3+}$ or nitrocytochrome $c^{3+}$ in 50 mM phosphate buffer phosphate. The concentration of the different cytochrome $c$ species was adjusted by quantitation of the Soret band absorption at 410 nm ($\varepsilon_{410} = 106,000$ M$^{-1}$cm$^{-1}$), known to be nearly identical for control and nitrated cytochrome $c^{3+}$ (41). For pH titration studies, the pH of the sample was adjusted to the reported values by addition of 0.1 – 1 M NaOH or 0.1 – 1 M HCl and spectra were collected at each pH point. Circular dichroism spectra were recorded using a Jasco J-810 Spectropolarimeter using thermostatted cells of 0.1-cm path.
Results
Preparation of nitro-Tyr-97 and nitro-Tyr-74- cytochrome c species

Horse heart cytochrome c contains 4 tyrosine residues. Tyr-67 and Tyr-48 are buried within the protein structure, while Tyr-97 and Tyr-74, instead, are solvent-accessible, hence both of them are preferentially nitrated by peroxynitrite-derived radicals (40,48). Accordingly, in the preparative method for obtaining nitrated cytochrome c herein reported (see Experimental Procedures), Tyr-97 and Tyr-74 were the two major modified residues obtained when cytochrome c\(^{3+}\) was treated with a peroxynitrite flow during 30 min, as shown in Fig. 1A. Indeed, early reaction products, corresponding to peaks “2” and “3” in Fig. 1, were compatible with two mono-nitrated species as evidenced by the 45 Da increase in molecular mass expected for the addition of a –NO\(_2\) group. Peptide mapping of tryptic fragments confirmed that peaks “2” and “3” correspond to nitro-Tyr-97 and nitro-Tyr-74 cytochrome c, respectively, in agreement with previous data (40,48). Repurification of the peaks through the same HPLC column (Fig. 1B) rendered pure forms of mononitrated cytochrome c (> 99 % purity) and with overall final yields in the range of 1 and 3 % of initial cytochrome c for the nitro-Tyr-74 and nitro-Tyr-97 species, respectively. Longer incubation times (> 30 min) of cytochrome c with the peroxynitrite flux induced the subsequent appearance of di- and tri-nitrated cytochrome c species eluting with earlier retention times (not shown).

Absorption and CD Spectroscopy of nitrated cytochrome c

The integrity of the sixth coordination position occupied by Met80 in the native state of cytochrome c can be followed by inspecting the charge transfer band at 695 nm (62). The decrease or loss of the 695 nm band in mono-nitrated cytochrome c in either Tyr-97 or Tyr-74 at pH 7.4 had suggested the possibility of an “early” alkaline transition (40) due to the decrease in pK\(_a\) (9.28 for native cytochrome c; (63)) determined by the addition of a -NO\(_2\) group to tyrosine, known to decrease the pK\(_a\) of the phenolic -OH group from ca.10 to 7.5 (64). Herein, we performed a detailed spectrophotometric pH titration of native, nitro-Tyr-97 and nitro-Tyr-74 cytochrome c which show that this band is lost in the three cases with pK\(_a\) values of 9.4, 8.7 and 7.3, respectively (Fig. 2). The extinction coefficient values at 695 nm of the nitro-cytochrome c species were somewhat smaller than that of the control protein, even under acidic pH (Fig 2, inset). These data indicate that nitration in Tyr-97 has a relatively mild effect in promoting the alkaline transition, while Tyr-74 nitration has a profound effect, lowering the pK\(_a\) value by ca. two pH units (Table I).

CD spectra were also recorded at different pH values in these samples. The native form of cytochrome c at pH 7 is characterized by a Cotton effect at 410 nm, corresponding to the maximum of the Soret band (Fig. 3A and) (65,66). Instead, at pH 11, the spectrum displays a positive band centered at 405 nm. In the case of NO\(_2\)-Tyr-74, this change is already noticeable at pH 9 (Fig. 3B), in agreement with the behavior of the 695 nm band in the absorption spectrum (32,41).

\(^1\)H NMR spectroscopy of nitrated cytochrome c

\(^1\)H NMR spectroscopy has been extensively employed to characterize the heme moiety and the axial iron ligands in a plethora of heme proteins, particularly cytochromes (33-35). We decided to follow the pH dependence of the nitrated cytochromes by this high resolution technique, to unequivocally characterize the different species formed. The usual ligands to the cytochrome c heme iron in the neutral species are indicated in Fig. 4A. When the spectra were recorded in conditions tailored
for the detection of fast relaxing nuclei (located close to the Fe(III) ion), several resonances were located outside the diamagnetic envelope (10-0 ppm). Figure 4B shows the spectrum of native cytochrome $c^{3+}$ at near neutral pH, where the resonances corresponding to two heme methyl groups and Met-80 are indicated. As already reported, spectra recorded at different pH values evidenced the transition from the native low spin form (III) at neutral pH to the alkaline low spin form (IV) (Fig. 4B) (13,18,19,24). While several resonances change at high pH (as those from methyl groups 3, 5 and 8), revealing an overall perturbation of the heme environment, detachment of Met-80 from the heme iron was unequivocally evidenced by the loss of the intense resonance at -20 ppm which corresponds to the ε-CH$_3$ group of this residue. This titration allowed us to determine a reference pKa value of 9.3 at 318 K for the unnitrated protein in our working conditions, for comparative purposes (Table I). This value is in good agreement with those previously reported (13,18-24), which vary between 8.9 and 9.5 pH units depending on the protein isoform, ionic strength, and temperature.

In Figure 4C and 4D, the pH titrations of nitro-Tyr-97 and nitro-Tyr-74 cytochrome $c$ followed by $^1$H NMR spectroscopy are shown. In both cases, raising the pH resulted in spectral perturbations similar to those reported for the native protein: the heme methyl resonances are located at smaller chemical shift values (20-25 ppm), and (most important), the upfield signal corresponding to Met-80 was lost. This demonstrates that both mono-nitrated species experience a transition similar to the one found for the native cytochrome. However, the pKa values for the transition are perturbed in both cases, being shifted to lower pH values. Nitration at Tyr-97 lowers the pKa value from 9.3 to 8.6, while Tyr-74 nitration drastically lowers the pKa to 7.2 (Table I).

A broad upfield resonance located at -9 ppm can be identified in the alkaline forms of both nitro-Tyr-97 and nitro-Tyr-74 cytochrome $c$, which closely resembles a signal assigned to Lys-72 in the alkaline form of native cytochrome $c$ (Fig. 4B-D). This residue replaces Met-80 as the axial ligand, preserving the low spin character at the Fe(III) center. Finding of this resonance in both nitrated forms further supports the similarity of the metal site structure in the alkaline form of the three samples.

Overall, NMR, absorption and CD spectroscopies reveal that nitration at both Tyr-97 and Tyr-74 shift the alkaline transition to lower pH values, the perturbation being substantially larger for Tyr-74. The NMR data demonstrate that Met-80 is detached from the heme iron, being replaced by a Lys residue, i.e., nitration does not alter the identity of the alkaline species, but leads to an earlier transition. In the case of nitro-Tyr-74, this effect results in a significant population of the alternative low spin form at neutral pH.

Analysis of Tyr to Phe mutants

Different mutations leading to Tyr to Phe substitution were obtained in order to analyze the perturbations in the same protein sites affected by nitration. Due to minor differences existent between the recombinant wild type protein and the commercial one purified from horse heart (59), we determined by NMR the pKa value for the alkaline transition of the recombinant protein, which resulted to be 9.1 ± 0.1 (similar to that of the commercial cytochrome of 9.3). The pKa values for the alkaline transition in the Y97F, Y74F, Y48F, Y67F point mutants were determined by NMR titrations.

The NMR spectra of the Y97F, Y74F and Y48F cytochrome $c$ mutants recorded at different pH values (Fig. 5) showed that the resonances corresponding to the neutral and alkaline species are identical to those observed for the wild type protein, with relatively small changes in the pKa of this transition, which is lowered in the three cases
by 0.4 – 0.5 pH units (ca. 8.6-8.7). Optical studies of the 695 nm band further supported that the spectral properties are nearly identical to those of wild type cytochrome c at physiological pH (not shown).

In the case of the Y67F mutant (Fig. 6A) the \(^1\)H NMR spectrum of the neutral form displays some perturbations compared to the spectrum of the wild type protein (Fig. 6B). This is not completely unexpected, based on the proximity of this residue to the heme site (Fig. 4A). The signal assignment was similar to the one reported for the wild type protein, as confirmed by COSY and NOESY experiments (not shown) (35). Regarding the alkaline transition, spectral changes were observed at higher pH values than in the native protein, resulting in a lower limit of 11.0 for the \(pK_a\) (Fig. 6A). At alkaline pH, the intensity of the heme 8-CH\(_3\) and 3-CH\(_3\) groups decreases together with that of Met-80, again revealing a transition which implies detachment of this axial residue. The newly formed species displays heme methyl signals around 20 ppm which resemble those of the alternative low spin form in the native protein. However, some additional resonances are also observed, revealing the presence of more species at high pH (see Figs. 5 and 6A).

The CD spectra of Y67F cytochrome c recorded at pH 7 and 11 in the near UV and visible regions display a similar form, but with quite different intensities. Since the NMR spectra recorded reveal a 60:40 ratio of the neutral and alkaline species at pH 11, the reduced negative ellipticity at 410 nm compared to the spectrum at pH 7 may well correspond to the contribution of a positive Soret band, as observed for the pure high pH form in the native protein (Fig. 3C). The effect of the Y67F replacement is in agreement with that reported for a similar mutation in rat cytochrome c, which was evaluated following the 695 nm band in the absorption spectrum (62).
Discussion

The alkaline transition of cytochrome $c$ has been extensively studied, as well as the influence of different chemical or physico-chemical perturbations on it (13,18,20,23,24). It has been shown that the alkaline form of cytochrome $c$ is stabilized by chemical modifications of amino acids, cleavage of certain parts of the polypeptide chain, perturbation of interactions inside the heme crevice, interactions with anionic phospholipids, increased temperature and the presence of denaturants, resulting in a lower $pK_a$ (29,41,67-72). Such conditions are associated with local or global destabilization of the protein fold, induced by deprotonation of one or more residues. The solution structure of the alkaline form of the K79A variant of yeast cytochrome $c$ has revealed that most protein regions are well-defined in the structure and are similar to the same regions in the native form; except for the segment encompassing residues Asn-70 to Ile-85, known as the $\Omega$-loop, which becomes floppy in the alkaline form (13,73).

Here, we have shown that nitration of Tyr-74 perturbs significantly the $pK_a$ of the alkaline transition down to the near-neutral pH region (Table I). The high pH form in this variant preserves the spectral features reported for the native protein at pH 11, such as the detachment of Met-80 from the heme iron and ligation of a Lys residue. In contrast, nitration of cytochrome $c$ at Tyr-97 shows a slightly lower $pK_a$ compared to the native protein (i.e. by 0.5 pH units). Both Tyr-97 and Tyr-74 side chains are solvent-accessible, in line with the high degree of peroxynitrite-dependent nitration displayed by both residues, and are both located far from the heme site (the Fe-OH-Tyr distances are 13.8 and 14.7 Å, respectively). However, the impact of nitration on the alkaline transition is quite different in each case. Examination of the structure of native cytochrome $c$ reveals that Tyr-74 is located in the $\Omega$-loop, while Tyr-97 is located in the opposite side of the protein structure (15). Despite the similar solvent accessibility, Tyr-97 is located in a more rigid area of the protein fold, and the influence in promoting early conformational changes may be rather modest. Thus, it is tempting to speculate that the drastic effect induced by nitration of Tyr-74 is related to the destabilization of the $\Omega$-loop. Accordingly, nitration at Tyr-74 is also the one which promotes the strongest gain-of-peroxidatic activity at physiological pH (40,48).

Tyr nitration is expected to affect the $pK_a$ of the phenolic moiety. Indeed, the $pK_a$ of 3-nitrotyrosine in solution is of 7.2, compared to a value of 10.1 for Tyr (64). In a previous study, we speculated that nitration of Tyr-74 was responsible of decreasing its $pK_a$ value and that this change served to shift the alkaline transition in the protein towards near-to-neutral pH values (40). This assumption would imply that deprotonation of Tyr-74 is responsible for triggering the conformational change coupled to the alkaline transition. However, the present study shows that the Y74F mutant exhibits an alkaline transition similar to the wild type protein, allowing us to rule out this hypothesis. Moreover, the $pK_a$ change induced by the Tyr to Phe substitution is similar in positions 48, 74 and 97, confirming that deprotonation of Tyr-74 does not trigger the alkaline transition in cytochrome $c$. Thus, we are left with the possibility that nitration of Tyr-74 induces a destabilization of the $\Omega$-loop. However, this hypothesis requires further elaboration to explain how this perturbation in a solvent-accessible...
residue is transmitted to the heme site and the fact that a relatively minor post-translational modification results in such a dramatic change of pKa.

Nitration of the aromatic ring of Tyr-74 is expected to present a steric strain with the Glu-66 side chain (i.e., the residue preceding Tyr-67) (Fig. 7). On its turn, the phenolic proton of Tyr-67 is involved in a H-bond with the S atom of Met-80 (15,74), i.e. providing a direct connection between the nitrated residue and an iron ligand (Fig. 7). Our observation that the alkaline transition in the Y67F mutant (pKa c.a. 11.0) is shifted to a much higher pH value compared to the native protein may be due to removal of this H-bonding interaction. This hypothesis is further supported by the NMR structure of the alkaline form of yeast cytochrome c (13), where (in addition to the different conformation adopted by the Ω-loop) Tyr-67 and Met-80 are distant from the heme site, and have lost the H-bond between them.

We therefore propose that Tyr-67 is involved in Met-80 detachment by wiring a conformational change in the Ω-loop to the metal site. Thus, Tyr-74 nitration perturbs the interaction with Glu-66, and this perturbation is transmitted via the Tyr-74-Glu66-Tyr-67-Met-80-Fe network (Fig. 7). The involvement of Glu-66 in this pathway is supported by recent results by Maity et al., who measured the shift in the pKa of the alkaline transition upon mutations at several positions of the sequence (73). They found that the E66A replacement is one of the few mutations not located in the Ω-loop that shifts the pKa of the alkaline transition.

This analysis allows us to postulate the following scenario for the effect of Tyr nitration in cytochrome c: while both Tyr-97 and Tyr-74 are almost equally nitrated based on their similar solvent accessibility, nitration of Tyr-74 has a profound effect on the heme site structure and the protein flexibility at neutral pH. This phenomenon is due to the stabilization of the alkaline form, resulting in a lower pKa, and therefore rendering a high concentration of cytochrome c molecules in which Met-80 has detached from the iron ion, and has been replaced by a Lys residue. This ligand switch gives rise to an increase of the peroxidatic activity at pH 7 (41) as the replacement ligand, Lys-72, is more weakly bound to the heme and readily displaceable by hydrogen peroxide and other peroxides, including peroxynitrite. Further, the gain-of-peroxidatic activity should favor the nitration of heme-adjacent Tyr-67 via iron-mediated reactions with either peroxynitrite or even hydrogen peroxide (in the presence of nitrite or nitric oxide) (40), supported by the detection of dinitrated cytochrome c species (i.e. in Tyr-74 and Tyr-67) during peroxynitrite exposure even under a large excess of remaining unmodified cytochrome c. This phenomenon indicates a preferential nitration of a second site (Tyr-67), after nitro-Tyr74 cytochrome c formation, which most likely occurs secondary to a direct and fast iron-catalyzed reaction of the species present in the alternative low spin conformation. We also propose a mechanism by which the conformational change in nitro-Tyr-74 cytochrome c is triggered (Fig. 7).

The biological relevance of this study relies on the fact that nitrated mitochondrial proteins, including cytochrome c, have been identified under a variety of disease conditions, and some of them are likely to participate in the pathogenic process. In the case of cytochrome c, we envision that the conformational changes induced by tyrosine nitration reported herein can
be tied to at least four processes: a) creating an intramitochondrial peroxidatic activity that can participate in oxidative sensing and signalling of apoptosis via oxidation of critical mitochondrial components including cardiolipin (26,29,56); b) by facilitating mitochondrial cytochrome c translocation in non-apoptotic cells to the cytosol and nucleus to exert yet to defined functions, but that may imply adaptative responses at the gene level (76); c) affecting cytochrome c interactions with cytosolic partners such as Apaf-1, since nitrocytochrome c is not capable of apoptosome activation (48) and can even interfere on unmodified cytochrome c dependent apoptosome activation. Interestingly, Tyr-97 is very close to Lys-7 that plays a critical role in Apaf-1 binding (77) and nitration seems to interfere with this process; and d) potentially impeding Tyr-97 phosphorylation (78), recently reported to occur in vivo (77).

At the structural level, we have shown that NMR of the paramagnetic active site in a metalloprotein is sensitive enough to monitor remote changes, and could be further exploited to study different post-translational modifications, mutations and physico-chemical factors and interactions that will influence cytochrome c structure and function. This work may be extended to many other redox metalloproteins known to be modified by nitration in vitro and in vivo (42) thus setting the ground for future studies in the area.

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2 It is worth to mention that in the moth/pigeon cytochrome c peptide model of antigen recognition (encompassing cytochrome c residues 88-103), a peptide containing nitro-Tyr-97 profoundly alters T-cell recognition (75), in a way that support that nitrated epitopes of autologous proteins could participate in autoimmunological processes. In this regard, nitro-Cyt c may contribute to autoimmune responses by exposing a self-antigen. The structural aspects behind the aberrant interactions of the nitrated cytochrome c peptide at the T-cell and/or major histocompatibility complex (MHC) levels are unknown.
ACKNOWLEDGEMENTS

This work was supported by grants from the Howard Hughes Medical Institute (to AJV and RR), International Centre of Genetic Engineering and Biotechnology (to RR) and NIH (R01-GM068682 to AJV) and Comisión Sectorial de Investigación Científica (CSIC), Universidad de la República (to LC). LAA is recipient of a doctoral fellowship from CONICET, and AJV is a staff member from CONICET. The Bruker Avance II 600-MHz NMR spectrometer was purchased with funds from ANPCyT (PME2003-0026) and CONICET. VT was partially supported by a fellowship from CSIC. We thank Dr. Carlos Batthyany (Institut Pasteur de Montevideo, Uruguay) for advice in the HPLC-based methods and BSc. Lucía Bonilla for assistance in the preparation of nitrated cytochrome c species. AJV and RR are Howard Hughes International Research Scholars.
References

Table I. pKa values determined for the alkaline transition in control, nitro-Tyr-97-, and nitro-Tyr-74-cytochrome c by NMR and optical spectroscopy.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pKa&lt;sup&gt;*&lt;/sup&gt;</th>
<th>NMR</th>
<th>695 nm band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.3 ± 0.1</td>
<td>695</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>Nitro-Tyr-97</td>
<td>8.6 ± 0.2</td>
<td>695</td>
<td>8.7 ± 0.2</td>
</tr>
<tr>
<td>Nitro-Tyr-74</td>
<td>7.2 ± 0.1</td>
<td>695</td>
<td>7.3 ± 0.1</td>
</tr>
</tbody>
</table>

* The data were fitted to the Henderson-Hasselbalch function to obtain the pKa values. NMR determinations were performed at 318 K and optical determinations at 298 K.
FIGURE LEGENDS

Figure 1. **Purification of mononitrated cytochrome c species.** Cytochrome c was nitrated by a flux of peroxynitrite as described under Experimental Procedures. (A) The reaction mixture (60 mg of protein in 3-ml of 10 mM sodium acetate) was separated by a preparative cation-exchange HPLC column, and three main peaks were found corresponding to unmodified- (peak “1”), nitro-Tyr-97- (peak “2”) and nitro-Tyr-74- (peak “3”) cytochrome c, as confirmed by MALDI-TOF and peptide mapping. (40) (B) Peaks 2 and 3 were repurified with the same HPLC column (2-5 mg of protein loaded), obtaining fractions of > 99% purity which were used for all subsequent NMR and optical spectroscopy studies.

Figure 2. **pH titrations of native and nitrated cytochrome c samples followed by electronic absorption at 695 nm.** Electronic absorption spectra of 0.5 mM cytochrome c (●), nitro-Tyr-97-cytochrome c (●) and nitro-Tyr-74-cytochrome c (▲). Spectra were recorded at 298 K in 50 mM phosphate buffer. Inset, absorption band centered at 695 nm for control and mononitrated cytochromes at pH 5.0. The data were fitted to the Henderson-Hasselbalch function to obtain the pKa values for each cytochrome c species. The correlation coefficient ($r^2$) for the data was ca. 0.99.

Figure 3. **Circular Dichroism spectra of cytochrome forms.** CD spectra in the near UV and visible regions corresponding to 10 µM (A) control, (B) nitro-Tyr-74 and (C) Y67F cytochrome c, recorded at pH 7 (solid line), pH 9 (dash-dot) and pH 11 (dotted).

Figure 4. **pH titrations of native and nitrated cytochrome c samples followed by NMR spectroscopy.** (A) Scheme showing the ligands of the heme iron atom. Groups that are referred in the text are labelled. Figure 4A was rendered with PyMol (DeLano Scientific, San Carlos, CA). (B-D) pH titrations of native cytochrome c (B) and of cytochrome c nitrated at Tyr-97 (C) or Tyr-74 (D) monitored by NMR. Spectra were acquired at 318 K in 100 mM phosphate buffer prepared in 100% D2O. Signal labelling is indicated in panel B. Resonances corresponding to the alkaline species are labelled as 8-CH3', 5-CH3' and Lys'.

Figure 5. **NMR Studies of cytochrome c wild type and Tyr to Phe single mutants at different pHs values.** pH titration of recombinant (A) wild type cytochrome c and (B) Y48F; (C) Y74F and (D) Y97F single mutants at 318 K. Spectra were acquired at 318 K in 100 mM phosphate buffer prepared in 100% D2O.

Figure 6. **NMR studies on the Y67F cytochrome c mutant at different pHs values.** (A) pH titration of the Y67F cytochrome c mutant. Spectra were acquired at 318 K in 100 mM phosphate buffer prepared in 100% D2O. (B) NMR spectra of recombinant wild type cytochrome c, at pH 7.1.

Figure 7. **Contact network proposed to lead to disruption of the Fe-S-Met 80 bond upon nitration at Tyr74.** Close up into the CE-NO2Tyr74 - COO-Glu66 - Glu66 - Tyr67 - OTyr67 - SMet80 - Fe pathway proposed to transmit the effect of nitration at Tyr-74 to the heme ring. The figure was rendered with PyMol (DeLano Scientific, San Carlos, CA) based on the pdb coordinates 1hrc.
Figure 1
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A

B

Peak 2

Peak 3
Figure 2
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Figure 3
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Figure 4
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Figure 5
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Figure 6
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Figure 7
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Nitration of solvent-exposed tyrosine-74 on cytochrome c triggers heme iron-methionine-80 bond disruption: Nuclear magnetic resonance and optical spectroscopy studies

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J. Biol. Chem. published online October 29, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M807203200

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