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Coupling of tyrosine deprotonation and axial ligand exchange in nitrocytochrome c

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Here we report a spectroscopic, electrochemical and computational study of cytochrome c showing that nitration of Tyr74 induces Tyr deprotonation, which is coupled to Met/Lys axial ligand exchange. The structural change results in altered electron shuttling capability and augmented peroxidatic activity of nitrocytochrome c at physiological pH.

Ortho-nitration of tyrosine residues is a post-translational modification that has been reported for a variety of proteins under basal physiological conditions, and that is significantly augmented under diverse pathological conditions.1 The accepted mechanism of nitration involves one-electron oxidation of Tyr to Tyro followed by reactions with either *NO or *NO2, resulting in *NO-dependent oxidative modifications. The size and electron withdrawing characteristics of the −NO substituent are likely to perturb structural and electronic features of the protein that may result in decreased or increased activity, as well as in the gain of a new function.2 Unveiling the mechanisms of functional modulation is crucial for a number of physiological and/or pathological conditions.

Here we report on the molecular mechanism underlying loss of electron shuttling capability and concomitant gain of peroxidatic activity of horse heart cytochrome c (Cyt) upon tyrosine nitration. Cyt is a 12 kD soluble monohemes protein present at mM levels in the mitochondrial intermembrane space,3 whose primary function is transporting electrons from complex III to the terminal O2-reductase in the respiratory electron transport chain. Specific and unspecific electrostatic interactions of Cyt with negatively charged counterparts have been extensively studied and found to be determinant for the optimization of electron transfer parameters such as electronic coupling and reorganization energy,4,7 as well as for inducing structural changes.8-14 Specifically, it has been proposed that Cyt/cardioplin interactions lead to structural changes at the level of the heme pocket that include detachment of the iron axial ligand Met80 and, eventually, to its replacement by either a lysine or a histidine residue.9-13 The first case resembles the so-called alkaline transition.15 The alternatively ligated isomers of Cyt present increased peroxidatic activity against cardioplin, suggesting a role in mitochondrial membrane permeabilization during the early events of apoptosis.

Mammalian Cyt contains four highly conserved Tyr residues at sequence positions 48, 67, 74 and 97. Treatment with peroxynitrite leads to preferential mononitration of either Tyr74 or Tyr97 which, in contrast to residues 48 and 67, are solvent exposed.16 These modifications have been reported to result in early alkaline transitions with apparent pH values of 7.3 and 8.7, respectively that contrast with the value of 9.4 determined for unmodified Cyt.15,17 NMR experiments confirmed that in all cases the transition implies replacement of Met80 by a Lys residue as axial ligand. Based on these results, it has been proposed that nitration of Tyr74 may be implicated in oxidative sensing and signalling of apoptosis via oxidation of cardioplin and other mitochondrial targets. Moreover, it has been speculated that the conformational transition does not involve deprotonation of the nitratred Tyr but, instead, is driven by steric perturbations of the flexible Ω loop comprising residues 70-85.17 While a plausible hypothesis, the molecular mechanism by which nitration of a solvent exposed residue elicits an early alkaline transition remains to be elucidated. For this purpose we performed acid-base titrations of ferric WT Cyt and of the mononitrated variants at positions 74 (NO2-Cyt74) and 97 (NO2-Cyt97) using UV-Vis absorption and resonance Raman (RR) detection. In the first case the evolution of the alkaline transition is determined from the disappearance of the charge transfer band at 695 nm (Figure S3). RR titrations performed under Soret excitation (413 nm laser line) are much more informative as the marker band region (ca. 1300-1700 cm−1) is highly sensitive to the redox state, spin and axial ligands of the heme iron.13,18-21

Figure 1 displays typical RR spectra obtained at different pH values.

Figure 1. Left: RR spectra of ferric NO2-Cyt74 recorded at different pH values (λex = 413 nm). Black: experimental spectra. Red: native spectral component. Blue: alkaline spectral component. Right: Relative concentrations of the native (red) and alkaline (blue) isomers of NO2-Cyt74 and NO2-Cyt97 as a function of pH, as determined by RR.

The spectral sets for the three protein variants (WT, NO2-Cyt74 and NO2-Cyt97) can be quantitatively simulated with varying
proportions of only two spectral components: (i) native low spin ferric Cyt with a Met/His axial coordination pattern and (ii) an alkaline form characterized by a low spin ferric heme with Lys/His axial coordination.\textsuperscript{20,21}

Table 1. Reduction potentials and pK\textsubscript{a} values of Cyt variants.

<table>
<thead>
<tr>
<th>Species</th>
<th>pK\textsubscript{a}\textsubscript{alk}</th>
<th>pK\textsubscript{a}\textsubscript{alk}</th>
<th>pK\textsubscript{a}\textsubscript{alk}</th>
<th>E\textsubscript{0}\textsubscript{n} (mV)</th>
<th>E\textsubscript{0}\textsubscript{alk} (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9.4±0.1</td>
<td>9.4±0.1</td>
<td>N.D.</td>
<td>-254±5</td>
<td>-148±5</td>
</tr>
<tr>
<td>NO\textsubscript{2}-Cyt\textsubscript{a}</td>
<td>9.2±0.2</td>
<td>9.4±0.1</td>
<td>6.2±0.1</td>
<td>-247±9</td>
<td>-4161±7</td>
</tr>
<tr>
<td>NO\textsubscript{2}-Cyt\textsubscript{b}</td>
<td>7.0±0.1</td>
<td>7.1±0.1</td>
<td>7.1±0.1</td>
<td>N.D.</td>
<td>-4173±5</td>
</tr>
</tbody>
</table>

\textsuperscript{*}UV-Vis at 695 nm \textsuperscript{2}RR at 413 nm. \textsuperscript{3}RR at 458 nm. \textsuperscript{4}pH 7. \textsuperscript{5}pH 10.

Figure 2. Left: RR spectra of free NO\textsubscript{2}-Tyr, NO\textsubscript{2}-Cyt\textsubscript{a} and NO\textsubscript{2}-Cyt\textsubscript{b} at pH 9 (\textlambda = 458 nm). Right: normalized RR intensity of deprotonated NO\textsubscript{2}-Tyr in NO\textsubscript{2}-Cyt\textsubscript{a} (green) and NO\textsubscript{2}-Cyt\textsubscript{b} (purple) as a function of the solution pH. See ESI for further details.

Quantification of the two species by spectral component analysis allows determining the pK\textsubscript{a} values for the alkaline transition in each case (Figure 1 and Table 1). RR titrations provide conclusive evidence that nitration of Tyr97 has no effect on the alkaline transition of Cyt, ruling out a mild effect suggested by previous titrations.\textsuperscript{17} The small discrepancy is mainly ascribed to the larger error and lack of specificity of the UV-Vis titration that is solely based on the disappearance of a very weak absorption band that in the case of NO\textsubscript{2}-Cyt\textsubscript{a} is even weaker. Nitration of Tyr74, on the other hand, results in a two units downshift of the pK\textsubscript{a} that is consistently reproduced by RR and UV-Vis titrations. In good agreement with previous NMR determinations,\textsuperscript{17} the RR experiments indicate that the neutral and alkaline forms are similar in the three protein variants in terms of coordination pattern and coarse structural features at the level of the heme pocket. Consistently, cyclic voltammetry experiments in solution (Figure S4) afford similar reduction potentials for the corresponding Met/His and Lys/His forms of the three protein variants, which in average are 255 and -161 mV, respectively (Table 1), thus underlying the loss of electron transport capability upon ligand exchange.

The acid-base equilibrium of free NO\textsubscript{2}-Tyr in aqueous solution can be easily monitored by UV-Vis absorption given that the acidic and basic forms of the nitrophenolic group present well separated maxima at 357 and 426 nm, respectively, and an isosbestic point at 379 nm, yielding pK\textsubscript{a}\textsubscript{alk} = 6.8 ± 0.1 (Figure S5). An almost identical value (pK\textsubscript{a}\textsubscript{alk} = 6.9 ± 0.1) is obtained by monitoring the RR intensity of the basic form as a function of pH recorded under 458 nm excitation. For NO\textsubscript{2}-Cyt UV-Vis titrations are hampered by the strong overlap of the weak bands of the nitrated residue with the strong absorption of the heme. RR spectra obtained with 458 nm, on the other hand, display well resolved vibrational bands of the heme group and of the basic form of the NO\textsubscript{2}-Tyr residue with comparable intensities, thereby allowing for reliable simultaneous titrations of the two chromophores in the proteins (Figure 2). As summarized in Table 1, the pKa value determined for NO\textsubscript{2}-Tyr97 is somewhat lower than for free NO\textsubscript{2}-Tyr in aqueous solution indicating that, in spite of being a surface residue, the environment is slightly different, probably due to partial burial into the protein matrix. For NO\textsubscript{2}-Tyr74, on the other hand, the pK\textsubscript{a}\textsubscript{alk} is identical within error to the free NO\textsubscript{2}-Tyr in aqueous solution, thus suggesting full exposure of this residue to the solvent. Based on these observations, one might expect larger perturbations of the protein structure upon nitration of Tyr97 compared to Tyr74, leading to a more pronounced labilization of the Met5-Fe bond that favours an earlier alkaline transition in the first case. The experimental results, however, contradict this idea. Moreover, while the alkaline transition and NO\textsubscript{2}-Tyr deprotonation appears to be completely decoupled for NO\textsubscript{2}-Cyt\textsubscript{a}, the pKa values of both processes are identical for NO\textsubscript{2}-Cyt\textsubscript{b}, thus suggesting that Lys/Met ligand exchange is triggered by electrostatic perturbation of the protein structure upon NO\textsubscript{2}-Tyr74 deprotonation. To gain deeper insight into these processes we performed molecular dynamics (MD) simulations (see ESI for details). Root mean square deviations of both Tyr74-protonated and deprotonated NO\textsubscript{2}-Cyt\textsubscript{a} with respect to the WT protein are very small, typically below 0.7 Å. Consistently, secondary structure elements as determined from the MD simulations show only subtle alterations (Figures S6-S7). Steered MD (SMD) simulations, on the other hand, show that the free energy change for displacing the axial ligand Met80 is essentially identical for WT and both the (NO\textsubscript{2}-Tyr74)-protonated and deprotonated forms NO\textsubscript{2}-Cyt\textsubscript{a} (Figure 3).

Figure 3. Free energy for displacing Met80 (left) and Lys73 (right) in the native and alkaline isomers, as estimated by SMD. Black: non-nitrated Cyt. Blue: protonated NO\textsubscript{2}-Cyt\textsubscript{a}. Red: deprotonated NO\textsubscript{2}-Cyt\textsubscript{a}.

As the 3D structure of the alkaline isomer of horse heart Cyt is not available, we created \textit{in silico} models starting from the NMR structure of alkaline iso-Cyt in which the sixth axial ligand is Lys73 (see ESI for details).\textsuperscript{22} SMD simulations performed on the model structures show that the free energy required for pulling the Lys73 ligand apart from the iron increases in the order protonated NO\textsubscript{2}-Cyt\textsubscript{a} < WT < deprotonated NO\textsubscript{2}-Cyt\textsubscript{a}, where the protonation state refers to the NO\textsubscript{2}-Tyr74 phenolic group. The alkaline transition of Cyt is a complex process that involves several steps, some of them common to the folding/unfolding pathway. Stopped-flow and NMR experiments suggest that the crucial events are partial unfolding of the \Omega loop 40-57 that leads to deprotonation of an unidentified internal group, followed by the Met/Lys ligand exchange reaction itself. The last step has...
been identified as rate limiting and requires deprotonation of the ligating surface Lys residue ($pK_{\text{a,sys}} = 11.4$) and rearrangement of the $\Omega$ loop 70-85. The equilibrium reaction can be described in terms of a minimal model:

$$
\text{Cyt-H}_{\text{Mes-Fe}} \xrightarrow[pK_{\text{a}} = 11.4]{k_f} \text{Cyt-Lys-Fe} \quad \xrightarrow{k_f} \text{Cyt(Lys-Fe)}
$$

(Scheme 1)

which leads to the expression $pK_{\text{a,sys}} = 11.4 - \log(k_f/k_b)$, with $k_f = 7.5 \text{ s}^{-1}$ and $k_b = 0.02 \text{ s}^{-1}$.26 Assuming that the rate constant of the back alternative ligation reaction ($k_b$) is determined by the activation barrier for breaking the Lys-Fe bond, a simple Eyring-type calculation based on the results presented in Figure 3 anticipates a 6-fold decrease of $k_b$ for (NO$_2$-Tyr74)-deprotonated NO$_2$-Cyt$_{74}$ compared to the non-nitrate-protonated Cyt. Assuming that $k_f$ is not significantly affected by Tyr nitration this yields $pK_{\text{a,sys}} = 8$ for NO$_2$-Cyt$_{74}$, which considering the approximations involved, is in reasonable agreement with the experimental value. Thus, the present experimental and computational results suggest that the early alkaline transition in NO$_2$-Cyt$_{74}$ arises from stabilization of the alkaline isomer by the deprotonated NO$_2$-Tyr74 residue rather than by destabilization of the native form. Note that for the alkaline isomer containing protonated NO$_2$-Tyr74 the activation barrier for displacing the ligating Lys is lower than for the deprotonated form by approximately 7 times the thermal energy at room temperature ($\Delta G^\circ$), thereby suggesting significant protein destabilization (and up-shift of $pK_{\text{a,sys}}$) upon protonation.

Figure 4. Peroxidatic activity of NO$_2$-Cyt$_{74}$ relative to WT-Cyt, as a function of pH determined by amplex red assay. The line was drawn using the parameters determined in Figures 1 and 2 for NO$_2$-Cyt$_{74}$.

For the minimal model shown in Scheme 1 the acid-base equilibrium with apparent $pK_a = 11.4$ has been assigned to the deprotonation of Lys73 and/or 79. While Lys deprotonation is clearly required for coordination to the heme iron, the high value of the apparent $pK_a$ underlines a more complex scheme that might include deprotonation of Tyr74 as crucial event, as suggested by the identity of the $pK_{\text{a,sys}}$ and $pK_{\text{a,sys}}$ values determined here for NO$_2$-Cyt$_{74}$. Independently of the detailed mechanism, we observe a one-to-one correlation between NO$_2$-Tyr74 deprotonation, alkaline transition and increase of peroxidatic activity relative to WT Cyt (see Figure 4 and ESI).

In summary, the present results demonstrate that nitration of Tyr74 in Cyt induces deprotonation of this residue concomitant with the so-called "alkaline transition" at neutral pH. The effect is ascribed to the stabilization of the alkaline form by the deprotonated NO$_2$-Tyr74, and results in a ca. 7-fold increase of the peroxidatic activity and in the loss of electron shuttling function due to a ca. 400 mV downshift of the reduction potential. The pH in the intermembrane space is typically 6.8, and it becomes only slightly more acidic under apoptotic signalling.27 Thus, a significant proportion of the alternative conformation of NO$_2$-Cyt$_{74}$ can be expected at any biologically-relevant pH.

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Notes and references