Structural basis of mitochondrial dysfunction in response to cytochrome c phosphorylation at tyrosine 48

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Regulation of mitochondrial activity allows cells to adapt to changing conditions and to control oxidative stress, and its dysfunction can lead to hypoxia-dependent pathologies such as ischemia and cancer. Although cytochrome c phosphorylation—in particular, at tyrosine 48—is a key modulator of mitochondrial signaling, its action and molecular basis remain unknown. Here we mimic phosphorylation of cytochrome c by replacing tyrosine 48 with p-carboxy-methyl-L-phenylalanine (pCMF). The NMR structure of the resulting mutant reveals significant conformational shifts and enhanced dynamics around pCMF that could explain changes observed in its functionality: The phosphomimetic mutation impairs cytochrome c diffusion between respiratory complexes, enhances hemeprotein peroxidase and reactive oxygen species scavenging activities, and hinders caspase-dependent apoptosis. Our findings provide a framework to further investigate the modulation of mitochondrial activity by phosphorylated cytochrome c and to develop novel therapeutic approaches based on its prosurvival effects.

Oxidative phosphorylation (OxPhos) relies on the electron transport chain (ETC) to generate the membrane potential that drives ATP synthesis (1). Components of the ETC oxidize substrates to reduce molecular oxygen, thereby producing water. Nevertheless, around 2% of the electrons flowing across the substrates to reduce molecular oxygen, thereby producing water. Components of the ETC oxidize substrates to reduce molecular oxygen, thereby producing water. Nevertheless, around 2% of the electrons flowing across the membrane protein complexes into supercomplexes (3, 7). Swapping, and modulation of the equilibria for the association of ROS production (6). The activity of the ETC is tightly regulated by posttranslational modifications of its components, isofrom swapping, and modulation of the equilibria for the association of the membrane protein complexes into supercomplexes (3, 7). Such associations allow substrate channeling while modulating ROS production (3, 7).

Oxidative stress response involves redox signals modulating protein phosphorylation (8). In the ETC, the major phosphorylation targets are NADH:UQ oxidoreductase, cytochrome c (Cc), and cytochrome c oxidase (CcO) (9). Besides being essential for oxidative respiration (Fig. 14), Cc acts as a redox regulatory protein within the mitochondrial intermembrane space (10). In addition, Cc aids in controlling ROS levels by oxidizing superoxide anions (11) and exhibiting peroxidase activity (12); the latter, however, also yields lipid peroxidation (13, 14). Furthermore, Cc plays a crucial role in programmed cell death, a process that is only partially understood (15–21). In this context, a fraction of Cc binds and oxidizes cardiolipin (CL) at the internal mitochondrial membrane, thereby facilitating the release of unbound Cc to the cytoplasm (16, 17). In mammalian cells, extramitochondrial Cc interacts with apoptosis activating factor 1 (Apaf-1) in the cytoplasm to spark the caspase proteolytic cascade (15). It has recently been shown that Cc can interact with several other proteins outside the mitochondria in humans and plants (18–21). The similarities between the Cc signaling networks in both organisms suggest that key programmed cell-death pathways are conserved throughout evolution (22).

Cc phosphorylation is an alleged modulator of the mitochondrial cell-death pathway (23, 24). Its deregulation is believed to be related to the onset of neurological disorders and cancer (24). Phosphorylation of Cc is easily reversed by phosphatases, hampering the isolation of the modified protein from tissues (25). Tyr-to-Glu substitutions of Cc, designed to emulate Tyr48 phosphorylation,

Significance

Cell response to physiological changes and oxidative stress involves the modulation of mitochondrial metabolism. Its dysfunction favors the development of hypoxia-dependent pathologies, including ischemia and cancer. A key modulator of mitochondrial activity is cytochrome c, whose cell function is regulated by tyrosine phosphorylation. However, how such modification affects cytochrome c structure and function is barely known. Here we report that a phosphomimetic mutant of cytochrome c exhibits enhanced dynamics, which could be responsible for the observed differences in cytochrome c functionality in oxidative stress and cell death. Thus, phosphorylation of cytochrome c becomes a target for further development of robust therapeutic approaches.

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Data deposition: The NMR assignments and coordinates for Y48pCMF cytochrome c reported in this paper have been deposited in the Biological Magnetic Resonance Data Bank [MRBIB entry code 25650] and Protein Data Bank [PDBe ID code 2N3Y] databases, respectively.

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impair both the electron donation to CcO and the activation of caspase-9 mediated by Apaf-1 in vitro (26, 27). Notably, nitration of this residue also hinders the ability of Cc to activate Apaf-1 (28–30). Thus, these posttranslational modifications alter both mitochondrial and cytoplasmic functions of Cc. Tyr48 phosphorylation also affects the physical-chemical properties of Cc, such as its alkaline transition and midpoint reduction potential \( (E_0) \) (27, 31). Understanding the origin of these effects and how they affect Cc activities requires solving the 3D conformation of the phosphorylated species. However, deciphering the effects of Tyr48 phosphorylation on the structure and dynamics of Cc is highly challenging because of its reactivity toward phosphatases. Indeed, no atomic-resolution structure has been reported for either phosphorylated Cc or any reliable mimic mutant.

Here we have elucidated the structure of a phosphomimetic Cc variant in which Tyr48 is replaced by the synthetic, noncanonical amino acid p-carboxy-methyl-L-phenylalanine (pCMF). We show that such replacement induces local perturbations of the Cc structure and enhanced internal dynamics of the mutation’s surroundings. We used biochemical assays to show that the Y48pCMF mutation impairs Cc channeling between cytochrome bc1 (Cbc1) and CcO, enhances peroxidase activity, and induces an antiapoptotic function of Cc.

**Results**

**Phosphorylation of Tyr48 Induces Local Structural Changes in Cytochrome c.** To understand how phosphorylation affects the structure of human Cc, we tackled the challenge of fully characterizing the phosphomimetic mutant Y48pCMF Cc in its reduced form, which is the redox state of Cc donating electrons to CcO in homeostasis and is essential for its apoptotic activity, because Cc becomes highly reduced upon its release from the mitochondria to the cytosol (32). Y48pCMF Cc maintains the overall secondary structure and global fold of wild-type (WT) Cc, as inferred from circular dichroism (CD) (Fig. 1B) and \(^{1}H\)–\(^{15}N\) heteronuclear single-quantum correlation (HSQC) NMR spectra (Fig. 1C), respectively. Also, the native heme axial coordination was preserved, as indicated by \(^{1}H\) NMR data (Fig. 1D). The NMR spectra of reduced Y48pCMF Cc were extensively assigned: Triple-resonance experiments (SI Appendix, Table S1) allowed us to detect and assign 91 backbone amide signals extensively assigned: Triple-resonance experiments \(^{1}H\)–\(^{13}C\) HSQC spectra of WT and Y48pCMF Cc at negative ppm values. Resonances for Met80 side-chain protons are shown for both Cc species. Assigned signals of all residues within this region are displayed for Y48pCMF Cc. The extra signal of WT Cc corresponds to the Q\(_B\) protons of ile53. (E) Superimposition of the aromatic region of the \(^{1}H\)–\(^{13}C\) HSQC spectra of WT and Y48pCMF Cc acquired in \(^{15}C\) natural abundance. Assigned aromatic resonances of Y48pCMF Cc are displayed in red.
natural abundance of $^{13}$C (Fig. 1E). The assignment of the $^{p}$CMF48 side-chain signals involved the complete assignment of the 2D $^1$H–$^{15}$N NOESY.

Numerous residues (Val20 to Asn31, Thr40 to Trp59, and Ile75 to Glu90) displayed signals attributable to a second, minor protein conformation, which had a 1:10 ratio in intensity. Hereafter, only the major form was considered for structure calculations.

We assigned 96% of all the $^1$H signals for the major form. Structural information derived from 2D and 3D NOESY maps supported the presence of five $\alpha$-helical regions (labeled $\alpha_1$ to $\alpha_5$) with typically strong HN–HN ($i$, $i$+$1$), medium-range HN–HN ($i$, $i$+$3$), and HN–HN ($i$, $i$+$4$) interresidual NOEs. These regions spanned the sequence stretches Val3 to Lys13, Ala50 to Asn54, Glu61 to Glu69, Pro71 to Tyr74, and Lys88 to Thr102, resembling those in the NMR structure of reduced WTCc (33). In total, we observed and assigned 2,176 meaningful NOEs, corresponding to 20.8 relevant restraints per residue on average (SI Appendix, Fig. S2). The $71 \phi$ and $71 \psi$ dihedral-angle constraints were derived from $^{15}$N, $^{13}$C, $^{15}$C$x$, $^{13}$C$p$, and HN chemical shifts, using TALOS+ (34). The heme moiety was included in the calculations as previously reported for WT cytochromes (35, 36), assuming an intact heme iron coordination as supported by X-ray absorption spectroscopy (XAS) data (next section).

Two hundred structures were calculated by CYANA (37), and the 20 structures with the lowest target-function (TF) value were selected to form a representative family. The range of TF values was 0.38 to 0.98 Å$, highlighting the high accuracy between calculated and experimental distances. Further refinement of the 20 lowest TF structures involved restrained energy minimization and restrained molecular dynamics (RMD) computations. A final restrained energy minimization was carried out on the structure with the lowest root-mean-square deviation (rmsd) from the average for each of the 20 trajectories. The overall quality of the 20 lowest TF ensembles was good, according to the PROCHECK G factor (38), MolProbity clashscores (39), and other structural quality indicators (SI Appendix, Table S2). Most residues were in the Ramachandran plot favored regions, whereas Cys14, Cys17, His18, Val20, Lys37, and Asn70 were in the generously allowed ones, as also observed for the solution structure of WT Cc (33, 40). Cys14, Cys17, and His18 are covalently bonded to the heme moiety, thereby straining their backbone conformation, as already described for c-type cytochromes (35, 36). As an additional control, we performed 20-ns unrestrained molecular dynamics (MD) simulations of the final minimized conformers without any geometrical restraint. The rmsd for the main-chain atoms was about 1.55 Å at the plateau, hardly drifting (0.122 pm·ns$^{-1}$), as expected for a stable structure (SI Appendix, Fig. S3).

The overall fold of Y48pCMF Cc is very similar to that of the WT species (SI Appendix, Fig. S4), with rmsd values for the backbone nuclei of 1.67 ± 0.10 Å (Fig. 2A and B). However, the two structures differ in the mutation-containing loop $\Omega_{IV}$ (residues 40 to 57, Fig. 2B). This is consistent with the chemical-shift differences (SI Appendix, Fig. S1) and the rmsd between the NMR solution structure of WT Cc [Protein Data Bank (PDB) ID code 1J3S] (33) and the refined lowest-TF Y48pCMF Cc (SI Appendix, Fig. S5A). An increased dynamics in the $\Omega_{IV}$-loop has recently been observed in the G41S variant of Cc (42). The $\Omega_{IV}$-loop (also known as foldon V) and helix $\alpha_5$ constitute the less-stable folding unit of Cc (43). Unlike Tyr48 in the WT species, the $p$CMF48 residue presents a very low number of $^1$H–$^{15}$N NOESY (SI Appendix, Fig. S2) and a high rmsd value within the family (Fig. 2C and SI Appendix, Fig. S5B). A decrease in the number of detectable NOEs can generally be attributed to either a partial assignment of the residue or an increased internal mobility. In our case, the highly dynamic behavior of the $p$CMF48 residue was confirmed from further analysis of internal motions within the nanosecond-to-picosecond timescale (see below).

Other regions of the Y48pCMF Cc structure that differed from those of WT Cc belong to the 19 to 36 $\Omega_3$-loop (part of foldon II) and the 71 to 85 $\Omega_5$-loop (foldon IV) comprising Met80 and helix $\alpha_5$. The rmsd values for backbone atoms of residues from Val20 to Gly29 of Y48pCMF Cc are higher (2.75 ± 1.50 Å) than those for the same residues of WT Cc (SI Appendix, Fig. S5A). In the second loop, the observed differences are mainly restricted to side chains. In fact, the rmsd values for backbone and heavy atoms are 1.18 ± 0.37 and 1.77 ± 0.71 Å, respectively. Residues included in the $\Omega_5$-loop indeed displayed a double conformation, suggesting the presence of conformational equilibria (SI Appendix, Fig. S5A).

The ensemble of structures for Y48pCMF Cc is very precise, except for residues surrounding the mutation. The backbone rmsd from the mean is 0.89 ± 0.01 Å for the whole protein and drops to 0.53 ± 0.12 Å when the mutation surroundings are excluded (residues 40 to 57) (SI Appendix, Fig. S5B). As expected, the highest rmsd values correspond to the 40 to 57 $\Omega_{IV}$-loop and the nearby residues Val20 to Gly29 in loop $\Omega_5$, whose global backbone...
rsmd values were 1.79 ± 0.53 and 0.78 ± 0.23 Å, respectively. These segments also exhibited a larger conformational variability in their secondary structure elements along the MD trajectory (SI Appendix, Fig. S3 B and C). High rsmd values along the Val20-to-Gly29 stretch are typical in Cc homologs (35, 36). They are also consistent with NMR signals revealing secondary conformations for the His26-to-Pro30 stretch. Moreover, the nearby Asn31 amide signal is weak, suggesting high mobility. Notably, all these residues contact the ΩNVC-loop, comprising the Y48pCMF mutation.

Further, the highest rsmd values mapped to the ΩNVC-loop. Consistently, signals from this region undergo a drastic reduction in their 1H–1H NOE cross-peaks. The intensities of the amide signals of Gly45 and Ser47 in Y48pCMF Cc were severely decreased, whereas those from Thr49, Ala51, Gly56, and Ile57 were undetectable. The ΩNVC-loop also contains some residues with high rsmd values (SI Appendix, Fig. S5B). The global rsmd value for backbone atoms of the 71 to 85 ΩNVC-loop is 0.76 ± 0.20 Å. Notably, the end of the ΩNVC-loop shows high rsmd values in WT cytochromes (35, 36).

Heme Iron Coordination Is Insensitive to Tyr48 Phosphorylation. We tested the effects of the mutation on the heme iron coordination environment and the axial coordination restraints used in our structure computations by using X-ray absorption spectroscopy, studying both WT and Y48pCMF Cc species (Fig. 2D and SI Appendix, Fig. S5 C–F). The absorption spectra of the two proteins are almost identical. The X-ray absorption near-edge structure regions of the absorption spectra for both proteins are superimposed (SI Appendix, Fig. S5C). The absence of any shift in the energy position of the absorption edge indicates that the mutation did not affect the electron density at the Fe center.

Likewise, the extracted extended X-ray absorption fine structure (EXAFS) signals of the WT and Y48pCMF Cc species were highly similar. However, small differences in high wave vector (k) values can be observed (SI Appendix, Fig. S5D), as well as in the slightly lower amplitude of the Y48pCMF protein. Whereas the corresponding Fourier transforms are also very similar (SI Appendix, Fig. S5E), the amplitude of the first peak is lower and broader in the Y48pCMF Cc species, suggesting a larger degree of disorder. The dynamic disorder for the two proteins should be similar, as the measurements were performed at cryogenic temperatures in both cases. Hence, the differences in disorder are due to a larger static disorder in Y48pCMF Cc. The main scattering paths contributing to this peak originate from the four nitrogen atoms of the porphyrin ring, although contributions of the nitrogen and sulfur axial ligands have also been included. The fit to the data requires the addition of the paths involving the eight porphyrin carbons closest to the iron atom, beyond the first coordination sphere. In addition, the multiple scattering paths involving these atoms were also included. Fits were performed in R space but also reproduced the spectra well in q space (SI Appendix, Fig. S5F). The parameters obtained from the best fit to the data revealed that the distances between the iron atom and its first coordinating ligands are insensitive to the Y48pCMF mutation (SI Appendix, Table S3). Specifically, the distances from the iron center to the axial S ligand are 2.26 ± 0.001 and 2.25 ± 0.001 Å in the WT and phosphomimetic mutant species, respectively (Fig. 2D). Data analyses also showed that the value for the Debye–Waller factor corresponding to the path involving the four porphyrin nitrogen atoms increased from 0.0012 ± 0.0006 Å2 in the WT species to 0.003 ± 0.001 Å2 in the Y48pCMF mutant. These data are consistent with the preserved chemical-shift pattern of the iron axial ligands and heme substituents observed in 1D 1H NMR (Fig. 1D), which have been reported to be sensitive indicators of the heme iron electronic structures (35, 36). The pattern of the observed NOEs for the heme substituents also supports an overall intact heme pocket, with the exception of the mutation site. Still, the ensemble of structures shows a small change in the orientation of Phe82 with respect to the porphyrin ring (SI Appendix, Fig. S6).

Phosphorylation of Tyr48 Enhances Internal Mobility in Cytochrome c. NMR relaxation measurements were performed to evaluate the dynamics of WT and Y48pCMF Cc. The Y48pCMF substitution slightly affected both relaxation rate (R1 and R2) parameters (SI Appendix, Table S4). The rotational correlation time of the phosphomimetic mutant (6.96 ± 0.02 ns) was higher than that of the WT form (6.33 ± 0.02 ns), in agreement with the small increase (p < 10−251, where p is the probability for the null hypothesis being the right one) in the average gyration radius, from 12.95 ± 0.07 to 13.04 ± 0.06 Å, as calculated by MD. Indeed, phosphorylation can alter protein dynamics at different timescales and cause conformational rearrangements, such as the formation of secondary conformations (44, 45).

Comparing R1, R2, and heteronuclear NOE (HetNOE) relaxation measurements recorded on the two proteins revealed that the ΩNVC-loop of Y48pCMF Cc exhibits a high mobility in the

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Fig. 3. Relaxation NMR measurements and dynamic properties of WT and Y48pCMF Cc. (A–C) Differences in heteronuclear NOE (A), relaxation rate R1 (B), and relaxation rate R2 (C) between the experimental values for the reduced forms of WT and Y48pCMF Cc, plotted as a function of the residue number. Gaps in data result from overlapping resonances, broadened resonances beyond the detection limit, and unassigned resonances. A scheme of the secondary structure elements is included (Top). (D) Map of the Y48pCMF Cc residues colored according to their dynamic properties. A scheme of the secondary structure elements is included (Top). (E) Internal mobility comparison between Y48pCMF and WT Cc. S2-order parameter values per residue for Y48pCMF (Upper) and WT (Lower). Cc are represented on the respective NMR ribbon structures using a blue–red scale. Undetectable backbone resonances are in gray. Heme atoms are in green, with the axial ligands depicted as sticks.
picosecond-to-nanosecond timescale (Fig. 3). Indeed, the Gly41-to-Lys55 segment showed a drastic drop of HetNOE values in the mutant species (Fig. 3A). Furthermore, the amide 

\[ R_2 \]

rates for the sequence stretch of Tyr46 to Lys55 in Y48pCMF Cc differ from those in WT Cc (Fig. 3B). In addition, \( R_2 \) analysis reveals three regions undergoing conformational exchange in the microsecond-to-millisecond timescale: His26 to Thr28, Thr40 to Trp59, and Ile75 to Thr78 (Fig. 3C). This behavior agrees with the reduced intensity or lack of detection of the amide signals belonging to these stretches, compared with the WT form. Furthermore, some regions within the protein displayed signals corresponding to double conformations in the 2D \(^1\)H NOESY spectra—namely, Val20 to Asn31, Thr40 to Trp59, and Ile75 to Glu90—indicating the presence of conformational equilibria between two different structures occurring on a slow timescale with respect to the NMR chemical shift. This dynamics involves residues located in a defined region surrounding the noncanonical amino acid (Fig. 3D).

Altogether, the NMR relaxation measurements of Y48pCMF Cc agree with the per-residue \( S^2 \)-order parameter values computed with TENSOR (46) (Fig. 3E).

Hydrogen–deuterium exchange experiments showed that a common core region is protected from solvent amide–hydrogen exchange in both Cc species. Nevertheless, a substantial number of amides become unprotected in pCMF48 Cc (SI Appendix, Fig. S7). The newly accessible amide protons in the phosphomimetic mutant (Gly29, Gly37, Arg38, Thr40, Gln42, and Trp59) are located in the surroundings of pCMF48 and the nearby \( \Omega \)-loop, in agreement with their high mobility in the microsecond-to-millisecond timescale.

Tyr48 Phosphorylation Modulates the Interaction of Cytochrome c with Its Mitochondrial Partners in the Electron Transfer Chain. Cc carries electrons from Cc1 to CcO within the ETC. To elucidate more details about the process, we analyzed the molecular recognition between the soluble N-terminal domain of plant cytochrome c1 (Cc1) or bovine CcO and the phosphomimetic human Y48pCMF Cc. The human and plant N-terminal domains of Cc1 have a 62% overall sequence identity and similar charge distributions on their molecular surfaces (47), whereas the bovine and human CcO are evolutionarily related proteins, with 91 and 96% sequence identity and homology, respectively.

Two binding sites for human and plant Cc on plant Cc1 have been recently reported (47, 48). The proximal site is located near the heme moiety and is compatible with electron transfer, whereas the distal site lies far from the heme group and probably constitutes a local energy minimum of the encounter ensemble.

To test how Tyr48 phosphorylation affects the conformation of the Cc1–Cc complex, we recorded \(^1\)H–\(^1\)N HSQC spectra upon titration of \(^1\)5N-labeled reduced Cc with unlabeled reduced Cc1. Several amide signals exhibited significant chemical-shift perturbations (CSPs), thus indicating a fast exchange rate within the NMR timescale (Fig. 4A and B). Average amide CSPs (\( \Delta \delta_{\text{avg}} \)) were larger than 0.075 ppm for 11 residues: Gln16, Lys27, Gly29, Ala50, Lys55, Ile58, Lys72, Gly77, Met80, Ile81, and Val83 (Fig. 4B). The fits of CSPs along the titration suggested that Cc1 binds two Y48pCMF Cc molecules, as previously found with the WT Cc species (47, 48) (Fig. 4C). Using NMR spectroscopy, the dissociation constant (K\( _D \)) values for the Cc1–Y48pCMF Cc complex were estimated to be 0.6 and 102 \( \mu \)M for the proximal and distal binding sites, respectively. All of the perturbed residues, except Lys55 and Ile58, surround the heme crevice, as previously described for the interaction between WT Cc and Cc1 (47, 48) (Fig. 4D). In fact, this region constitutes a very well conserved interaction surface in c-type cytochromes (49–52). Lys55 and Ile58, in turn, are located in the \( \Omega \)-NY-loop, which undergoes a conformational exchange in free Y48pCMF Cc (Fig. 4D). In addition, significant CSPs (\( \Delta \delta_{\text{avg}} \geq 0.05 \) ppm) were detected for Lys7, Lys13, His26, Lys39, Ala43, Thr78, Lys86, Lys88, and Glu89. Interestingly, Lys8, Lys13, Lys27,
The nanomolar range and to a second site with less affinity (54). Complex IV or C
mammalian C
binding studies performed by gel filtration between several pH 8.5. ITC measurements of the oxidized C
micromolar-ranging for the two independent binding sites (47, 48).

To assess the functional ability of Y48
To determine the binding affinity and stoichiometry of the interaction between the two redox proteins, isothermal titration calorimetry (ITC) experiments were performed on both redox states. The isotherms obtained by titrating reduced Y48pCMF Cc with reduced Cc1 are displayed (Fig. 4E, Left). For the interaction between Cc and Cc1, the isotherm clearly shows the two components with opposite signs in their respective enthalpy terms, thereby indicating the presence of two binding sites, in agreement with the NMR data herein presented and previous reports on the WT species (47, 48). Indeed, all data fit a model with two independent binding sites in the corresponding Cc partner (SI Appendix, Table S5). The interaction between Y48pCMF Cc and Cc1 was entropic-driven. At pH 7.4, the Kp value at the proximal site was half that observed for WT Cc, whereas that at the distal site was four times higher (SI Appendix, Table S5). This could be ascribed to the extra negative charge at position 48, which alters the surface electrostatic potential of the hemeprotein (SI Appendix, Fig. S8). Both Cc1–Y48pCMF Cc and Cc–Cc complex in their oxidized states showed similar thermodynamic and equilibrium parameters at both acidic and basic pH values. The only exception was the Kp value for the oxidized Cc1–Y48pCMF Cc complex, at the proximal site of Cc1, which was approximately four times lower than that for the oxidized Cc–WT Cc adduct at pH 8.5. ITC measurements of the oxidized Cc1–Y48pCMF Cc complex were not run at pH 7.4 because the pK of the alkaline transition, which is specific to the ferric state (53), shifts to neutral pH upon Tyr48 phosphorylation (31). Hence, the WT and phosphomimetic forms of oxidized Cc can differ in their sixth axial ligand at pH 7.4 (SI Appendix, Table S5).

In addition, we analyzed the binding affinity of the cross-complex between the reduced species of bovine Cc1 and human Y48pCMF Cc by ITC. The isotherm inflection point lies at a 2:1 Y48pCMF:Cc:Cc ratio, thereby indicating the presence of two binding sites on Cc1, as previously found for WT Cc (47, 54, 55). The best fit was achieved when the model used distinct Kp values for the two independent binding sites (SI Appendix, Fig. S9). The resulting isotherms likewise fit to such a model, yielding different micromolar-ranging Kp values (Fig. 4E, Right, and SI Appendix, Table S6) and enthalpy-driven interactions. Both the Cc1 proximal and distal site affinities for Y48pCMF Cc were lower than those for WT Cc. For Y48pCMF Cc, the interaction parameters at both acidic and basic pH values were consistent with such data (SI Appendix, Fig. S10B). Interestingly, the Cc1–Cc complex was positively regulated by the human membrane proteins hypoxia-inducible domain family members 1A and 2A (HIGD1A and HIGD2A), which promote cell survival under hypoxia. HIGD2A was successfully expressed in cell-free expression systems combined with n-dodecyl-β-D-maltoside, as previously reported for HIGD1A (57) (SI Appendix, Fig. S11 A–C). HIGD1A significantly increased the rate of CcO-catalyzed oxidation of Y48pCMF Cc (Fig. 5B), as reported for WT Cc (58). Strikingly, HIGD2A induced an even stronger positive effect than HIGD1A (Fig. 5B). Nevertheless, the HIGD-dependent increase in CcO activity was slightly lower with Y48pCMF Cc (Fig. 5B).

To confirm the HIGD-mediated regulation of the CcO activity in a cellular context, we isolated mitochondria from different yeast strains grown either with glucose (YPD medium), which supports fermentation and respiration, or with the non-fermentable carbon sources lactate and galactose (YP-Gal) (59). Under both metabolic conditions, the isoformic respiratory supercomplex factors 1 (Rcf1, formerly Aim31) and 2 (Rcf2, formerly Aim38) are constitutively expressed (Fig. 5C, Inset), in agreement with their role in CcO activity and supercomplex stability (59–61). Rcf1 is a yeast ortholog of the human HIGD1A and HIGD2A proteins, whereas Rcf2 is specific to yeast. The external membranes of isolated mitochondria were then permabilized to allow the entry of exogenous WT or Y48pCMF Cc. Under these conditions, mitochondria isolated from a yeast strain deficient in both Rcf1 and Rcf2 (Δrcf1/2), as verified by Western blot, displayed an endogenous CcO activity lower than that isolated from WT yeast (WT

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Moreno-Beltrán et al.
Tyrosyl-Phosphorylated Cytochrome c Acts as an Improved Peroxidase-Like Enzyme, in Particular When Bound to Cardiolipin-Containing Liposomes. Assembly of the mitochondrial protein membrane complexes Cbc1 and CcO into OxPhos supercomplexes enables more efficient electron flow and decreases ROS levels generated by the ETC (63). The phospholipid CL, located in the inner mitochondrial membrane, also stabilizes the resulting supercomplexes (64) in a HIGD1/Rcf-independent manner (59). In addition, CL interacts with Cc in a two-step binding reaction (65): In the first step, the so-called A site at the Cc surface makes transient electrostatic contacts with the membrane; in the second step, hydrophobic forces drive the formation of a tight and stable Cc–CL complex, with one of the CL acyl chains entering the hydrophobic groove of Cc (termed the C site) (66–68). C-site binding then triggers Cc-regulated CL peroxidation under oxidative stress and induces early apoptosis (40). Interestingly, several residues of Cc (Lys22, Lys27, and His33) become protonated at low pH to form an extra binding site—the so-called L site—that facilitates the electrostatic interaction of Cc with mitochondrial membranes (69).

Within this framework, we analyzed how tyrosine phosphorylation can fine-tune the affinity of Cc toward CL-containing liposomes, analyzing binding of Cc species to liposomes in Hepes buffer at a physiological pH value by electrophoretic mobility shift assays (EMSA) in native agarose gels (Fig. 6 A and B). To compare the binding properties of Cc species to the CL-containing liposomes of 4:1 DOPC:TOCL (1,2-diOleoyl-sn-glycero-3-phosphocholine:1,1',2,2'-tetraoleoylcardiolipin) or to liposomes of DOPC alone, we measured the mobility profiles of Cc at different Cc:lipid ratios. Although both WT and Y48pCMF Cc bound to DOPC:TOCL and DOPC vesicles, their binding affinity for the CL-free DOPC liposomes seemed to be lower (Fig. 6 A and B). Notably, the presence of free hemeprotein at high lipid concentrations suggested that Y48pCMF Cc has a lower affinity than WT toward DOPC:TOCL liposomes (Fig. 6B), as recently observed for the phosphomimetic mutant S47D Cc (70). Another interesting finding is the unspecific interaction of Cc—either the WT or Y48pCMF species—with DOPC vesicles (Fig. 6 A and B).

ITC experiments corroborated these EMSA data. ITC experiments were performed under aerobicosis to mimic physiological conditions, despite using degassed samples. This may result in partial oxidation of Cc upon binding to liposomes, as previously reported (71). ITC analysis yielded the apparent \( K_D \) values for the first binding event of \( 427 \mu M \) (WT Cc) and \( 780 \mu M \) (Y48pCMF Cc) (Fig. 6C). Data for the interaction of WT Cc with liposomes are consistent with the \( K_D \) values reported before (68, 72–74), but they diverge from the lower \( K_D \) values reported in the literature...
for CL could affect their peroxidase activity. In the absence of CL-containing liposomes, Y48pCMF Cc exhibited a threefold higher peroxidase activity than WT Cc (Fig. 6D). However, the presence of DOPC:TOCL vesicles, at a 1:100 Cc:lipid ratio, increased the enzymatic activity of both WT and Y48pCMF Cc, similar to that observed for other phosphomimetic Cc mutants (70). Note that the slightly lower peroxidase activity increment observed for Y48pCMF Cc is likely due to its higher population of free protein compared with WT Cc (Fig. 6 B and D).

**Discussion**

Here we tackle the structural and functional characterization of the Y48pCMF variant of human Cc. This mutation mimics protein phosphorylation at Tyr48 by adding a negative charge and slightly increasing the side-chain size while keeping the aromatic ring. A recent spectroscopic analysis of Y48pCMF Cc showed a singular shift of the typical alkaline transition pKₐ to physiological pH values (31), as is the case with the Y48E Cc mutant (26, 27). Here, we report NMR-based structure computations that indicate that Tyr48 phosphorylation maintains the core foldon of Cc but increases internal motions in the loops Ω₁, Ω₂, and Ω₃. Specifically, the Ω₂–Ω₃ loop, the most unstable folding unit of the hemeprotein, becomes looser and reaches conformational equilibria in Y48pCMF Cc. Enhanced motions at the Ω₂–Ω₃ interfaces could be associated with the shift in the alkaline transition pKₐ. Indeed, these two loops hold the residues that provide the iron axial ligands, which are observed to at least partially lose their metal coordination in the alkaline form (76). This is in contrast to XAS data that suggest that axial coordination remains untouched. However, the cryogenic temperatures at which the XAS spectra were recorded may

(75) and the A/C two-site binding model described by Kinnunen and coworkers (66, 67). Such discrepancies are still unresolved, and further research is necessary to harmonize data from all these different binding assays in a unified and single model. The interaction with CL resulted in apparent ΔH values equal to 2.75 kcal mol⁻¹ for WT Cc and 38.97 kcal mol⁻¹ for Y48pCMF Cc. The differences in K₀ indicate a small change in binding energies (of ~6 kcal mol⁻¹). Hence, the changes in ΔH are indicative of enthalpy–entropy compensation effects, which are compatible with the electrostatic change resulting from the extra carboxylate group of the Y48pCMF species. Further, an exothermic process can be observed, with longer peak equilibrium times upon successive lipid additions. In this case, the apparent ΔH values with the WT and Y48pCMF Cc species were equal to ~6.97 and ~47.84 kcal mol⁻¹, respectively, but the apparent K₀ value (of ~1 mM) was practically the same with the two Cc species. Altogether, our EMSA and ITC assays indicate that Y48pCMF Cc binds to CL-containing liposomes with a slightly lower affinity than WT Cc.

WT Cc undergoes CL-dependent conformational changes that allow H₂O₂ to access the heme crevice (74). Hence, we addressed whether the affinity differences between WT and Y48pCMF Cc

**Fig. 6.** Liposome-binding assays with caspase-3 activity induced by WT and Y48pCMF Cc. (A and B) EMSA of Cc in the presence of increasing concentrations of lipids. DOPC:TOCL (4:1) or DOPC liposomes were incubated with WT (A) or Y48pCMF (B) Cc. Note that free Cc species moved to the cathode, whereas liposome-bound Cc migrated to the anode. Lanes marked by rectangles correspond to the Cc:lipid ratio at which the peroxidase activity was determined (see below). (C) Calorimetric assays for lipid binding to Cc. (Upper) ITC thermograms, corresponding to titrations of DOPC:TOCL 4:1 liposomes (black), WT Cc (blue), or Y48pCMF Cc (red). (Lower) Isotherms with WT Cc (blue dots) or Y48pCMF Cc (red dots). Continuous lines represent the best fits to a sequential binding, as computed with NanoAnalyze software (TA Instruments) with a stoichiometry of 30 molecules of lipid per molecule of Cc. All data represent the mean ± SD of three independent experiments. (D) Relative peroxidase activities of WT Cc (blue) or Y48pCMF Cc (red) in the presence of liposomes containing DOPC (empty bars) or DOPC:TOCL (4:1) (filled bars). (E) Relative caspase-3 activity in HEK293 cell extracts devoid of endogenous Cc upon addition of exogenous WT Cc (blue) or Y48pCMF Cc (red). A lack of caspase autoactivation was verified in a run without the addition of Cc (gray).

**Fig. 7.** Schematic diagram illustrating the changes induced in cell function by phosphomimetic Y48pCMF Cc. The negative charge at position 48 decreases CcO activity by disrupting Cc channeling in OxPhos supercomplexes (Left), enhances ROS scavenger activity by increasing the peroxidase activity of CL-bound Cc (Middle), and promotes the antiapoptotic function of Y48pCMF Cc by inhibiting its ability to activate the caspase-3 cascade (Right).
The recent X-ray diffraction structure (80), which displays a catalytic step can be tuned by conformational changes of Cₚ (77). However, phosphorylation at Tyr48 hinders the pro-apoptotic activity of extramitochondrial Cₚ (77). In this context, the ca. 60-mV decrease in entropy compensation herein reported. Indeed, the binding of dissociation enthalpies and the strong (or moderate) enthalpy considerations, the high affinity of reduced Y₄₈ₚ represents a long-lived intermediate but also the shift in the alkaline transition pKa of oxidized Y₄₈CMP Cₚ (31). Therefore, the transition from reduced to oxidized Y₄₈CMP Cₚ, which is accompanied by a change in the iron axial ligand at physiological pH, is expected to have a high-energy barrier (82). Hence, such conformational changes might be the limiting step for Y₄₈CMP Cₚ oxidation inside the complex.

Under nutrient and oxygen depletion, a dysfunctional mitochondrial ET Complex and OXPhos can lead to many human diseases, including pathologies such as ischemia and cancer (24). The nexus in both disorders could be the control of mitochondrial ET rate by Cₚ phosphorylation (23), which has indeed been reported to efficiently fine-tune respiratory rates (81). Even though HIGD-mediated assembly of Cₚ₁ and Cₚ into OXPhos supercomplexes preferably occurs under low-glucose conditions, the decrease in both the Cₚ activity and binding affinity toward Cₚ₁ and Cₚ resulting from the enhanced dynamics of Y₄₈CMP Cₚ could explain the disruption of Cₚ channeling inside the Cₚ₁–Cₚ₉ supercomplex, thus slowing down the ET Complex flow (Fig. 7).

Intriguingly, not only respiratory supercomplex formation (60, 61)—whose structure has recently been solved (83, 84)—but also Cₚ phosphorylation, and the resulting decrease in the ETC rate, could help to keep ROS levels low and guarantee cell survival.

Tyr48-phosphorylated Cₚ could be targeted as a biomarker of mitochondrial dysfunction for associated pathological states such as ischemia/reperfusion and cancer. Deciphering the details of the phosphorylated Cₚ-controlled complex network requires accurate structural and dynamic analyses to eventually develop robust therapeutic approaches to foster or silence pro-survival action of phosphorylated Cₚ reported here.

Methods

Methods and associated references are available in SI Appendix, Methods. All biological samples were obtained from bacteria, yeast, or human cell extracts, in full compliance with University of Seville Ethical Committee bylaws.

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The Cₚ–Cₚ₉ complex keeps its interface solvent, according to the recent X-ray diffraction structure (80), which displays a protruding lysine side chain in Cₚ₉ pointing to Tyr48 in Cₚ. A salt bridge involving phosphorylated Tyr48 would restrain the ΔNᵥ interface. The increase in the Kᵥ of the complex between Y₄₈CMP Cₚ and Cₚ₉ (this work) and the decrease in the Michaelis constant (Kₘ₉) of phosphorylated Cₚ with Cₚ₉ (81) indicate that Kₐ₉ and main gate protein Cₙ₁ catalytic step. Our ab initio calculations agrees with the larger Cₚ-O(disc) oxidation rate of Y₄₈CMP Cₚ compared with that of WT Cₚ at the limiting protein concentrations in the enzymatic activity assays used here. In this context, the ca. 60-mV decrease in the E₉ₒ of Y₄₈CMP Cₚ (31) may facilitate the electron flow from Cₚ to Cₚ₉, although it may impair Cₚ reduction by Cₙ₁. In other words, Y₄₈CMP Cₚ exhibits a reduced ability as oxidizing agent (it is less capable than WT Cₚ of receiving electrons from Cₙ₁) but displays an increased capacity as reducing agent (it is a better electron donor to Cₚ₉ than WT Cₚ). It is well-known that the catalytic step can be tuned by conformational changes of Cₚ upon binding to Cₚ₉ (52). Strikingly, these changes resemble the prominent internal dynamics of Y₄₈CMP Cₚ, which could also explain not only the change in E₉ₒ but also the shift in the alkaline transition pKₐ of oxidized Y₄₈CMP Cₚ (31). Therefore, the transition from reduced to oxidized Y₄₈CMP Cₚ, which is accompanied by a change in the iron axial ligand at physiological pH, is expected to have a high-energy barrier (82). Hence, such conformational changes might be the limiting step for Y₄₈CMP Cₚ oxidation inside the complex.

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Moreno-Beltrán et al. PNAS | Published online March 27, 2017 | E3049
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