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Supporting Information

Orthogonal Translation Meets Electron Transfer: In Vivo Labeling of Cytochrome *c* for Probing Local Electric Fields

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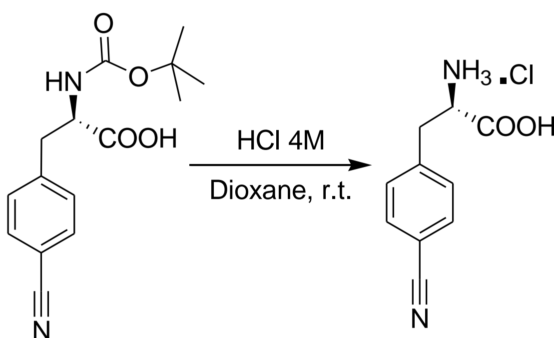
1. Reagents

1.1 Reagents and chemicals

Reagents and chemicals were purchased from Sigma Aldrich and used without further purification.

1.2 Non-canonical amino acid analogues

N-Boc-4-cyano-L-phenylalanine was purchased from ABCR Germany (AB156069). Boc protecting group was removed by treatment with HCl 4M in dioxane at room temperature (Scheme 1, supporting information). The purity of the *p*-cyano-L-phenylalanine (*p*CNF) hydrochloride product was monitored by 1H NMR, 13C NMR and MS-ESI. All characterizations match previously reported data.[1,2]



Scheme S1: deprotection of N-Boc-*p*-cyano-(*L*)-phenylalanine

Procedure: In an Ar-purged flask containing 1.5 g (5.2 mmol) of N-Boc-*p*-cyano-(*L*)-phenylalanine, 10 mL of HCl 4 M were added in an ice bath, while stirring. After 2 h at 0°C, the ice bath was removed and the reaction mixture was kept at room temperature for another hour. The solvent was removed by vacuum evaporation and lyophilization. The product *p*-cyano-(*L*)-

phenylalanine hydrochloride was obtained in 92 % yield (1.1 g, 4.8 mmol). ^{1}H NMR (400 MHz, D_2O, ppm) δ 7.76 (m, 2H) 7.47 (m, 2H) 4.31 (dd, *J*=7.5, *J*=5.9, ^{1}H) 3.84 (m, 2H); ^{13}C NMR (100 MHz, D_2O, ppm) δ 171.4, 140.58, 133.37, 130.52, 119.72, 110.86, 54.30, 36.11; MS-ESI $[m/z]^+$= 191.0817; calcd for $[C_{10}H_{11}N_2O_2]^+$=191.0821.

1.3 Plasmids

The genes encoding horse heart CytC with N-terminal hexahistidine tag and TEV restriction site together with yeast cytochrome c heme lyase (CCHL) are arranged in a pET22b expression vector (Novagen) under the control of a T7 promotor. Eight single codons (corresponding tot he amino acids AA=Phe10, Phe36, Tyr48, Trp59, Tyr67, Tyr74, Phe82, Tyr97) of the CytC gene sequence were mutated to an amber (TAG) stop codon by site-directed mutagenesis to generate pET22b+CytC(Codon→TAG)+CCHL. The plasmid sequences have been verified by DNA sequencing analysis.

The plasmid pEVOL-*p*CNF-RS[3,4] bearing the orthogonal non-sense suppressor tRNA/aminoacyl-tRNA synthetase pair and a chloramphenicol resistance was kindly provided by Dr. Peter Schultz laboratory.

Plasmid sequence of pET22b+CytC(Codon→TAG)+CCHL:

tggcgaatgggacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccct
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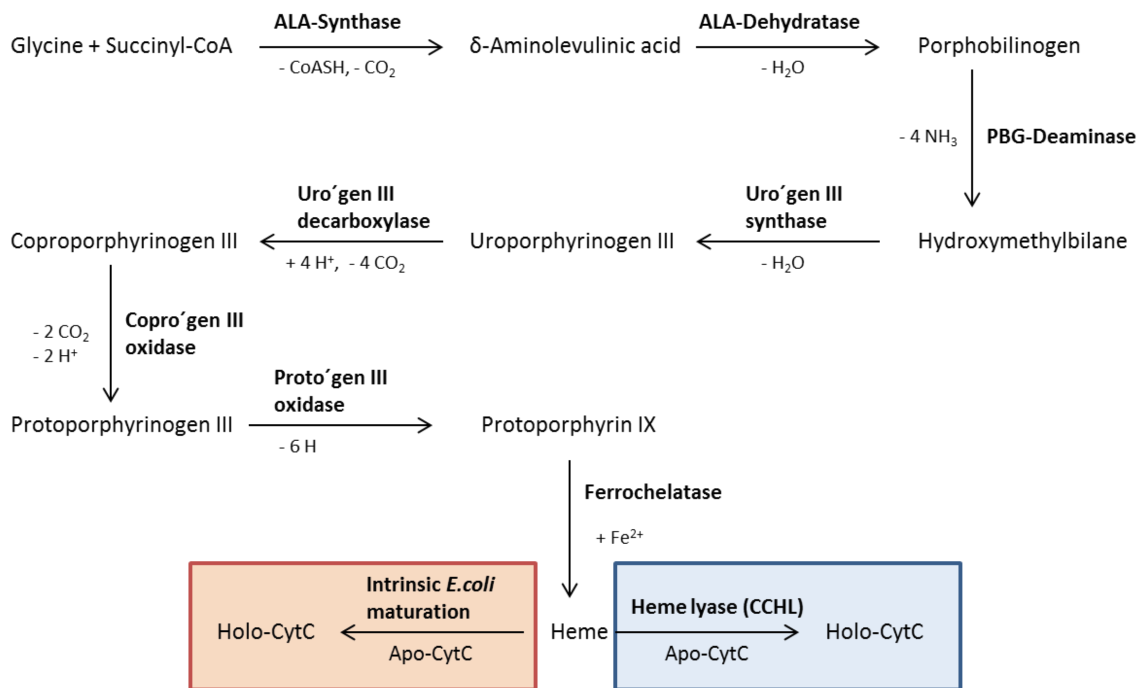
aactatatccggat

2. *Biosynthesis of cytochrome c and sequences*

Heterologous expression of horse heart CytC is a complex system as several enzymes are involved in the biosynthesis of the heme cofactor.[5] In the performed experiments we co-expressed the yeast cytochrome c heme lyase (CCHL) for enhanced catalysis of the attachment of the heme cofactor to CytC.[6] Both synthesis of heme and attachment to CytC are crucial steps for generating soluble and catalytically active CytC.



Scheme S2: Biosynthesis of heme[5] and recombinant expression of horse heart CytC in *E.coli*. The cofactor can be attached to CytC by the intrinsic maturation system of *E.coli* or by a heterogously coexpressed heme lyase from yeast.[6,7]

Wild-type cytochrome c (wt-CytC) amino acid sequence:

His-Tag TEV-restriction site

MGSSHHHHHHSSG•ENLYFQ•GDVEKGKKIF•VQKCAQCHTV•EKGGKHKTGP•NLHGL**F**GRKT•GQAPGF

TYTD•ANKNKGITWK•EETLMEYLEN•PKKYIPGTKM•IFAGIKKKTE•REDLIAYLKK•ATNE

Wt-CytC nucleotide sequence:

5'ATGGGTAGCAGCCATCATCATCATCATCACAGCAGCGGCGAAAATCTCTATTTTCAGGGTGACGTAGA

AAAGGGCAAGAAGATCTTTGTACAGAAATGTGCTCAGTGCCACACGGTGGAGAAGGGTGGTAAGCACA

AAACCGGTCCAAATCTCCATGGGCTC**TTT**GGTCGCAAAACGGGCCAGGCTCCGGGTTTCACCTATACCGA

TGCCAACAAAAACAAGGGTATTACCTGGAAAGAAGACGCTGATGGAATATCTTGAAAATCCGAAAAA

GTATATTCCGGGTACGAAAATGATCTTCGCCGGGATTAAGAAGAAAACGGAGCGTGAAGACCTTATCGC

ATATCTCAAGAAAGCGACGAACGAGTAA **3´**

In the gene encoding *p*CNF36-CytC mutant, codon **TTT** of CytC DNA sequence was mutated to stop codon **TAG**, which resulted in the substitution of **F** in the expressed protein to ***p*CNF**.

CCHL sequence:

MGWFWADQKTTGKDIGGAAVSSMSGCPVMHESSSSSPPSSECPVMQGDNDRINPLNNMPELAASKQPG

QKMDLPVDRTISSIPKSPDSNEFWEYPSPQQMYNAMVRKGKIGGSGEVAEDAVESMVQVHNFLNEGCWQ

EVLEWEKPHTDESHVQPKLLKFMGKPGVLSPRARWMHLCGLLFPSHFSQELPFDRHDWIVLRGERKAEQQP

PTFKEVRYVLDFYGGPDDENGMPTFHVDVRPALDSLDNAKDRMTRFLDRMISGPSSSSSAP

3. Bacterial strains and culture conditions

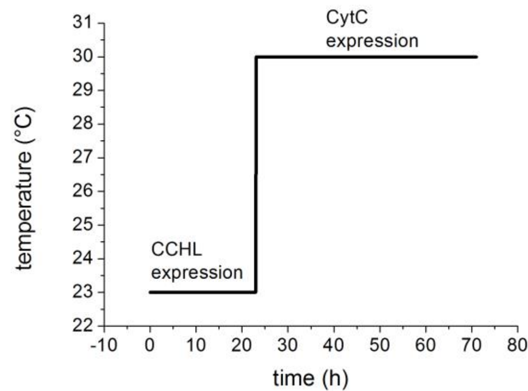
3.1 Strains

E. coli strain BL-21(DE3) was transformed with pET22b+CytC+CCHL or co-transformed with pEVOL-*p*CNF-RS and pET22b+CytC(Codon→TAG)+CCHL respectively. Chloramphenicol (25 µg/mL) and ampicillin (50 µg/mL) were used for selection.

3.2 Incorporation of the non-canonical amino acid analog

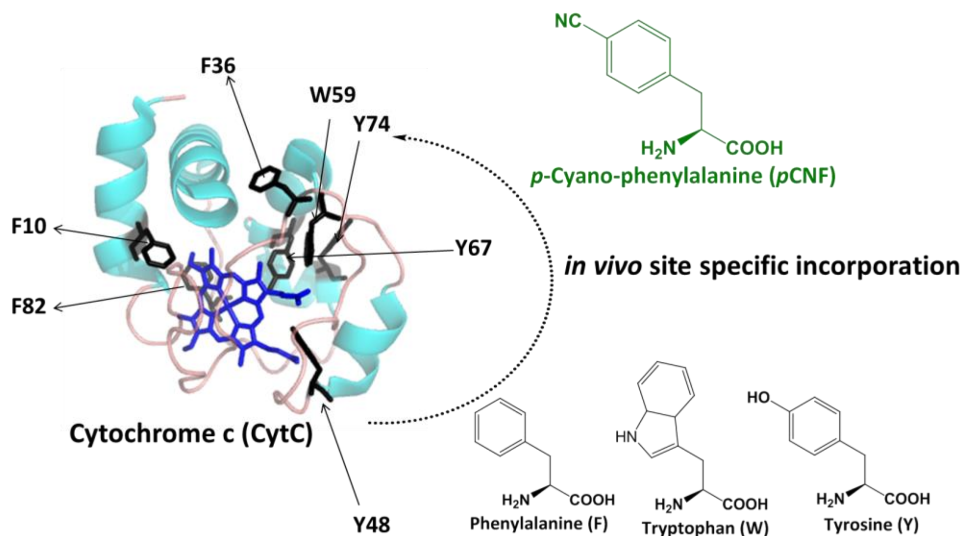
E. coli strain BL-21(DE3) was co-transformed with the pEVOL-*p*CNF-RS and pET22b+CytC(Codon→TAG). After overnight incubation on plates, single colonies were transferred into 5 ml LB medium and grown at 37°C overnight. This culture (2 mL) was used to inoculate 2 L of ZYP5052[8] self-inducing media containing chloramphenicol (37 µg/mL) and ampicillin (100 µg/mL) in a 5 L erlenmeyer flask. 0.2 % Arabinose to induce *p*CN-RS expression, 0.028 g/L ferric ammonium citrate and 0.1 mM δ-aminolevulinic as heme precursors, 0.1 mM *p*CNF to supplement basal expression of CytC and 2 mM $MgSO_4$ were added. The culture was grown at 23°C while shaking for 24 h to express CCHL. Afterwards *p*CNF concentration was increased to 2 mM and the culture was grown at 30°C for another 48 h under constant shaking (200 rpm) for ensuring high *p*CNF-RS activity and CytC expression rate. Cells were harvested by centrifugation for 20 min at 8000 rpm using an Eppendorf Centrifuge 5810R. A similar procedure was used to express wt-CytC using a pET22b+CytC plasmid with no mutation on horse heart CytC sequence. The two-step temperature ramp expression protocol was developed in order to secure expression of active CytC maturing machinery (CCHL for heme attachment to CytC and enzymes involved in heme synthesis). Furthermore ZYP-5052 media contains a phosphate buffer,

which enables on one hand for the growth to a high optical density as well as long incubation times, which secures proper maturing of CytC.



Scheme S3: Schematic representation of the temperature ramp up expression of CCHL heme lyase and CytC

4. Stop codon read-through determination by SDS-PAGE and Western Blot



Scheme S4: View of CytC (PDB ID: 1HRC) and targeted residues (black) for substitution with *p*-cyano-phenylalanine. The cofactor heme is highlighted in blue.

It is reported, that incorporation rate via SCS of the non-canonical amino is influenced by the nucleotides surrounding the nonsense codon (here amber stop codon).[9] Detection of CytC via Western Blot was performed using a monoclonal CytC antibody (7H8.2C12) from Thermo Scientific.

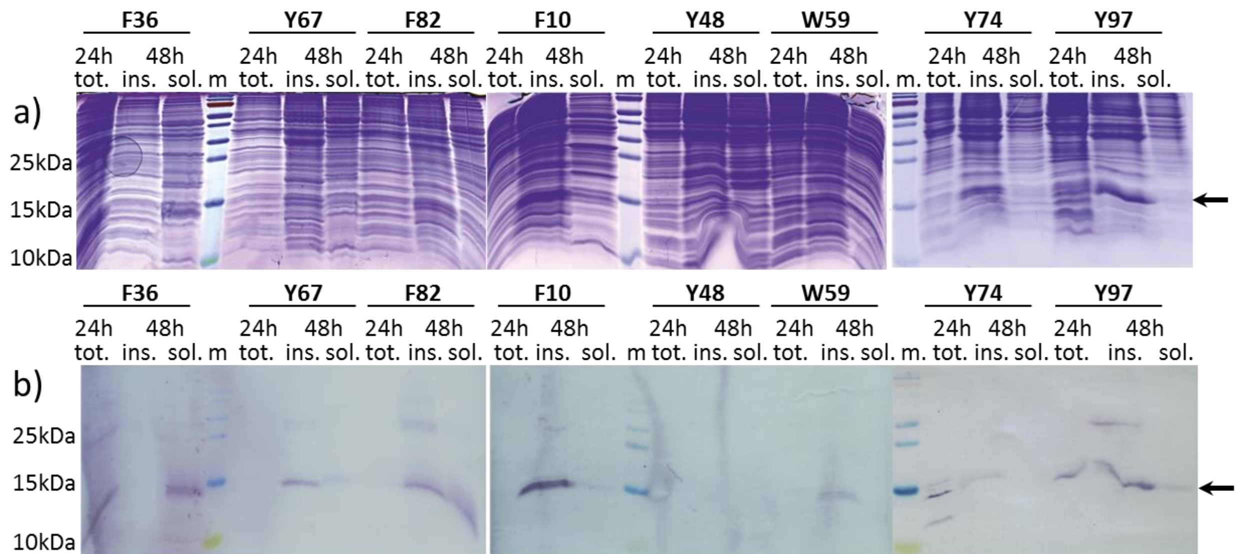


Figure S1: Detection of stop-codon read-through at various positions in CytC by SDS-PAGE Gels (20 %) and Western Blots using a monoclonal CytC antibody (7H8.2C12) from Thermo Scientific. Loaded are the total fractions (tot.) after 24 h expression at 23°C and the soluble (sol.) and insoluble (ins.) fraction after lysis of cells incubated another 48 h at 30°C. m: PageRuler Prestained Protein Ladder. Wt-CytC (M_{calc}=14396.1 Da), F$\rightarrow$*p*CNF-CytC (M_{calc}=14421.1 Da), Y$\rightarrow$*p*CNF-CytC (M_{calc}=14405.1 Da), W$\rightarrow$*p*CNF-CytC (M_{calc}=14382.1 Da). Appearance of corresponding bands is in the region of the 15 kDa band of the PageRuler Prestained Ladder and indicated with an arrow.

Substitution at position tryptophan 59 (W59) and tyrosine 74 (Y74) resulted in insoluble CytC´s. CytC variants with *p*CNF at the positions phenylalanine 36 (F36), F82, F10, tyrosine 67 (Y67) and Y97 showed at least partial expression in the soluble fraction. Eight out of nine positions allowed efficient read-through, with the exception of position 48 resulting in no detectable product.

5. Purification of proteins

5.1 Lysis

The pellets of 1 L liquid culture were re-suspended in 80 mL lysis buffer (pH 8.0) containing 50 mM TRIS, 150 mM NaCl, 20 mM imidazole, 40 µg/mL DNase I and RNase A and 1 mM phenylmethylsulfonyl fluoride. Lysis was carried out in 5 cycles of 2 min sonication (Sonoplus HD3200, KE76 electrode) using a 60 % amplitude at an interval of 1 s pulse and 4 s pause.

5.2 Purification from soluble fraction

Cell debris were collected by centrifugation (12 000 x g, 40 min). The supernatant was purified by a pre-equilibrated Ni-NTA affinity column on the ÄKTApure FPLC system from GE Healthcare using a linear gradient of elution buffer (TRIS 50 mM, NaCl 150 mM, 500 mM imidazole, pH 8.0). Fractions absorbing at 550 nm were collected, dialyzed against phosphate buffer (Na_3PO_4 100 mM, pH 7.4) and subsequently concentrated. The dialyzed sample was loaded onto a pre-equilibrated S-Sepharose cation-exchange column and recombinant CytC was eluted with a high-salt buffer (Na_3PO_4 100 mM, NaCl 500 mM, pH 7.4) using a linear gradient. Fractions with absorption at 550 nm were collected, dialyzed against phosphate buffer (Na_3PO_4 100 mM, pH 7.4) concentrated and stored at 4°C.

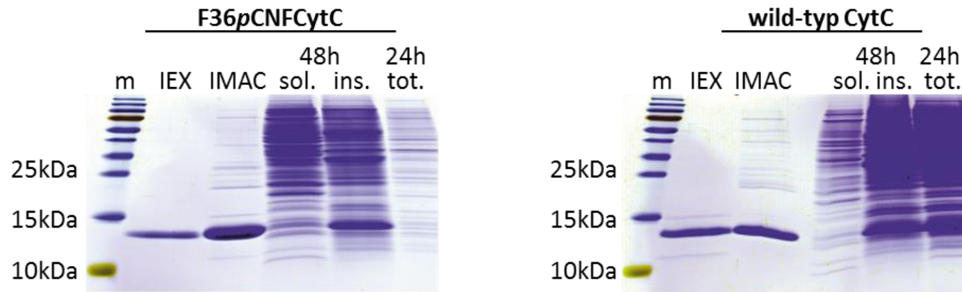


Figure S2: SDS-PAGE gels showing the purification steps of wt-CytC and and F36pCNF-CytC. Loaded are the total fractions (tot.) after 24 h expression at 23°C and the soluble (sol.) and insoluble (ins.) fraction after lysis of cells incubated another 48 h at 30°C as well as the purified proteins after immobilized metal (Ni^{2+}) affinity chromatography (IMAC) and (cat)ion-exchange chromatography (IEX). m: PageRuler Prestained Protein Ladder.

5.3 Purification from insoluble fraction and refolding

Insoluble fractions were resuspended in denaturing solubilization buffer (50mM TRIS, 150 mM NaCl, 6M GndCl, pH 8.0) to solubilize inclusion bodies. Samples were sonicated (KE5 electrode, 52 % amplitude, 1 sec pulse, 2 sec pause, 2 min) and centrifuged at 12 000 x g for 40 min to remove insoluble material. The supernatant was purified by affinity chromatography (Ni-Column) in presence of GndCl 6M in both binding and elution buffers. After purification, the samples were dialyzed overnight against buffer (50 mM TRIS, 150 mM NaCl, pH 8.0). After dialysis a precipitate was formed. Precipitate was isolated by centrifugation (12 000 x g, 30 min) and both fractions soluble and pellet were analyzed by SDS-PAGE gel and Western Blot.

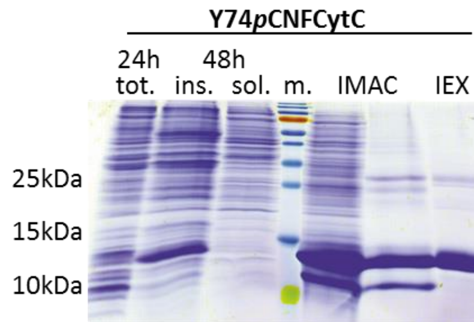


Figure S3: SDS-PAGE gels showing the purification steps CytC(Y74→*p*CNF). Loaded are the total fractions after 24 h expression at 23°C and the soluble and insoluble fraction after lysis of cells incubated another 48 h at 30°C as well as the purified proteins after immobilized metal (Ni^{2+}) affinity chromatography (IMAC) and (cat)ion-exchange chromatography (IEX). m: PageRuler Prestained Protein Ladder.

6. Analytical characterization

6.1 Protein concentration

The final concentrations of recombinant *p*CNF-CytC mutants and wild-type CytC were determined by using an extinction coefficient of 29.0 mM at 505 nm.[10]

6.2 HPLC-ESI-MS

The mass of the purified proteins was analyzed by LC-ESI-MS. 10 µL of the purified protein with a concentration of 0.1-0.3 mg/mL were injected. First, proteins were pre-seperated on a C5 column (Supelco Analytical, Sigma-Aldrich) by reversed-phase chromatography with a gradient of 20 % - 90 % acetonitrile and afterwards transmitted to the mass spectrometer Exactive (Thermo Scientific, Waltham, MA, USA). Ionization was conducted via electrospray ionization (ESI) and mass analysis via orbitrap. The obtained spectrum was deconvoluted with the program MagTran to obtain the molecular mass.

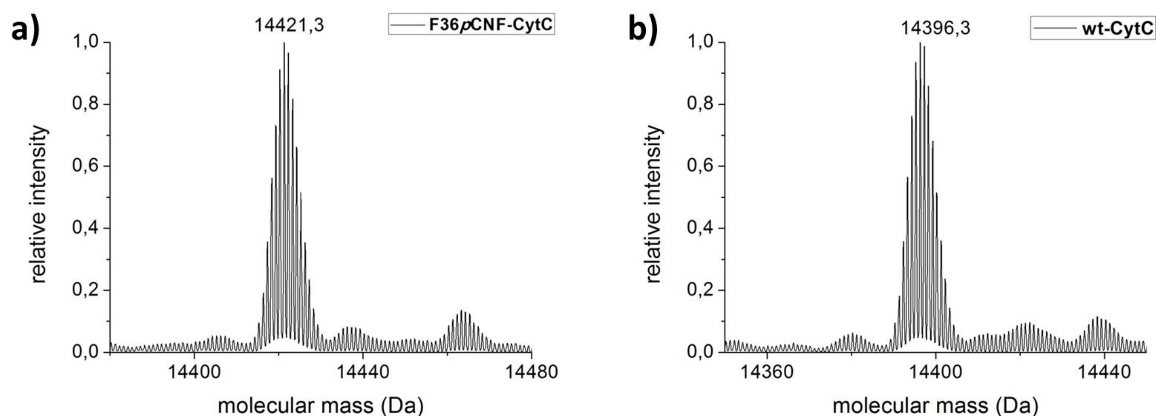


Figure S4: deconvoluted ESI-MS profiles of purified wt-CytC (Mcalc=14396.1 Da) and *p*CNF-CytC (Mcalc=14421.1 Da).

6.3 UV-absorption, CD-spectra and thermal unfolding of CytC variants

Electronic spectra (UV-visible) were recorded on a JASCO J-815 spectrometer at room temperature in the wavelength range 300-800 nm using a standard 10 mm path length quartz cuvette. Circular Dichroism (CD) spectra were collected in a JASCO J-815 spectrometer employing a 1 mm quartz cuvette. Secondary structure of oxidized CytC samples was analyzed by recording far-UV CD spectra (199–255 nm) at 25°C. Protein concentration was 25 µM in 100 mM sodium phosphate buffer (pH 7.4), supplemented with 50 µM potassium ferricyanide. Thermal unfolding was monitored between 20°C and 98°C by recording the CD signal at 220 nm.

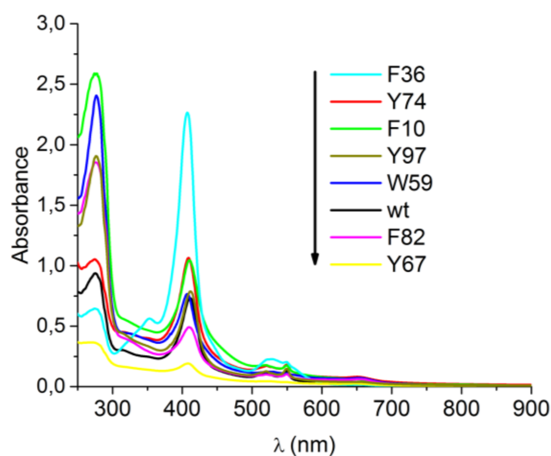


Figure S5: Absorption spectra for heterologously expressed CytC variants with *p*CNF incorporated at the indicated positions at 25°C in 100 mM phosphate buffer pH 7.4.

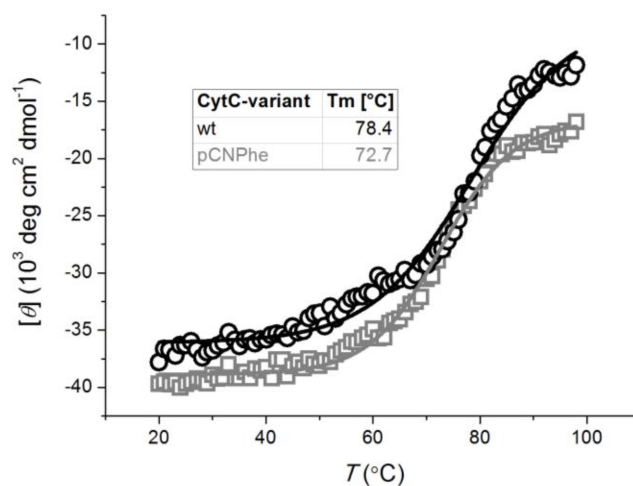


Figure S6. Effect of the *p*CNF substitution on the thermal stability of oxidized CytC mutants (○)wt-CytC; (□)*p*CNF-CytC. The Tm values of the *p*CNF-CytC and wt-CytC species were calculated from theoretical fitting (indicated as solid lines) of the 220 nm CD signal at increasing temperature, using a two state native-denatured model.

6.4 Infrared absorption spectroscopy

IR spectra were measured with a spectral resolution of 4 cm^{-1} using a Bruker Tensor 27 FT-IR spectrometer controlled by the OPUS Data Collection Program. Each spectrum consisted of 400 scans. All measurements were carried out at room temperature (25°C).

6.5 Electrochemistry

Cyclic voltammetric measurements of immobilized CytC variants were performed with a multistat CH instrument controlled by the CH Labs-CHI 660b software. The scan rate was varied in the range from 0.1 - 1 Vs^{-1}. The proteins were immobilized on Ag electrodes coated with self-assembled monolayers of mercaptohexanoic acid as described previously.[11] The solution contained 12.5 mM potassium sulfate and 12.5 mM sodium phosphate buffer at pH = 7.0. Solutions were purged with Ar prior to the experiments.

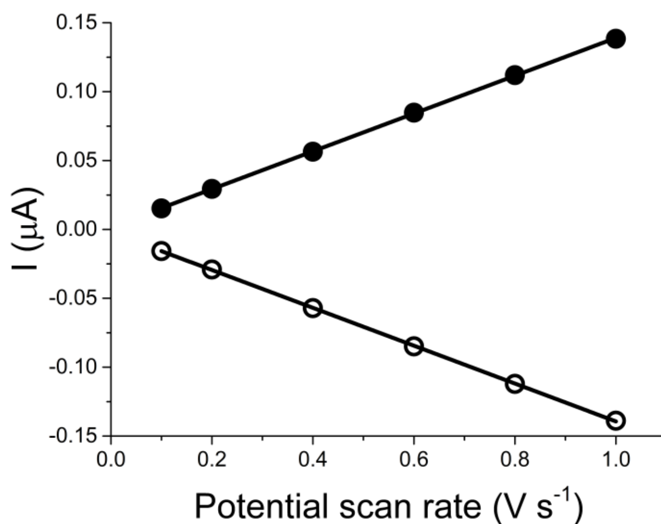


Figure S7. Variations of the anodic (○) and cathodic (●) peak currents of *p*CNF-CytC adsorbed on C_5-coated roughened Ag electrode as a function of the potential scan rate.

7. Summary of expressed CytC congeners

Substituted residue	**Position**	**Orientation of *para* position**	**Expressed**	**Purified from**	**Prone to aggregation**	**Remarks**
Wild-type	-	-	Mostly soluble	Supernatant	No	
F10	Partly buried	Protein interior	Partly soluble	Supernatant	No	
F36	Surface	Protein interior	Soluble	Supernatant	No	2 methionines oxidized
Y48	Partly buried	Protein interior	-	-	-	
W59	Partly buried	-	Insoluble	Inclusion bodies	Yes	
Y67	Buried	Protein interior	Partly soluble	Supernatant	Yes	
Y74	Surface	Solvent	Insoluble	Inclusion bodies	Yes	
F82	Surface	Protein interior	Partly soluble	Supernatant	Yes	

Table S1. Summary of expression and properties of CytC variants with *p*CNF incorporated at different positions.

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