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

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Cytochrome *c* (cyt *c*), a redox protein involved in diverse fundamental biological processes, is among the most traditional model proteins for analyzing biological electron transfer and protein dynamics both in solution and at membranes. Studying the role of electric fields in energy transduction mediated by cyt *c* relies upon appropriate reporter groups. Up to now these had to be introduced into cyt *c* by in vitro chemical modification. Here, we have overcome this restriction by incorporating the noncanonical amino acid *p*-cyanophenylalanine (*p*CNF) into cyt *c* in vivo. UV and CD spectroscopy indicate preservation of the overall protein fold, stability, and heme coordination, whereas a small shift of the redox potential was observed by cyclic voltammetry. The C≡N stretching mode of the incorporated *p*CNF detected in the IR spectra reveals a surprising difference, which is related to the oxidation state of the heme iron, thus indicating high sensitivity to changes in the electrostatics of cyt *c*.

Cytochrome *c* (cyt *c*) is a ubiquitous small globular protein that contains a c-type heme covalently attached to the polypeptide backbone by two thioether bonds. The essential role of cyt *c* in the chemistry of life and cell viability is well established: it participates in fundamental biological processes such as respiration, photosynthesis, apoptosis, detoxification and gas sensing.[1,2] These critical biological roles are, on the one hand, mediated by cycling between the reduced (Fe^{2+}) and the oxidized (Fe^{3+}) forms of the heme iron and, on the other, by distinct structural states of the protein, thus revealing that protein folding is essential for determining the biological function of cyt *c* and its localization in the cell. In particular, the electron transfer (ET) function of cyt *c* has been studied in detail both for the protein in solution, in complex with its natural reaction partners, and in electrochemical environments.[1,3–5] Electrochemical devices are particularly instructive as, upon coating the working electrode with membrane models, one can mimic the natural reaction conditions at the mitochondrial membrane where the physiological redox process takes place. Thereby it was shown that high local electric fields are likely to affect the interplay between protein dynamics and ET and, hence, the efficiency of the redox process.[3,5]

Nonetheless, a quantitative description of how local electric fields control the ET reaction mechanism is still missing. To fill this gap, local electric field strengths can be determined by exploiting the vibrational Stark effect (VSE), which requires the incorporating of an appropriate reporter group (e.g., nitrile, carbonyl, azide) site-specifically into the protein.[6,7–12] A frequently used approach is based on engineered protein variants carrying a single reactive amino acid (i.e., cysteine) for subsequent chemical addition of the label.[7,8,12] However, this method has severe intrinsic limitations, such as partial denaturation under coupling conditions, mixed-labeled and unlabeled protein samples, long reaction times, and oxidation of sensitive residues.[13] Furthermore, because of the steric demands of the labels (e.g., mercaptobenzonitrile),[12] the site of modification is restricted to exposed side chains, and the reporter group is located in the solvent and thus outside the protein. Alternatively, semisynthetic approaches have been employed by combining a cleaved fragment of the native protein with a synthetic peptide including the modified amino acid.[9,10] This approach is typically restricted to small proteins, but has been used to generate labeled cyt *c*.[9]

To overcome the limitations associated with in vitro labeling and peptide synthesis, this study aimed at in vivo incorporation of a non-canonical amino acid (ncAA) carrying a nitrile function as a Stark reporter group. We chose *p*-cyanophenylalanine (*p*CNF), which has already been used to modify cyt *c* by a semisynthetic route[9] and, by in vivo labeling, has been incorporated into the more flexible myoglobin carrying a noncovalently bound heme.[11] We used stop codon suppression (SCS) with an in-frame rare stop codon (TAG) for site-directed incorporation of *p*CNF into horse-heart cyt *c* in a bacterial expression system.[14] An orthogonal nonsense suppressor tRNA/aminoacyl-tRNA synthetase (aaRS) pair derived from the *Methanococcus janaschii* (Mj) tyrosyl tRNA/aaRS pair[15] was employed and tested at eight positions for the read-through of an in-frame stop codon with *p*CNF in cyt *c*.

Unlike myoglobin, which can be assembled by simply adding the cofactor to the apoprotein, a crucial step in cyt *c* biosynthesis is the covalent attachment of the heme group to apo-cyt *c*. Although this can be attained by the intrinsic maturation machinery of *Escherichia coli* under anaerobic conditions, previous work indicates that protein yield could be greatly improved by co-expressing yeast cytochrome *c* heme lyase (CCHL), which catalyzes the covalent attachment of heme to apo-cyt *c* (Figure 1).[16,17] This reaction is likely to be nega-

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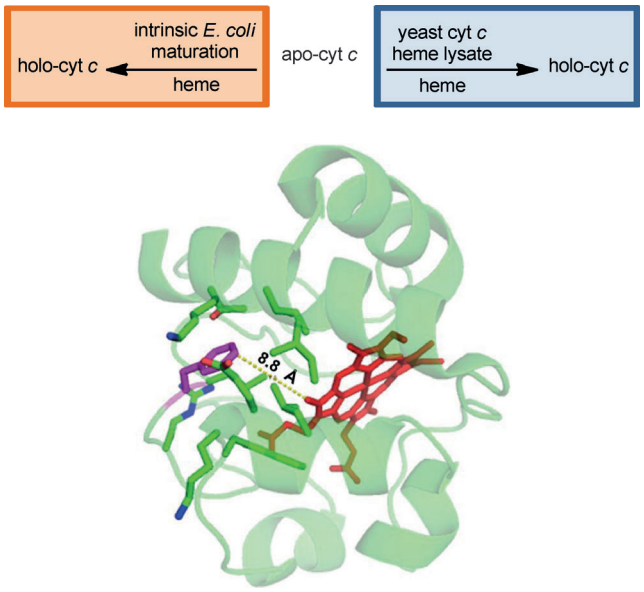

Figure 1. Heterologous biosynthesis, and tertiary structure of horse-heart cyt *c* (PDB ID: 1HRC). The distance between the *para*-carbon atom of Phe36 (magenta) and the heme (red) group is 8.8 Å. Residues within 5 Å of Phe36 are shown as sticks.

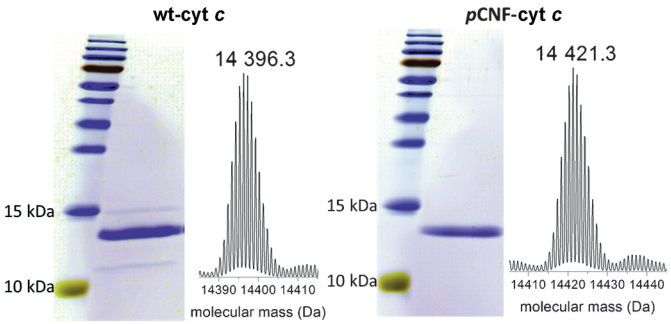

Figure 2. SDS-PAGE gels and deconvoluted ESI-MS profiles of purified wt-cyt *c* ($M_{W\,(calcd)}$ = 14396.1 Da) and *p*CNF-cyt *c* (14421.1 Da).

tively affected by any structural changes of the substrate, as inferred from the conservation of the cyt *c* tertiary structure during evolution.[18]

Successful cyt *c* expression and simultaneous *p*CNF incorporation by SCS in a bacterial host requires that both the orthogonal aminoacyl-tRNA synthetase (here *p*CNF-RS) and CCHL remain active under the experimental conditions for cyt *c* expression and maturation. As precipitation of CCHL in inclusion bodies at elevated temperatures (37 °C) has been reported,[17] the bacterial strain was first incubated at reduced temperatures (24 h, 23 °C) to express folded CCHL before incubation at higher temperatures (48 h, 30 °C) to express engineered cyt *c* (Scheme S3 in the Supporting Information). Improved expression and solubility of mature cyt *c* variants were observed. Under these conditions eight out of nine positions chosen for site-specific ncAA incorporation allowed efficient stop codon read-through (Figure S1, Scheme S4, and Table S1). Cyt *c* variants with *p*CNF substituting either phenylalanine (Phe36, Phe82, or Phe10) or tyrosine (Tyr67 or Tyr97) could be purified from soluble fractions whereas those substituting Trp59 or Tyr74 had to be purified from inclusion bodies under denaturing conditions with subsequent refolding. After immobilized metal-ion affinity chromatography (IMAC) and ion exchange chromatography (IEC), only the wild-type cyt *c* (as a control) and the variants with substitution of Phe10 or Phe36 yielded purified proteins that were stable in solution at 4 °C over several days. ESI-MS data confirmed the successful substitution of *p*CNF (Figure 2). Our data are in line with a previous study that demonstrated the importance of Phe82, Tyr67, Phe10, Tyr97, and Trp59 for cyt *c* folding and stability.[18]

Cyt *c* with *p*CNF substitution at Phe36, termed *p*CNF-cyt *c*, was chosen for further investigations, as it showed stability at 4 °C for several weeks and gave even higher yield (~10 mg L^{-1}) of purified protein than wt-cyt *c* (5 mg L^{-1}).

To check for a possible impact of *p*CNF incorporation on the structural integrity of cyt *c*, we applied UV–visible absorption and UV circular dichroism (CD) spectroscopy (Figure 3 A). The UV–visible spectra of purified *p*CNF-cyt *c* and wt-cyt *c* display intense Soret (412 nm) and less intense Q bands (520 and 550 nm) at the same positions, thus indicating that the proteins exist predominantly in the reduced forms with the heme

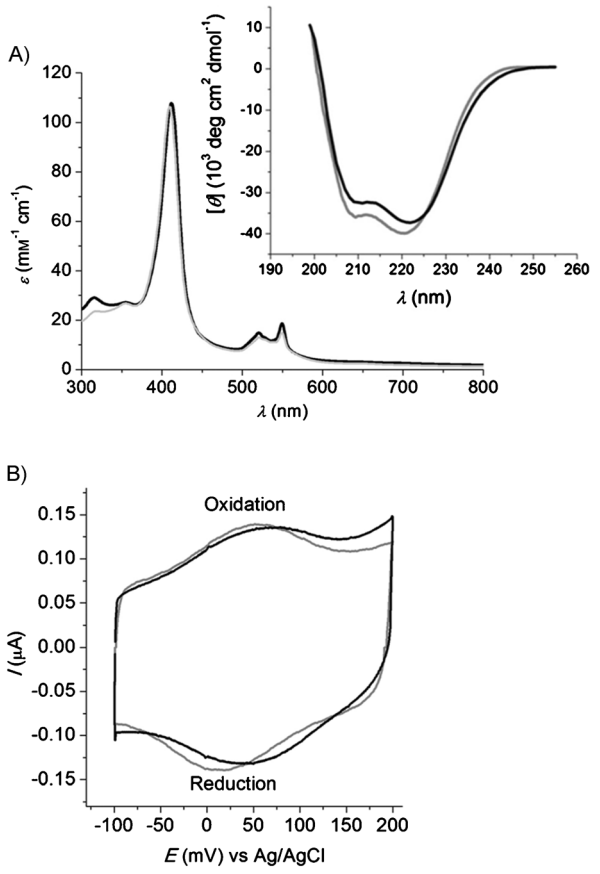

Figure 3. A) UV/Vis absorption spectra of proteins after purification, and CD spectra of oxidized forms (inset). B) CVs measured on Ag electrodes coated with C5-SAM. The black and gray traces in (A) and (B) refer to the wt-cyt *c* and *p*CNF-cyt *c*, respectively. Gaussian curve-fitting was applied to analyze the represented data to determine peak-to-peak separations and midpoint potentials.

pocket structure not altered by the Phe→*p*CNF substitution. The far-UV CD spectra (Figure 3A, inset) of wt-cyt *c* and *p*CNF-cyt *c*, show the same typical pattern of α-helical proteins with the two characteristic minima at 222 nm and at 208 nm of similar intensity. The overall dichroic patterns of the proteins are nearly identical. However, a slight decrease in thermal stability was detected in the mutant (Figure S6).

The redox potentials of *p*CNF-cyt *c* and wt-cyt *c* were determined for the proteins immobilized on Ag electrodes coated by a self-assembled monolayer of 6-mercaptohexanoic acid (C5-SAM). Cyclic voltammograms obtained at a scan rate of $1\,V\,s^{-1}$ (Figure 3B) revealed well-defined redox peaks with small peak-to-peak separations of ≈40 mV, thus indicating a nearly reversible process. When varying the scan rate, peak current increases linearly, thus demonstrating that cyt *c* remains bound to the electrode surface (Figure S7). For wt-cyt *c* a positive midpoint potential of about +50 mV (vs. Ag/AgCl) was derived from the CV traces, close to previously determined values.[19] The variant *p*CNF-cyt *c* displayed a slightly lower midpoint potential of about +40 mV. The shift in redox potential might be the result of a subtle structural perturbation in the heme pocket (too small to be detected in the UV–visible and CD spectra). Note that cyt *c* is to sensitive to amino acid substitutions or modifications.[5,9,20] Alternatively, the redox potential change might be a consequence of a change in electrostatics due to introducing an additional dipole moment by the F→*p*CNF substitution (vide infra).

The latter interpretation is supported by the IR spectra of reduced and oxidized *p*CNF-cyt *c*: a weak but clearly distinguishable peak in the C≡N stretching region (Figure 4). The second

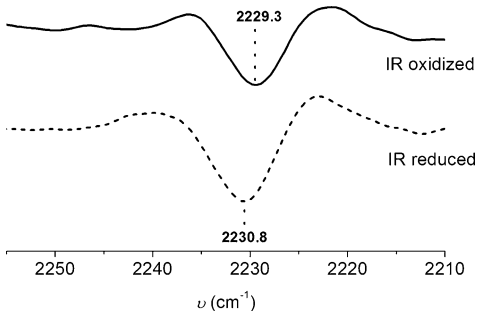

Figure 4. Second derivatives of the IR spectra of ferric and ferrous *p*CNF-cyt *c*.

derivative spectra reveal peak positions at 2230.8 and 2229.3 cm^{-1} (reduced and oxidized forms, respectively). Both frequencies fall in the range of a non-hydrogen-bonded nitrile function,[21] thus implying that the cyano group of *p*CNF is buried in the protein. Most surprisingly, however, C≡N stretching responds to changes in the oxidation state of the distal heme (8.8 Å, Figure 1). Thus, we conclude that the alteration of the electrostatics in the heme pocket, associated with the transition between the ferric and the ferrous heme, is transmitted to the protein surface where it is sensed by the VSE reporter group. Using the Stark tuning rate determined for benzonitrile,[10] the 1.5 cm^{-1} difference for C≡N stretching frequencies correspond to the remarkable change in the electric field strength along the nitrile bond ($2.5\times10^{6}\,V\,m^{-1}$) upon heme reduction. These results demonstrate the long-range effect of local charge changes. Conversely, this long-range electrostatic communication might also account for the 10 mV shift of the redox potential of the heme due to the incorporation of the additional dipole moment at position 36. It must be noted that such redox-state sensitivity was not observed for benzonitrile attached to the thiol side chain of a nearby Cys (K39C).[12]

We have reported here a general approach for the in vivo incorporation of the sensitive VSE reporter group (nitrile function) into cyt *c* including a covalently bound redox cofactor. The approach can, in principle, be extended to larger proteins and substitutions of buried amino acids typically not accessible by semisynthetic approaches and chemical modifications of exposed residues. The site-specific in vivo incorporation of *p*CNF was achieved by using an optimized two-temperature ramp expression protocol to increase the efficiency of cyt *c* maturation machinery. For the F36*p*CNF variant, overall protein folding and stability as well as the structure of the redox center were preserved. Our approach proved to be highly beneficial as it offers the generation of sufficient amounts of homogenous samples, although not all variants were stable in solution. Thus, further systematic studies are required to identify additional substitution sites for mapping the electric field in cyt *c*. To this end, position 36 might be an ideal candidate for introducing other functional groups for spectroscopic or functional studies. The redox-linked long-range modulation of local electric fields discovered in this work motivates the extension of VSE spectroscopic studies to proteins immobilized on electrodes coated with membrane models. Such studies might allow determination of the role of interfacial electric fields on the structure and function of cyt *c*.

Experimental Section

E. coli strain BL21(DE3) was co-transformed with the pEVOL-*p*CNF-RS[15,22] and pET22b + cyt *c*(Codon→TAG) + CCHL. Cells were grown in ZYP-5052 medium[23] in the presence of *p*CNF and arabinose at 23 °C for 24 h, followed by 48 h incubation at 30 °C in the presence of IPTG. A similar protocol was used for expression of wt-cyt *c*. After lysis, all cyt *c* variants (except CytC(Y74→*p*CNF), see the Supporting Information) were purified from the soluble fraction by IMAC and IEC. Analytical characterization of the proteins was performed by SDS-PAGE, western blotting and HPLC-ESI-Orbitrap-MS. Details of the spectroscopic and electrochemical experiments are given in the Supporting Information.

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