SUPPORTING INFORMATION

Methionine ligand lability of Type 1 cytochromes *c*: detection of ligand loss using protein film voltammetry

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Materials and Methods

Protein samples of *Pseudomonas aeruginosa* cytochrome c_{551} (PA cyt *c*) and *Hydrogenobacter thermophilus* cytochrome c_{552} (HT cyt *c*) and mutants were prepared as described previously.[1-5] Horse heart cytochrome *c* was obtained from Sigma and purified as described by Brautigen, et al.[6] Restriction and DNA modifying enzymes were purchased from Gibco BRL or New England Biolabs. A cloning kit for PCR products (TA cloning kit) including the pCR2.1 cloning vector was purchased from Invitrogen. *Escherichia coli* strain Nova Blue (Novagen) was used for ligation and sequencing steps. The plasmid pEC86, harboring cyt *c* maturation genes, *ccmABCDEFG*[7] was a gift from Dr. Linda Thöny-Meyer, and the plasmid pETPA[2] was a gift from Dr. Francesca Cutruzzolà. Oligonucleotide synthesis and DNA sequencing were performed at the Core Nucleic Acid Laboratory at the University of Rochester.

DNA manipulations were carried out generally as described in Sambrook et al.[8] The HT Q64V and Q64Y mutant were prepared The HT Q64V and Q64Y mutants were prepared using the polymerase chain reaction overlap extension method.[9] The pSHC552 plasmid,[10] containing the *Ht* cyt c_{552} gene preceded by an *E. coli*-compatible signal sequence, was the template. Mutagenic primers used to mutate Gln64 to Val (underlined codon) were: 5'-GCCTCCTGTAAATGTAACCGATGCG-3' and 5'-CGGTTACATTTACAGGAGGCATGGG-3'. Mutagenic primers used to mutate Gln64 to Tyr (underlined codon) were 5'-GCCTCCTTATAATGTAACCGATGCG-3' and 5'-CGGTTACATTATTAGGAGGCATGGG-3'. The fresh PCR products were cloned into the pCR2.1 vector. Cleavage with *Bam*HI and *Nde*I removed the gene inserts, which were cloned into pSHC552 to yield pSHC552Y64 (or pSHC552V64) (Amp^r). The sequences of the mutant genes were confirmed by DNA sequencing. The expression plasmid pSHC552Y64 (or pSHC552V64) and pEC86 (Cm^r) were used to transform BL21(DE3)Star competent cells to chloramphenicol and ampicillin resistance for expression of HT Q64Y (HT Q64V). Expression and purification of HT mutants was as described previously for the mutant HT Q64N.[4]

Preparation of the N64V mutation of the PA gene was by the megaprimer procedure.[11] The pETPA vector for PA expression[2] was used as the template for PCR amplification. The mutagenic primer was 5'-GCCGCCGGTCGCGGTCAGCGA-3' (mutation site is underlined). The sequence of the resulting plasmid, pETPAN64V, was verified by DNA sequencing. pETPAN64V and pEC86 were used to transform BL21(DE3)Star competent cells (Novagen) to chloramphenicol and ampicillin resistance. Expression and purification procedures for PA N64V were as described for wild-type PA.[2]

All electrochemical and spectroscopic experiments were performed in a combination of phosphate and citrate buffer with various pH values, except where noted. Salt (sodium chloride) was added when needed to modulate the ionic strength.

All experiments were carried out on the countertop. Before each experiment, buffer was degassed by bubbling nitrogen or argon for at least 40 minutes. And during the course of experiment, a nitrogen/argon blank was maintained using tubing. The thermostat electrochemical cell was housed in a solid faraday cage. A stationary PGE working electrode (physical area = 3.14mm^2) was used with a platinum wire used as the counter-electrode, and a saturated calomel electrode (SCE), located in a Luggin sidearm containing 100 mM Na_2SO_4 and maintained at room temperature was used as the reference. All potentials quoted are versus the standard hydrogen electrode (SHE); Voltammeric data were recorded with Autolab electrochemical analyzer (PGSTAT 12m Eco Chemie, Utrecht, The Netherlands) controlled by GPES software (Eco Chemie).

Before preparation of a film, the PGE electrode was polished with aqueous alumina slurry (Buehler 1μ m) and sonicated thoroughly. This generated a rough surface, rich in a variety of functionalities such as –CHO and –COOH groups that interact with proteins molecules. Protein films were prepared at room temperature by applying directly the concentrated protein stock (~250 μM in phosphate buffer, at near neutral pH) onto the electrode surface, followed by washing away the non-adsorbed protein sample

During the course of experiment, the electrochemical signal was stable, and only a minimum peak lost was observed.

In bulk-phase voltammetry experiments, the graphite working electrode was prepared as described above, but immersed in a cell solution containing: 150 μM purified HH cytochrome *c*, 50 mM Tris, 100 mM NaCl, pH 8.0.

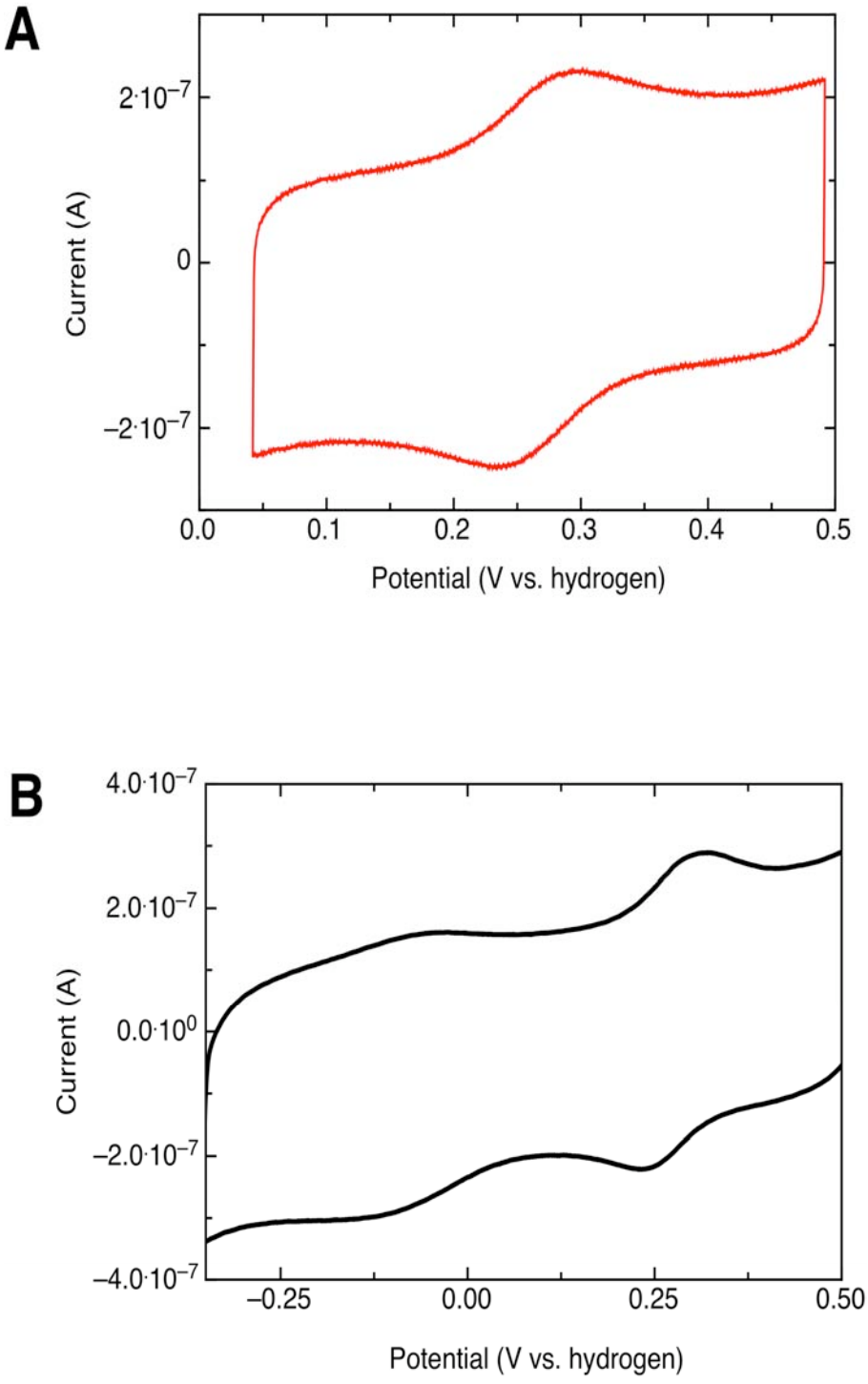

Figure S1. (A) A representative voltammogram of bulk phase electrochemisitry of horse heart cytochrome *c*, at an PGE electrode. Details of the experiment, scan range: [cyt *c*] = 150 μM +480 mV to 40 mV, *v* = 20 mV/s, Temperature = 20^0C. (This data is a reproduction of the trace shown in Figure 1D, reproduced here for comparison.) (B) An identical experiment, but with an expanded scan range, revealing the low-potential form of the HH cyt *c*.

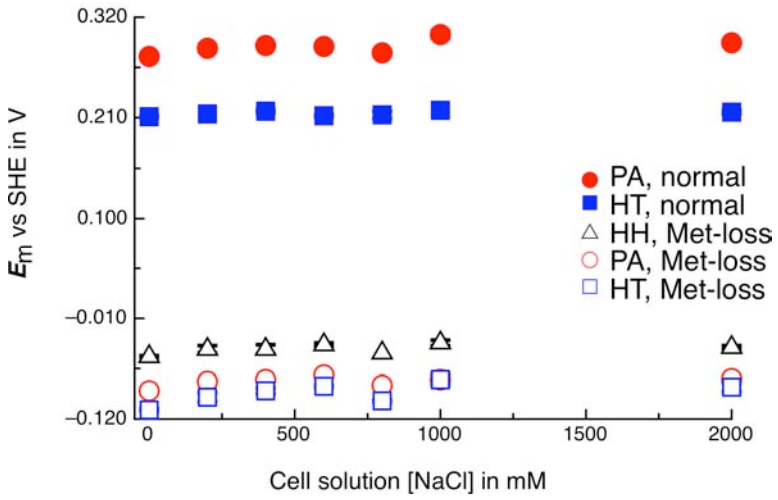

Figure S2. Modulation of observed potentials at PGE electrodes by variation of the bulk electrolyte concentration (NaCl).

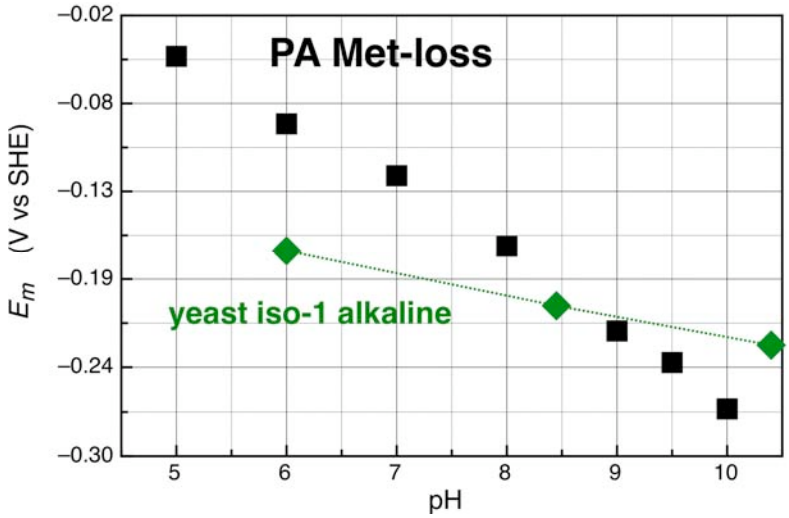

Figure S3. pH dependence of the midpoint potential observed here for the PA cyt *c* (solid squares), as compared to reported potentials for the alkaline form (Refs. 14, 15 in the main text).

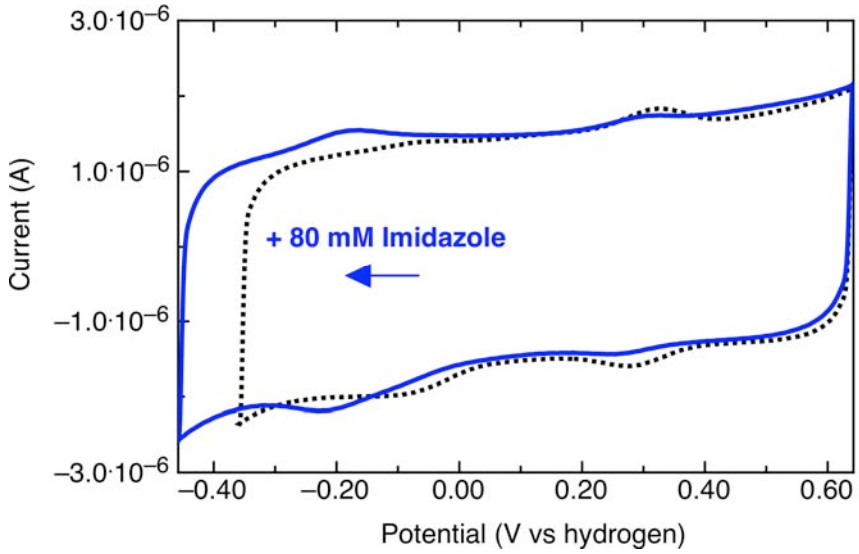

Figure S4. Impact of addition of 80 mM imidazole upon PA cyt *c* immobilized on a PGE electrode. Dotted line, PA PFV prior to imidazole addition; solid blue line, with imidazole. All experimental parameters are the same as Figure 1.

References

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