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Design and engineering of a man-made diffusive electron-transport protein☆

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ABSTRACT

Maquettes are man-made cofactor-binding oxidoreductases designed from first principles with minimal reference to natural protein sequences. Here we focus on water-soluble maquettes designed and engineered to perform diffusive electron transport of the kind typically carried out by cytochromes, ferredoxins and flavodoxins and other small proteins in photosynthetic and respiratory energy conversion and oxido-reductive metabolism. Our designs were tested by analysis of electron transfer between heme maquettes and the well-known natural electron transporter, cytochrome *c*. Electron-transfer kinetics were measured from seconds to milliseconds by stopped-flow, while sub-millisecond resolution was achieved through laser photolysis of the carbon monoxide maquette heme complex. These measurements demonstrate electron transfer from the maquette to cytochrome *c*, reproducing the timescales and charge complementarity modulation observed in natural systems. The ionic strength dependence of inter-protein electron transfer from 9.7×10^6 $M^{-1}s^{-1}$ to 1.2×10^9 $M^{-1}s^{-1}$ follows a simple Debye–Hückel model for attraction between +8 net charged oxidized cytochrome *c* and −19 net charged heme maquette, with no indication of significant protein dipole moment steering. Successfully recreating essential components of energy conversion and downstream metabolism in man-made proteins holds promise for *in vivo* clinical intervention and for the production of fuel or other industrial products. This article is part of a Special Issue entitled Biodesign for Bioenergetics — the design and engineering of electronic transfer cofactors, proteins and protein networks, edited by Ronald L. Koder and J.L. Ross Anderson.

1. Introduction

Small, diffusive water-soluble proteins, accommodating one or a few oxidation–reduction cofactors, connect larger and less mobile protein complexes in the electronic circuits of natural respiratory and photosynthetic energy conversion metabolism. Decades of research describe the design elements that confer recognition and binding specificity between the transporter and the electron-donor and acceptor-protein partners [1–4]. Short and long-range interactions between reacting protein pairs promote the formation of dynamic encounter complexes, bringing the electron-exchanging cofactors close enough, and for sufficient time, to support electron tunneling between the proteins [5]. Cytochrome *c* (cyt *c*), was the first such electron-transporter protein to be described [6] in a family that has since grown to encompass other cytochromes, flavodoxins, ferredoxins and metal-binding proteins. Despite the differences in cofactors, operating redox potentials, cellular compartments and protein partners, it seems clear that these diffusive transporters share the same broad operational characteristics and engineering tolerances that combine to support the millisecond rates typical of energy conversion and catalysis [7].

Table 1 summarizes key functional characteristics for a range of inter-protein electron transfers between diffusing *c* cytochromes and natural partners cyt *c* oxidase, cyt *c* peroxidase [8], cyt bc_1 and photosynthetic P870 [9,10]. Driving forces for the interfacial electron-tunneling step are often modestly favorable, but near zero or energetically uphill electron transfer is possible if followed by a sufficiently downhill intra-protein step [11]. Reported cofactor separations in the electron-transfer complexes range from 4 to 14 Å, comparable to the range of intra-protein electron-tunneling distances between cofactors within the same protein [5]. Inter-protein electron-transfer turnover rates from ~100 to 1000 s^{-1} are typically orders of magnitude slower than the intrinsic electron tunneling of the complex, suggesting that diffusion between redox partners or complex formation can be a limiting step.

Maquettes are man-made proteins designed from first principles, sidestepping evolutionary complexity in sequence and structure but highly adaptable in supporting a range of oxidoreductase functions. These elementary α-helical proteins bind a range of redox-active cofactors, including *b*-type hemes [16] and many other natural and unnatural tetrapyrroles [17,18], FeS clusters [19,20], flavins [21], quinones [22] and a variety of light activatable cofactors [17]. With modest sequence alterations, maquettes replicate a diverse range of natural oxidoreductase functions including the promotion of ultrafast intra-protein electron

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Table 1
Key engineering parameters for several well-characterized inter-protein *c*-type cytochrome electron-transfer complexes [10,12–15]. Minimum distance between cofactor edges is estimated from published structures [13,15]. Log of electron-tunneling rates estimated from empirical expression [5], assuming protein reorganization energy of 1 eV [34].

Redox pair	ΔE_m (eV)	Min. cofactor distance (Å)	K_d (μM)	log k_{ET} (s^{-1})
cyt *c* → CcO	−0.07 (*spheroides*)	11 (*thermophilus*)	0.35	5.9
cyt *c* → CcP	~−1.0	14	20	6.8
cyt bc_1 → cyt *c*	~0	9	9.5	6.7
cyt c_2 → P870	−0.16	8	1	8.2
Isocyt c_2 → P870	−0.22	8	40	8.5

transfer in photo-activated charge separation [21], and the suppression of electron transfer into the minute time scale to promote stable heme dioxygen binding [23].

Inter-protein electron transfer offers a new functional frontier for designed proteins. So far, preliminary experiments have shown electron transfer from a heme maquette to natural bovine cyt *c* on a millisecond time scale (Fig. 2) [17] and from ferredoxin-NADPH reductase to a heme *a* maquette [24]. In recent work, Ghirlanda and coworkers describe a related α-helix protein ligating 4Fe4S clusters that reduces cyt *c*[25]. Complimentary pairs of designed redox proteins also demonstrate inter-protein electron transfer [26]. Here, we deepen the enquiry to define the engineering principles governing critical electrostatic interactions between redox proteins, bringing cofactors within electron-transfer distance. We have improved the temporal resolution from the millisecond regime afforded by stopped-flow method [17] to the microsecond time domain using classic ferrous heme-CO flash photolysis [27]. This allows us to resolve rapid electron transfers from the maquette at rates approaching the diffusion limit. A series of time-resolved measurements of electron transfer from ferrous heme maquettes with contrasting surface charge patterns, to ferric bovine cyt *c* at various ionic strengths, reveal a process broadly similar to electron-transfer interactions between physiological redox partners.

Successful recreation of basic component parts of respiratory and photosynthetic energy conversion in maquettes confirms a practical understanding of the underlying engineering and construction of their natural counterparts. It also holds promise for a range of practical applications, ultimately *in vivo*. Examples include the interception and diversion of high or low potential electrons in living cells directly towards production of useful chemicals and fuels (analogous to synthetic biology's goal of redirecting metabolic pathways), and amelioration of genetic or age-related failures in respiratory energy conversion in humans [28].

2. Materials and methods

2.1. Maquette preparation

Reagent grade chemicals from Sigma-Aldrich were used, unless otherwise noted. Protein maquettes were expressed and purified as described in [17]. Heme cofactors were incorporated from DMSO stock solutions with concentrations quantified using the hemochrome method [29]. Maquette proteins were dissolved in CHES buffer (20 mM CHES, 100 mM NaCl, pH 9.0) and protein concentrations were measured *via* tryptophan absorbance at 280 nm ($\varepsilon = 5600\ M^{-1}cm^{-1}$). 2.5 equivalents of heme stock solution were added and allowed to incubate at room temperature for 5 min. A PD-10 gravity column (GE Healthcare) was equilibrated with pH 7.9 phosphate buffer for subsequent electron-transfer experiments. The heme-bound protein was passed through the column to exchange buffer and remove unbound heme. Finally, the protein was diluted to the desired experimental concentration by following the 412 nm oxidized heme Soret band of the bound cofactor ($\varepsilon = 117{,}000\ M^{-1}cm^{-1}$).

The redox difference spectra of heme bound to both maquette designs considered here can be compared with the redox difference spectrum of an equivalent amount of free heme (supplementary Figure 1). In both maquettes, absorption around 560 nm is dominated by a bis-His coordinated, cytochrome b-like alpha band, with a minor contribution (5 to 10%) of a single His coordinated, myoglobin-like absorption band. In the presence of either maquette, the broad free-heme spectrum contribution is less than 10% in this wavelength region, indicating that almost all the heme present is bound at the designed histidine sites.

2.2. Oxidation of cytochrome c

Bovine heart cytochrome *c* was dissolved in phosphate buffer (50 mM Na_2HPO_4, NaCl 110 mM, pH 7.9). 3 stoichiometric equivalents of $K_3Fe(CN)_6$ were added to guarantee that all cyt *c* was in the oxidized state prior to electron-transfer experiments. The oxidized protein solution was then passed through a PD-10 gravity column (GE Healthcare) to remove residual $K_3Fe(CN)_6$. Cyt *c* solutions were diluted to the desired experimental concentration by following the 409 nm Soret band ($\varepsilon = 107{,}000\ M^{-1}cm^{-1}$).

2.3. Degassing and CO binding

Prior to electron-transfer experiments, several mL of heme-bound maquette solution and cyt *c* solution were placed in septum-sealed quartz cuvettes with stir bars. The maquette was placed under either an argon or carbon monoxide (CO) gas flow, while cyt *c* was placed under argon. After 30 min, an oxygen scrubbing mixture of 80 nM glucose oxidase, 12 nM catalase, and 1 mM glucose was added to the maquette solution. The maquette protein was reduced by careful titration of sodium dithionite as monitored at 318 nm to minimize excess unreacted dithionite. For CO bound samples, dithionite was added until full CO binding was observed spectroscopically, with a dithionite excess of only a few μM. The maquette was then placed under argon flow for 5 min with stirring to remove excess CO. Cyt *c* and CO bound or CO free maquette were transferred under positive argon pressure to 10 mL gastight syringes (SGE Analytical) equipped with stainless steel Luer valves (Cadence Science) used as load syringes in the stopped-flow apparatus.

2.4. Electron-transfer measurements without CO photolysis

Electron-transfer measurements were run on an RSM-1000 UV–Vis stopped-flow spectrophotometer (OLIS, Inc.). For experiments without CO photolysis, the spectrophotometer was equipped with a rapid-scan monochromator sampling the Q-band spectral region at 1000 spectra per second. Roughly 3900 scans (3.9 s) were acquired after mixing the maquette and cytochrome *c* reactants. All experiments were performed at 20 °C.

Cyt *c* reduction was monitored at 549 nm, corresponding to the reduced α band of cyt *c*. Note that a maquette binds two hemes, each potentially available to transfer an electron to cyt *c*. Static measurements of the reduced and oxidized forms of the maquette and cyt *c* provide an extinction coefficient change of 19,040 $M^{-1}cm^{-1}$ per electron transferred. Although any anaerobic compromise during handling in the stop-flow may lessen the concentration of reduced maquette (but not oxidized cyt *c*) prior to mixing, the amount of reduced maquette oxidized by excess cyt *c* can be monitored using this redox extinction coefficient for cyt *c* at 549 nm. Reaction profiles were fit to second-order reaction trajectories; for comparison in Fig. 2B, the trajectories were normalized to fraction completed to 0 at early time and 1 at late time.

2.5. Electron-transfer measurements with CO photolysis

All experiments were performed at 20 °C. A 10 Hz Q-switched frequency-doubled Nd:YAG laser (532 nm, DCR-11 Spectra Physics) was focused with a cylindrical lens onto the 20 mm × 2 mm stopped-flow cell of an Olis RSM-1000 spectrophotometer. A mechanical shutter (Thor Labs) triggered by the stopped-flow TTL output selected a laser single pulse. A 600 μM fixed slit sampled an individual wavelength at