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A [illegible] 4F –4 [illegible] Clostridium acetobutylicum F F [illegible]

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–oxidation (redox) cofactors is to mediate electron transfer in biological enzymes catalyzing redox-based chemical transformation reactions. There are numerous examples of enzymes that utilize redox cofactors to form electron transfer relays to connect catalytic sites to external electron donors and acceptors. The compositions of relays are diverse and tune transfer thermodynamics and kinetics towards the chemical reactivity of the enzyme. Diversity in relay design is exemplified among different members of hydrogenases, enzymes which catalyze reversible H_2 activation, which also couple to diverse types of donor and acceptor molecules. The [FeFe]-hydrogenase I from Clostridium acetobutylicum (CaI) is a member of a large family of structurally related enzymes where interfacial electron transfer is mediated by a terminal, non-canonical, His-coordinated, [4Fe–4S] cluster. The function of His coordination was examined by comparing the biophysical properties and reactivity to a Cys substituted variant of CaI. This demonstrated that His coordination strongly affected the distal [4Fe–4S] cluster spin state, spin pairing, and spatial orientations of molecular orbitals, with a minor effect on reduction potential. The deviations in these properties by substituting His for Cys in CaI, correlated with pronounced changes in electron transfer and reactivity with the native electron donor–acceptor ferredoxin. The results demonstrate that differential coordination of the surface localized [4Fe–4S]His cluster in CaI is utilized to control intermolecular and intramolecular electron transfer where His coordination creates a physical and electronic environment that enables facile electron exchange between electron carrier molecules and the iron–sulfur cluster relay for coupling to reversible H_2 activation at the catalytic site.

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There is broad interest in understanding reduction–oxidation (redox) enzymes that catalyze the diverse chemical transformations that comprise biological energy transformation. Many of these reactions are relevant to energy storage in energy carrier

intramolecular electron transfer fundamental to driving a broad range of chemical reactions in biological systems.

Site-di erentiated iron–sulfur clusters, where a canonical Cys residue is exchanged for another residue such as His, Asp, Glu, or Ser are prevalent in redox enzymes.[6,7] They are often found as part of electron relays in proteins that perform unique chemical reactions, such as electron bifurcation where a pair of electrons are individually transferred from a single site down spatially and energetically separated pathways,[8–10] DNA binding and regulation of gene expression,[11,12] and the catalytic activation of small molecules (e.g., H_2).[13–15] Studies have shown that site-di erentiated coordination of iron–sulfur clusters can result in changes to the redox potential (E_m), geometry, and electronic properties. These properties contribute to function in electron transfer embodied in the Marcus theory for non-adiabatic electron transfer (eqn (1)).[16] The electron transfer rate constant (k_{ET}) is determined by the tunneling matrix element $|T_{DA}{}^2|$, reorganization energy (λ), the total Gibbs free energy change (ΔG^0) for the electron transfer reaction, and temperature (T).

$$k_{\blacksquare} = \frac{2\pi}{\hbar} \frac{\left|T_{\blacktriangle}{}^2\right|}{\sqrt{4\pi\lambda k_{\blacksquare} T}} \frac{\left(\lambda+\Delta G^0\right)^2}{4\lambda k_{\blacksquare} T} \quad (1)$$

In this context, site di erentiation of iron–sulfur clusters can be envisioned as a means to control electron transfer by tuning of ΔG^0 (i.e., E_m), the density of electronic states $|T_{DA}{}^2|$ (i.e., geometry and bonding e ects on spin states and spin pairing) and/or λ (i.e., solvation).

One example of site di erentiation is found in the [FeFe]-hydrogenase from Clostridium pasteurianum (CpI) and the structurally homologous enzyme from C. acetobutylicum (CaI). These enzymes catalyze H_2 activation at a conserved organometallic, iron–sulfur cluster, or H-cluster, that is integrated with a conduit of iron–sulfur clusters, termed the F-clusters. F-

iron–sulfur clusters, a truncated construct was created that consisted of the protein fold around the distal [4Fe–4S]His and [2Fe–2S] clusters. The construct was composed of the first 128 N-terminal residues of CaI (hereafter referred to as distal domain, DDHis) and recombinantly expressed in Escherichia coli (Fig. 1B). Following purification and iron–sulfur cluster reconstitution, 6.2 ± 0.2 mol Fe/protein was determined, confirming complete incorporation of both iron–sulfur clusters. A variant of the distal domain (DDCys) wherein the His-ligand was substituted by Cys was also constructed, allowing for explicit determination of properties unique to His coordination. Again, a value of 6.1 ± 0.4 mol Fe/protein was determined, showing that the His residue is not explicitly required for proper incorporation of a [4Fe–4S] cluster in this site. The iron–sulfur clusters in both constructs were found to be highly stable once incorporated into the protein scaffold, i.e., clusters remained intact indefinitely across a broad range of pH values (6.5–10.5) under anaerobic condition con79 iron

than for assays employing MB (H_2 uptake) and/or reduced MV (Table S1).

cluster remained largely unchanged (Fig. 4B). Similar low-field resonances were observed for WT CaI, albeit broadened and weaker in intensity (Fig. 4C). Like DDCys, the low-field resonances

Fig. 5B illustrates the e ect of changes in the site-di erentiated coordinating residue on the lowest unoccupied molecular orbitals (LUMOs) of the distal [4Fe–4S] cluster. The LUMO is the lowest energy molecular orbital that does not host an electron and, in the oxidized state, can be considered as the most likely place for hosting the extra electron that would be received by the cluster upon reduction. In the comparison of MO's of [4Fe–4S]Cys to [4Fe–4S]His or [4Fe–4S]HisH, it is evident that the MO's for the [4Fe–4S]Cys extend only to the S-atom of the coordinating Cys residue. However, in the case of [4Fe–4S]His and [4Fe–4S]HisH, the MO's are observed on the Nδ and coordinating Nε atoms of the His imidazole ring, and thus extended closer to the nearby FS4B [4Fe–4S] cluster. Interestingly, there is a coordinated water in the crystal structure of CpI that bridges the His93 Nδ atom to the carbonyl carbon of Cys146, which coordinates to the adjacent [4Fe–4S] cluster. This water coordination is expected to be changed or be lost when His is replaced with Cys. Hence, our MO analysis for the ααββ spin pairing combination of [4Fe–4S]His suggests that His coordination creates a tunnelling network to direct electron transport to and from the neighbouring [4Fe–4S] cluster in the F-cluster relay (Fig. 5C).

Disc ssion

The e ect of site-di erentiation on [4Fe–4S] clusters, specifically where a mixture of Cys and His coordinating residues is observed, has been shown to have di erential e

Author contributions

The manuscript was written through contributions of all

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