Replacement of an Electron Transfer Pathway in Cytochrome *c* Peroxidase with a Surrogate Peptide[†,‡]

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ABSTRACT: A proposed electron transfer pathway in cytochrome *c* peroxidase was previously excised from the structure by design. The engineered channel mutant was shown to bind peptide surrogates without restoration of cyt *c* oxidation. Here, we report the 1.6 Å crystal structure of (*N*-benzimidazole-propionic acid)-Gly-Ala-Ala bound within the engineered channel. The peptide retains many features of the native electron transfer pathway: placement of benzimidazole at the position of the Trp-191 radical, hydrogen bonding to Asp235, and positioning of the C-terminus near the point where wild type C*c*P makes closest contact to cyt *c*. The inability of this surrogate pathway to restore function supports proposals that electron transfer requires the Trp-191 radical.

Electron transfer (ET) between redox active cofactors in proteins can be mediated by general effects of the intervening medium or by specific pathways that enhance the ET rate between donor and acceptor (*1*−*3*). These pathways are often comprised of covalently linked segments of the protein backbone and provide efficient enhancement of ET provided that they are short and contain minimal jumps across noncovalent links. In addition, ET pathways often include an intervening redox active center, such as a tryptophan or tyrosine side chain, and these sites can play an ephemeral role in superexchange-coupled pathways (*4*) or as a true radical intermediate in ET hopping pathways (*5*−*7*).

One of the first and most well-known ET pathways is that between the heme of cytochrome *c* peroxidase (C*c*P) and cytochrome *c* (cyt *c*). C*c*P is a heme enzyme that catalyzes the oxidation of cyt *c* by H_2O_2 (*8*). In the reaction cycle, H_2O_2 reacts with the ferric heme of C*c*P to generate an intermediate, compound ES, containing a ferryl iron center (Fe^{4+}═O) and a cation radical on Trp-191 (*9*−*11*). Cyt *c* reduces this oxidized intermediate in two separate ET steps to regenerate ferric C*c*P (*12*).

The backbone of residues Trp-191, Gly-192, Ala-193, and Ala-194 in C*c*P are believed to provide an efficient ET pathway between the heme of cyt *c*, and the Trp-191 radical of C*c*P. In the crystal structure of the complex between yeast cyt *c* and C*c*P, the methyl group of Ala-194 is positioned within 4.0 Å of the heme edge of cyt *c*, while the Trp-191 side chain is in van der Waals contact with the heme of C*c*P (*13*). Results of a number of studies support the proposal that both ET steps from cyt *c* proceed by direct reduction of the Trp-191 radical (*14*−*17*), presumably via this pathway, while other studies have suggested the existence of multiple binding sites on C*c*P for cyt *c*, implying that more than one ET pathway may contribute (*18*−*20*). Despite many years of study, this issue has not been completely resolved.

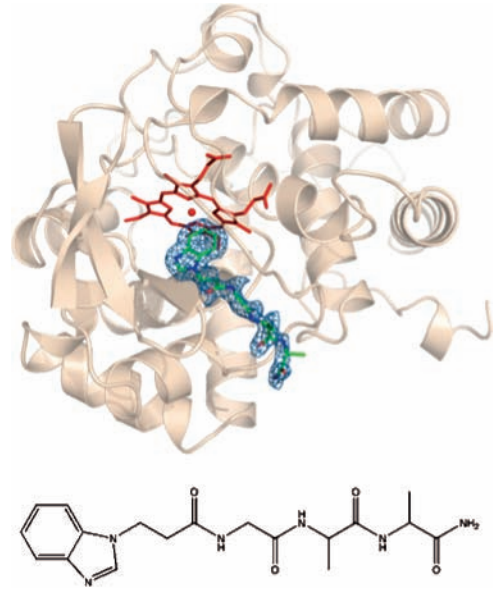

FIGURE 1: Crystal structure at 1.6 Å resolution of the C*c*P ET channel mutant bound to BzGAA (shown below). The $F_o - F_c$ omit electron density map for the peptide is shown at 1.0 σ (blue) and the peptide model in green.

We have previously described an engineered variant of C*c*P in which the proposed ET pathway was excised from the structure, leaving a water filled channel in its place (*21*). Upon screening a library of potential peptide surrogates, (*N*-benzimidazole-propionic acid)-Gly-Ala-Ala (BzGAA) (Figure 1) was found to be kinetically trapped by the ET channel mutant following a reconstitution procedure that involved partial denaturation and refolding in the presence of the peptide (*22*). However, reconstitution by the peptide resulted in no significant recovery of cyt *c* oxidation activity (k_{cat} < 0.02% of wild type) even though it retained its ability to react with H_2O_2 to form an oxidized intermediate (see Supporting Information). This suggests that the trapped peptide lacks some critical feature that is necessary for this pathway to function efficiently (*22*). In order to understand the origin of the functional lesion in the engineered ET

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[‡] The coordinates for the structure described here have been submitted to the Protein Data Bank as entry 3EXB.

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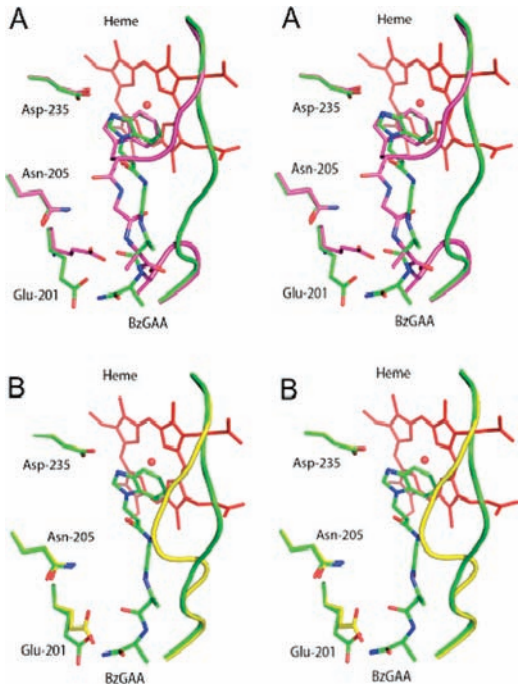

FIGURE 2: Stereoviews of the structures of (A) the BzGAA/ET channel mutant complex (green) overlaid on the native ET pathway of wild type C*c*P (magenta, PDB entry 1zby), and (B) the ET channel mutant in the peptide-bound (green) and peptide-free (yellow) state (PDB entry 1kxn). Labeled residues make hydrogen bonds with the native ET pathway.

pathway and to provide a rational basis for further surrogate design, we describe here the crystal structure, at 1.6 Å resolution, of the ET channel mutant containing BzGAA.

The ET channel mutant was purified, reconstituted with BzGAA, and crystallized using a procedure (see Supporting Information) adapted from that previously described (*22*). Low temperature (100 K) X-ray diffraction data, extending to 1.6 Å resolution (Table S1, Supporting Information), were collected from crystals of the reconstituted complex. Following model building and refinement (R = 16.8, R_{free} = 19.0), the $F_o - F_c$ omit electron density map (Figure 1) clearly showed the presence of the peptide in the ET channel. Aside from local changes at the site of the installed peptide, the overall structure of the ET channel/BzGAA complex is very similar to wild-type C*c*P. The benzimidazole group occupied a position that overlaid with that of the wild-type Trp-191 radical center almost exactly (Figure 2a), and included a hydrogen bond between the benzimidazole and Asp-235. This interaction is analogous to the one between Trp-191 and Asp-235 in the wild type enzyme and known to be functionally important (*23*−*25*). The tail of the BzGAA peptide backbone extends through the engineered ET channel following the overall position of the native ET pathway. In addition, the C-terminal methyl group of BzGAA is approximately 2.3 Å from the Ala-194 methyl of wild type in the superimposed structures (Figure 3). Thus, the overall placement of the engineered ET pathway is very similar to that of the native structure. Each backbone amide and carbonyl group in both structures make hydrogen bonds with the protein or are exposed to solvent, yet the native and surrogate pathways have different hydrogen bonding interactions (Figure S1, Supporting Information). Comparison of the ET channel mutant structures with and without bound peptide (Figure 2b) indicated that peptide binding caused a

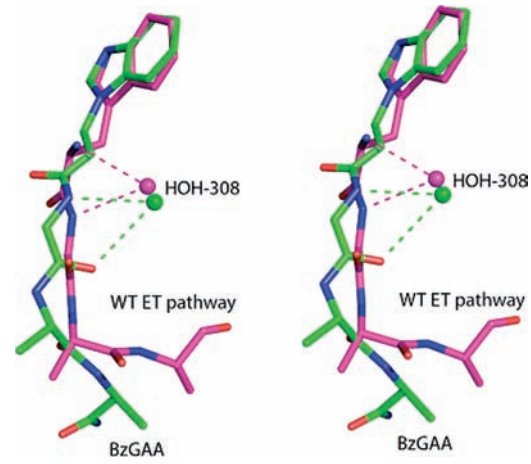

FIGURE 3: Stereoview showing the conformation of the BzGAA peptide (green) and native ET pathway. HOH-308 occupies a similar position in both structures, but shifts its hydrogen bonding pattern with the backbone due to the absence of the N-terminal amide in the peptide.

small perturbation in the protein backbone at Gly-190 due to steric clash with the bound peptide. This segment of the protein also exhibited higher than average B-values, suggesting that it is somewhat disordered. These results suggest that the overall driving force for occupation of this channel is the electrostatic interaction of the charged benzimidazole headgroup with the nascent binding site of the removed Trp-191 cation radical, and this is consistent with previous cavity complementation studies (*26*−*28*).

The structure suggests several possible explanations for the inability of BzGAA to restore efficient cyt *c* oxidation to the ET channel mutant. The BzGAA peptide was positioned in the ET channel with a reasonably good approximation of the native pathway, but the details of its backbone conformation differ somewhat from wild type. In the native pathway, the backbone between Gly-192 and Ala-193 is very nearly planar and is also coplanar with the aromatic ring of the Trp-191 radical (Figure 3). In contrast, the backbone of BzGAA has rotated so that the glycine carbonyl in BzGAA is approximately 90° from that of Gly-192 in the native pathway. This rotation may be the result of a change in hydrogen bonding to a buried water molecule. In the wild-type structure, HOH-308 accepts hydrogen bonds from the backbone amides of Trp-191 and Gly-192. However, the absence of the N-terminal amide nitrogen in the ET channel/BzGAA structure results in the analogous water forming hydrogen bonds with the glycine amide and the carbonyl oxygen of the following Ala in BzGAA. This difference may be responsible for the observed backbone reorientation. It is not well understood if the orientation and planarity of this segment plays a significant role in the ET coupling efficiency between C*c*P and cyt *c*, but backbone conformation and hydrogen bonding are known to play a modulating role in other systems (*29*). However, it is unlikely that this conformational change would completely abolish cyt *c* oxidation as seen in this system. It is also possible that the installation of the BzGAA peptide into the ET channel mutant disrupts the binding interaction between C*c*P and cyt *c*. However, comparison of the peptide-bound and -free states of the ET channel mutant to the structures of the C*c*P-cyt *c* complexes suggests that a complete disruption of the protein−protein interaction would not be expected (Figure

S2, Supporting Information). The interactions of C*c*P with horse and yeast cyt *c* differ somewhat, both in their structure (*13*, *17*) and binding affinity (*30*), and while the kinetic properties for oxidation of horse and yeast cyt *c* differ, the enzyme is capable of oxidizing both forms (*31*). Thus, we propose that the complete loss of functional turnover is due to some factor other than the interaction of C*c*P with cyt *c*. Full characterization of the binding thermodynamics of this system is in progress and will be necessary to resolve this issue.

Previous ET and mutagenesis studies have suggested the importance of the intact Trp-191 radical for efficient ET from cyt *c* (*9*, *32*), and this suggests that ET from cyt *c* proceeds through the proposed pathway. However, mutations at Trp-191 have resulted in the additional disruption of the functionally important hydrogen bond to Asp-235 at the active site, preventing unambiguous conclusions. The Trp-191 side chain has also been replaced with a number of small molecule surrogates by cavity complementation, many of which retain the hydrogen bond to Asp-235 (*26*−*28*). However, in these cases, the covalent linkage between the radical surrogate and the ET pathway was broken, again leaving it uncertain whether radical loss or covalent coupling was most important. Thus, the replacement of the native ET pathway in C*c*P with BzGAA provides a novel approach to probing this important structural element.

Upon analysis of this structure, we thus propose that the most functionally significant difference between the native enzyme and the reconstituted ET channel mutant is the nearly isostructural replacement of the Trp-191 radical site with a benzimidazole moiety. We have previously shown that the peptide Trp-Gly-Ala-Ala is not trapped by the ET channel mutant under the conditions (pH < 7) required for successful reconstitution by BzGAA (*21*). Under these conditions, benzimidazole is a protonated cation, and the protein structure surrounding Trp-191 provides an electrostatic environment optimized to stabilize the Trp-191 cation radical (*26*−*28*).

The structure reported here shows that the BzGAA peptide replacement for the ET pathway retains a critical interaction with the protein, and provides a potentially well-coupled tunneling pathway, while simply removing the energetically accessible radical state. Complete loss of functional activity in the BzGAA/ET channel mutant thus strongly supports proposals that the Trp-191 radical intermediate is required for efficient turnover of cyt *c* via the proposed ET pathway. Further studies of these and other ET pathways using photochemically triggered or electrochemically coupled wires will provide a novel approach to study of ET in proteins and enzymes.

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SUPPORTING INFORMATION AVAILABLE

Materials and Methods, Table S1, and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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