Structures of benzylsuccinate synthase elucidate roles of accessory subunits in glycyl radical enzyme activation and activity

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Structures of benzylsuccinate synthase elucidate roles of accessory subunits in glycyl radical enzyme activation and activity

Michael A. Funk1, Evan T. Judd1, E. Neil G. Marsh2, Sean J. Elliott2, and Catherine L. Drennan3,4,5,6

1Department of Chemistry, 2Department of Biology, and 3Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139; 4Department of Chemistry, Boston University, Boston, MA 02215; and 5Departments of Chemistry and Biological Chemistry, University of Michigan, Ann Arbor, MI 48109

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Anaerobic degradation of the environmental pollutant toluene is initiated by the glycyl radical enzyme benzylsuccinate synthase (BSS), which catalyzes the radical addition of toluene to fumarate, forming benzylsuccinate. We have determined crystal structures of the catalytic α-subunit of BSS with its accessory subunits β and γ, which both bind a [4Fe-4S] cluster and are essential for BSS activity in vivo. We find that BSSα has the common glycyl radical enzyme fold, a 10-stranded β/α-barrel that surrounds the glycyl radical cofactor and active site. Both accessory subunits β and γ display folds related to high potential iron–sulfur proteins but differ substantially from each other in how they interact with the α-subunit. BSSγ binds distally to the active site, burying a hydrophobic region of BSSα, whereas BSSβ binds to a hydrophilic surface of BSSα that is proximal to the active site. To further investigate the function of BSSγ, we determined the structure of a BSSαγ complex. Remarkably, we find that the barrel partially opens, allowing the C-terminal region of BSSα that houses the glycyl radical to shift within the barrel toward an exit pathway. The structural changes that we observe in the BSSαγ complex center around the crucial glycyl radical domain, thus suggesting a role for BSSγ in modulating the conformational dynamics required for enzyme activity. Accompanying proteolysis experiments support these structural observations.

Microbial metabolism | bioremediation | radical chemistry | iron–sulfur cluster | crystallography

Microbial degradation of hydrocarbons is a crucial component of the restoration of ecosystems after an influx of natural or human-made pollution. Aromatic hydrocarbons, primarily toluene and its derivatives, are some of the most abundant molecules present in groundwater pollution and thus are a prominent target for bioremediation efforts. The persistence of these molecules is due to their solubility and inertness: compounds such as toluene, phenol, and ethylbenzene contain no easily oxidized carbons, and dearomatization of these compounds requires strong oxidants or reductants. Nevertheless, microorganisms have developed diverse strategies to activate and degrade these compounds under both aerobic and anaerobic conditions (1).

Benzylsuccinate synthase (BSS) catalyzes the first step in an aerobic toluene degradation, the generation of (R)-benzylsuccinate from toluene and fumarate (Fig. 1A) (2). This unusual reaction is thought to proceed through radical mediated C–C bond formation (3–6), with the radical source being an enzyme-bound glycyl (Gly) radical cofactor (7) that transiently forms a catalytically essential enzyme-bound thyl (Cys) radical. Close family members of BSS, henceforth called the X-succinate synthase group, catalyze conjugation of fumarate and 2-methyl-naphthalene, p-cresol, and hydrocarbons including n-hexane (8, 9). The X-succinate synthase group is a member of the larger Gly radical enzyme (GRE) family (Fig. S14), which includes class III ribonucleotide reductase, pyruvate formate lyase (PFL), glycerol dehydratase (GDH), and 4-hydroxyphenylacetate decarboxylase (HPAD) (10). These enzymes share structural features including a 10-stranded β/α-barrel, a Cys loop containing the cysteine residue essential for catalytic hydrogen atom transfer, and a Gly radical loop housed within the C-terminal Gly radical domain that functions in radical storage.

The Gly radical is generated posttranslationally through direct hydrogen atom abstraction by a GRE-activating enzyme (AE) (11), a member of the radical S-adenosyl-l-methionine superfamily (Fig. 1B) (12). A structure of an AE bound to a peptide mimic of the GRE Gly radical domain (13) shows that the Gly radical domain must be extended away from the GRE to fit into the AE active site. However, once activated, the Gly radical domain must return to the inside of the barrel where the Gly radical can undergo hydrogen atom transfer with the catalytic Cys during each round of turnover, and where it is sequestered away from oxidants and reductants that could quench the radical. Consistent with this idea, all structural data thus far on GREs show the Gly radical domain buried deep within the GRE core adjacent to the Cys loop. Thus, two states of any GRE must exist: one with the Gly radical domain extended out of the protein core for posttranslational modification by the AE and one closed for catalysis. Using CD spectroscopy and analysis of enzyme activity, the Gly radical domain of PFL was shown to undergo structural changes when binding to its activase PFL-AE (14), with PFL-AE either catalyzing egress of the Gly radical domain or shifting the equilibrium of closed and open states. Despite these glimpses of the activation process, the mechanism by which this large conformational change is initiated, reversed, and regulated remains enigmatic.

BSS is distinguished among characterized GREs as one of the few that is composed of multiple subunits; a stable (αβγ)2 dimer is proximal to the active site. To further investigate the function of BSSγ, we determined the structure of a BSSαγ complex. Remarkably, we find that the barrel partially opens, allowing the C-terminal region of BSSα that houses the glycyl radical to shift within the barrel toward an exit pathway. The structural changes that we observe in the BSSαγ complex center around the crucial glycyl radical domain, thus suggesting a role for BSSγ in modulating the conformational dynamics required for enzyme activity. Accompanying proteolysis experiments support these structural observations.

Significance

Glycyl radical enzymes perform many chemical transformations that form the bedrock of microbial anaerobic metabolism. The structure of benzylsuccinate synthase reveals the architecture of an enzyme capable of removing aromatic hydrocarbons from polluted environments. These structures also illustrate a strategy for controlling the generation and utilization of radicals by glycyl radical enzymes through the use of accessory subunits.


The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4PKC and 4PKF).

1To whom correspondence should be addressed. E-mail: cdrennan@mit.edu.

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heterohexamer can be isolated from cultured \textit{Thauera aromatica} strain T1 grown on toluene (15) or expressed recombinantly in \textit{Escherichia coli} (16). The 98-kDa $\alpha$-subunit contains the catalytic machinery and forms a homodimeric complex, a common feature of GREs. BSS$\beta$ and $\gamma$ are much smaller, 9 and 7 kDa, respectively, and have been investigated on their own (16) and in complex with the $\alpha$-subunit (16, 17). Electron paramagnetic resonance, Mössbauer, and UV-visible spectroscopy from these studies indicate that BSS$\beta$ and BSS$\gamma$ each contain a [4Fe-4S]$\beta_\gamma$ cluster ligated by four conserved cysteines in these proteins. In both subunits, the cluster is a [4Fe-4S]$\alpha$ species that can be reduced to form the [4Fe-4S]$\alpha$ species after treatment with dithionite. The only structural information available for multimeric GREs comes from HPAD. The structure of the catalytic subunit of HPAD, HpdB, in complex with its small subunit, HpdC, was recently solved, revealing that HpdC is bound at the protein’s surface away from the active site and the Gly radical domain (18).

In this study, we investigate the structure of BSS, the mechanism of posttranslational activation, and the role of BSS$\beta$ and BSS$\gamma$ in this system. Although in vivo experiments have demonstrated that both subunits are essential for in vivo toluene utilization (19), their functions in the system have not been established. We have determined structures of two complexes, BSS$\alpha$$\beta$$\gamma$ and BSS$\alpha$BSS$\gamma$, which reveal structural and dynamic changes in the catalytic $\alpha$-subunit in response to binding of BSS$\beta$. These results suggest BSS$\beta$ acts as a regulator of activation and may additionally be involved in regulating access to the enzyme’s active site. Excitingly, we have observed two different states of the Gly radical domain in BSS. The movements seen in these structures are consistent with large conformational changes occurring during the activation of GREs by their cognate AE.

Results

\textbf{BSS$\alpha$ Adopts a Conserved Gly Radical Enzyme Fold.} We crystallized and determined the structure (Table S1) of BSS from \textit{T. aromatica} strain T1, a denitrifying bacterium. BSS$\alpha$, BSS$\beta$, and BSS$\gamma$ were coexpressed in \textit{E. coli} and purified as an ($\alpha$$\beta$$\gamma$)$_2$ complex. The catalytic $\alpha$-subunit adopts the core fold observed in other GREs (Fig. 2 and Fig. S2): a 10-stranded $\beta$-barrel with five strands oriented in one direction and the other five in the opposite direction. The barrel encloses two loops that harbor the catalytic Gly and Cys residues. The Cys loop contains the catalytic Cys449 residue and connects two $\beta$-strands that insert through the center of the $\beta$-barrel. The four backbone amide nitrogens from residues Val491-Ser495 point inward to form a shallow pocket that coordinates a chloride anion (Fig. S3A). This site is occupied by the carboxylate of a neighboring Glu in HPAD and GDH, but Ser or Ala replace this Glu in X-succinate synthases and could play a role in binding fumarate as well. One wall of the active site cavity is formed entirely of hydrophobic residues, providing a surface for toluene binding. Adjacent to the Cys loop is the Gly loop, found at the innermost tip of the C-terminal Gly radical domain, the key structural element believed to undergo a large conformational change during activation.

The structure of the BSS$\alpha$$\beta$ complex reveals two exterior binding sites for the small $\beta$- and $\gamma$-subunits (Fig. 2), with no changes to the core barrel architecture of BSS$\alpha$, compared with other GREs that do not have additional subunits. Within one $\alpha$$\beta$$\gamma$ unit, the average distance between BSS$\beta$ and BSS$\gamma$ is 45 Å, with the closest contact distance being 13 Å. The subunits bind to different regions of BSS$\alpha$ in both primary and tertiary structure and have radically different binding interfaces. A number of unique structural features are present at the surface of BSS$\alpha$ that enable binding to the small subunits as discussed below.

\textbf{BSS Small Subunits Resemble High Potential Iron-Sulfur Protein.} Despite having no recognizable sequence homology to any protein of known structure, BSS$\beta$ adopts a fold similar to that of a high potential iron-sulfur protein (HiPIP) (Fig. S4). Three short $\beta$-strands make a stable platform for the coordination of the [4Fe-4S]$\beta_\gamma$ cluster by four Cys ligands. A $\beta_10$ helix abuts the cluster and contains two of the Cys thiolate ligands, and long loops at the N and C termini wrap around the cluster, shielding it from solvent on two faces. Comparison with a characterized HiPIP reveals that several hydrophobic residues that normally exclude solvent from the cluster environment and enable stabilization of the [4Fe-4S]$^{2+}$ state are missing in BSS$\beta$ or replaced by hydrophilic residues (Fig. S4A). The region around Arg69 in particular is directly open to solvent molecules, and several waters are observed adjacent to the cluster, 3.7 Å from $\gamma$ of Cys44. BSS$\gamma$ contains an extension of the basal $\beta$-sheet by several residue pairs compared with HiPPIs, which provides a larger surface area for making contacts to the $\alpha$-subunit (Fig. 3B). Additionally, BSS$\beta$ contains an extended hairpin loop and a C-terminal $\beta_10$ helix (Fig. S4A), both of which are involved in intersubunit interactions as described below.

BSS$\beta$ also resembles the single small subunit of HPAD, which is known as the HpdC or HPAD$\gamma$ in that system (Fig. S4B). HpdC contains two small, concatenated HiPIP domains connected by a short linker. BSS$\beta$ superposes well with both of these domains (Cα rmsd of 0.78 and 0.77 for the N- and C-terminal domains, respectively, based on a core excluding unshared regions), although neither contain the $\beta$-sheet extension or the long N-terminal loop, which shield the cluster in BSS$\beta$. The [4Fe-4S]$\beta_\gamma$ cluster in BSS$\beta$ is therefore intermediate in terms of sequestration from solvent between the less buried clusters present in HpdC and the more buried clusters of HiPPIs. The C-terminal
region is also unique to BSSβ but does not contribute to cluster shielding.

Although BSSγ is clearly present in the structure of the BSSαβγ complex, the N and C termini are disordered, and there is no bound [4Fe-4S] cluster observable in the electron density. A close symmetry mate precludes packing of the intact subunit in both of the two crystal forms that we have obtained (Fig. S5 A and B). BSSγ is only 19% identical to BSSβ, but they share the basic HiPIP fold along with the extended β-sheet and hairpin loop (Fig. S5 C). Modeling of the disordered residues in BSSγ based on the structure of BSSβ suggests that the cluster in BSSγ would be more exposed to solvent than in BSSβ (Fig. S5D).

To investigate whether BSSβ and BSSγ display typical redox properties of HiPIP proteins, we performed protein film voltammetry on the BSSαβγ and BSSαγ complexes (Fig. 4 A and B). Consistent with the less hydrophobic environment observed for the [4Fe-4S] cluster of BSSδ, a redox potential of ~80 mV is observed, which is in the lower end of the range for the HiPIP +3/+2 couple (50–500 mV, average ~300 mV) (20). BSSδ and BSSγ have similar potentials, as evidenced by the small differences between the voltammograms of the BSSαβγ and BSSαγ complexes, such that the contributions of the two [4Fe-4S] clusters cannot be readily distinguished in the case of the αβγ complex. Unlike true HiPIP proteins (20), the [4Fe-4S] clusters of both αγ and αβ complexes engage in redox chemistry that is coupled to a single proton over a pH range of 6–9 (Fig. 4C). However, the [4Fe-4S] clusters in the BSS small subunits are resistant to degradation by oxygen (Fig. S6), a characteristic shared with HiPIP proteins (21).

**BSSα-BSSγ Interactions.** Previous studies on BSS have shown that expression and folding of BSSα depends on the coexpression of BSSγ but not BSSβ (16). The structure of the complex reveals a mostly hydrophobic binding site for BSSγ (Fig. 3D), exposure of which might induce the aggregation or misfolding seen during expression of BSSα alone. The hydrophobic patch on BSSα is positioned at the end of the dimer-forming helices and includes Ile273 and Phe278 in BSSα. These residues are conserved within the X-succinate synthase group—all of which contain putative γ-subunits—but not in more distant GRE families, including HPAD (Fig. S5E). An intersubunit β-sheet is present with a single strand from BSSα and the two BSSγ strands that make the [4Fe-4S] cluster platform. The nature and arrangement of interactions leave very little area exposed to solvent at the main interface. There are additional ionic interactions between a highly acidic loop in BSSγ and the main dimerization helix of BSSα, which contains two Arg residues (Fig. 3E).

**BSSγ-BSSδ Interactions.** In contrast to BSSα-BSSγ interactions, the binding surface for BSSγ on BSSα is not hydrophobic (Fig. 3B), nor is it confined to any single region in the primary sequence (Fig. S2). The primary interaction surface of BSSγ, the side and underside of the β-sheet, packs into a central indentation on BSSα, creating a hydrophilic interface with extensive polar side-chain contacts and more than 20 buried water molecules. Several of the structural features that differentiate BSSγ from traditional HiPIPs are observed to be important for binding to BSSα. In particular, the extended nature of the β-sheet with a kink in the central β-strand creates an unusually strong β-sheet curvature that allows for numerous binding interactions with BSSα, including contacts to the Gly radical domain. Also, the C-terminal 310 helix of BSSγ makes multiple contacts with the Gly radical domain (Fig. 3B). Together, the C terminus and sheet of BSSγ cover ~220 Å2 (of ~1,400 Å2) of solvent-accessible surface at the Gly radical domain. With this binding mode, BSSγ appears to stabilize the Gly radical domain inside the protein as any movement of the domain would require a change in conformation or dissociation of BSSγ.

Part of the BSSγ interaction surface includes a cavity proposed in GREs to be the physiological access route for substrate into the completely buried Cys loop at the active site (22). Using CAVER (23), a tunnel that is similar to that described previously in GREs can be identified in BSS (Fig. 3C). However, in BSS, this putative substrate entryway is obstructed by a hairpin loop from BSSβ that inserts into the top of the cavity. Notably, this hairpin loop is another region that differs between BSSβ and

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**Fig. 3.** BSS small subunits and subunit interfaces. (A) Comparison of BSSγ (magenta) to a model HiPIP (gray, Protein Data Bank ID code 1ISU (31)), with fully Cys-ligated [4Fe-4S] clusters shown in sticks. The C terminus and last visible residue at the N terminus are labeled for BSSδ. BSSα binds a broad surface on BSSα (green) that includes regions of the Gly radical domain (yellow). A small cavity on the surface of BSSα is occupied by a β-hairpin loop from BSSβ. Waters connecting the two subunits by hydrogen bonds are shown as red spheres. (C) A putative substrate access tunnel is shown in a white surface extending up from the catalytic Cys loop toward the enzyme surface. (D) BSSγ (blue) binds BSSα at a hydrophobic surface near the BSSα dimer inter- face. (E) Additional ionic interactions are present between BSSγ and BSSα.

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**Fig. 4.** BSS protein film voltammetry. (A) Voltammetric response of BSSγCys10 (E0 = 76 mV). (B) Voltammetric response of BSSγE0 = 89 mV. Raw cyclic voltammograms (outer) and curves with the background electrode capacitance corrected (inner) are shown. (C) Dependence of E0 on pH for BSSγ and BSSγCVN compared with the pH dependence of the E0 of Chromatium vinosum HiPIP (CVIN), the Rhodococcus gelatinosus HiPIP (RGEL), and the Ectothiorhodospira vaculata iso-2 HiPIP (EV1) as determined previously (20).

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traditional HiPIP proteins. When bound to BSSα, the hairpin loop appears to be very stable and is perfectly structured to fit into the BSSα cavity and possibly gate substrate entry.

**Global Structural Changes in the BSSα–BSSγ Complex.** To further investigate the role of BSSβ in this system, we sought to obtain a structure lacking the subunit. We successfully expressed and purified the BSSαγ complex in *E. coli*, obtained crystals in a new crystal form, and determined the structure by molecular replacement using the structure of BSSα as a basis (Table S1). The overall structure of BSSα in complex with BSSγ alone retains many of the features of the complex with BSSβ, especially in the N-terminal half-barrel and dimer interface, both of which are unchanged from BSSαβγ (Fig. 5A). However, the C-terminal half-barrel exhibits large crystal-to-crystal variation in the amount and quality of electron density around both core helices and peripheral loops and is consistently shifted relative to the BSSαβγ complex. Difference distance matrix analysis (Fig. S7) of the BSSαβγ and BSSαγ structures revealed a number of structural changes with distances unbiased by the choice of alignment strategy (24). With BSSβ absent, BSSα expands in a clamsHELL-like motion by 5–7 Å, centering at helix 9 (residues 748–758), which is partially disordered, and at strand 9 (residues 763–771) (Fig. S7). These motions open a gap in the center of the protein that allows the Gly radical domain to slip out of the active site by ~2 Å (Fig. 5B). Residues 55–65 of BSSα form a loop, henceforth referred to as loop 1, which contacts both the Gly radical domain and the C-terminal tail of BSSβ (Fig. 5B). Loop 1 moves alongside the Gly radical domain away from the active site by ~3 Å in the BSSαγ structure relative to BSSαβγ, possibly facilitating release of the Gly radical domain from the interior of the protein. At the active site, the Ca–Sγ distance between Gly829 and Cys493 lengths in the αγ complex by 0.60 to 4.45 Å. Although the Gly radical domain remains within the barrel opening, the absence of BSSβ removes a major obstacle from its exit pathway.

The observed rearrangements described above prompted an analysis of the distribution of B factors in BSSα with and without BSSβ present. Comparison of the two models clearly shows an increase in thermal motion around the C-terminal helix bundle and Gly radical domain in the BSSαγ complex (Fig. 5C). The N-terminal half-barrel and the long helices that form the dimer interface, in contrast, retain similar B factors and show hardly any conformational changes from the BSSαβγ structure. The highest B factors are along the center of the protein where the opening of the protein core destabilizes strand 9 and permits a range of motion to helix 9 and the surrounding loops.

**Limited Proteolysis of BSS Complexes Supports Increased Flexibility and Surface Accessibility in the Absence of BSSβ.** Our crystallographic data indicate that BSSαγ is more open and flexible than BSSαβγ, consistent with the hypothesis that BSSβ regulates access to the active site in BSSα. An alternate explanation for the observed structural differences is that changes in the crystal lattice result in a greater degree of disorder in the BSSαγ structure. The BSSαγ crystal form does have fewer contacts to symmetry mates (four within 4 Å for αγ vs. nine within 4 Å for αβγ) and shows a 10–20% increase in solvent content (39–43% in αβγ vs. 47% in αγ) as well as an increase in average Ca B factor (29.0 Å² for BSSαβγ and 39.5 Å² for BSSαγ). To differentiate between the effects of BSSβ and those of crystal packing on the flexibility of BSSα, we examined the flexibility of BSSα in solution by limited trypsin proteolysis. In agreement with the proposed role of BSSβ, BSSαγ is degraded to numerous small fragments by trypsin in 1 h, whereas BSSαβγ is much more resistant to degradation over the same time period (Fig. 5D). Residues in BSSα are thus more accessible to trypsin when BSSβ is absent. Although BSSβ shields some regions in BSSα from proteolysis directly, the surface area covered by BSSβ is only 1,500 Å². This number is about 5% of the total BSSα surface area and not large enough to explain the almost complete degradation of BSSα in the absence of BSSβ compared with almost none in its presence. The enhanced degradation of BSSα in the absence of BSSβ therefore supports the conclusion that the BSSαγ complex is dynamic in solution as well as in our crystal structure.

**Discussion**

The transformation of hydrocarbons into biologically degradable compounds is chemically challenging, as these molecules lack functional groups that serve as handles for entry into biological metabolism. To degrade inert substrates like toluene, microorganisms use enzymes capable of using free radicals to functionalize these molecules for further oxidation. A fundamental question in the study of radical enzymes is how they control the generation and reactivity of radical species. Within the GRE family, structures have revealed a common mechanism for harnessing radicals: sequester them within a shell of protein residues that allows the Gly radical domain to slip out of the active site by ∼2 Å (25). For microbes that live both anaerobically and aerobically, burying the Gly radical within the protein core also provides some protection against oxygen-induced cleavage of the backbone at the radical site. This protection strategy, however, creates
a problem for these enzymes: how to regulate access to the active site such that substrates are permitted in under the right conditions and other molecules are kept out. Furthermore, inactive GREs must undergo a large conformational rearrangement to interact with their cognate AE, but already activated enzymes must contain the Gly radical within the core of the protein to prevent radical quenching. This dichotomy of access and sequestration makes GREs an excellent target for studying the regulation of large conformational changes in enzymes.

Here we present structures of BSS complexes that illustrate a strategy for controlling conformational rearrangement in GREs: stabilization of a naturally flexible Gly radical domain by an accessory subunit. In the GRE structures so far determined, the Gly radical domain is consistently more flexible than the surrounding GRE core, as evidenced by a 5- to 10-Å increase in the B factors for the domain relative to the average B factor for the remainder of the protein (Fig. S8). The BSSαγ complex recapitulates this relative increase in B factors in the Gly radical domain, suggesting that this complex is capable of accessing the open state and undergoing activation. In contrast, the BSSαβγ complex appears to be the outlier, having lower-than-average B factors overall and essentially no difference in B factors for the Gly radical domain relative to the rest of the protein. As seen in the BSSαβγ structure, the Gly radical domain in BSSαγ appears more prone to enter the open state than in other GREs, and by binding to BSSβγ, the resulting complex is stabilized in the catalytic state. Thus, BSSαγ appears to modulate movement of the Gly radical domain, affecting both activation by the AE and protection of the stable Gly radical. Also, through its interactions with the Gly radical domain, the β-subunit could regulate BSS activity by adjusting the distance between the Gly radical and catalytic Cys residue such that radical transfer is slowed, blocked, or facilitated. Alternatively, or in addition, BSSβγ may regulate activity by gating entry of substrates to the active site through movement of the β-hairpin loop.

The comparison of BSS with HPAD deserves special attention because of the general similarity of these two enzymes: HPAD is the closest relative to BSS with a solved structure; their substrates are similar in shape and properties; and both have small, accessory, [4Fe-4S] cluster-containing subunits. Although the [4Fe-4S] cluster subunit of HPAD, HpdC, has greater sequence similarity to BSSβγ, this subunit does not interact with the Gly radical domain, occupying instead a space near the dimer interface more reminiscent of how BSSβγ binds to this region of BSSαγ (Fig. S9). Despite the differences in the location of their small subunits, BSS and HPAD are similar in that both have protein features absent in PFL and GDH that appear to prevent the Gly radical domain from easily interacting with the cognate AE (Fig. S9). In HPAD, the Gly radical domain is blocked by an additional loop from the catalytic subunit, which overlaps with some of the contacts made by the β-sheet of BSSβγ (Fig. 6A and Fig. S9). This additional loop is not present in any other structurally characterized GREs and appears to be a short insertion into the preformed architecture of the GRE C-terminal helix bundle. Another loop that extends from a pair of helices near the dimer interface, which we will now call loop 2, is present in all structurally characterized GREs but is expanded in HPAD to make extensive contact with the Gly radical domain (Fig. S9). In BSS, loop 1 and the C terminus of BSSβγ make interactions with the Gly radical domain not seen in any other GRE (Fig. 6B and Fig. S9). Loop 2 is present in BSS but makes no contacts to the Gly radical domain. Although these additional protein elements around the Gly radical domain in BSS and HPAD are different, in both cases, they make the Gly radical domain considerably less solvent-accessible than the other enzymes in their class (Fig. S9). HPAD and BSS thus seem to have developed two different strategies for reducing the accessibility of the Gly radical loop. Notably, PFL, which appears to have the least encumbered radical domain (Fig. S9), has a repair mechanism to restore enzyme activity upon oxygen damage. In particular, repair involves a “spare part” protein, YfdD, which is post-translationally activated by PFL-AE and replaces the oxygen-damaged glycyl radical domain, yielding active enzyme (26). As more GRE enzymes are characterized, it will be interesting to see if structural features surrounding the glycyl radical domain correlate with the likelihood of oxygen exposure or the presence of repair mechanisms.

Although our structural data allow us to propose roles of the BSS subunits in regulating enzyme conformational dynamics and in modulating complex solubility, the particular function(s) of the [4Fe-4S] clusters in these subunits is less obvious. The clusters of both BSSβ and BSSγ do appear to play a structural role in that their removal leads to dissociation of the subunits from BSSα (16), which in turn leaves BSSα prone to unfolding and clearance by cellular degradation machinery in vivo (16) and sensitive to proteases in vitro. In terms of whether these clusters also play a redox role in addition to the above structural role, we find that the overall fold of both subunits is derived from a HiPIP, a family of [4Fe-4S] cluster proteins known for their redox potentials (100–450 mV) and for their ability to stabilize clusters in the +3 oxidation state (20, 27). A universal pattern of hydrophobic shielding of the [4Fe-4S] clusters in HiPIPs is believed to be responsible for shifting the +3/+2 redox potential into a biologically relevant range and shifting the +2/+1 couple out of a biological window, with potentials of ~1.75 V (28). In contrast to ferredoxins, which use the +2/+1 couple, dithionite is typically unable to reduce HiPIP proteins (28). Here we find that BSSβγ and BSSγ are hypermodified HiPIPs. Unlike most HiPIPs, the [4Fe-4S] clusters of BSS are not highly shielded from solvent, can be reduced by dithionite to the [4Fe-4S]2+/1 couple, and have redox couples that are proton-coupled. Like most HiPIP proteins, BSSβγ and BSSγ are relatively stable in air and display high redox potentials, albeit on the lower end of the HiPIP range. In short, our data suggest that the [4Fe-4S] clusters of BSS can transition from oxidized to reduced to superreduced ([4Fe-4S]3+/2+ & [4Fe-4S]2+/1+), an unusual, but not unprecedented, property for a protein with a HiPIP fold (28). These atypical redox properties support the consideration of redox roles as well as structural roles for the clusters of BSS.
For HPAD, it has been proposed that the [4Fe-4S] clusters are redox active and could act to reductively quench the Gly radical by electron transfer, returning the enzyme to the inactive state (18). Efficient, reversible reduction of the Gly radical has been observed in vivo in PFL in the presence of DTT or β-mercaptoethanol (29), suggesting that Gly radical reduction could be a means of preventing permanent damage to the GRE by quenching the radical before O2-catalyzed backbone cleavage can occur. However, no data are available to support the existence of this type of protective mechanism in vivo. In addition, the current structural data are inconsistent with such an electron transfer function, with long distances between the closest cluster and the Gly radical of over 40 Å in HPAD and over 30 Å in BSS, whereas typical distances for electron transfer are in the range of 8–12 Å (30). Notably, the distance between BSSp and the Gly of BSSr is likely to change when the radical domain undergoes its conformational change, potentially giving BSSp access to the Gly radical. Alternatively, the redox properties of the [4Fe-4S] clusters could be used to sense the redox state of the cell and communicate with BSSr either by initiating a conformational change or by altering the solubility of BSSr.

In summary, although movement of the Gly radical domain out of the core of the protein was previously proposed (13), our structures of BSS provide experimental evidence that this domain can move as a unit from the protein interior toward the protein surface. Before these structures, it was not known whether other adjustments to the protein core would be required for this motion of the Gly radical domain. We now see that the C-terminal half-barrel exhibits a 5- to 7-Å clambshell-like motion centered at strand and helix 9 to expand the core enough for the Gly radical domain to egress. These structures also allow us to present hypotheses as to the function of these [4Fe-4S] cluster-containing accessory subunits that include modulation of conformational dynamics and structural stability as a means of regulating enzyme activation and activity. Given the potential applications of GRES in environmentally and industrially important transformations of inert hydrocarbons, these findings provide a valuable framework for considering the protein design features that harness radical-based reactivity in this significant class of anaerobic enzymes.

Materials and Methods

C-terminally His-tagged BSSs was coexpressed with either BSSG alone or BSSG and BSSβ and purified as previously described (16). Crystals of the BSSpγ complex were grown by vapor diffusion. The structure was solved by selenomethionine single-wavelength anomalous dispersion at a 3.1-Å resolution and then refined against 2.0-Å resolution data from a native crystal. Crystals of the BSSα complex were grown by vapor diffusion, and the structure was solved by molecular replacement. Trypsin degradation assays and electrophoresis were performed in a MBraun anaerobic chamber. Detailed protocols can be found in Supplemental Material and Methods.

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