Substrate and enantiomer selectivity in the GRE-type dehydratases

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ABSTRACT

Glycyl radical enzymes (GREs) represent a diverse superfamily of enzymes that utilize a radical mechanism to catalyze difficult, but often essential, chemical reactions. In this work, we present the first biochemical and structural data for a GRE-type diol dehydratase from the organism Roseburia inulinivorans (RiDD). Despite high sequence (48% identity) and structural similarity to the GRE-type glycerol dehydratase from Clostridium butyricum (CbGD), we demonstrate that the RiDD is in fact a diol dehydratase. In addition, the RiDD will utilize both (S)-1,2-propanediol and (R)-1,2-propanediol as a substrate, with an observed preference for the (S) enantiomer. Based on the new structural information we develop and successfully test a hypothesis that explains the functional differences we observe.

In contrast to the well-characterized B12-dependent dehydratases (1,2), only one glycyl radical enzyme (GRE) or GRE-type dehydratase has been characterized to date (3). While pyruvate formate lyase (PFL) and anaerobic ribonucleotide reductase (ARNR) are among the most comprehensively studied GREs (4-9), recent work provides evidence that the catalytic diversity of GREs is considerably greater than originally anticipated (10). It is now recognized that GREs can catalyze the formation of C-C bonds (benzyl succinate synthase)(11), C-C bond cleavage (PFL and 4-hydroxyphenylacetate decarboxylase - a.k.a. 4-HPAD)(5,12), ribonucleotide reduction (ARNR)(8), dehydration reactions (glycerol dehydratase)(3), and C-N bond cleavage (choline TMA-lyase)(13-15). Taken together, these observations underscore a superfamily of enzymes that utilize a conserved structural core to perform vastly different radical-catalyzed reactions. Many of these reactions are important to human health or represent difficult chemical conversions that may be significant to the development of future biocatalysts.

At the present time, the only structural information available for any GRE-type dehydratase is that of the glycerol dehydratase from Clostridium butyricum (3,16) (CbGD). The presence of a homologous 1,2-propanediol dehydratase in Roseburia inulinivorans was proposed based on genomic and metabolic analyses of the organism (17). However, the enzyme was never isolated, and only propanol, production was observed when R. inulinivorans was cultured under anaerobic conditions (17). In addition to the presence of
the proposed GRE-type dehydratase gene, the operon containing the diol dehydratase (WP_007885173) also contained another gene that was consistent with that of an “activating enzyme” (ABC25540). These observations are significant because all of the GREs that have been investigated to date are expressed in an inactive form that is post-translationally activated by an “activating enzyme” (AE). The generally accepted mechanism for the activation of all GREs involves a “radical SAM” enzyme that is specific to the GRE partnering enzyme (10). The activation process that is catalyzed by the AE involves a uniquely coordinated [4Fe-4S] cluster and S-adenosylmethionine (SAM) as a co-substrate. The [4Fe-4S] cluster (formerly in the 1+ oxidation state) catalyzes the reductive cleavage of SAM to generate a highly reactive 5’-deoxyadenosyl radical (5′-dA•) that is positioned to facilitate the abstraction of a hydrogen atom from a conserved glycine residue on the GRE (10). The activation reaction, and subsequent dehydration investigated herein, are highly sensitive to molecular oxygen and must be performed under strictly anaerobic conditions. In vitro, the catalytic [4Fe-4S] can be reduced from the 2+ to the 1+ oxidation state using a biological reducing system, such as a NADPH flavodoxin/oxidoreductase and flavodoxin system (18). In addition, chemical reductants such as sodium dithionite or a light-driven reducing system are also effective electron donors (19,20). The activation process, or generation of the catalytic glycyl radical, is further complicated by recent observations that demonstrate the substrate for the GRE accelerates the activation rate (21). Regardless of the electron source, once the glycyl radical is generated, all GREs will catalyze their primary metabolic reaction, regenerating the radical with each turnover. Precisely how or when GREs are inactivated in vivo remains an outstanding question in the field.

In this work, we have expressed and purified the predicted diol dehydratase from R. inulinivorans (RiDD) as well as the RiDD activating enzyme (RiDD-AE) (homologously expressed in E. coli). In addition to assay data, we have determined the crystal structures for the ethanediol- and propanediol-bound RiDD. Structural alignment of the crystallographic models with our previously published models for the glycerol dehydratase from C. butyricum (CbGD) reveals a similar overall fold and a highly conserved active site structure. However, subtle variations in the active site amino acid residues provide a viable explanation for the observed functional differences. This hypothesis is tested through site directed mutagenesis herein.

RESULTS

Overall structure of the RiDD. All of the glycyl radical enzyme (GRE) structures published to date represent a model of the inactive enzyme. This is not surprising considering that maintaining the glycyl radical during crystal growth is simply not feasible. Despite this technical detail, the crystal structures for GREs have provided significant insight into enzyme mechanism (3,22-28). Numerous computational predictions have also been made based on this structural information (29-31). In this work we were able to crystallize and model the RiDD with ethanediol or 1,2-propanediol bound in the active site (Table 1).

The overall fold of the RiDD is most similar to the CbGD. Figure 1 (Panel A) shows an overlay comparing the model of the RiDD, reported herein, aligned with the GRE-type glycerol dehydratase from Clostridium butyricum (CbGD) (3). Figure 1 (Panel B) also highlights the location of the conserved glycine residues in both enzymes and the high degree of structural homology found in the C-terminal domain. With a small exception, discussed below, we do not observe any significant changes in the overall position of the backbone atoms when the ethanediol- and propanediol-bound models are compared with each other or the CbGD structures. However, there is a 48-amino acid insert that is present in the RiDD (Figure 1, Panel A). Specifically, when compared to the CbGD, the RiDD contains a 48 amino acid insert (residues 592-640 of the RiDD). Excluding this insertion and the first twenty N-terminal amino acids of
the RiDD, the structural alignment of monomer A from the CbGD model (PDB ID 1R9D) with the A monomer of the RiDD model results in a root mean square deviation (RMSD) of 0.6 angstroms for the backbone alpha carbon atoms. The additional 48 amino acid insert that is found in the RiDD extends an existing helix and forms an additional helix, loop, and helix motif. This region of the RiDD structure is highlighted in dark blue in Figure 1, Panel A, and forms an important crystallographic contact by interacting with the identical domain in a symmetry-related molecule (data not shown). Interestingly, three hydrophobic amino acids (I623, I627, and L631) form a largely hydrophobic interface between the helical domains of this insert with a buried surface area of ~470 Å². The precise function of the additional domain is not clear, however, much of this region could not be modeled for the (S)-1,2-propanediol-bound model due to a lack of electron density. This observation is consistent with the change we observe in the space group for the propanediol-treated crystals (Table 1) suggesting that substrate binding may be communicated to this region of the enzyme. A recent report on the GRE choline lyase (CutC) also demonstrated that substrate binding can influence the overall structure for that enzyme (15).

The RiDD forms a dimer in solution. Sedimentation velocity analysis of the RiDD in solution, without substrate, shows a c(s) distribution dominated by a 9.2 S species (70.5%), which is close to the predicted values of 8.8 S and 8.7 S for the dimers produced by non-crystallographic and crystallographic symmetry operations, respectively (Figure 2, Panel A). The dimer with the largest surface area is shown in Figure 2, Panel B. The difference in the observed and expected S values suggests that the substrate-free dimer may adopt a slightly more compact conformation than that observed in the crystal lattice of substrate bound RiDD. We also observe a 5.6 S (16.7%) and 13.4 S (5.5%) species that closely match the predicted values for the monomer (5.8 S) and tetramer (13.6 S). Finally, there is a minor 3.6 S species (6.0%) in the c(s) distribution that likely corresponds to misfolded monomer. These results suggest that the RiDD may undergo a concentration dependent association to form a dimer and a relatively unstable tetramer. The asymmetric unit we observe for the RiDD is a dimer, however, precedence in the literature for PFL(26-28) and the CbGD(3) indicate that the dimer in our asymmetric unit is not functionally relevant. In fact, the buried surface area for the interface of the dimer in our asymmetric unit is only ~350 Å². The dimer that is shown in Figure 2, Panel B, has an interface that is substantially larger consisting of ~800 Å² of buried surface area based on PISA analysis(32). Interestingly, in the unit cell of both RiDD crystal forms it is possible to construct a tetramer out of two of the dimers shown in Figure 2 and two of the smaller interfaces.

Enzyme activation and dehydratase assays. All GREs are activated by their corresponding activating enzyme (AE) in a S-adenosyl-L-methionine (SAM)-dependent manner (10). The activation reaction is not trivial as all aspects of the procedure, including the glycyl radical, are sensitive to molecular oxygen. Previous work from this laboratory has demonstrated the SAM-dependent activation of the Clostridium butyricum glycerol dehydratase (CbGD) by the CbGD activating enzyme (CbGD-AE) (3,33), but the activation of the Roseburia inulinivorans diol dehydratase (RiDD) by the RiDD-AE has not been reported. The protein sequence of the RiDD-AE indicates that this activating enzyme contains only a single [4Fe-4S] cluster. Consistent with this observation and unlike what we observe for the CbGD-AE (33), iron analysis of the anaerobically expressed, purified, and reconstituted RiDD-AE used in this investigation revealed 3.73 ± 0.26 iron atoms per mole of protein. In this regard, the RiDD-AE is more similar to PFL-AE (34) in that both activating enzymes (PFL-AE and RiDD-AE) contain only the catalytic [4Fe-4S] cluster. In order to further address whether a [4Fe-4S] cluster was present in the RiDD-AE and functioned in the activation of the RiDD, we applied electron paramagnetic resonance (EPR) spectroscopy in order to probe the electronic environment of the [4Fe-
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The EPR spectrum of the purified RiDD-AE in the presence of 1.0 mM sodium dithionite is consistent with that of a reduced [4Fe-4S]$^{1+}$ cluster (Figure 3, Panel A). Specifically, we observe a predominantly axial signal with $g_{||}$ of 2.016 and $g_{\perp}$ of 1.923 at 10 K. The addition of excess SAM to the purified RiDD-AE resulted in a shift in the signal to give a $g_{||}$ of 2.029 and $g_{\perp}$ of 1.863 at 10 K (Figure 3, Panel B). These observations are typical of what has been extensively observed for the [4Fe-4S] cluster of other radical SAM AEs and has been recently reviewed elsewhere (10).

When the RiDD is added to the reduced RiDD-AE in the presence of SAM, the Fe-S cluster signal is lost and a radical signal, centered at $g = 2.006$, is observed (Figure 3, Panel C). All of the glycyl radicals that have been characterized to date have been centered at $g = 2.00$, therefore the slightly shifted value we observe for the g-tensor of the RiDD radical is slightly different. In addition to the g-tensor, the lineshape and splitting of glycyl radicals is typically indicative of two-fold splitting due to the remaining proton on the glycine residue (14,34). Additional results are described below that further address the differences in the radical signal we observed for the activated RiDD and we expanded upon this in the discussion. However, it is becoming increasingly apparent that it is important to utilize the physiological electron donor when studying radical SAM enzymes (35) and we are utilizing sodium dithionite as an electron source in this work. Regardless, we do not observe any dehydrogenation activity unless we perform the SAM and RiDD-AE dependent activation of the RiDD. The complete loss of the [4Fe-4S] signal was also somewhat surprising, however, spin integration of this signal against a Cu-EDTA standard indicated that sodium dithionite is incapable of fully reducing this cluster. Specifically, while iron analysis indicates that each monomer of the RiDD-AE contains an intact [4Fe-4S] cluster, spin integration against a Cu-EDTA standard results in 0.3 spins per monomer of RiDD. This confirms that less than 30% of the [4Fe-4S] clusters are in the reduced, or 1+, oxidation state. This also explains the complete loss of the EPR signal for the cluster at 10 K, after SAM is added and the radical species has been generated, as all of the spin density has been converted to the radical signal. Given the difficulty working with these enzymes and evidence that the physiological electron donor is important for complete reduction of the [4Fe-4S] cluster in other radical SAM enzymes (35), future efforts will be made to isolate the appropriate donor(s) from R. inulinivorans and optimize reduction of the catalytic cluster.

“Pre-activation” of the RiDD. Given the observations above, it was important to establish a linear range for RiDD turnover using the partially activated enzyme, prior to investigating the dehydration of 1,2-propanediol. Various concentrations of pre-activated RiDD were investigated using a previously reported coupled assay (3) with a small modification. Specifically, we pre-activated a batch of RiDD prior to adding known amounts of this activated RiDD to the dehydration assay. The decrease in the absorbance at 340 nm (Figure 4) is due to the NADH-dependent reduction of propanal to propanol by the coupling enzyme, yeast alcohol dehydrogenase (YADH). Based on the rate of NADH consumption by the coupling enzyme at the various RiDD concentrations investigated (Figure 4, inset), the average specific activity under these experimental conditions is calculated to be $5.5 \pm 0.4$ µmoles min$^{-1}$ mg$^{-1}$. If the RiDD is not pre-activated, then a significant lag time is observed before the production of propanal, and subsequent NADH-dependent reduction to propanol by YADH, is observed (data not shown). However, the specific activity calculated from the maximum slope observed at the highest concentrations of substrate (S)-1,2-propanediol is very similar to what was observed for the pre-activated enzyme (~5.0 compared with 5.5 µmoles min$^{-1}$ mg$^{-1}$). Notably, increasing the 1,2-propanediol concentration results in a decrease in the lag time required to achieve maximum turnover, suggesting a faster activation rate in the presence of substrate, a proposal that is consistent with recent observations for PFL.
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Moreover, as shown in Figure 5, the RiDD catalyzes the dehydration of (S)-1,2-propanediol considerably faster than (R)-1,2-propanediol. However, due to the degree of error and technical challenges associated with these experiments it is impossible to determine a reliable $K_m$ for the two substrates at this time. In addition, as we demonstrate below, there may be additional factors that complicate the kinetic analysis. Regardless, what can be interpreted from these data is that the RiDD is in fact a 1,2-propanediol dehydratase with a preference for the “S” enantiomer.

Direct detection of dehydration products by gas chromatography. Gas chromatography (GC) was also employed to look directly at the products of the RiDD-catalyzed dehydration reaction. As previously reported, it was possible to obtain good separation of the compounds 1,2-propanediol, acetone, and propionaldehyde (36). It is important to note that this experiment is performed on a significantly larger scale and at a much higher concentration of 1,2-propanediol (5% v/v). No coupling enzyme was included, thus, allowing the products of the dehydration reaction to accumulate. Samples were analyzed by GC/FID analysis to directly quantitate the production of propanal. In particular, when glycerol was used as a substrate for the RiDD, no activity was observed when up to 8 mM glycerol was tried as the substrate. Surprisingly, when 1,2-propanediol was the substrate we observed acetone production in addition to propanal production with both enantiomers of 1,2-propanediol (Figure 6). An explanation for acetone production is not immediately apparent, however, these observations further confirm that the RiDD is in fact selective for 1,2-propanediol. These data may have mechanistic implications as we do not observe any acetone production when 1,2-propanediol is the substrate for the CbGD.

Active site structure of the RiDD compared to the CbGD. The active site structure of the RiDD may provide an explanation for the functional observations described above. Figure 7 shows the models for the RiDD determined in this work. Ethanediol, presumably a contaminant of the low molecular weight PEG we used to crystalize the RiDD, is observed in the active site of crystals obtained for the “as-isolated” enzyme (Figure 7, Panel A). Fortunately, it was possible to transfer these crystals, in a stepwise fashion, to mother liquor containing 5% 1,2-propanediol (racemic mixture). The diffraction quality did decreased to 2.4 angstroms (Table 1), but the 1,2-propanediol was clearly observed in the active site (Figure 7, Panel B). Refinement with either enantiomer, visual inspection of the difference density, and slightly lower R-values indicated that (S)-1,2-propanediol is bound and therefore has been modeled as such. Notably, the hydroxyl groups of the ethanediol and 1,2-propanediol are similarly positioned in the active site of the RiDD models (Compare Panel A with Panel B in Figure 7). The binding mode of the ethanediol further supports modeling (S)-1,2-propanediol in the active site as alignment of the backbone carbon atoms of the “R” enantiomer for 1,2-propanediol would cause a steric clash with the side chain of F344. Furthermore, when the 1,2-propanediol-bound RiDD model is aligned with the glycerol-bound model of the CbGD, both substrates are in similar positions within the active site (Figure 8). However, there is a very distinct difference between the active site structure of the RiDD and the CbGD. Specifically, amino acids Y339 and S642 are within hydrogen bonding distance of each other and S642 is also within hydrogen bonding distance of the third hydroxyl group of glycerol. The equivalent residues in the RiDD are F344 and V696. In fact, based on the alignment shown in Figure 8, the side chain of V696 occupies the space that would be required to accommodate glycerol.

Site-directed mutagenesis of the RiDD and glycerol dehydration. In order to test the hypothesis that residues F344 and V696 are responsible for the preventing glycerol binding and turnover in the wild-type RiDD, we constructed the F344Y/V696S RiDD variant. This variant was also useful in re-examining the radical signal we observe for the wild-type RiDD. Specifically, before performing a coupled assay using glycerol as the substrate, we first investigated the SAM-dependent
activation of the F344Y/V696S RiDD by the RiDD-AE in a side-by-side activation experiment with the wild-type CbGD and RiDD. These data are shown in Figure 9. Similar to what we observed for the wild-type RiDD, the g-tensor for the activated F344Y/V696S RiDD signal is also somewhat shifted by comparison to the radical signal observed for the CbGD. Both the wild-type RiDD and F344Y/V696S RiDD are weaker than the radical signal observed for the activated CbGD, consistent with the partial reduction we observe for the dithionite-treated RiDD-AE. Interestingly, the lineshape for the activated F344Y/V696S RiDD is more similar to what we observe for the CbGD. In the absence of access to the physiological reduction partners for the RiDD-AE, we are hesitant to read too much into these observations. However, although this is a minor difference, but it clearly implies spin-orbit coupling in the RiDD that is different from the CbGD. If the unpaired electron in the activated RiDD is coupled to a different atom, relative to the typical glycyl radical observed for GREs, this might provide new mechanistic insight and is being investigated further. Regardless, in all cases, we do not observe any dehydration activity for any of these enzymes unless the enzymes are activated first.

Given that the F344Y/V696S RiDD could be activated, we asked whether or not the enzyme could dehydrate glycerol. We did in fact observe NADH consumption in a coupled assay when glycerol is the substrate for either the CbGD or the F344Y/V696S RiDD variant (Figure 10). However, the specific activity for glycerol dehydration by the F344Y/V696S RiDD variant is considerably less than what is observed for the activated CbGD. The F344Y/V696S RiDD did purify with slightly lower yields suggesting it might be less stable than the wild-type enzyme, but nonetheless this variant catalyzed glycerol dehydration at maximum rate of 0.81 ± 0.02 µmoles min⁻¹ mg⁻¹. Although this rate is almost 5-fold slower when compared to the CbGD, these observations suggest that these two amino acids play a role in the substrate selectivity. The slower rate of activity suggests that the conformational dynamics of the active site and dehydration mechanism are considerably more complex and in both cases (CbGD and RiDD) it may be more appropriate to utilize a physiological electron donor in all future investigations.

**DISCUSSION**

The functional diversity of glycyl radical enzymes is impressive (9,37,38) and has important implications for many aspects of human health and general quality of life. For example, the inhibition of some of these reactions will prevent anaerobic growth by certain pathogenic microorganisms (39). In addition, many of the reactions are industrially significant, making GREs of interest as potential biocatalysts. However, there are a number of unanswered questions regarding the activation, mechanism, and deactivation of GREs that remain to be addressed.

*Overall structure and the C-terminal glycyl radical domain.* The RiDD model exhibits the anticipated 10-α/β-barrel fold and aligns best with the structure reported for the CbGD. The enzyme exists predominantly as a dimer in solution. The presence of some tetrameric species in solution was unexpected and potentially interesting in light of the observation of two additional protein-protein interfaces in the unit cell of the RiDD crystals. One of these interfaces is facilitated by an additional stretch of amino acids unique to the RiDD, although the function of this domain and interaction is unclear. Regardless, the conserved glycyl radical domain is found in the C-terminal domain of the 10-α/β-barrel fold. The general consensus is that significant conformational changes are required in order to move the glycyl radical loop out of the core of the GRE structure and into the active site of the activating enzyme. Recent work with CutC has provided evidence that the substrate itself may help induce some of the conformational changes required for activation(15). In addition to the CbGD, the overall fold of the RiDD is also more similar to the large subunits of benzyl succinate synthase (BSS) and 4-hydroxyphenyl acetate decarboxylase (4-HPAD) (24,40), consistent with what has been reported for the CbGD. However, both the BSS and 4-HPAD have additional peptide
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Subunits, presenting an additional challenge when moving the glycyl radical loop out of the C-terminal domain and into the active site of their respective activating enzymes (25). Funk et al. noticed that contacts between the C-terminal glycyl radical domain and “loop 2” of the N-terminal domain in the CbGD resulted in burying approximately an additional 100 Å (25). They speculated that loop 2 would have to move in order to facilitate the opening of the glycyl radical domain in the CbGD. We observe a similar interaction in the RiDD structures reported here, however, we do see a subtle difference in the positioning of loop 2 when the RiDD structures are aligned with the CbGD model. Specifically, with the exception of backbone residues 106 to 109 in the RiDD, the N-terminal and C-terminal glycyl radical domain align with an RMSD of less than 0.5 Å. Interestingly, the backbone alpha carbon atoms of residues 106-109, found within loop 2, are 2-3 Å away from the equivalent atoms in the CbGD model. This observation provides additional support for the hypothesis put forth by Funk et al. regarding the activation of the CbGD. This interaction is not present in the structural models of pyruvate formate lyase (PFL). However, PFL remains somewhat unique in that it has also been shown to exhibit “half-sites” reactivity (19,34,41) and appears to have a “repair peptide”, YfID, that can be post-translationally activated by PFL-AE. The activated YfID can replace any glycyl radical domains in PFL that have been oxygen damaged (42). These data indicated significantly greater freedom of movement for the C-terminal domain of PFL.

Generation of the glycyl radical. At the present time, there is no evidence to suggest that there is any allosteric communication between active sites in the physiological dimer we observed for the RiDD. In addition, there is no evidence that the glycyl radical must be generated at both active sites in order to observe activity in any GRE. However, one common feature that all of the GREs have been shown (or predicted) to contain is a catalytic dyad consisting of a conserved glycine and cysteine residue. The thiol group of the conserved cysteine is positioned close to the alpha carbon atom of the glycine residue (typically within ~3.5 angstroms). The location and position of the catalytic dyad facilitates radical transfer from the alpha carbon atom of the glycine residue to the sulfur atom of the cysteine residue via hydrogen atom abstraction. The latter radical is positioned to abstract a hydrogen atom from the primary metabolic substrate. For the RiDD reported here, the sulfur atom of C438 is within 3.6 angstroms of the C1 carbon atom of 1,2-propanediol (Figure 7, Panel B). At the present time, we have not identified or isolated the physiological electron donor for the RiDD-AE and therefore we are hesitant to speculate on the precise identity of the radical signals we observe for the wild-type and F344Y/V696S variant of the RiDD at 70 K. However, chemical reductants can lead to artifacts and the importance of the electron source for radical SAM enzymes has been underscored in recent work (35). Interestingly, the lineshape of the EPR spectra we observe for the activated F344Y/V696S variant looks more similar to the radical signal we observe for the activated CbGD, suggesting that these amino acids may have some influence on the generation and/or environment of the glycyl radical in the RiDD. Future work will absolutely require the identification and isolation of the physiological electron donor to the RiDD-AE in R. inulinivorans.

Mechanism of GRE-dependent dehydration. At least two mechanisms have been proposed for the dehydration of glycerol as catalyzed by the CbGD (3,31). Both mechanisms are also relevant to 1,2-propanediol dehydration and both begin with the radical on the catalytic cysteine residue (C433 or C438 in the CbGD or RiDD, respectively). This radical is positioned to abstract a hydrogen atom from substrate (either 1,2-propanediol or glycerol). The former mechanism, proposed by O’Brien et al., involves abstraction of the pro-S hydrogen atom from the primary carbon atom followed by the migration of the new substrate radical and hydroxyl group in opposite directions. The substrate/intermediate radical re-abstracts a hydrogen atom from the catalytic cysteine to produce a hydrated aldehyde that spontaneously decomposes to water and 3-
hydroxy propionaldehyde. In the O’Brien proposal the migrating hydroxyl is stabilized by a pair of conserved, and appropriately protonated, histidine side chains (H164 & H281 of the CbGD). In a purely computational investigation, Feliks et al. proposed that these histidine residues, H164 in particular, could facilitate the direct donation of a proton and loss of water. In such a “direct dehydration” mechanism, only the substrate/intermediate radical migrates from the primary to secondary carbon atom of substrate before re-abstracting a hydrogen atom from C433 and resetting the enzyme for another round of turnover.

Prior to this work, there was no evidence to suggest that the mechanism of glycerol dehydration would be significantly different from 1,2-propanediol dehydration in the GRE-dependent dehydratases. In fact, as both mechanisms for the CbGD had predicted, it was reasonable to expect that a proton on the C1 carbon atom of 1,2-propanediol would be abstracted first in the mechanism of the RiDD. However, the production of some acetone with either enantiomer of 1,2-propanediol by the RiDD calls this proposal into question. Although both the RiDD and the CbGD prefer the “S” enantiomer of 1,2-propanediol the CbGD will not utilize the “R” enantiomer at all and does not produce any acetone. This suggests that the two dehydratases have subtly different molecular dynamics. In particular, the RiDD may be a “sloppy” enzyme by comparison to the CbGD with a slightly more flexible active site. In theory, the F344Y/V696S RiDD should call this proposal into question. Although both the RiDD and the CbGD prefer the “S” enantiomer of 1,2-propanediol the CbGD will not utilize the “R” enantiomer at all and does not produce any acetone. This suggests that the two dehydratases have subtly different molecular dynamics. In particular, the RiDD may be a “sloppy” enzyme by comparison to the CbGD with a slightly more flexible active site. In theory, the F344Y/V696S RiDD should have identical interactions with glycerol and catalytic activity when compared to the CbGD or wild type RiDD, clearly this is not the case. In this light, acetone production by the RiDD may simply reflect this flexibility and may be the result of some improperly oriented substrate leading to hydrogen atom abstraction from the C2 atom of 1,2-propanediol and direct removal of the hydroxyl group on the C1 carbon atom of 1,2-propanediol.

Taken together, these data also indicate that both dehydration mechanisms (direct dehydration versus hydroxyl/radical co-migration) may be valid, depending on the dynamics of the active site and precisely how the substrate is positioned. In fact, multiple binding modes for have been proposed to explain the mechanism-based inactivation by glycerol that has been observed for the B12-dependent diol dehydratase (43). Specifically, although glycerol is not a chiral compound, glycerol can bind to the active site of the B12-dependent diol dehydratase in two different orientations. One orientation mimics the geometry and binding mode of S-1,2-propanediol (G_S), while the other binding mode is similar to that of R-1,2-propanediol (G_R). Because of the different geometry, the hydrogen bond strength varies between the G_S or G_R orientation. Hence, it was proposed that undesirable side reactions leading to the suicide inhibition of the B12-dependent diol dehydratase occur with a much higher probability for glycerol that is bound in the G_S orientation (43). Consistent with this proposal, the B12-dependent diol dehydratase will utilize both enantiomers of 1,2-propanediol but has a preference for the R isomer (44). Interestingly, the results reported here are consistent with the CbGD having a more selective binding site with glycerol or S-1,2-propanediol bound in a single orientation, while the RiDD, has slightly more conformational flexibility.

Conclusions. The catalytic power of GREs is significant interest, especially in light of anaerobic metabolism in the intestinal microbiome(15,22,23) and growing demand for biocatalysts capable of performing difficult chemical rearrangements. While B12-dependent glycerol and diol dehydratases have been well studied, this is the first report of a GRE-dependent diol dehydratase. Interestingly, a similar mechanistic parallel is observed when comparing the activity of the GRE-dependent glycerol dehydratase, CbGD, with the GRE-independent diol dehydratase, RiDD. Specifically, similar to what has been reported for the B12-dependent enzymes, the diol dehydratase may be more amenable to
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re-engineering (43). However, in order to address this thoroughly, as well as the mechanism of all GREs, it will be imperative to utilize the physiological donors (i.e. flavodoxin or ferredoxin from the native organism) when providing reducing equivalents for the activating enzymes.

EXPERIMENTAL PROCEDURES

Enzyme expression, purification and crystallization. Unless explicitly stated, all experimental procedures were carried out under strictly anaerobic conditions and all solutions were made anaerobic by purging with oxygen-free argon on a vacuum manifold. In addition, all sample preparation for spectroscopic studies or enzyme assays was carried out in a Coy™ anaerobic chamber containing an atmosphere of 5% hydrogen with the balance being nitrogen and less than 1 ppm oxygen. The genes for the Roseburia inulinivorans diol dehydratase (RiDD), the RiDD activating enzyme (RiDD-AE), and the 3-hydroxyprionaldehyde (3-HPA) reductase (DhaT, a.k.a. 1,3-propanediol dehydrogenase) from C. butyricum (NCBI accession #’s WP_007885173, ABC25540, and AAM54730.1 respectively) were all codon-optimized for expression in E. coli and cloned into the commercially available pTRCHis™ vector introducing a 6x polyhistidine tail at the N-terminus (specifically MSHHHHHHSGS) prior to the first amino acid of the native sequence. The diol dehydratase from Roseburia inulinivorans (RiDD), the RiDD activating enzyme (RiDD-AE), and DhaT were purified using the same affinity column procedures reported for the C. butyricum glycerol dehydratase (CbGD) and C. butyricum glycerol dehydratase activating enzyme (CbGD-AE)(3). Briefly, this involves affinity purification using the cobalt-loaded Talon™ affinity resin followed by gel filtration using an S-200 column equilibrated with 50 mM TRIS pH 8.0 and 500 mM KCl. Identical buffer was used to further purify the RiDD using gel filtration Superdex S-200 resin (GE Healthcare Life Sciences). In the case of the RiDD-AE, a chemical reconstitution of the [4Fe-4S] cluster was performed as previously described (45) and the enzyme was subjected to further purification using anion exchange chromatography. Specifically, sodium sulfide (0.2 mM), 2-mercaptoethanol (5 mM), and ferrous ammonium sulfate (0.25 mM) were added to the solution and the entire mix was gentle stirred overnight at 4 °C. Precipitate was removed by centrifugation and the supernatant was loaded onto a 5 mL Sepharose QFF column. The protein was then eluted from the column by applying a linear salt gradient (from 0 to 0.5 M KCl) in 20 mM TRIS pH 8.1. Elution typically occurred around 150 mM KCl. Essentially, the reconstituted activating enzymes were subjected to anion exchange chromatography in order to remove adventitiously bound iron and sulfide. All of the purified enzymes were concentrated and kept in liquid nitrogen. Protein concentrations were determined using the modified Biuret method (46) and all proteins were judged homogeneous by SDS-PAGE analysis.

EPR spectroscopy. Electron paramagnetic resonance (EPR) spectroscopy was employed in order to monitor SAM-dependent changes in the spectrum of the [4Fe-4S]1+ cluster in the RiDD-AE as well as the SAM-dependent generation of a catalytic radical on the RiDD. All EPR samples contained 1 mM sodium dithionite and were recorded using a microwave frequency of 9.602 GHz, modulation amplitude of 6.3 G, and a modulation frequency of 100 kHz as previously described (3). The concentrations of enzyme or SAM added were as noted in the figure legend. Spectra were recorded at 10 K as well as 70K for all samples unless stated otherwise.

Coupled enzyme assay for the CbGD and RiDD. The coupled enzyme assay for the CbGD and the RiDD were performed an anaerobic quartz cuvettes essentially as previously described (3) at 25 °C, except that, where indicated, the RiDD was “pre-activated” prior to addition of the substrate as described below. Similar assays utilizing a coupling enzyme have been reported by those studying the B12-dependent glycerol and diol dehydratases (47). Essentially, an aldehyde reductase, that utilizes NADH or NADPH to
reduce the aldehyde to the corresponding alcohol, is used as a coupling enzyme. Depending on the specific dehydration reaction being investigated, several coupling enzymes have already been well characterized (16,48-52). Unless stated otherwise, the assay contained 1 mM SAM, 1 mM sodium dithionite, 100 mM KCl and 200 µM NADH in 50 mM HEPES pH 7.5. The assay also contained 28 µM of yeast alcohol dehydrogenase (YADH). The total volume of the coupled assay was 0.5 mL and the substrate as well as the enzymes (RiDD and RiDD-AE) were all added to the concentrations indicated in the appropriate figures. Prior to initiating the assay the spectrophotometer was blanked against assay buffer. In order to “pre-activate” the RiDD, this enzyme was incubated with the RiDD-AE (2:1 RiDD-AE:RiDD) in the presence of 2 mM SAM and 2 mM sodium dithionite for 10 minutes in order to allow for generation of the glycyl radical, a variety of enzyme concentrations were tried in these experiments and the assay was initiated by addition of the substrate. In general, the coupled assay takes advantage of a catalytic excess of the reductase YADH in order to rapidly reduce the propionaldehyde product of the dehydratase reaction. We independently investigated the specific activity of commercially available YADH (Sigma Chemical Company) by measuring the NADH-dependent reduction of propionaldehyde in the assay buffer described above and found that it was greater than 400 µmol min⁻¹ mg⁻¹. In order to address glycerol dehydrogenase by the CbGD and the F344Y/V696S variant of the RiDD, the YADH was replaced with purified DhaT in the coupled assay.

**Direct monitoring of 1,2-propanediol dehydration by gas chromatography (GC).** The direct detection of 1,2-propanediol, propionaldehyde, and acetone was performed using a gas chromatograph (GC) equipped with a 30 m x 0.329 mm Carbowax 20m column. Due to the sensitivity of the GC/FID measurement and the sample collection protocol, the assay had to be performed at a much larger scale. Specifically, these assays were performed in 20 mL of assay solution with a concentration of RiDD of 1 µM. Yeast alcohol dehydrogenase as well as NADH, are omitted from this assay in order to allow to the propionaldehyde to accumulate. A stir bar was also included in the experiment and provided stirring at rate of approximately 75 rpm. In all these experiments the reaction was initiated by the addition of RiDD-AE to 2 µM in the presence of 2 mM SAM and 2 mM sodium dithionite. Propanediol (either S-1,2-propanediol or R-1,2-propanediol) was added to 5% (v/v) at the beginning of the assay. At the time points indicated, 1 mL samples were extracted anaerobically and immediately treated with 250 µL of 1M formic acid. The acid-treated samples were all centrifuged in a microfuge at 14,000 rpm for ten minutes in order to remove any precipitated material. The supernatant was then injected into the GC and separated using a temperature gradient that ramped from 60 °C to 120 °C over a 20 minute period. The GC was equipped with a flame ionization detector and a series of standards for 1,2-propanediol, acetone, and propionaldehyde were used to calibrate the integrated peak areas.

**Crystallization, data collection, and structure determination.** Initial crystallization conditions for the RiDD were identified in sitting drop experiments and then optimized in a hanging-drop tray. The final conditions for diffraction quality crystals of the RiDD were 0.125 M sodium acetate, 42 % PEG 400 (w/v), and 0.025 M HEPES pH 7.5. Crystallization was performed in hanging drop experiments with 800 µL of precipitating solution in the well. Protein (40 mg/mL) and precipitation solution were mixed (2 µL each) to initiate the reaction and then the tray was placed at 4 °C for 12 hours before being moved to an incubator at 18°C. Crystals of the RiDD took approximately one week to form and no further cryo-protection was required for freezing. Ethanediol was serendipitously observed in the active site of the RiDD for the native crystals, but it was possible to soak 1,2-propanediol (racemic mixture) into the crystals by simply transferring the crystals to mother liquor containing 5% 1,2-propanediol in a stepwise manner.
Data were collected at the Advanced Photon Source through SER-CAT on beam line 22BM at 0.98 Å. The program PHENIX (53) was used to solve the initial phase problem by using a poly-alanine model of the CbGD (PDB ID 1R9D) and molecular replacement. Iterative rounds of model building and refinement using COOT (54) and PHENIX were performed with experimental phase restraints and a 5.0 % R\text{free} test set (55) that was generated by PHENIX and used throughout all stages of model building and refinement. A summary of the data collection and refinement statistics for the ethanediol- and 1,2-propanediol-bound models is shown in Table 1.

Site-directed mutagenesis. The F344Y/V696S variant of the RiDD was generated using the QuickChange II kit (Agilent Technologies) by following the manufacturers instructions. Mutagenic oligonucleotides were synthesized by Integrated DNA Technologies (Coralville Iowa) and both mutations were verified by sequencing at the Georgia Genomics Facility.

Sedimentation velocity experiments. The RiDD was dialyzed into 10 mM HEPES (pH 7.5) and 150 mM KCl and then diluted to a final concentration of 5 µM for analysis. RiDD (400 µL) or reference buffer (410 µL) was loaded into 12 mm double-sector Epon centerpieces equipped with quartz windows, and equilibrated at 20 °C in an AN60 Ti rotor for 1 h. Sedimentation velocity data were collected in an Optima XLA analytical ultracentrifuge, using a rotor speed of 50,000 rpm at 20 °C. Data were recorded at a wavelength of 280 nm using a radial step size of 0.003 cm. The partial specific volume of RiDD (0.73478 mL/g) was calculated from the amino acid sequence. The program SEDNTERP was used to calculate the density (1.00609 g/mL) and viscosity (0.01009 P) of the buffer(56). SEDFIT was used to model and analyze the sedimentation data. Modeled data were fit as a continuous sedimentation coefficient c(s) distribution using the baseline, meniscus, frictional coefficient, systematic time-invariant noise and radial-invariant noise(57). The root-mean-square deviation (rmsd) value for the experiment was less than 0.004 OD. Theoretical sedimentation coefficients (S) values were calculated from the atomic coordinates of RiDD using HYDROPRO(58).

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AUTHOR CONTRIBUTIONS WNL conceived and coordinated the study and wrote the paper. JWL, SK, and PR contributed substantially to conception and design, acquisition of data, NK exclusively assisted with the sedimentation experiments, all of these authors also contributed to analysis and interpretation of the data presented in this work. FG, DJK, and IEG provided technical assistance and contributed to the preparation of the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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FIGURE LEGENDS

Figure 1. Cartoon representation of the RiDD model aligned on the CbGD model (Panel A) and a structural alignment of the C-terminal domain for both models (Panel B). The CbGD model is colored green and the RiDD model is colored magenta, with the exception of a 48-amino acid insert that is colored blue. The conserved glycine residue in both structures is represented by red space-filling atoms in Panel B.

Figure 2. Sedimentation velocity study for the RiDD (Panel A) and a cartoon representation (Panel B) of the proposed RiDD dimer overlayed on top of the asymmetric unit observed for the model of the CbGD (PDB ID 1R9D). The c(s) sedimentation distribution reveals species corresponding to monomer (M, 5.6 S), dimer (D, 9.2 S) and tetramer (T, 13.4 S). The peak at 3.6 S likely represents a small amount of misfolded monomer. The color scheme for the cartoon representation of the RiDD and CbGD models is the same as shown in Figure 1 except that the atoms of the conserved glycine in the RiDD model are highlighted as cyan space-filling spheres.

Figure 3. EPR spectra monitoring the SAM-dependent generation of a catalytic radical on the RiDD by the RiDD-AE. EPR spectra of 0.1 mM RiDD-AE alone (Panel A), in the presence of 1.25 mM SAM (Panel B), and following the addition of an equimolar amount of the RiDD (Panel C). The spectra presented in Panel A and Panel B were recorded at 10 K with a microwave power of 0.25 mW, while the data presented in Panel C was recorded at 70 K with a microwave power of 0.05 mW. All of the spectra represent the sum of three scans and were recorded with a microwave frequency of 9.582 GHz, a modulation amplitude of 6.477 G, and the modulation frequency was 100 kHz.

Figure 4. Monitoring RiDD activity using pre-activated enzyme. The RiDD was pre-activated as described in the Materials and Methods and then added to a coupled assay where the NADH-dependent reduction of propanal by YADH (1 mg/mL) was monitored in real time at 340 nm using the extinction coefficient of 6220 M⁻¹ cm⁻¹ in order to follow the change in NADH concentration. Raw data for assays that were performed with (a) 0, (b) 0.3 µM, (c) 0.5 µM, (d) 1.4 µM, (e) 2.7 µM, and 5.5 µM concentrations of the RiDD. The inset shows a plot of the rate of NADH consumption versus the concentration of RiDD (molecular weight ~94 kDa) for multiple experiments. Assays were performed in triplicate and error bars represent the standard deviation across the data set.

Figure 5. Substrate saturation plot showing the specific activity, calculated from the maximum rates of NADH consumption in the coupled assay, that were observed for the RiDD at different concentrations of (S)-1,2-propanediol (Trace “S”) or (R)-1,2-propanediol (Trace “S”). Data points represent the average value for three experiments and were fit to the Hill equation with a 95% confidence range displayed by the dotted lines.

Figure 6. Gas chromatography (GC) data following the conversion of 1,2-propanediol to propionaldehyde and acetone by the RiDD. Assays were performed and samples for GC analysis were prepared as described in the Materials and Methods. Samples were taken and analyzed at the indicated time points. The data show the amount of propionaldehyde (■) and acetone (☐) produced when S-1,2-propanediol was used as substrate as well as the propionaldehyde produced (●) and acetone (○) produced when R-1,2-propanediol was used as the substrate for the RiDD in the assay. All experiments were performed in triplicate and the error bars represent the standard deviation across all data points for a given data set.
Figure 7. Wall-eyed stereoview showing a stick representation of the active site model determined for the *R. inulinivorans* diol dehydratase (RiDD) with ethanediol (Panel A) or *S*-1,2-propanediol bound (Panel B). In both cases, the $2F_o-F_c$ composite omit map (*Green cage*) was generated using the simulated annealing protocol with 7% of the model omitted per cycle. The electron density is contoured at 1.3 $\sigma$ and the carbon, oxygen, nitrogen, and sulfur atoms are colored tan, red, blue, and cyan, respectively. Ethanediol and *S*-1,2-propanediol are labeled EDO and PGO respectively. The dashed lines highlight atoms within hydrogen bonding distance of the hydroxyl groups of ethanediol (Panel A) or propanediol (Panel B).

Figure 8. Wall-eyed stereo view of a cartoon diagram showing an overlay of the RiDD model with ethanediol bound (this work, tan carbon atoms), the RiDD model with *S*-1,2-propanediol bound (this work, yellow carbon atoms) and the CbGD model with glycerol bound (*green carbon atoms*). The amino acid residues for the RiDD and CbGD models are labeled in black and brown text, respectively. The dashed lines indicate atoms that are within 3.7 angstroms of one another.

Figure 9. EPR spectra recorded at 70 K for the activated CbGD, RiDD, and the RiDD double mutant F344Y/V696S. All of the spectra represent the sum of three scans and were recorded with a microwave power of 0.05 mW, microwave frequency of 9.582 GHz, modulation amplitude of 6.477 G, and a modulation frequency of 100 kHz. The intensity of the RiDD and the F344Y/V696S variant were considerably less that what was observed for the CbGD and have been increased five fold for a better comparison.

Figure 10. Substrate saturation plot showing the specific activity, calculated from the maximum rates of NADH consumption in the coupled assay, for the CbGD (*left axis, solid squares*) and the F344Y/V696S variant of the RiDD (*right axis, solid circles*) when glycerol was used as the substrate. The coupled assay was performed as described in the Experimental Procedures with the notable exception that purified DhaT was used as the coupling enzyme instead of YADH. Experiments were performed in triplicate and error bars represent the standard deviation across all data points.
Table 1. Data collection and refinement statistics

|                      | ethanediol-bound | 1,2-propanediol-bound |
|----------------------|------------------|-----------------------|
| PDB ID code          | 5I2A             | 5I2G                  |
| Space Group          | C222₁            | C2                    |
| Wavelength           | 0.98             | 0.98                  |
| Resolution Range (Å) | 50.0-2.1         | 50.0-2.4              |
| Unit Cell Dimensions (Å) |  
|                       | a = 138.2, b = 183.2,  
|                       | c = 228.3       | a = 204.3, b = 83.2,  
|                       | c = 138.2       |                       |
| Outer Shell          | 2.18-2.1         | 2.45-2.4              |
| Unique Observations  | 166,139          | 74,266                |
| Completeness (%)     | 98.9(90.3)       | 98.8(88.9)            |
| R<sub>merge</sub> (%) | 0.06(0.29)       | 0.12(0.33)            |
| Redundancy           | 6.9(3.4)         | 7.2(3.5)              |
| I/σ                  | 22.2(2.8)        | 22.1(4.9)             |
| Protein Atoms        | 13,127           | 12,512                |
| Solvent Atoms        | 1,153            | 370                   |
| R<sub>cryst</sub> (%) | 15.2             | 14.5                  |
| R<sub>free</sub> (%)  | 19.0             | 20.3                  |
| rmsd bonds (Å)       | 0.007            | 0.008                 |
| rmsd angles (°)      | 1.01             | 1.04                  |
| average B factor (Å<sup>2</sup>) | 30.2       | 36.4                  |
| Ramachandrian outliers (%) | 0.3          | 0.4                   |
| Rotamer outliers (%)  | 0.6              | 0.7                   |

<sup>a</sup>Numbers in parentheses denote values for the outermost resolution shell.  
<sup>b</sup><i>R</i><sub>sym</sub> =  
\[ \frac{\sum_{hkl} \left( I_{hkl} - \langle I_{hkl} \rangle \right)}{\sum_{hkl} \langle I_{hkl} \rangle} \], where <i>I</i><sub>hkl</sub> is the intensity of an individual measurement of the reflection with indices hkl and \( \langle I_{hkl} \rangle \) is the mean intensity of that reflection.
Figure 1, LaMattina et al.
LaMattina et al. Figure 2.
Figure 3, LaMattina et al.
Figure 4, LaMattina et al.
Figure 5, LaMattina et al.
Figure 6, LaMattina et al.
Figure 7, LaMattina et al.
Figure 8, LaMattina et al.
Figure 9, LaMattina et al.
Figure 10, LaMattina et al.
1,2-propanediol Dehydration in Roseburia inulinivorans; Structural Basis for Substrate and Enantiomer Selectivity
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