The Crystal Structure and Reaction Mechanism of Escherichia coli 2,4-Dienoyl-CoA Reductase*

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Escherichia coli 2,4-dienoyl-CoA reductase is an iron-sulfur flavoenzyme required for the metabolism of unsaturated fatty acids with double bonds at even carbon positions. The enzyme contains FMN, FAD, and a 4Fe-4S cluster and exhibits sequence homology to another iron-sulfur flavoprotein, trimethylamine dehydrogenase. It also requires NADPH as an electron source, resulting in reduction of the C4-C5 double bond of the acyl chain of the CoA thioester substrate. The structure presented here of a ternary complex of E. coli 2,4-dienoyl-CoA reductase with NADPH and a fatty acyl-CoA substrate reveals a possible mechanism for substrate reduction and provides details of a plausible electron transfer mechanism involving both flavins and the iron-sulfur cluster. The reaction is initiated by hydride transfer from NADPH to FAD, which in turn transfers electrons, one at a time, to FMN via the 4Fe-4S cluster. In the final stages of the reaction, the fully reduced FMN provides a hydride ion to the C5 atom of substrate, and Tyr-166 and His-252 are proposed to form a catalytic dyad that protonates the C4 atom of the substrate and complete the reaction. Inspection of the substrate binding pocket explains the relative promiscuity of the enzyme, catalyzing reduction of both 2-trans,4-cis- and 2-trans,4-trans-dienoyl-CoA thioesters.

Metabolism of unsaturated fatty acids requires auxiliary enzymes in addition to those used in β-oxidation. After a given number of cycles through the β-oxidation pathway, those unsaturated fatty acyl-CoAs with double bonds at even-numbered carbon positions contain 2-trans,4-cis double bonds that cannot be modified by enoyl-CoA hydratase. Therefore, an auxiliary enzyme, 2,4-dienoyl-CoA reductase (DCR); EC 1.3.1.34, is used that utilizes NADPH to remove the C4-C5 double bond (1, 2). DCR is unusual in that it lacks stereospecificity, catalyzing the reduction of both natural fatty acids with cis double bonds, as well as substrates containing trans double bonds (3).

Two structurally unrelated forms of DCR have been isolated, both of which use NADPH as reducing equivalents to catalyze reduction of a 2,4-dienoyl-CoA to an enoyl-CoA. The Escherichia coli enzyme contains both FMN and FAD noncovalently bound to a single polypeptide, reducing the substrate by a hydride transfer mechanism in which reducing equivalents from NADPH are supplied indirectly to substrate (1). It functions as a dodecamer, having a molecular mass of 73 kDa, and has been shown recently to contain a 4Fe-4S cluster (4). The enzyme produces 2-trans-enoyl-CoA (1), which can be incorporated into the β-oxidation pathway. In contrast, three homologous eukaryotic forms have been identified, two mitochondrial and one peroxisomal. Two of these lack flavin and iron-sulfur cofactors but are homotetramers with a total molecular mass of 124 kDa. (The third has not been characterized.) The substrate is reduced by a direct hydride transfer mechanism from NADPH to form trans-3-enoyl-CoA (1). Thus, eukaryotic DCR requires an auxiliary enzyme, Δ3,Δ2-enoyl-CoA isomerase, to convert trans-3-enoyl-CoA to trans-2-enoyl-CoA (1), which can be subsequently fed into the β-oxidation pathway. Lastly, eukaryotic DCR has been shown to metabolize fatty acids with double bonds at odd as well as even positions (5, 6), whereas there is currently no evidence to support this for the E. coli enzyme.

In addition to DCR from E. coli, sequence analysis shows both Gram-negative and Gram-positive bacteria to have open reading frames encoding for DCR-like proteins; for example Yersinia pestis and Streptomyces coelicolor proteins have 72 and 62% sequence identities, respectively, with E. coli DCR. These conserved residues include the four cysteine residues that coordinate the iron-sulfur cluster and the cofactor binding sequence motifs. E. coli DCR also shares homology with a large family of TIM barrel-containing flavoenzymes, including the single-domain TIM barrel enzyme NADH oxidase from Thermoanaerobium brockii, which shares a 30% identity and much larger multi-domain proteins such as trimethylamine dehydrogenase (TMDh) from Methylophilus methylphilus; this enzyme also contains a 4Fe-4S cluster and shares a 26% sequence identity with DCR spanning the entire length of both polypeptides (7). A preliminary report on the structure of a functional homolog of DCR, 2-enoate reductase from Clostridium tyrobutyricum, has been presented (8). This enzyme shares a 32% sequence identity with E. coli DCR, binding FMN, FAD, and a 4Fe-4S cluster; however, 2-enoate reductase differs from DCR because it functions as a dodecamer, uses NADH rather than NADPH as an electron source, and reduces 2-enoates that are not activated by conjugation to CoA. Because the 2-enoate reductase structure reported lacks a bound substrate analog, little is known about its enzyme mechanism, including how the flavin and iron-sulfur cofactors participate in electron transfer from NAD(P)H to substrate. To this end, the crystal structure of E. coli DCR has been solved in the presence of substrate and...
gives insights into the catalytic and electron transfer mechanisms of prokaryotic forms of DCR.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Protein was expressed and purified as described previously (7). Briefly, plasmid pNDH, which encodes E. coli DCR, was transformed into E. coli BL21(DE3)pLysS. Cells were cultured in Luria broth medium and induced by isopropyl-1-thio-β-D-galactopyranoside before being pelleted and resuspended by sonication into a 20 mM phosphate buffer solution, pH 7.4, including 10 mM β-mercaptoethanol. The suspension was centrifuged, and the supernatant was loaded onto a DEAE-cellulose column (Sigma). A 100 mM phosphate buffer solution, pH 7.4, including 10 mM β-mercaptoethanol was used to elute protein, which was subsequently transferred to a 2.5-ADP-Sepharose 4B column (Amersham Biosciences). Protein was eluted with a 20 mM phosphate buffer solution containing 5 μM 2-trans–4-trans-decadienoyl-CoA and 10 mM β-mercaptoethanol; the free ligand was removed by passing through another DEAE-cellulose column. Protein was stored at −80 °C in 20% glycerol, 100 mM phosphate buffer, pH 7.4, 1 mM EDTA, and 5 mM β-mercaptoethanol.

**Protein Crystallization**—Purified protein was crystallized by vapor diffusion using the hanging-drop method (9), with the reservoir solution containing 30% polyethylene glycol 5000 monomethyl ether, 0.2 M sodium acetate, 0.1 M ammonium sulfate, and 90 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5. Precipitant solution containing 18% polyethylene glycol 5000 monomethyl ether, 180 mM sodium acetate, 90 mM ammonium sulfate, and 90 mM 2-(N-morpholino)ethane sulfonic acid, pH 6.5, was mixed with protein at 15 mg/ml in a 1:1.5 volume ratio. Protein in storage solution was thawed and concentrated in the presence of a 2-fold molar excess of NADP⁺ before crystallization set up. Rod-shaped crystals having a yellow/brown color appeared within about 1 week, reaching a maximum size of 40 × 40 × 500 μm. No cryoprotectant was used before flash cooling the crystals in liquid nitrogen. Crystals typically diffraeted to 2.2 Å.

Two heavy atom derivatives of DCR were used for phasing; first, a derivative was obtained by soaking crystals in reservoir solution containing 10% saturated mercuric acetate. An osmium derivative was obtained by soaking crystals in reservoir solution containing 100% saturated osmium hexachloride for 10 min. In both cases, no additional cryo-protectant was used before flash cooling the crystals in liquid nitrogen. Crystals diffracted to 2.4 Å.

**Data Collection and Structure Determination**—A complete native data set of DCR was collected in-house using an R-AXIS IIC image plate equipped with a Rigaku RU 200 generator, an MSC X-stream cooling system set at −180 °C, and Osmic blue confocal mirrors. High-resolution data were collected at the Advanced Photon Source (Argonne, IL), beam-line 14-BM-C. Data were processed using the HKL suite of programs (10). Difference Patterson map analysis was performed in XtalView (11), and phases were calculated using either SHARP (12) for single wavelength anomalous (SAD) phases or SOLVE (13) for multiple isomorphous replacement with anomalous scattering phases. Cross-Fourier calculations for locating additional heavy atom sites were performed using GRASP (19). Electron transfer pathway calculations were performed using HARLEM (20).

**RESULTS**

**Structure Determination**—Native crystals diffraeted to 2.4 Å using the in-house x-ray source, with data collected at the synchrotron extending to 2.2 Å. The mercury derivative diffracted in-house to 2.7 Å, and the osmium derivative to 2.4 Å at the synchrotron. The Harker sections of the native anomalous difference Patterson map from in-house data showed a strong peak, above 10 σ, corresponding to the 4Fe-4S cluster; however, the resolution was insufficient to resolve individual iron peaks. SAD phasing was performed in SHARP using the four iron atoms from an ideal 4Fe-4S cluster, which were positioned to give the best visual agreement with the Patterson map. The phases were modified in SOLOMON, and the resulting map showed clear density indicating a large TIM barrel domain in the structure, with traceable density outlining two additional domains extending into other parts of the molecule. Cross-Fourier analysis of mercury and osmium derivatives showed them to contain one and two heavy atom sites per asymmetric unit, respectively. These data were scaled and phased in SOLVE using both isomorphous and anomalous components, then combined with the SAD phases from SHARP using the “combine” CNS script, prior to performing density modification in SOLOMON. These steps resulted in minor improvements in electron density for a medium-sized and a small domain, with the most pronounced improvement around what was subsequently shown to be the FAD moiety in the small domain. An initial Co-atom trace was made, and this model was included in a further round of solvent flattening using SigmA phase combination (21). Subsequent poly-alanine and partial structure models were included in this phase refinement step to further improve the quality of the electron density map. Data collection and final refinement statistics are listed in Table I.

**Overall Protein Structure**—The overall fold of the enzyme is composed of three domains: an N-terminal TIM barrel (residues 1–368) which binds FMN, the 4Fe-4S cluster, and the substrate; a flavodoxin-like fold (residues 369–467 and 626–671) which binds FAD; and an NADP(H)-binding domain (residues 468–625) (Fig. 1). These domains will be referred to as the N-terminal, middle, and C-terminal domains, respectively. There are two disordered regions including residues 370–372 and residues 452–455, both of which form part of the loops on the surface of the protein; however, both N and C termini are well defined, with the C terminus ending as an α-helix. The arrangement of the three domains and the overall polypeptide fold resemble that seen in TMDh (22). The structure-based sequence alignment with TMDh shows DCR to have an α-helical insert in the C-terminal domain, which forms part of the protein’s surface (Fig. 2). A large loop, which forms part of the dimer interface of TMDh in the C-terminal domain (residues 607–630), is missing in DCR, as is the C-terminal extension. Superimposition of DCR with one monomer of TMDh gives an r.m.s. deviation of ~1.5 Å over 467 Cα carbon atoms (corresponding mostly to residues within the N-terminal and middle domains), showing that the high degree of sequence similarity extends to structural similarity. The FMN and 4Fe-4S cofactors that appear in both proteins are spatially conserved, and the bound ADP of TMDh occupies the same spatial position as the ADP portion of FAD in DCR. The conserved cysteine residues (334, 337, 341, and 353) coordinate the four iron atoms of the iron-sulfur cluster (Fig. 2).

**Active Site**—Inspection of an [F Donetsk – F Donetsk] map over the si-face of the isoalloxazine ring of FMN in the early stages of refinement showed low tunnel electron density, indicating the presence of substrate/product bound to the active site. This density could be fitted with one molecule of substrate, 2-trans,4-trans-decadienoyl-CoA (used for elution from the affinity column during protein purification), which was included in the model for subsequent rounds of model building and refinement. However, what was surprising during the mid-stages of model building was that the density bifurcated in the middle of the
Further rounds of model building and refinement showed this fork to stem from the C5 atom of the acyl chain of the substrate, and that the C4-C5 double bond had been reduced by β-mercaptoethanol (included during protein purification and also in the storage buffer). The strong electron density in the map corresponding to the sulfur atom of β-mercaptoethanol allowed correct placement of the remaining acyl chain and of β-mercaptoethanol, showing the product to have R-configuration at the chiral center (carbon 5). Fig. 3A shows an OMIT map confirming bifurcation at the C5 atom.

The carbon-carbon double bond of substrate that is reduced during catalysis (atoms C4-C5 of the acyl chain) is sandwiched between the si-face of the isoalloxazine ring of FMN, centered around atom N5, and two residues of the polypeptide chain, Tyr-166 and His-252. The phenolic oxygen atom of Tyr-166 is about 3.5 Å away from the C4 atom of substrate, whereas the C atom of the imidazole ring of His-252 is in van der Waals contact with the C2 and C3 atoms of substrate, and its N atom forms a hydrogen bond to Tyr-166. These residues form part of a binding pocket illustrated in Fig. 3B. Interactions between substrate and protein at the catalytic site are outlined in Figs. 4 and 5A.

Substrate Binding Site—Fig. 5A illustrates the substrate binding site. The catalytic residue His-252 forms a hydrogen bond with the pantothenate region of the substrate, with the N8 atom of the imidazole ring being 3.5 Å away from the N4 atom of the pantothenate region. Three additional hydrogen bonds are formed between the protein and the pantothenate moiety of the substrate; a carbonyl backbone contact from His-252 to N8, the indole ring of Trp-577 to O9, and the guanido group of Arg-255 to O10, respectively. The ADP moiety of substrate is bound to a relatively basic region on the protein’s surface, with the

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**Table I: Data collection and refinement statistics**

|                          | Native<sup>a</sup> | Native<sup>b</sup> | Osmium<sup>a</sup> | Mercury<sup>b</sup> |
|--------------------------|--------------------|--------------------|-------------------|--------------------|
| Data collection          |                    |                    |                   |                    |
| Wavelength (Å)           | 0.90               | 1.54               | 0.90              | 1.54               |
| Unit cell dimensions (Å) |                    |                    |                   |                    |
| a                        | 65.73              | 65.60              | 65.44             | 65.44             |
| b                        | 108.47             | 109.23             | 108.39            | 108.50            |
| c                        | 110.51             | 110.30             | 109.67            | 111.66            |
| Resolution (Å)           | 2.2 (2.26)         | 2.4 (2.48)         | 2.4 (2.47)        | 2.7 (2.75)        |
| Completeness (%)         | 94.8 (61.2)        | 91.4 (71.4)        | 83.7 (59.8)       | 92.4 (68.1)       |
| Mean (Fo/Fc)             | 30.3 (7.8)         | 43.8 (5.6)         | 18.4 (4.2)        | 10.3 (2.5)        |
| Total no. of reflections | 898148             | 985006             | 255483            | 216402            |
| Unique no. of reflections| 42467              | 31874              | 31925             | 23012             |
| R<sub>sym</sub> (%)      | 5.7 (24.9)         | 5.5 (18.4)         | 7.0 (35.7)        | 9.0 (25.3)        |
| R<sub>free</sub> (%)     | 11.0               |                    |                   |                    |
| Phasing statistics       |                    |                    |                   |                    |
| Method                   | SAD                | MIRAS<sup>d</sup>  |                   |                    |
| Resolution (Å)           | 2.4                | 2.5<sup>e</sup>    |                   |                    |
| No. of sites             | 4                  | 2                  | 1                 |                    |
| Phasing power (cent./acent.)<sup>f</sup> | 1.60 | 0.370.40 | 0.30/0.33 | |
| FOM<sup>g</sup>          | 0.28<sup>acent.</sup> | 0.36 | | |
| Combined FOM<sup>g</sup> |                    | 0.22               |                   |                    |

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<sup>a</sup> Data collected at Advanced Photon Source, beamline 14-BM-C.
<sup>b</sup> Data collected in-house.
<sup>c</sup> Values in parentheses are from the highest resolution shell.
<sup>d</sup> MIRAS, multiple isomorphous replacement with anomalous scattering.
<sup>e</sup> Automatically set by SOLVE.
<sup>f</sup> cent./acent., centric/acentric.
<sup>g</sup> FOM, figure of merit.
3′-phosphate in close proximity to Arg-580 and Lys-566; the latter residue forms a salt bridge. However, the high B-factors of the phosphate atoms suggest that this is a relatively weak association. The adenine ring is sandwiched between Pro-122 and Arg-255 and forms a hydrogen bond from the N6 atom to the guanido group of Arg-175, whereas the pyrophosphate linkage is exposed to solvent. The -adenylic phosphate forms an intramolecular hydrogen bond to the O10 atom of the pantothenate chain. Glu-164 forms a hydrogen bond to the thioester carbonyl oxygen atom of the acyl chain, which may promote the positive character of the C5 atom of the acyl chain through resonance of the conjugated double bonds.

**FMN Binding Site**—As seen in Fig. 1, the FMN cofactor is well buried within the protein’s core. The si-face of the isoalloxazine ring is centered against the C3 atom of the acyl chain of substrate, with atoms C2 and C4 in van der Waals distance with the N10 (3.6 Å) and N5 (3.4 Å) atoms of FMN, respectively (Fig. 4). Met-25 is positioned on the opposing re-face of the isoalloxazine ring. Fig. 5B shows residues that interact with the FMN cofactor. Unlike TMDh in which the isoalloxazine ring of FMN is tethered to the polypeptide through a covalent bond between Cys-30 and the C6 atom of FMN, the FMN of DCR is not covalently attached to the enzyme; His-26 replaces the thiol residue of TMDh (Fig. 2). The ribityl chain forms hydrogen bonds with the guanidinium group of Arg-214 and the backbone carbonyl oxygen atom of Ser-24. The guanidinium group of Arg-214 also hydrogen bonds with the O2 atom of the isoalloxazine ring, as does the amide side chain of Gln-100. The N3 atom of the isoalloxazine ring forms a hydrogen bond to an amide group of Gln-100, and the O4 atom hydrogen bonds to the backbone nitrogen atom of Gly-58. Oxidized FMN is able to hydrogen bond to the backbone nitrogen atom of His-26 from the N5 atom of the isoalloxazine ring. The phosphate group is surrounded by backbone nitrogen atoms of Arg-288, Ala-310, and Arg-311. The guanido group of Arg-311 also forms hydrogen bonds to the phosphate.

**FAD Binding Site**—Similar to FMN, the isoalloxazine ring of FAD is well buried; however, the ribityl and pyrophosphate...
moieties project to the protein surface such that the adenosine region is exposed to solvent (Fig. 1). The isoalloxazine ring is positioned such that the two phenyl groups of Phe-412 and Phe-424 and the methyl side chain of Ala-415 are on its si-face, with the phenyl groups pointing edge-on to the plane of the isoalloxazine ring, and the nicotinamide and adjacent ribose ring of NADP(H) are on its re-face. There are no hydrogen bonds between protein or water molecules and the ribityl chain of FAD (Fig. 5); however, the O3/H11032 atom of the ribityl chain forms a hydrogen bond with the H9252-adenylic phosphate group in the pyrophosphate linkage. Both phosphate groups of the pyrophosphate linkage make several hydrogen bonds with backbone nitrogen atoms, Ala-384, Gln-411, and Gly-648, with the γ-amide side chain of Gln-411 also forming a hydrogen bond to the α-adenylic phosphate. Both hydroxyl groups of the adenosyl ribose ring form hydrogen bonds with the carboxylate of Asp-403, whereas the N1 and N6 atoms of the adenine ring form hydrogen bonds with backbone atoms of Val-448.

NADP(H) Binding Site—Superimposition of the DCR structure with that of glutathione reductase shows that the middle and C-terminal domains of DCR resemble the N-terminal and middle domains of glutathione reductase (23), having an r.m.s. deviation of 1.6 Å for 43% of C atoms in the two domains of glutathione reductase. The superimposition shows both FAD and NADP(H) occupying spatially conserved sites. During the mid-stages of model building of DCR, there was sufficient electron density above the re-face of the FAD flavin moiety to allow NADP+ to be included in the model. Unfortunately, however, it was not possible to accurately position the nicotinamide ring because there was not enough electron density to unambiguously fit the ring. Subsequent rounds of refinement failed to improve density surrounding this part of the

Fig. 4. Stereo diagram of the substrate-binding site and possible electron transfer pathway of E. coli DCR. The β-mercaptoethanol modified form of 2,4-decadienoyl-CoA is sandwiched between the si-face of the isoalloxazine ring of FMN and the side chains of Tyr-166 and His-252. Hydrogen bonds are denoted by long dashed lines. The electron transfer pathway from FAD to FMN proposed by calculations using HARLEM (20) is illustrated by short dotted lines, with the shortest FAD to iron-sulfur distance being 8.6 and 5.7 Å between iron-sulfur and FMN. The iron-sulfur cluster is shown as a cube with orange (iron) and yellow (sulfur) balls; the color scheme is the same as in Fig. 3. Distances given are in Å.

Fig. 5. Balls-and-sticks representation of the binding sites of substrate (A), FMN (B), and FAD (C). For clarity, only those residues that form hydrogen bonds with the ligand/cofactor are shown; those residues that form significant van der Waals interactions are discussed in the text. For simplicity, the diagram shows a flattened projection with water molecules removed. The figure was generated using LIGPLOT (34) and rendered using MOLSCRIPT (35). Light gray balls represent carbon atoms, mid-gray are oxygen, dark gray are nitrogen, large mid-dark gray balls are sulfur, and black balls are phosphorus.
molecule, although the well-defined electron density of the adjacent ribose ring and the surrounding peptide limits positioning of the nicotinamide ring to a small area. Therefore, it remains unclear whether the A- or B-side of the nicotinamide ring interacts with the re-face of the FAD isoalloxazine ring.

**Electron Transfer Pathway**—Fig. 4 shows the arrangement of cofactors and substrates found in the crystal structure. The relative positions of the electron source (NADPH) and the electron sink (substrate) suggest that electrons from NADPH must travel through FAD, 4Fe-4S, and FMN to reduce substrate. Inter-cofactor distances appear substantially short enough to allow rapid electron transfer, with the FAD to 4Fe-4S minimal distance being 8.6 Å between N3 of the isoalloxazine ring and the nearest iron atom, sufficiently below the 14 Å electron tunneling limit proposed by Page et al. (24). The distance between the iron-sulfur cluster and FMN is somewhat shorter, being 5.7 Å between the C8 methyl group carbon of FMN and the nearest iron atom of the cluster. Analysis of a potential electron transfer pathway using the software program HARLEM (20) suggests that the amide group of the Gln-339 side chain may participate in electron transfer between FAD and the iron-sulfur cluster by dividing electron transfer between the two cofactors from one large through-space transfer to two smaller through-space steps. However, the FAD to 4Fe-4S distance is sufficiently short that this is unlikely to be essential (24). There are no other side chains or water molecules in the proposed pathway that appear close enough to participate in electron transfer.

**DISCUSSION**

The overall topology of *E. coli* DCR is much like that of TMdh (22), having an R.m.s. deviation of 1.5 Å over 467 Ca carbon atoms, corresponding to ~70% of amino acids in DCR. This extends to cofactor arrangement, with FMN and the 4Fe-4S cluster in spatially conserved positions, and shows the ADP moiety of FAD in DCR to occupy the same position as ADP in TMdh. Though structurally similar, DCR and TMdh contrast each other in that DCR uses NADPH to reduce substrate, whereas TMdh oxidatively demethylation substrate using water, meaning that electron transfer within the enzymes goes in opposite directions. The topology of the N-terminal TIM barrel of DCR shows that this region of the polypeptide belongs to the old yellow enzyme (OYE) family of proteins, with DCR and OYE having an R.m.s. deviation of ~1.2 Å for ~62% of Ca carbon atoms from OYE. OYE is a simple TIM barrel flavoenzyme that has FMN noncovalently bound toward one end of the core of the fold, with the active site directly above the si-face of the isoalloxazine ring (25). Similar to DCR, OYE uses NADPH to supply reducing equivalents to substrate; however, the nicotinamide ring also binds to the active site of OYE so that the enzyme displays ping-pong kinetics (26). Detailed kinetic studies on *E. coli* DCR have not yet been carried out, but the structure suggests that it is unlikely to exhibit ping-pong-like kinetics. Similar to DCR, glutathione reductase catalyzes NADPH-dependent reduction of substrate but uses a pair of redox-active cysteine residues to reduce its substrate, glutathione disulfide. Superimposition of these two structures show that the middle and C-terminal domains of DCR have a similar fold to the N-terminal and middle domains of glutathione reductase (23) (R.m.s. deviation of 1.6 Å for ~43% of amino acids of the Ca carbon atoms in the two domains of glutathione reductase). The FAD and NADP⁺ cofactors are bound in spatially conserved positions with the re-face of the isoalloxazine ring of FAD facing toward the nicotinamide cofactor. In the DCR crystal structure, similar to glutathione reductase complexed with NADP⁺, there is insufficient electron density to allow the nicotinamide ring of NADP⁺ to be modeled in (23). However, when glutathione reductase is crystallized in the presence of NADPH, the nicotinamide ring displaces Tyr-197 so that the B-face of the nicotinamide ring interacts with the re-face of FAD, in a parallel displaced fashion (27). In the case of DCR, it would be interesting to see whether, similar to glutathione reductase, the electron density corresponding to the nicotinamide ring becomes visible when NADP⁺ is substituted for NADPH. DCR lacks an aromatic residue that could block access to the isoalloxazine ring, as is observed for glutathione reductase, so it remains to be seen what may be causing the ring to be disordered. A more distantly related enzyme, dihydroxyprymidine dehydrogenase (28), also contains the middle and C-terminal domains of DCR, as well as the N-terminal TIM barrel. However, they are arranged in a different manner, with dihydroxyprymidine dehydrogenase having additional domains and three extra iron-sulfur clusters. Dihydroxyprymidine dehydrogenase catalyzes NADPH-dependent reduction of uracil and thymine, although because of the spatial arrangement of cofactors, the electron transfer pathway is most likely much more complicated than that of DCR. Although functionally diverse, their structural similarity suggests that all of these enzymes are related, and that DCR may have evolved through fusion of ancestral genes encoding primitive forms of OYE and glutathione reductase.

*E. coli* DCR reduces the C4-C5 double bond of the 2,4-dienoyl-CoA thioester. To achieve this, two reducing equivalents are supplied by NADPH to FAD via a direct hydride transfer mechanism, with the 4Fe-4S cluster and FMN moiety forming a conduit for electrons to the substrate. Inspection of the active site shows modified product to be sandwiched between the si-face of FMN and the side chains of amino acids Tyr-166 and His-252 (Fig. 4). The close proximity of Tyr-166 to the C4 atom of substrate (3.5 Å) strongly suggests that Tyr-166 acts as the catalytic residue that protonates the C4 atom during substrate reduction. At the same time, the imidazole group of His-252, which is within hydrogen bonding distance to Tyr-166 (2.7 Å), is likely to stabilize the phenolate ion formed during this step of catalysis. Sequence alignment of all available prokaryotic DCR sequences shows Tyr-166 and His-252 are conserved and lends support to the importance of these residues in catalysis. The fact that substrate has been fortuitously modified by β-mercaptoethanol at the C5 atom of the acyl chain lends support that the C5 atom is reduced by a hydride transfer from FMN, therefore priming the C4 atom for protonation by Tyr-166.

The overall reaction mechanism can be divided into three stages: initially, reduction of FAD by NADPH, then electron transfer from FAD to FMN via the 4Fe-4S cluster, and finally reduction of substrate. Initiation of electron transfer requires reducing equivalents to be transferred from NADPH to FAD via a direct hydride transfer mechanism, with the nicotinamide ring interacting with the re-face of the isoalloxazine ring. This results in fully reduced FAD, which in turn transfers electrons to the iron-sulfur cluster, either directly or possibly via Gln-339. Because a 4Fe-4S cluster can only accept one electron at a time, and substrate is reduced by hydride transfer from fully reduced FMN, it is reasonable to assume that electron transfer is divided into two steps. First, one electron is transferred from FAD to FMN via the iron-sulfur cluster, such that both flavins are in semiquinone states. Then the second electron from FAD is transferred to FMN, resulting in fully oxidized FAD and fully reduced FMN. Support for the hypothesis that all redox centers are involved in substrate catalysis comes from the observation that DCR can be reduced to a five-electron reduced state using NADPH (4). Fig. 6 outlines a possible mechanism of substrate
reduction from fully reduced FMN. The C4-C5 double bond follows a Michael addition reaction because the acyl chain contains conjugated double bonds at carbon atoms 2 and 4 and the carbonyl oxygen at carbon 1, therefore stabilizing a partial positive charge at atom C5. This property is further enhanced by a hydrogen bond (3.3 Å) between the thioester carbonyl oxygen of substrate and the carboxylate of Glu-164. This observation is supported by studies with 5-phenyl-2,4-pentadienoyl-CoA, which show that C5 is polarized when bound to the active site of the enzyme (4). This property of the active site, promoting the labile nature of the C5 atom of the substrate, may explain why the crystalline protein contains substrate modified by mercaptoethanol, a potent nucleophile used during the purification stage and included in the storage buffer.

The relatively neutral pH at which protein is purified and crystallized suggests that substrate is modified by mercaptoethanol after binding to the active site, because these conditions reduce the nucleophilic character of the sulfhydryl group when free in solution. The microenvironment of the active site promotes the labile nature of the C5 atom of substrate, allowing mercaptoethanol to react under non-optimal pH conditions. In the normal reaction pathway, the C5 atom undergoes nucleophilic attack from a hydride ion off the N5 atom of reduced FMN (Step I, Fig. 6). The guanidinium group of Arg-214 lies within hydrogen bonding distance (3.4 Å) of the N1 atom of the isoaflavoxine ring of FMN and therefore could potentially act as the general acid/base necessary to protonate/deprotonate the N1 atom during the catalytic cycle. Although uncommon, an arginine residue has been proposed to act as a catalytic base in the pectate lyase family (29) and an acid in quinol:fumarate reductase (30). However, the pH optimum of 7.4 for DCR (31) is well below that of pectate lyases, pH 10.5, suggesting that Arg-214 is maintained in a protonated state in DCR. Instead, a water molecule (WAT1) that lies within hydrogen bonding distance (3.5 Å) to the N1 atom of the isoaflavoxine ring of FMN most likely protonates/deprotonates the N1 atom during the catalytic cycle. Furthermore, the reduced FMN may exist in anionic state during reduction as has been proposed for TMDh (32), suggesting that Arg-214 would be ideally positioned to provide a counter ion. Concomitant with this is the protonation of C4 by Tyr-166, which acts as a general acid (Step II, Fig. 6). A hydrogen bond between the Ne atom of His-252 and Tyr-166 stabilizes the negative charge on the phenol ring (Step III, Fig. 6).

A recent preliminary report of 2-enoate reductase from Clostridium tyrobutyricum (8) shows the overall fold to be much like that of DCR, with NADH and FAD bound to the corresponding medium and small domains of DCR, respectively; the FMN and iron-sulfur cluster are bound to the TIM barrel domain in a similar manner as well. Enolase reductase performs...
similar chemistry to DCR in that a carbon-carbon double bond is reduced by a 1,2-proton addition; however, enoate reductase does not require substrate to be conjugated to CoA, functions as a dodecamer rather than a monomer, and uses NADH rather than NADPH as the electron source. Because the reported crystal structure of enoate reductase lacks bound substrate or inhibitor at the active site, catalytic residues, His-175 and Tyr-181, have been proposed based on homology to OYE. Inclusion of the sequence of enoate reductase with the structure-based sequence alignment of DCR with TMDh (Fig. 2) shows that His-175 is not conserved in DCR, instead being Met-161. Instead, the alignment suggests that Tyr-181 in enoate reductase may act as the general acid such as that of Tyr-166 in DCR, and that Tyr-281 may hydrogen bond to Tyr-181 to allow protonation of substrate, akin to the function of His-252 in DCR. The structure of enoate reductase complexed with substrate or analog should clarify which residues participate in catalysis. The large size of the substrate binding pocket (Fig. 3B) explains why the enzyme can accommodate the relatively bulky β-mercaptoethanol modified form of decadienoyl-CoA at the active site and may explain the relative substrate promiscuity between the 4-trans- and 4-cis-dienoyl-CoA isomers. Further structural and biochemical studies using various substrate and NADPH analogs will likely yield more insight into the function of this and members of this large family of enzymes.

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REFERENCES

1. Dommes, V., and Kunau, W. H. (1984) J. Biol. Chem. 259, 1781–1788
2. You, S. Y., Cusot, S., and Schulz, H. (1989) J. Biol. Chem. 264, 16489–16495
3. Cuebas, D., and Schulz, H. (1992) J. Biol. Chem. 267, 14140–14144
4. Liang, X., Thorpe, C., and Schulz, H. (2000) Arch. Biochem. Biophys. 380, 373–379
5. Smeland, T. E., Nada, M., Cuebas, D., and Schulz, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6673–6677
6. He, X. Y., Shoukry, K., Chu, C., Yang, J., Sprecher, H., and Schulz, H. (1995) Biochem. Biophys. Res. Commun. 215, 15–22
7. He, X. Y., Yang, S. Y., and Schulz, H. (1997) Eur. J. Biochem. 248, 516–520
8. Steinbacher, S., Stumpf, M., Weinkauf, S., Bödheck, F., Bachler, A., and Simin, H. (2002) in Flavin and Flavoproteins 2002 (Chapman, S., Perham, R., and Scrutton, N., eds) pp. 941–949, Agency for Scientific Publications, Berlin
9. McPherson, A. (1999) Crystallography of Biological Macromolecules, pp. 176–196, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
10. Owinoowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
11. McRee, D. E. (1999) Practical Protein Crystallography, Academic Press, San Diego
12. La Fortelle, E., and Brizioge, G. (1997) Methods Enzymol. 276, 472–494
13. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. D Biol. Crystallogr. 55, 849–861
14. Furey, W., and Swaminathan, S. (1997) Methods Enzymol. 277, 590–620
15. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grose-Kuntoleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. D Biol. Crystallogr. 54, 905–921
16. Abrahams, J. P., and Leslie, A. G. W. (1996) Acta Crystallogr. D Biol. Crystallogr. 52, 30–42
17. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard (1991) Acta Crystallogr. A 47, 110–119
18. van den Akker, F., and Hol, W. G. (1999) Acta Crystallogr. D Biol. Crystallogr. 55, 206–218
19. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins 11, 281–296
20. Curry, W. B., Grabe, M. D., Kurnikov, I. V., Skourtis, S. S., Beratan, D. N., Regan, J. J., Aquino, A. J., Beroza, P., and Osuchic, J. N. (1999) J. Bioenerg. Biomembr. 27, 285–293
21. Read, R. J. (1996) Acta Crystallogr. A 42, 140–149
22. Lim, L. W., Shamana, N., Mathews, F. S., Steenkamp, D. J., Hamlin, R., and Xuong, N. H. (1986) J. Biol. Chem. 261, 15140–15146
23. Karpus, P. A., and Schulz, G. E. (1987) J. Mol. Biol. 195, 701–729
24. Page, C. C., Moser, C. C., Chen, X., and Dutton, P. L. (1999) Nature 402, 47–52
25. Fox, K. M., and Karpus, P. A. (1994) Structure 2, 1089–1105
26. Brown, B. J., Deng, Z., Karpus, P. A., and Massey, V. (1998) J. Biol. Chem. 273, 32753–32762
27. Pai, E. F., Karpus, P. A., and Schulz, G. E. (1988) Biochemistry 27, 4465–4474
28. Dohrntzach, D., Schneider, G., Schnackertz, K. D., and Lindqvist, Y. (2001) EMBO J. 20, 650–660
29. Charnock, S. J., Brown, I. E., Turkenburg, J. P., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. D Biol. Crystallogr. 55, 849–861
30. Lancaster, C. R., Gross, R., and Simon, J. (2001) Eur. J. Biochem. 278, 32753–32762
31. Krulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
32. Levitt, M., and Gerstein, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5013–5020