

Precursor of ether phospholipids is synthesized by a flavoenzyme through covalent catalysis

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Edited by Emil F. Pai, Ontario Cancer Institute/Princess Margaret Hospital, Toronto, ON, Canada, and accepted by the Editorial Board October 5, 2012 (received for review August 31, 2012)

The precursor of the essential ether phospholipids is synthesized by a peroxisomal enzyme that uses a flavin cofactor to catalyze a reaction that does not alter the redox state of the substrates. The enzyme crystal structure reveals a V-shaped active site with a narrow constriction in front of the prosthetic group. Mutations causing inborn ether phospholipid deficiency, a very severe genetic disease, target residues that are part of the catalytic center. Biochemical analysis using substrate and flavin analogs, absorbance spectroscopy, mutagenesis, and mass spectrometry provide compelling evidence supporting an unusual mechanism of covalent catalysis. The flavin functions as a chemical trap that promotes exchange of an acyl with an alkyl group, generating the characteristic ether bond. Structural comparisons show that the covalent versus noncovalent mechanistic distinction in flavoenzyme catalysis and evolution relies on subtle factors rather than on gross modifications of the cofactor environment.

peroxisomal disorder | phospholipid biosynthesis | plasmalogen | rhizomelic chondrodysplasia punctata

Textbooks typically describe a phospholipid molecule as consisting of a glycerol backbone with a polar head and two acyl chains linked via an ester bond to the glycerol *sn*-1 and *sn*-2 carbons. This description can be misleading, however, in that a significant fraction of eukaryotic and archaeobacterial cell membranes contain a different group of molecules, the ether phospholipids, which represent up to 20–50% of mammalian membranes. These phospholipids' distinctive property is the ether bond that connects an alkyl (rather than an acyl) chain to the *sn*-1 carbon of the glycerol moiety (1, 2). Ether phospholipids facilitate membrane fusion processes and function as effective antioxidants, signaling molecules, and storage depots for docosahexaenoic (ω -3) and arachidonic (ω -6) acids. The key fact is that these phospholipids are essential for normal growth and development (3, 4).

The initial and critical steps in the generation of ether phospholipids occur in the peroxisomes through a curious route. First, an acyl-transferase enzyme uses acyl-CoA and dihydroxyacetone phosphate (DHAP) to generate acylDHAP. This molecule is subsequently processed by alkylDHAP synthase (ADPS) in formation of the ether bond, the most crucial and chemically demanding step of the entire biosynthetic pathway (5–7). In particular, ADPS replaces the acyl group of acylDHAP with a long-chain fatty alcohol, generating alkylDHAP (Fig. 1). Defects in the foregoing metabolic steps have devastating consequences that lie at the basis of genetic diseases. In patients with a peroxisomal biogenesis disorder, such as Zellweger syndrome, peroxisomes lack matrix enzymes (so-called “ghost” organelles), and thus ether phospholipids cannot be effectively synthesized. Rhizomelic chondrodysplasia punctata is caused by more specific single-enzyme deficiencies. Patients with this autosomal recessive disorder die within the first or, at best, second decade of life (8). Disease types 2 and 3 are caused by mutations in the acyl-transferase and

ADPS enzymes, whereas type 1 arises from mutations in PEX7, the protein mediating the peroxisomal import of ADPS (9–12).

Here we report a structural and mechanistic investigation of mammalian ADPS in WT and mutated forms. The biochemical hallmark of the enzyme is that it uses a redox cofactor, flavin adenine dinucleotide (FAD), to catalyze a reaction that does not alter the redox state of the substrates (10, 13). Our findings indicate that FAD functions as a chemical trap for the substrate DHAP moiety so as to favor the acyl-alkyl exchange. They also demonstrate that pathological mutations target residues directly involved in catalysis and cofactor binding. These findings raise intriguing hypotheses about the covalent versus noncovalent mechanistic dichotomy in flavoenzyme catalysis and evolution.

Results

V-Shaped Active Site. The structure of *Cavia porcellus* ADPS (93% sequence identical to the human enzyme) was solved at 1.9-Å resolution (Fig. 2*A* and *B* and *SI Methods*). This protein is a 130-kDa dimer of identical subunits each comprising three domains (N-terminal, FAD-binding, and cap domains). The active site is located in front of the flavin ring at the interface between the FAD and cap domains and comprises residues belonging to both monomers, indicating that the dimeric assembly is essential for catalysis (Fig. 24). Mammalian ADPS shares 33% sequence identity with the *Dictyostelium discoideum* enzyme, the crystal structure of which is known (14). Domain superpositions indicate a significant change in the relative domain orientation, with a 14° rotation of the *D. discoideum* cap domain with respect to the same domain of ADPS (Fig. S1). This change is associated to another key difference; the so-called “HHH” loop is clearly visible in the electron density of the mammalian protein, whereas it is disordered in the *Dictyostelium* structure (Fig. 2*A* and *B*). This loop is named after the strictly conserved His-His-His motif (residues 615–617), which is essential for activity (14). The HHH loop of ADPS forms a sharp bend that wedges into the active site to directly contact the flavin (Figs. 2 and 3). This conformation is coupled to the ordering of residues 347–359 of a twofold-related subunit. Thus, the structure of ADPS visualizes the conformation of all elements directly involved in catalysis, a critical issue for investigation of the enzyme mechanism.

Author contributions: S.N., A.J.R.H., D.E.E., and A.M. designed research; S.N., V. Piano, S.R., A.A., and V. Pandini performed research; S.N., A.A., M.W.F., A.J.R.H., D.E.E., and A.M. analyzed data; and S.N. and A.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. E.F.P. is a guest editor invited by the Editorial Board.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4BBY, 4BC7, 4BC9, and 4BCA).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1215128109/-DCSupplemental.

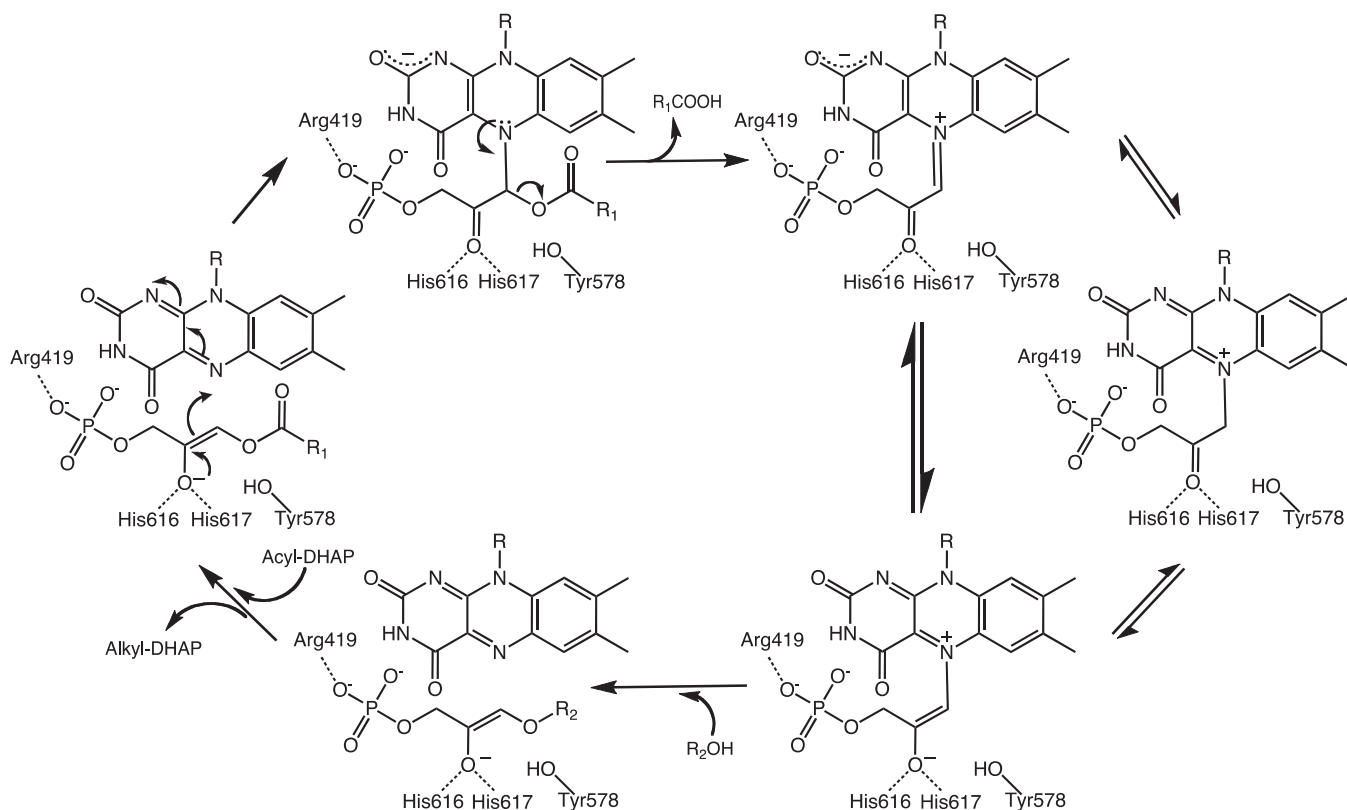


Fig. 1. Mechanistic proposal for the ADPS reaction. The acylDHAP substrate is shown in its enolic form. The crucial covalent intermediate can have different tautomeric forms. The exact protonation states of several titratable groups purportedly involved in the reaction (Tyr578, His616-His617, N1 and N5 of the flavin, oxygens of DHAP, fatty acid product, and fatty alcohol substrate) are not firmly defined and are likely to change during the reaction. The data do not rule out adduct formation on C4a rather than on N5 of the flavin (see Fig. 4C, *Right* for atomic numbering); however, a C4a adduct intermediate would be difficult to reconcile with the subsequent fatty acid elimination step. The predicted negative charge of the covalent adduct can be stabilized by a network of H-bonding interactions involving protein backbone atoms, an ordered water molecule, and the N1 and O2 atoms of the flavin, as shown in Fig. S2A.

AcylDHAP, the substrate of ADPS, comprises a fatty chain hydrophobic tail, the central three-carbon unit of DHAP, and the negatively charged phosphate group (Fig. 1). This tripartite nature perfectly matches the architecture of the substrate-binding site. ADPS has a V-shaped tunnel that runs across the cap-FAD domain interface from the gating helix to Arg419 (Fig. 3). The longest arm of the tunnel represents the binding site for the aliphatic portion of the substrate. Indeed, its electron density indicates that this segment of the active site tunnel is occupied by a long-chain molecule (possibly a PEG molecule used for crystallization). The tunnel length matches that expected for 16- to 18-carbon ligands, in line with the specificity for hexadecanoyl- and octadecanoyl-DHAP; the enzyme is inactive with shorter-chain substrates (5). The shorter arm of the V-tunnel has a more spherical shape and is lined by several hydrophilic residues, including Arg419 and two Thr residues. These features make it well suited to host the substrate phosphate group. The two arms are connected through a narrow constriction defined by His616, His617, the flavin ring, and Tyr578. Importantly, the constriction is located exactly in front of reactive locus of the cofactor, the N5 atom (Fig. 3). This arrangement of the flavin with His and Tyr side chains generates the catalytic armamentarium underlying the ether bond formation carried out by ADPS.

Pathological Mutations Targeting Functionally Crucial Residues. We produced and studied five active-site mutations, three of them (see Fig. S2) found in patients affected by rhizomelic chondrodysplasia punctata (10, 12). Above all, the point emerging from these experiments is that all mutations completely inactivate

the enzyme. We found that three of these mutations (Thr309Ile, Leu469Pro, and Arg515Leu) were defective in their ability to bind FAD, associated with poor stability of the apoenzyme (Fig. S2A and B). The other two mutations (Arg419His and Tyr578Phe) exhibited normal stability, and their crystal structures showed no large conformational changes. However, none of the mutations was enzymatically active, and incubation with acylDHAP caused no spectral perturbation (*Reactivity of the Flavin Cofactor*). Arg419His targets a positively charged residue that is part of the short arm of the active site V-tunnel (Fig. 3). Crystal structure analysis showed that substitution of the long Arg side chain with a less bulky (but polar) His group causes a small (up to 1.5 Å) shift in the central portion of helix α 15, located near the site of mutation (Fig. S2C). Otherwise, the protein does not exhibit any alteration with respect to the WT conformation. Similarly, the Tyr578Phe structure simply shows that the hydroxyl group of the Tyr side chain can be removed without causing any structural changes (Fig. 2B). The Arg419His and Tyr578Phe mutants are very informative, in that their lack of reactivity indicates that the mutated residues are essential for substrate binding and/or for performing an essential step of the catalytic reaction.

Reactivity of the Flavin Cofactor. The present study is based on an improved expression system that produces a protein with enhanced stability and, above all, far less propensity for aggregation compared with previously used recombinant enzymes (13). These properties make the enzyme more amenable to biochemical studies, although it must be noted that ADPS substrates are

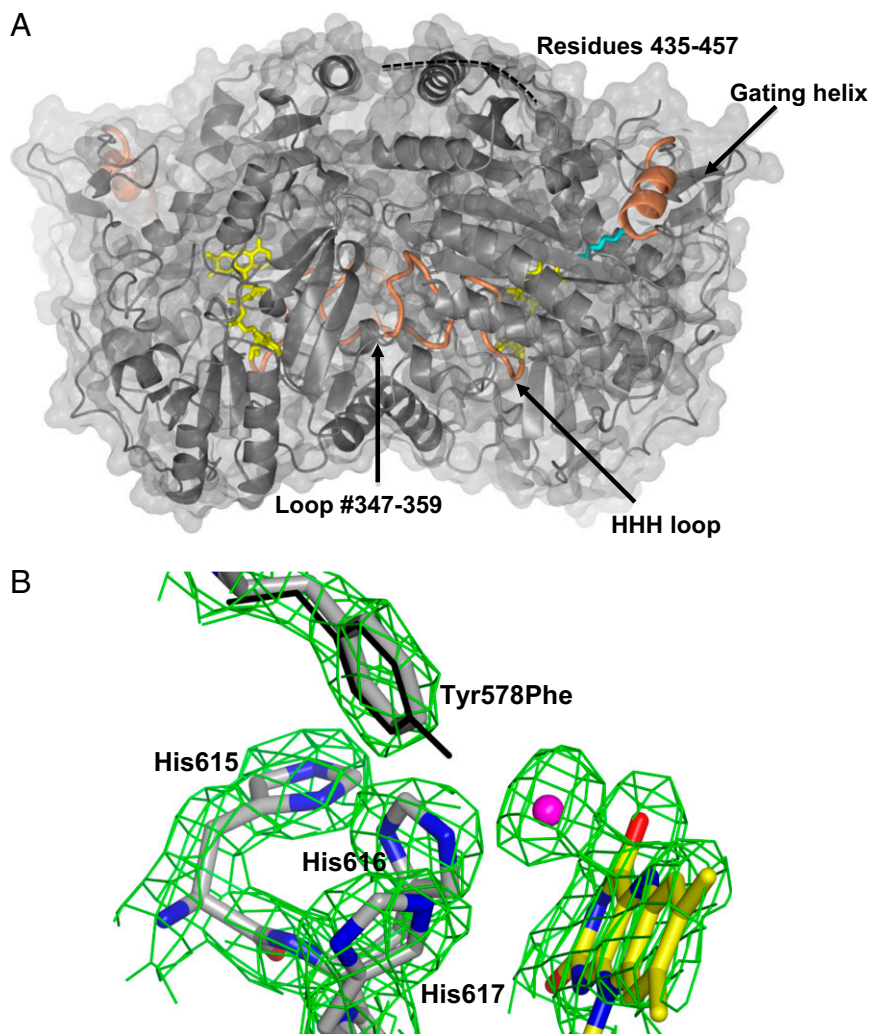


Fig. 2. Crystal structure of ADPS. (A) The dimer with the molecular twofold axis vertical in the plane of the paper. The FAD is shown in yellow; the bound aliphatic ligand, in cyan. Loop 347–359, the “HHH loop” (615–622), and the gating α -helix (126–137) are shown in orange. # indicates that loop 347–359 is part of the active site of the twofold related subunit. ADPS is associated to the inner side of the peroxisomal membrane (13). This localization enables the liposoluble fatty alcohol substrate and fatty acid product to directly diffuse to and from the membrane (29). By analogy with the *Dictyostelium* ADPS structure, the site of membrane association is predicted to be on the upper side of the dimer, in the area embraced by the disordered 435–457 segment (14). (B) The final 2Fo–Fc–weighted electron density map (contoured at the 1.4 σ level) of the active site in the Tyr578Phe mutant. Protein carbons are shown in gray; flavin carbons, in yellow; oxygens, in red; nitrogens, in blue; and chloride, in magenta. The Tyr578 side chain of the WT protein is superimposed and shown with thin bonds. The only detectable change is the strong electron density peak in direct contact with the flavin N5 atom. We have interpreted this peak as a chloride ion bound to flavin, the negative charge of which is compensated for by the His side chains of the HHH loop. This observation supports the role of these His residues in stabilization of the negatively charged enolate form of the substrate (Fig. 1).

water-insoluble and tend to cause slow protein unfolding and aggregation. The key initial observation was that incubation of the enzyme with palmitoylDHAP led to rapid bleaching of the

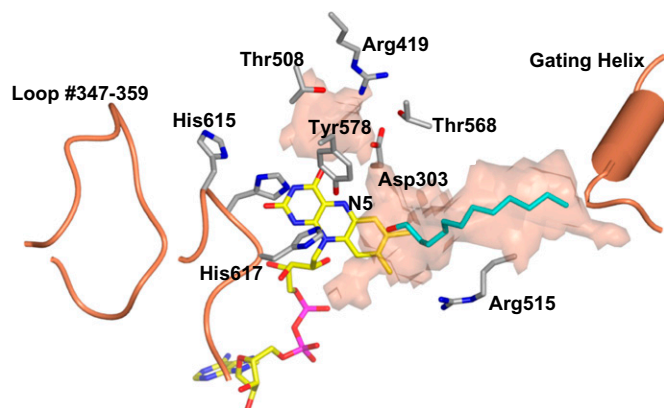


Fig. 3. Active site of ADPS. Protein carbons are shown in gray; flavin carbons, in yellow; ligand carbons, in cyan; oxygens, in red; nitrogens, in blue; and phosphorus, in magenta. The substrate-binding site tunnel was calculated with a 1.4-Å radius probe. The semitransparent surface outlines the “probe-accessible” region of the tunnel. # indicates residues from the opposite subunit of the dimeric enzyme. The orientation is the same as that of the subunit shown in Fig. 2A.

cofactor. The absorbance spectrum indicates that the flavin becomes either two electron-reduced or covalently modified by the substrate (Fig. 4A). This bleached form decays to the original oxidized state within 10–20 min under both aerobic and anaerobic conditions, implying that the entire process is completely oxygen-independent. We found that regeneration of the oxidized enzyme resulted in the formation of two molecules, palmitic acid (detected by MS analysis of the reaction mixture) and DHAP, effectively detected using an assay based on triose phosphate isomerase (Fig. S3). Importantly, the bleached cofactor appeared to be stable only when enzyme-bound, given that protein unfolding led to the immediate formation of oxidized flavin. Again, unfolding under anaerobic condition did not prevent flavin reoxidation. These data suggest that the two electron-reduced form of the flavin probably is not the intermediate, and support the idea that the bleached form of the cofactor is a covalent adduct generated by the reaction with substrate. The adduct undergoes rapid hydrolysis in water, but is insensitive to oxygen.

These findings led us to explore the possibility that the covalent intermediate may result from the nucleophilic addition of acylDHAP on the cofactor (Fig. 1). Along this line, we probed ADPS for its reactivity with nitroalkanes, anionic forms of which are potentially able to nucleophilically attack the flavin (15, 16). We found that ADPS reacts with both nitroethane and nitrooctane, as indicated by the bleaching of the flavin on incubation of the enzyme with these compounds (Fig. S4A and B).

position 5 of the flavin is replaced by a carbon (5-deazaFAD). The rationale for this experiment was two-pronged: (i) This analog probes the role of the flavin N5 locus, and (ii) 5-deazaflavin is known to be reactive toward nucleophilic reagents, thereby representing a potential tool for probing the enzymatic mechanism (17–19). The 5-deazaFAD–reconstituted enzyme exhibited very low activity (3–5% compared with the native protein), likely reflecting the presence of a small fraction of FAD-bound protein, given that the same degree of activity was observed for the apoenzyme.

A most enlightening result was obtained by incubating the 5-deazaFAD ADPS with the substrate. The addition of palmitoylDHAP led to slow bleaching of the longer-wavelength absorption band in the absorbance spectrum (Fig. 4B), and, most importantly, the spectral changes lasted for several hours. In contrast, as soon as the protein was unfolded, the released 5-deazaFAD acquired the standard spectrum of the oxidized state, a process that cannot be a simple oxygen-mediated reoxidation, because oxygen does not react with this flavin analog (17). Thus, 5-deazaFAD reacts with the substrate to form a complex that is very stable as long as it remains protein-bound (*SI Methods*). Moreover, the observed spectral changes are fully consistent with those exhibited on formation of covalent adducts between carbon 5 of protein-bound 5-deazaflavins and nucleophilic reagents (17, 19) (Fig. 4B).

We further characterized this complex by native MS, which is a technically demanding analysis because the reconstituted enzyme preparations (although mainly dimeric and cofactor-bound) contain molecules that are monomeric and/or in the apo form. These heterogeneities cause broadening of the peaks in the acquired mass spectra, hampering accurate mass assignments. We repeated the analysis several times using two different protein preparations. The observed mass shifts (800 ± 50 Da for the dimeric enzyme) consistently indicated that an entire palmitoylDHAP molecule is bound to each protein chain (Fig. 4C). The fundamental conclusion that can be drawn from these MS experiments is that, consistent with the absorbance spectroscopy results, 5-deazaflavin forms a tight, most likely covalent, complex with the substrate to the extent that the enzymatic reaction cannot proceed any further, in line with the observed lack of activity with 5-deazaFAD.

Discussion

Taken together, our structural and biochemical data indicate that at the heart of the ADPS reaction lies the formation of a covalent adduct with the substrate to enable acyl–alkyl exchange (Fig. 1). The reaction is predicted to start with formation and/or preferential binding of the enolate form of the substrate, which can subsequently attack the flavin. A key finding supporting this model is that the pro R proton (or hydrogen) at the C1 carbon of the DHAP moiety exchanges stereospecifically with solvent during the reaction (6, 7). Tyr578 is the residue most likely responsible for the acid/base catalysis underlying substrate tautomerization. Indeed, Tyr578Phe is inactive, and incubation with the substrate does not lead to flavin bleaching, implying that the substrate is unable to react with the flavin bound to the mutant. The His residues of the HHH loop are ideally located to stabilize the negatively charged oxygen of the enolic substrate (Fig. 2B). Consistent with this, mutations of each of the three loop histidines (His615, His616, and His617) to Ala have been shown to completely abolish activity (13).

Formation of the enolate form of DHAP allows the key step of the reaction: the nucleophilic attack of C1 of DHAP onto the flavin. This step is followed by the release of the fatty acid product to form the covalent complex between hydroxyacetonephosphate and the flavin (Fig. 1). Much data support the formation of this crucial covalent intermediate. First, the intermediate exhibits spectral features fully compatible with those of a flavin covalent

adduct (Fig. 4A). Second, the fatty acid product retains both carboxyl ester oxygens on cleavage from DHAP (20–22). Third, decay of the intermediate releases DHAP through an hydrolytic process that does not require oxygen or other electron acceptors. Fourth, the ADPS-bound flavin is capable of reacting with the nucleophilic nitroalkanes to form a covalent adduct on the N5 position, which can be accommodated in the active site without alterations in flavin conformation and environment (Fig. S4C). Fifth, protein-bound 5-deazaFAD is able to react with the substrate, consistent with the known reactivity of this flavin analog toward nucleophilic agents. In this case, however, the presence of a CH group instead of a nitrogen in position 5 of the flavin prevents elimination of the fatty acid and continuation of the reaction (Fig. 4C). Finally, two structural features underlying this mechanism are of special interest (Figs. 3 and 5). The binding site for the DHAP phosphate enables proper “in-phase” binding of the DHAP C1 atom with respect to the flavin, and the fine geometry of the V-shaped active site tunnel positions a constriction in direct contact with flavin N5, where the substrate–flavin covalent bond must form.

The fatty alcohol binds in the hydrophobic arm of the active site in place of the exiting fatty acid, which is consistent with a ping-pong mechanism (7). The hydroxyacetonephosphate–flavin intermediate does not react with either borohydride or cyanide, indicating that the enzyme stabilizes mainly the ketonic and/or enolic form of the compound rather than the imine (Fig. 1). Thus, the alcohol substrate, possibly after deprotonation by Tyr578, can react with the intermediate by simply substituting for the flavin to generate the ether-containing product. By analogy, the hydroxyacetonephosphate–flavin intermediate can be expected to react with water to release DHAP. This reaction occurs rapidly on

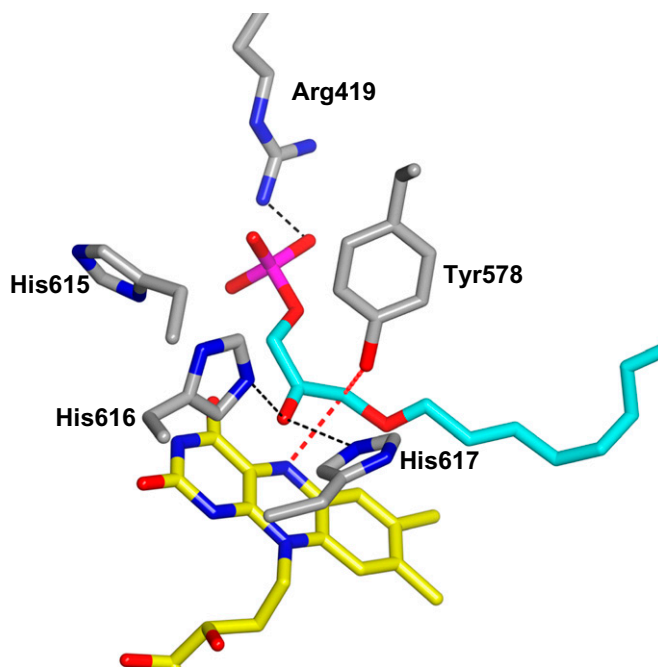


Fig. 5. Substrate binding to ADPS. To aid data analysis, a 3D model for the bound alkylDHAP product was generated based on the prediction that the alkyl chain binds in the long arm of the catalytic tunnel, whereas the phosphate group binds in the other arm, establishing a direct electrostatic interaction with the essential Arg419. We found that modeling of the alkylDHAP product in its *cis* enolate form (5, 20) fits very well in the active site and requires only a small shift of Tyr578, which is relatively flexible in the crystal structures. Possible H-bond interactions are depicted as black dashed lines. The red lines indicate contacts of the substrate C1 atom with Tyr578 and flavin N5. Atoms are colored as in Fig. 3.

solvent exposure (e.g., protein unfolding) or more slowly in the active site, which thus is able to protect the intermediate from unwanted reactions with the solvent.

This mechanism is most unusual for a flavoenzyme. A well-known example of covalent flavoenzyme catalysis is that of UDP-galactopyranose mutase, the reaction of which, however, requires a reduced flavin, as opposed to the catalytically competent oxidized flavin of ADPS (23). Covalent trapping of a substrate group through an imine intermediate is a catalytic “strategy” that seems rather more typical for pyridoxal phosphate-dependent proteins. Numerous flavoenzymes are known to react covalently with mechanism-based inhibitors, often through poorly understood reactions. Importantly, covalent flavin adducts are not necessarily irreversible. Covalent but reversible inhibitor formation has been reported for monoamine oxidases (24), and reversible adducts with nucleophilic reagents have been documented for enzyme-bound 5-deazaflavins (17, 19). A fascinating property of ADPS is its ability to turn this potential for covalent reactivity into a tool for catalysis. The subtle distinction between redox and covalent reactivity is illustrated by the observation that the flavin-binding site of ADPS shares many features with these sites of flavoenzyme oxidases and dehydrogenases of the vanillyl-alcohol oxidase structural family, of which ADPS is a member (25). Among the common features (Fig. S24) are a Asp–Pro pair (Pro202–Asp203) in contact with the N5 atom of the cofactor and conserved H-bonding interactions between the flavin and protein backbone atoms (Ser319). These H bonds might help stabilize the predicted negatively charged flavin of the covalent intermediate formed by reaction with the substrate (Fig. 1 and Fig. S24).

Another related observation is the recurrent presence in alcohol dehydrogenases of a His residue located with respect to

the flavin as His617 of ADPS (26) (Fig. S5). In the dehydrogenases, the His side chain is crucial for catalysis by abstracting a proton from the substrate hydroxyl group to promote substrate dehydrogenation through hydride transfer to the cofactor. In contrast, the histidine of ADPS stabilizes the enolic form of DHAP to promote formation of a covalent intermediate with the flavin. Apparently, no drastic alterations in the geometry of the flavin site are needed to implement an unusual covalent catalysis starting from a widespread “redox” flavoenzyme scaffold.

Methods

Protein expression, purification, and crystallization, as well as crystal structure analysis, were performed following standard protocols (27), as described in *SI Methods*. X-ray data were collected at the European Synchrotron Radiation Facility in Grenoble, France and the Swiss Light Source in Villigen, Switzerland. Enzyme activities were assayed as described elsewhere (14, 28). Reconstitution with 5-deazaFAD took advantage of the relatively weak binding of the protein to FAD, as described in *SI Methods*. UV-Vis absorbance spectra were recorded with either an Agilent HP8453 diode array or a Varian Cary 100 spectrophotometer using a 100- μ L cuvette with a path length of 1 cm. Native MS experiments with 5-dezaFAD-reconstituted ADPS were performed on a modified Waters Q-ToF 2 mass spectrometer. Buffer exchanges into 150 mM ammonium acetate (pH 7.5) were performed using 30-kDa molecular weight cutoff spin-filter columns (Vivaspin500; Sartorius Stedim Biotech). Samples were sprayed at a concentration of 10 μ M using gold-coated borosilicate capillaries created in house using a Sutter P-97 puller and an Edwards Scancoat six sputter coater. MassLynx V4.1 (Waters) was used for experimental mass determination.

ACKNOWLEDGMENTS. This study was supported by Telethon Grant GGP12007. A visiting professorship was supported by Fondazione Cariplo Grant 2008.3148 (to D.E.E.).

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