Structure and Reaction Mechanism of Basil Eugenol Synthase

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Phenylpropenes, a large group of plant volatile compounds that serve in multiple roles in defense and pollinator attraction, contain a propenyl side chain. Eugenol synthase (EGS) catalyzes the reductive displacement of acetate from the propenyl side chain of the substrate coniferyl acetate to produce the allyl-phenylpropene eugenol. We report here the structure determination of EGS from basil (Ocimum basilicum) by protein x-ray crystallography. EGS is structurally related to the short-chain dehydrogenase/reductases (SDRs), and in particular, enzymes in the isoflavone-reductase-like subfamily. The structure of a ternary complex of EGS bound to the cofactor NADPH and a mixed competitive inhibitor EMDF (1,7,8-triethyl-7,8-methylene)-dihydroferulate) provides a detailed view of the binding interactions within the EGS active site and a starting point for mutagenic examination of the unusual reductive mechanism of EGS. The key interactions between EMDF and the EGS-holoenzyme include stacking of the phenyl ring of EMDF against the cofactor’s nicotinamide ring and a water-mediated hydrogen-bonding interaction between the EMDF 4-hydroxy group and the side-chain amino moiety of a conserved lysine residue, Lys132. The C4 carbon of nicotinamide resides immediately adjacent to the site of hydride addition, the C7 carbon of cinnamyl acetate substrates. The inhibitor-bound EGS structure suggests a two-step reaction mechanism involving the formation of a quinone-methide prior to reduction. The formation of this intermediate is promoted by a hydrogen-bonding network that favors deprotonation of the substrate’s 4-hydroxyl group and disfavors binding of the acetate moiety, akin to a push-pull catalytic mechanism. Notably, the catalytic involvement in EGS of the conserved Lys132 in preparing the phenolic substrate for quinone methide formation through the proton-relay network appears to be an adaptation of the analogous role in hydrogen bonding played by the equivalent lysine residue in other enzymes of the SDR family.

INTRODUCTION

The phenylpropenes are a diverse group of plant secondary metabolites characterized by a phenyl ring bearing a propenyl side chain (Figure 1A). A variety of phenylpropenes occur in angiosperms, whereas a more limited subset of these compounds exist in gymnosperms. In plants, the phenylpropenes function in defense and interspecies communication. Because some phenylpropenes are toxic to animals and microorganisms, these compounds are typically produced and stored in plant vegetative tissues to act as deterrents against herbivores and microbial pathogens [1]. Moreover, some volatile phenylpropenes are emitted by flowering plants and serve as attractants for insect pollinators [2]. Historically, humans have exploited both the aromatic and toxic properties of the phenylpropenes in perfumes, flavorings, preservatives, and general antiseptics.

The phenylpropenes are derived from coumaryl, coniferyl, and sinapyl alcohol, which are also intermediates in the lignin and lignan biosynthetic pathways. As precursors for phenylpropene production, the monolignol alcohols undergo first acetylation of the C9 hydroxyl group [3] and then reductive cleavage of the acetate moiety to yield the propenyl side group [4,5]. This reduction reaction is catalyzed by enzymes that produce an allyl propene (with the double bond between C9 and C8) or an “iso” propene (with the double bond between C8 and C7). An example of the former is basil (Ocimum basilicum) eugenol synthase (EGS), which converts coniferyl acetate to eugenol, and an example of the latter is petunia (Petunia hybrida) isoegenol synthase (IGS), which converts coniferyl acetate to isoegenol (Figure 1). Further modifications required for formation of the known natural phenylpropenes include additional hydroxylation of the benzene ring, methylation of any of the hydroxyl groups on the benzene ring, and formation of a methylenedioxy bridge (Figure 1). Some of these modifications may occur before the formation of the propenyl moiety, but a free hydroxyl group at the para position appears to be a requirement for the reduction reaction [4]. The biosynthetic routes to the phenylpropenes generate considerable chemical diversity.

The basil EGS and petunia IGS are closely related to a number of other NADPH-dependent enzymes that act on phenylpropenoid-derived substrates. These enzymes constitute the PIP family, named after the three initially identified members, pinosylvinolliciresinol reductase (PLR) [6], isoflavone reductase (IFR) [7], and phenylcoumaran benzylic ether reductase (PCBER) [6]. Other enzymes in this family are leucoycinidin reductase [8],...
and pterocarpan reductase [9]. Notably, several PIP enzymes in addition to EGS and IGS catalyze the reductive cleavage of a carbon-oxygen bond that occurs in a phenyl-ring substituent positioned para to the 4-hydroxyl group. The PIP-enzyme catalyzed reductions all involve A-type stereospecificity of hydride transfer from the NAD(P)H cofactor (donation of the nicotinamide C4 pro-R hydride). However, the reaction mechanism of the PIP enzymes remains to be fully characterized. In particular, the cleavage of a carbon-oxygen bond represents an unusual application of a nicotinamide-cofactor dependent reduction, which more typically adds a hydride anion and proton across a double bond in the substrate. The involvement of a quinone-methide (conjugated enone) intermediate in the bond cleavage [4,9–11] is frequently assumed, although solid experimental support has not been reported. Therefore, a direct reductive displacement [4] of the oxygen function by hydride ion cannot be excluded.

We describe here the crystal structure of basil EGS in apo and holo forms, and also as a ternary complex with the cofactor NADP(H) and a designed inhibitor, (7S,8S)-ethyl (7,8-methylene)dihydroferulate. Previous crystallographic studies of PIP-family enzymes yielded only apo-structures [6,7], and thus, reliable pictures of cofactor and substrate binding and catalytic mechanism were significantly hampered. Our EGS structures now clearly reveal the interactions formed by the substrate within the enzyme’s active site and identify possible catalytic residues. These studies, together with the analysis of the activity of the protein following in vitro mutagenesis of specific residues, provide substantial support for a reaction mechanism involving a quinone-methide intermediate and the participation of a key lysine residue in an unusual push-pull like two-step catalytic mechanism.

RESULTS AND DISCUSSION
Crystallographic structure elucidation for basil EGS complexed with NADP+
A structure solution was obtained initially for the orthorhombic crystal form of holo-EGS, which contains two EGS/NADP(H) complexes per asymmetric unit. A three-dimensional model was refined at 1.7-Å resolution resulting in crystallographic R-factors of 0.244 and 0.267 (working and FreeR, respectively; see Table 1). This refined structure then served as the search model for MR analysis of the monoclinic crystal form. The monoclinic structure also contains two EGS/NADP(H) complexes per asymmetric unit and was refined at 1.6-Å resolution resulting in crystallographic R-factors of 0.210 and 0.229 (Table 1). For each of the other EGS crystal forms, MR solutions were obtained with either the monoclinic or orthorhombic crystal structure of EGS serving as the search model. A 2-fold rotationally symmetric homodimer is consistently observed as the asymmetric unit in all crystal forms analyzed and solved to date. However, the inter-monomer association within the dimer is not extensive, and in agreement with elution behavior on gel-exclusion chromatography, monomeric EGS is likely the functionally relevant form. In all cases, the entire polypeptide chain of EGS is visible in electron-density maps, except for four residues at the N-terminus, and additionally the NADP(H) cofactor is extremely well ordered.

Overall structure of basil EGS and structure comparisons with other IFR-like proteins
EGS is very similar in polypeptide-chain fold to three other PIP-family proteins that have been structurally characterized, pinocembrin-licoricresinol reductase (PLR) [6], isoflavone reductase (IFR) [7], and pinor-esinol-lariciresinol reductase [9]. Notably, several PIP enzymes in addition to EGS and IGS catalyze the reductive cleavage of a carbon-oxygen bond that occurs in a phenyl-ring substituent positioned para to the 4-hydroxyl group. The PIP-enzyme catalyzed reductions all involve A-type stereospecificity of hydride transfer from the NAD(P)H cofactor (donation of the nicotinamide C4 pro-R hydride). However, the reaction mechanism of the PIP enzymes remains to be fully characterized. In particular, the cleavage of a carbon-oxygen bond represents an unusual application of a nicotinamide-cofactor dependent reduction, which more typically adds a hydride anion and proton across a double bond in the substrate. The involvement of a quinone-methide (conjugated enone) intermediate in the bond cleavage [4,9–11] is frequently assumed, although solid experimental support has not been reported. Therefore, a direct reductive displacement [4] of the oxygen function by hydride ion cannot be excluded.

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Structurally, the PIP-family proteins organize around an N-terminal, Rossman-fold domain, containing a core, six-stranded parallel β-sheet flanked on each face by an α-helical layer (Figure 2A). One edge of the core β-sheet provides the extended binding surface for the NADP⁺ cofactor (as discussed further below). The C-terminal polypeptide-chain segment of the PIP-family proteins forms a predominantly α-helical domain, and this C-terminal segment also contributes three additional β-strands.

Table 1. Summary of data collection and refinement statistics for EGS structures

|                     | EGS-NADP⁺ monoclinic | EGS-NADP⁺ orthorhombic | EGS-NADPH | Apo-EGS† | EGS-NADP⁺-EMDF | EGS(Lys132Gln)-EMDF |
|---------------------|-----------------------|-------------------------|-----------|----------|-----------------|---------------------|
| Space group         | P2₁                   | P2₁/2;2;1               | P2₁       | P2₁/2;2;1 | P2₁             | P2₁                 |
| Unit-cell parameters (Å) | 53.8                  | 79.3                    | 54.3      | 79.4     | 54.0            | 53.8               |
| a (Å)               | 85.9                  | 85.9                    | 86.0      | 86.3     | 85.4            | 86.2               |
| b (Å)               | 76.2                  | 99.2                    | 76.4      | 98.2     | 76.9            | 76.8               |
| c (Å)               | 107.3                 | 90                      | 107.7     | 90       | 107.5           | 107.6              |
| β (°)               |                       |                         |           |          |                 |                    |
| Monomers per asymmetric unit | 2                     | 2                       | 2         | 2        | 2               | 2                  |
| Resolution range (Å) | 43-1.60 (1.78-1.60)    | 100-1.72 (1.84-1.72)    | 34-1.60   | 1.69-1.60| 43-1.80 (1.88-1.80) | 34-1.60 (1.69-1.60) | 44-1.80 (1.90-1.80) |
| Number of reflections measured | 316974                | 492798                  | 268704    | 443874   | 354814          | 167942              |
| Merging R-factor*   | 0.083 (0.676)         | 0.110 (0.663)           | 0.105 (0.286) | 0.086 (0.477) | 0.129 (0.443) | 0.109 (0.341) |
| Mean (I/σ(I))*     | 9.9 (2.4)             | 10.0 (2.9)              | 8.9 (1.9) | 23.0 (2.5) | 8.3 (1.6)       | 7.9 (1.6)          |
| Completeness*       | 0.972 (0.959)         | 0.972 (0.961)           | 0.912 (0.592) | 0.956 (0.732) | 0.975 (0.950) | 0.921 (0.826) |
| Redundancy*         | 3.74 (3.74)           | 6.73 (5.07)             | 3.3 (2.2) | 7.4 (5.1) | 4.1 (2.4)       | 2.9 (2.2)         |
| Number of reflections used | 84786                | 73101                   | 80436     | 60262   | 85858           | 57422              |
| R-factor*           | 0.205 (0.322)         | 0.229 (0.361)           | 0.201 (0.277) | 0.224 (0.327) | 0.259 (0.318) | 0.248 (0.312) |
| Free R-factor*      | 0.226 (0.329)         | 0.256 (0.411)           | 0.221 (0.306) | 0.242 (0.341) | 0.286 (0.328) | 0.280 (0.361) |
| Number of amino-acid residues | 620                   | 620                     | 620       | 620     | 620             | 620                |
| Number of water molecules | 472                  | 547                     | 674       | 388     | 374             | 350                |
| Residues with most favorable conformation (%) | 93.0 | 93.2 | 93.2 | 92.1 | 93.6 | 92.7 |
| PDB entry           | 2QW8                  | 2QX7                    | 2R6J      | 2QYS    | 2QZZ            | 2R2G               |

* Merging R-factor = \( \sum_{i} \sum_{h} |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{i} \sum_{h} I_i(hkl) \)

Values in parentheses describe the highest resolution shell.

† In the apo-EGS crystal structure, a small amount of nicotinamide cofactor is likely present (less than 20% occupancy, as judged from residual electron density at the expected NADP(H) binding site).

[7], and PCBER [6]. EGS is also shown (the structures of the other PIP-family enzymes were determined in the absence of cofactor).

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Figure 2. Structure of EGS and comparison with other PIP-family enzymes. (A) Orthogonal views of EGS/NADP⁺ monomer. The polypeptide chain of EGS is represented as a ribbon, with coloring varying from blue for the N-terminus to red for the C-terminus. The atoms of the NADP⁺ cofactor are drawn as balls and sticks, and are colored coded according to element (carbon: gray; nitrogen: blue; oxygen: red; phosphorus: orange). (B) Superposition of the polypeptide-chain backbones of EGS and other PIP-family enzymes (color coding as shown in inset). The NADP⁺ cofactor of EGS is also shown (the structures of the other PIP-family enzymes were determined in the absence of cofactor).

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to the Rossman-fold domain. The C-terminal domain is presumed (see below) to function in substrate binding. Indeed, this domain together with the last α-helix of the Rossman-fold domain surround a cavity located immediately adjacent to the nicotinamide ring of the NADP⁺ cofactor. Within the IFR-like PIP family, the substrate-binding domains (residues 154–314 in EGS) appear more structurally divergent than the nicotinamide- cofactor binding domains (residues 1–153 in EGS). For example, from comparisons of polypeptide-chain backbones (Figure 2B), EGS differs from PCBER by 1.40 Å (rmsd) overall, but by only 0.83 Å for the Rossman-fold domain alone; and similarly, EGS differs from IFR by 1.63 Å overall and 1.09 Å for the Rossman-fold domain alone. Curiously, PCBER, PLR, and IFR-like EGS-all form 2-fold rotationally symmetric homodimers, but the various homodimeric associations are distinct in each case. An additional structural element unique to EGS (absent in the other PIP-family proteins) is a proline-rich extension at the C-terminus. This tail segment passes across the mouth of the active-site region, and the side chain of the C-terminal phenylalanine residue participates directly in forming the substrate-binding pocket. The positioning of the tail segment in EGS precludes the formation of the homodimeric associations observed in PCBER and IFR.

Binding of the NADP(H) cofactor to EGS

The structures of EGS complexed with NADP⁺ or NADPH provide the first structural characterization of nicotinamide-cofactor binding by the PIP family of enzymes (previous crystallographic analyses had yielded only apo-enzyme structures). The cofactor is bound through a large number of polar and non-polar interactions (Figure 3). The adenine ring adopts the anti conformation and is sandwiched between the ε-guanido group of Arg39 and the carboxamide group of Gln87. The adenine-ribose conformation and is sandwiched between the C3'-endo conformation, and its hydroxyl groups are involved in hydrogen bonds with residues 111's carbonyl oxygen, Ser110's side chain hydroxyl moiety, and the side-chain amino group of Lys132. The nicotinamide ring adopts the anti conformation with its B-face stacked against the side chain of Phe154 and its A-face directed towards the substrate-binding pocket. The nicotinamide-nitrogens of residues 18 and 19. The protein residues directed towards the substrate-binding pocket. The positioning of the tail segment in EGS precludes the formation of the homodimeric associations observed in PCBER and IFR.

Structural comparison of EGS with UDP-galactose 4-epimerase

The PIP-family enzymes belong to a larger superfamily of NAD(P)-dependent dehydrogenases, the short-chain dehydrogenases/reductases (SDRs) [13]. The most similar member of the larger superfamily is UDP-galactose 4-epimerase [14] (PDB entry 1KVQ), which provided a template for the binding modes of both the nicotinamide cofactor and substrate in the earlier structural analyses of the apo-forms of IFR, PCBER and PLR [6,7]. Indeed, UDP-galactose 4-epimerase possesses a C-terminal domain that is similar topologically to the C-terminal domains of the PIP-family proteins (Figure 4A). In the UDP-galactose 4-epimerase crystal structure, a cavity within the C-terminal domain is positioned next to the nicotinamide ring of the NAD⁺ cofactor and is occupied by the substrate, UDP galactose. The corresponding cavity in EGS is much smaller in volume and the side chains lining the cavity are more non-polar in character. These properties of the EGS substrate-binding pocket are consistent with the smaller size and greater hydrophobicity of the EGS substrate, the acetate ester of coniferyl alcohol. Notably, the conformation of NAD⁺ bound to UDP-galactose 4-epimerase differs markedly from that of NADP⁺ bound to EGS, particularly in the conformation of the nicotinamide ring (Figure 4B). In the UDP-galactose 4-epimerase/NAD⁺ complex, the nicotinamide ring adopts the syn conformation, consistent with the class-B oxidoreductase activity of the enzyme. In contrast, the anti-conformer of the nicotinamide ring observed in EGS is consistent with the class-A reductase (donation of the pro-R hydride) activity of the PIP-family enzymes.

The orientation of the nicotinamide ring in EGS appears to be influenced largely by interactions of the carboxamide group with the polypeptide-chain backbone; an observation also made previously for the SDRs [15]. Also, EGS possesses an additional loop (residues 38–42) that forms a binding pocket for the 2'-phosphate group of NAD(P)H. This loop is absent in UDP-galactose 4-epimerase, but occurs with variable length in all PIP-family proteins.

Binding of the EGS-inhibitor EMDF

The co-crystal structure of EGS complexed with a specifically designed inhibitor, EMDF ((7S,8S)-ethyl (7,8-methylene)-dihydro-
and UDP-glucose bound to UDP-galactose-4-epimerase. The carbon atoms of the nicotinamide cofactors, EMDF bound to EGS, and 4-bromo-cinnamyl acetate (unpublished data), which lacks a substituent at the 3-position. In addition, the acetate ester of sinapyl alcohol, which bears hydroxymethyl groups at both the 3- and 5-positions, would be expected to be incapable of binding to EGS. The inhibitor’s C1 substituent bearing the cyclopropyl and ethyl-ester moieties projects into a cavity formed by the C-terminal domain of the protein. This cavity is capped by the side chains of Tyr157, Tyr161, Pro258, Leu262 and Phe314. With the exception of the C-terminal Phe314, these capping residues form a relatively rigid cage, as indicated by their invariant positioning in all EGS crystal structures and low crystallographic temperature factors. The capping region appears to lack sufficient volume to accommodate a C1 substituent larger than an acetate-esterified propenol. This is consistent with the limited ability of EGS to utilize as substrate other esters of coniferyl alcohol, for example coniferyl coumarate [4], which bears a much bulkier substituent.

Curiously, at the inhibitor-binding site described above, some residual electron density is invariably observed, even with crystal samples prepared with EGS protein that had not been purposely exposed to a potential ligand. This density might be due to low-occupancy binding of a small, eugenol-resembling compound that originated from bacterial growth-media derived from yeast extracts. However, soaking experiments of EGS crystals with extracts. However, soaking experiments of EGS crystals with the 3-hydroxymethyl group within its binding pocket is perhaps limited ability of EGS to utilize as substrate coumaryl acetate (unpublished data), which lacks a substituent at the 3-position. In addition, the acetate ester of sinapyl alcohol, which bears hydroxymethyl groups at both the 3- and 5-positions, would be expected to be incapable of binding to EGS. The inhibitor’s C1 substituent bearing the cyclopropyl and ethyl-ester moieties projects into a cavity formed by the C-terminal domain of the protein. This cavity is capped by the side chains of Tyr157, Tyr161, Pro258, Leu262 and Phe314. With the exception of the C-terminal Phe314, these capping residues form a relatively rigid cage, as indicated by their invariant positioning in all EGS crystal structures and low crystallographic temperature factors. The capping region appears to lack sufficient volume to accommodate a C1 substituent larger than an acetate-esterified propenol. This is consistent with the limited ability of EGS to utilize other esters of coniferyl alcohol, for example coniferyl coumarate [4], which bears a much bulkier substituent.

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Figure 5. Binding of the competitive inhibitor EMDF by EGS. (A) Orthogonal views of the 7S,8S- stereoisomer of the competitive inhibitor EMDF bound to EGS. Hydrogen-bond interactions formed by the EMDF molecule (cyan colored carbons) are represented as magenta dashed lines. The blue-colored contours envelope regions greater than 2.5σ in the initial F_{obs} - F_{calc} electron-density map. The direction of view used in the right panel (approximately perpendicular to the plane of the nicotinamide ring) is maintained roughly in figures 5B–D and 6B. The chemical structure of EMDF is shown in the inset. (B) Modeled binding of coniferyl acetate to EGS. The atom coloring is the same as in (A), with magenta carbon atoms for the coniferyl acetate. The chemical structures of coniferyl acetate and EMDF are compared in the inset. The close interaction between the EMDF C7-atom and the hydride donor of the nicotinamide (C4) is shown as a yellow dashed line. (C) Binding of EMDF to the Lys132Gln variant of EGS. Hydrogen-bond interactions formed by the EMDF molecule (cyan colored carbons) are represented as magenta dashed lines. Hydrogen bonds involving the side chain of Gln132 are shown as orange dashed lines. The blue-colored contours envelope regions greater than 2.0σ in the initial F_{obs} - F_{calc} electron-density map. (D) Binding of EMDF to the Lys132Arg variant of EGS (stereo representation). The blue-colored contours envelope regions greater than 2.0σ in the initial F_{obs} - F_{calc} electron-density map for the EGS-Arg132/EMDF complex (green). The altered positioning of the Arg132 side-chain and neighboring residues (most notably Phe85, Ile88, and Ile129) and the disordering of the C-terminal tail (residues 310–314) are apparent with respect to the holo-EGS-Arg132 structure (magenta). For comparison, the position of the wild-type Lys132 side chain and the key bridging water molecule shown in Figure 5A are also shown (yellow).

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in the EMDF preparation) was excluded, due to poorer steric complementarity with the EGS active site.

### 3D structure determination and in vitro mutagenesis suggests a reaction mechanism for EGS

Although the binding of EMDF exploits shape features of the EGS active site that are inaccessible to the coniferyl acetate substrate, the structure of the EGS-NADPH-EMDF complex nevertheless provides a useful framework for probing the EGS enzymatic mechanism. Together with the observed effects on catalytic activity of specific amino-acid replacements (Table 2), as described below, the structure provides compelling support for the involvement of a quinone-methide intermediate both in promoting carbon-oxygen bond cleavage of the acetate moiety and in serving as the actual substrate of the reduction reaction via NADPH-mediated hydride transfer.

A prominent active-site residue is Lys132, which occurs in all PIP-family enzymes as well as most SDRs [17]. The structure of the EGS-NADPH-EMDF complex shows that the ε-amino group of Lys132 forms interactions with both the nicotinamide-ribose of NADPH, and potentially, the substrate molecule (Figure 5A). The Lys132 interaction with the substrate is particularly intriguing, as it is not proximal to the site of hydride addition (as suggested in the case of IFR [7]), but instead involves the p-hydroxyl group via a bridging water molecule. Notably, a p-hydroxyl group is a distinguishing feature of the substrates of all PIP-family enzymes, and is a requirement for reduction by EGS [4]. For both PLR and IFR, alanine replacements of the lysine that is equivalent to EGS-Lys132 abolish enzyme activity [6,7]. In EGS, Lys132Ala and Lys132Gln mutants are completely inactive, whereas the Lys132Arg mutant retains partial (71%) activity (Table 2). Crystallographic analyses confirmed that for the Ala132 and Gln132 mutants, both NADPH and EMDF binding are little affected (Figure 5C), despite the loss of the binding interactions normally contributed by Lys132. These results therefore point to a catalytic role for Lys132. In particular, the involvement of a catalytic group acting at the p-hydroxyl group clearly argues for the formation of a quinone-methide as a reaction intermediate as opposed to direct nucleophile replacement by a NADPH derived hydride anion.

The formation of a quinone methide can be promoted by abstraction of the proton from the p-hydroxyl group of substrate.

### Table 2. Relative activity of EGS variants

| EGS variant     | Relative activity* (%) |
|-----------------|------------------------|
| Wild type       | 100                    |
| Lys132Ala       | 0                      |
| Lys132Gln       | 0                      |
| Lys132Arg       | 71 (+6)                |
| Tyr157Phe       | 32.5 (+4.4)            |
| Tyr157Ala       | 21 (+3)                |
| Ile261His       | 3.1 (+0.2)             |
| Phe314Tyr       | 112 (+8)               |
| Phe314Ala       | 54 (+5)                |
| Phe314Tyr-Ala-Gln-Pro-Ser-Thr | 115 (+11) |
| I311–314        | 34.9 (+0.7)            |

*The standard error of each activity measurement (derived from duplicate or triplicate determinations) is given in parentheses.

Detailed inspection of the hydrogen-bonding network involving the ε-amino group of Lys132 (Figure 6A) suggests that this group exists formally in the unprotonated–NH₂ state and is the donor in hydrogen-bond interactions with the 2'-hydroxyl group of the nicotinamide-ribose and the backbone carbonyl oxygen of residue 110. With an available lone pair of electrons, the amino group can serve as the acceptor in a hydrogen bond with the bridging water molecule, and most importantly, thereby act as a general base. The water molecule (as a hydroxide ion) can in turn facilitate deprotonation of the substrate’s p-hydroxyl group. Intriguingly, in the monoclinic structure of unliganded EGS-NADPH [8], a nitrate anion from the crystallization medium occupies the site of the bridging water molecule, and may mimic the hydroxide ion that develops during the catalytic reaction.

The loss in activity of EGS Lys132-mutants can be interpreted in terms of the proposed mechanistic model, in conjunction with results from structural analyses. In the EGS (Lys132Gln)-NADPH-EMDF complex, the Gln132 side chain retains an interaction with the nicotinamide ribose (also through an intervening water molecule), but is unable to form a direct or water-mediated interaction with the p-hydroxyl group of EGS. Likewise, Ala132 obviously lacks a catalytic group capable of promoting deprotonation of the substrate’s p-hydroxyl group, and therefore, the observed inactivity of the Lys132Ala and Lys132Gln mutants can be readily explained. The Lys132Arg mutant is partially active, and in this case, with the higher pKₐ and greater length of the Arg side chain, the positively charged guanidinium moiety could possibly participate directly (i.e. without the requirement for an intervening water molecule) in lowering the pKₐ of the substrate’s p-hydroxyl group. Interestingly, preliminary structural analysis of the holo and EMDF-bound forms of the Lys132Arg mutant shows that the Arg132 guanidinium moiety is displaced slightly by the EMDF guaiacol ring (Figure 5D), thus diminishing the potential influence of the Arg132 on catalysis.

One difficulty with the proposed catalytic role for a lysine residue is the relatively high pKₐ (normally ~10.4 in solution) of the side-chain amino group, which would disfavor acquisition of the initial free-base state required for proton abstraction from the substrate. However, the pKₐs of ionizable groups in proteins can be greatly influenced by the local structural environment, in particular, involvement in hydrogen-bonding networks and hydrophobic interactions. Such factors have been suggested to account for the catalytic-base activity of the lysine ε-amino group in a number of enzymes. A notable example is isochorismate synthase [18], in which a catalytic lysine is proposed to deprotonate and thereby activate a nucleophilic water molecule. Furthermore, from theoretical calculations on the conserved Lys-Tyr-Ser catalytic triad in an SDR-type alcohol dehydrogenase, the catalytically important lysine residue is suggested to exist in a partially unprotonated state, and in this state, participate in a proton-relay network that involves hydroxyl groups on the catalytic tyrosine and nicotinamide ribose [19]. This network functions ultimately to abstract a proton from the alcohol substrate. Intriguingly, although EGS lacks the catalytic tyrosine of the SDR enzyme, Lys132 in EGS corresponds exactly to the catalytic lysine of the SDRs, and the p-hydroxyl group of EGS-bound EMDF occurs at roughly the same position as the Tyr ε-hydroxyl group of the catalytic tyrosine of SDRs (Figure 6B).

For the EGS-catalyzed reaction with the coniferyl acetate substrate, formation of the quinone-methide intermediate would be concomitant with displacement of an acetate ion (Figure 6C). In concert with proton abstraction from the p-hydroxyl group, EGS may therefore exploit an additional driving force for generation of the reaction intermediate—promoting the loss of the acetate. In
Determinants of the regioselectivity of the EGS-catalyzed reduction reaction

In the structure of the EGS-NADP$^+$-EMDF complex, the C4 atom of the cofactor’s nicotinamide ring, which serves as the donor of the p-hydroxy group (PUSH) coupled with expulsion of acetate ion from the C1 substituent (PULL). The result of the extended quinone methide intermediate serves as a hydride acceptor at C7 to yield the product eugenol. For NAD(P)H-mediated reduction reactions, the acceptance of hydride by a substrate is typically accompanied by the acquisition of a proton at an adjacent atom, to maintain charge neutrality. As discussed above, EGS lacks an appropriately positioned proton donor near the site of hydride addition (C7); protonation instead occurs at the p-hydroxyl group, in concert with rearrangement of the double-bond system of the quinone methide and deprotonation of the phenyl ring. Furthermore, the reduction of a double bond within a quinone methide intermediate more closely resembles a typical reaction catalyzed by a nicotinamide cofactor enzyme, and indeed the reaction catalyzed by the PIP-family member IFR [10].

In summary, a mechanistic scheme emerges in which binding of the coniferyl acetate substrate within the active site of EGS leads to deprotonation of the p-hydroxy group coupled with expulsion of acetate ion from the C1 substituent. The resultant extended quinone methide intermediate serves as a hydride acceptor at C7 to yield the product eugenol. For NAD(P)H-mediated reduction reactions, the acceptance of hydride by a substrate is typically accompanied by the acquisition of a proton at an adjacent atom, to maintain charge neutrality. As discussed above, EGS lacks an appropriately positioned proton donor near the site of hydride addition (C7); protonation instead occurs at the p-hydroxyl group, in concert with rearrangement of the double-bond system of the quinone methide and deprotonation of the phenyl ring. Furthermore, the reduction of a double bond within a quinone methide intermediate more closely resembles a typical reaction catalyzed by a nicotinamide cofactor enzyme, and indeed the reaction catalyzed by the PIP-family member IFR [10].

**Figure 6. Hydrogen-bonding interactions in the EGS active-site and proposed reaction mechanism of EGS.** (A) Hydrogen-bonding network involving the Lys132 side-chain amino group, the 4-hydroxyl group of EMDF, and the bridging water molecule. Inferred hydrogen-atom positions are shown in blue. Hydrogen-bond interactions are represented as magenta dashed lines. (B) Comparison of the hydrogen-bond interactions made by the catalytic lysine residue in EGS (Lys132) and the SDR UDP-galactose-4-epimerase (Lys153). The inset shows the coloring used for the carbon atoms of EMDF bound to EGS, and UDP-glucose bound to UDP-galactose-4-epimerase. Hydrogen bonds are drawn as thin dashed lines. Water molecules are drawn as red spheres. Those outlined in the pale red form part of a postulated proton-relay network in UDP-galactose-4-epimerase, whereas the presumed catalytic water molecule in EGS is outlined in green and marked with an asterisk. (C) Proposed reaction mechanism of EGS (and IGS) involving a quinone-methide intermediate. The catalytic base (B) that promotes deprotonation of the 4-hydroxyl group of the substrate is the hydroxide ion that is activated by the side-chain amino group of Lys132. The loss of acetate generates the quinone-methide intermediate. The attack at C7 of this intermediate by the NADPH-derived hydride yields the allyl phenylpropeno eugenol, whereas hydride attack at C9 presumably yields the isopropylneno eugenol.

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Particular, steric restrictions within the enzyme’s active site (as discussed above) appear to disfavor the binding of an extended C1 substituent on the substrate. Furthermore, due to the predominantly non-polar character of the capping region of the EGS active site, only a single residue, Tyr157, is available for hydrogen-bond interactions with the polar oxygen atoms of the acetate moiety. In fact, the cluster of aromatic side-chains in this region of the active site (see Figure 5A) may provide a favorable environment for stabilizing the carbocationic character of C7 and C9 [20] within the extended quinone-methide. The proposed model for disfavoring binding of the acetate group is consistent with the diminished catalytic activity of mutants of EGS that carry smaller active-site capping residues (Tyr157Ala, Tyr157Phe, Phe314Ala, and Phe311–314; see Table 2).

Both the Tyr157Ala and Tyr157Phe mutants retain partial enzyme activity (21% and 33%, respectively, Table 2), and therefore, the hydrogen-bonding capacity of Tyr157 is apparently not essential for catalysis. The lack of a suitable proton donor within the active site of EGS to catalyze the carbon-oxygen bond cleavage may account for the requirement of EGS for an esterified substrate: coniferyl alcohol carries a much poorer leaving group (free hydroxide ion) than its acetylated form (the resonance-stabilized acetate ion) (see Figure 6C). The absence of a well-defined binding site for the acetate, underscored by the finding that soaking of EGS-NADP$^+$ crystals with a high concentration (0.5 M) of sodium acetate yielded no ordered binding of acetate ion, may also contribute to the apparent irreversibility of the reaction that generates eugenol and acetate from coniferyl acetate.

In summary, a mechanistic scheme emerges in which binding of the coniferyl acetate substrate within the active site of EGS leads to deprotonation of the p-hydroxy group (PUSH) coupled with expulsion of acetate ion from the C1 substituent (PULL). The resultant extended quinone methide intermediate serves as a hydride acceptor at C7 to yield the product eugenol. For NAD(P)H-mediated reduction reactions, the acceptance of hydride by a substrate is typically accompanied by the acquisition of a proton at an adjacent atom, to maintain charge neutrality. As discussed above, EGS lacks an appropriately positioned proton donor near the site of hydride addition (C7); protonation instead occurs at the p-hydroxyl group, in concert with rearrangement of the double-bond system of the quinone methide and deprotonation of the phenyl ring. Furthermore, the reduction of a double bond within a quinone methide intermediate more closely resembles a typical reaction catalyzed by a nicotinamide cofactor enzyme, and indeed the reaction catalyzed by the PIP-family member IFR [10].
studies and analyses of additional, newly discovered IGS-type enzymes [Koeduka et al., submitted] are underway to probe the enzymic determinants of the regioselectivity of the EGS/IGS catalyzed reactions.

Modeling of NADP(H) and substrate binding in other IFR-like proteins

The EGS residues that are involved in interactions with the NADP(H) cofactor (see above) are highly conserved in the other IFR-like enzymes, and thus these enzymes can be expected to maintain a cofactor-binding site very similar to that observed in EGS. Most of these other enzymes have also been characterized as A-type reductases. On the basis of the hole-EGS structures, the NADP(H) cofactor can be readily modeled into the apo-structures of the other IFR-like enzymes, although small, accommodating adjustments to the surrounding protein are necessary.

The substrates of the PLR, PCBER and IFR enzymes all possess a phenyl ring with a C4-hydroxy group, and the expected sites of hydride addition occur near the C7 atom of the substrate. For these enzymes, substrate binding can be modeled on the basis of the positioning of the guaiacol moiety of EMDF bound to EGS. As expected, in all cases, the substrate C7-atom is positioned very close to the C4 atom of the cofactor’s nicotinamide ring. The substrate-binding pocket in general appears less enclosed in the other IFR-like proteins than in EGS, consistent perhaps with the larger size of the C1 substituents of the cognate substrates. The more open binding pockets are due primarily to the absence of the C-terminal tail that occurs in EGS, as well as the substitution of smaller residues within the active-site capping region.

Moreover, the substrates for PLR and PCBER contain a cyclic-ether linkage adjacent to the site of hydride addition, and will therefore generate a transient alkoxide intermediate upon carbon-oxygen bond cleavage and ring opening. These enzymes may employ two means to promote protonation of the alkoxide intermediate: a potential proton donor or hydrogen-bonding group within the active site (His271 in PLR; Ser263 and Ser267 in PCBER), and a more open binding site that may allow access to the intermediate by bulk water.

MATERIALS AND METHODS

Protein expression and purification

A DNA fragment encoding the entire amino-acid sequence (residues 1–314) of Ocimum basilicum EGS1 [4] was inserted between the NcoI and BamH1 sites of the expression vector pHI88, which, under the control of a T7 promoter, yields the target protein fused to an N-terminal octahistidine tag [21]. For heterologous over-expression of the EGS protein, the plasmid pHIS8(EGS) was transformed into the expression host E. coli pHIS8(EGS) with the PCR method [22]. The DNA sequences of the mutant constructs were confirmed by sequencing of the entire EGS insert in both the forward and reverse directions.

Site-directed mutagenesis of O. basilicum EGS

Site-directed mutants of the EGS gene were created in the plasmid pHIS8(EGS) with the PCR method [22]. The DNA sequences of the mutant constructs were confirmed by sequencing of the entire EGS insert in both the forward and reverse directions.

Chemoenzymatic synthesis of coniferyl acetate ((E)-4-hydroxy-3-methoxycinnamylacetate)

Coniferyl alcohol (180.2 mg, 1.0 mmol), Candida antarctica lipase B (CAL-B, 25 mg, Aldrich, 30,000 U/g, vinyl acetate (401 μL, 5.0 mmol), and dry diethyl ether (Et2O, 50 mL, 0.2 M) were stirred in a 125-mL Erlenmeyer flask at room temperature for 2 h (Figure 7). The reaction mixture was filtered through glass wool and concentrated in vacuo. The reaction yielded quantitatively the desired product (222 mg colorless oil). 1H NMR (500 MHz, CDC13) δ = 6.91 (m, 3H), 6.59 (d, J = 15.9 Hz, 1H), 1.15 (dt, J = 6.62, 15.9 Hz, 1H), 4.72 (d, J = 6.62 Hz, 2H), 3.92 (s, 3H), 2.11 (s, 3H); 13C NMR (125 MHz, CDC13) δ = 171.2, 146.8, 146.1, 134.7, 129.0, 121.0, 120.9, 114.7, 108.6, 65.5, 56.1, 21.3. LCMS [M-H]- calculated for C19H18O4: 221.08, found: 221.2.

Chemical synthesis of (7S,8S)-ethyl (7,8-methylene)-dihydroferulate (EMDF)

The compound (7S,8S)-ethyl (7,8-methylene)-dihydroferulate (EMDF, (1S,2S)-ethyl 2-(4-hydroxy-3-methoxyphenyl) cyclo-propanecarboxylate) was synthesized in three steps (Figure 8).

4-((2-methoxyethoxy)methyl)-3-methoxy benzaldehyde (step 1). K2CO3 (1.38 g, 10.0 mmol, 1.0 eq.) was added to solution of vanillin (1.52 g, 10.0 mmol, 1.0 eq.) in dry acetone (70 mL, 0.15 M), and the mixture was stirred under argon at 0°C for 30 min. Then MeOCH2CH2OCH2Cl (MEM-Cl, 1.49 g, 12.0 mmol, 1.2 eq.) was added drop wise, and the mixture was stirred an additional 5 h. The mixture was then concentrated in vacuo to ~20 mL, combined with 20 mL of water, and extracted with diethyl ether (3×3 mL). The organic layers were pooled, washed with brine, dried with MgSO4, and concentrated in vacuo.

The resulting yellow oil (2.38 g, 98% yield) was used without further purification. 1H NMR (500 MHz, CDC13) δ = 9.86 (s, 1H), 7.43 (s, 1H), 7.42 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 8.5 Hz, 2H), 5.41 (s, 2H), 3.93 (s, 3H), 3.86 (t, J = 4.7 Hz, 2H), 3.55 (t, J = 4.7 Hz, 2H), 3.35 (s, 3H); 13C NMR (125 MHz, CDC13) δ = 191.0, 152.0, 150.0, 131.1, 126.5, 114.6, 109.4, 94.0, 71.4, 68.2, 59.0, 56.0; LCMS [M+Na]+ calculated for C14H17O5Na: 263.09, found: 263.3.

(E)-2-methoxy-4-vinylbenzene (step 2). To a suspension of methyltriphenylphosphorinum bromide (MeP(PPh3)Br, 3.57 g, 10.0 mmol, 2.0 eq.) in anhydrous tetrahydrofuran (THF, 30 mL, 0.33 M), under argon at room temperature, was added potassium bis-trimethylsilylimide (KN(TMS))2, 4.5 mL, 9.0 mmol, 1.8 eq. 0.5 M in toluene). After 30 min, a solution containing 4-((2-methoxyethoxy)methyl)-3-methoxy benzaldehyde (1.20 g, 5.0 mmol, 1.0 eq.) was added to the yellow colored ylide solution via cannula. The reaction was completed within 30–60 min (as monitored by TLC, hexanes/ethyl acetate).

Figure 7. Chemoenzymatic synthesis of coniferyl acetate. Coniferyl acetate was obtained as a colorless oil with a yield of nearly 100%. doi:10.1371/journal.pone.0000993.g007
The reaction mixture was quenched with saturated NaCl, and extracted with diethyl ether (2×100 mL). The organic layers were combined, washed with water (2×100 mL), brine (2×100 mL), dried over Na₂SO₄, filtered, concentrated in vacuo, and subjected to Dry Column Vacuum Chromatography (90% hexanes/ethyl acetate) to afford a colorless oil (2.01 g, 84% yield).

**EGS enzyme assay**

EGS enzyme activity was measured by gas chromatography/mass spectrometry as described previously [4]. The assay mixture (total volume 0.15 mL) contained 0.05 M MES-KOH (pH 6.5), 1 mM NADPH, 1 mM coniferyl acetate, and 2 μg of EGS. Reaction mixtures were incubated at 25°C for 15 min followed by extraction with 1 mL of hexane. For determination of the specific activities of crude preparations of EGS, enzyme concentrations were assessed from western blots with an EGS antibody. For detailed kinetic analyses, substrate concentrations ranged from 0.1 to 5.0 mM, and for EMDF-inhibition determinations, the inhibitor concentrations used were 0, 0.4 and 0.8 mM (Figure 9).

**Crystallization of basil EGS**

Wild-type EGS from basil (*Ocimum basilicum*) in complex with NADP⁺ was crystallized at 4°C from buffered solutions of protein mixed with polyethylene glycol (PEG) and a salt. The typical crystallization solutions employed were 0.1 M sodium succinate (pH 5.5), 5 mM NADP⁺ (Sigma Aldrich), 0.3 M KCl, 2 mM diithiothreitol and 21% (w/v) PEG 3350 or 0.1 M MOPS (pH 6.5-7.0), 5 mM NADP⁺, 0.3 M KNO₃, 2 mM diithiothreitol and 28% (w/v) PEG monomethylether 5000. These conditions yielded a number of distinct (but related) crystal forms. Morphologically, all of the crystal forms grew as thin plates. The typical unit-cell parameters a = 53.8, b = 85.9, c = 76.2 Å, β = 107.3° and orthorhombic (space group P2₁2₁2₁, with unit-cell dimensions a = 79.3, b = 83.9, c = 99.2 Å) and both diffracted X-rays to high resolution (typically 1.6 to 2.0 Å). Similar crystallization conditions were also employed for wild-type EGS complexed with NADPH (the reduced form of the cofactor) or without added cofactor (apo-EGS), and for Lys132-mutant EGS proteins complexed with NADP⁺. (It should be noted that the apo-EGS protein likely contained a small amount of nicotinamide cofactor incorporated during expression in *E. coli*.) Micro-seeding
EMDF were obtained by soaking EGS/NADP\(^+\) crystallization solution supplemented with 5–10 mM EMDF. Crystals were transferred briefly to a cryoprotectant solution.

X-ray diffraction data

Crystals were transferred briefly to a cryoprotectant solution (consisting of reservoir solution supplemented with 17–20\% v/v glycerol) prior to immersion in liquid nitrogen. X-ray diffraction data were measured from frozen crystals at beamlines 8.2.1 and 8.2.2 of the Advanced Light Source (Lawrence Berkeley National Laboratory) on ADSC Quantum 210 or 315 CCD detectors. Diffraction intensities were indexed, integrated and scaled with the programs XDS and XSCALE [23] or Mosflm [24] and Scala [25].

Figure 9. Double reciprocal plot for EGS activity in the presence and absence of EMDF. The plots illustrate the mixed nature of the EMDF EGS inhibitor. \(K_i\) was estimated from nonlinear fitting to a modified Michaelis-Menten equation.

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was useful for promoting crystal growth of the mutant forms of EGS. Ternary complexes of EGS, NADP\(^+\) was useful for promoting crystal growth of the mutant forms of EGS. Ternary complexes of EGS, NADP\(^+\) and the EGS inhibitor EMDF were obtained by soaking EGS/NADP\(^+\) crystals in crystallization solution supplemented with 5–10 mM EMDF.

X-ray diffraction data

Crystals were transferred briefly to a cryoprotectant solution (consisting of reservoir solution supplemented with 17–20\% v/v glycerol) prior to immersion in liquid nitrogen. X-ray diffraction data were measured from frozen crystals at beamlines 8.2.1 and 8.2.2 of the Advanced Light Source (Lawrence Berkeley National Laboratory) on ADSC Quantum 210 or 315 CCD detectors. Diffraction intensities were indexed, integrated and scaled with the programs XDS and XSCALE [23] or Mosflm [24] and Scala [25].

X-ray structure determination of EGS

Initial crystallographic phases were determined for the orthorhombic crystal form of the EGS/NADP\(^+\) complex through molecular-replacement (MR) with the program Modeller [27] based on the structure of phenylcoumaran benzyl ether reductase (PCBER, PDB entry 1QYC [6]). The program ARP/wARP was used for automated rebuilding of the initial structural model against a two-fold, non-crystallographic-symmetry averaged map. Subsequent structural refinement used the program CNS [28]. Xfit [29] was used for graphical map inspection and manual rebuilding of the atomic model. Programs from the CCP4 [30] suite were employed for all other crystallographic calculations. Structural depictions were generated with the program PyMol (Delano Scientific, San Carlos, CA).

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Author Contributions

Conceived and designed the experiments: JN EP GL TB TK. Performed the experiments: GL TB MB JT SS TK. Analyzed the data: JN EP GL TB MB JT SS TK. Contributed reagents/materials/analysis tools: JN EP GL TB TK. Wrote the paper: JN EP GL TB.

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