Isomerization of the Phytohormone Precursor 
12-Oxophytodienoic Acid (OPDA) in the Insect Gut

**A MECHANISTIC AND COMPUTATIONAL STUDY**

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12-Oxophytodienoic acid (OPDA) is isomerized in the gut of herbivorous insects to tetrahydrodricranenone B (iso-OPDA). The transformation is achieved by a glutathione S-transferase present in the gut epithelium. Experiments with 9-[2H]-iso-OPDA demonstrated the complete retention of the deuterium atom in the product 11-[2H]-OPDA consistent with an intramolecular 1,3-hydrogen shift. Homology modeling based on the x-ray structure of a glutathione S-transferase from *Anopheles gambiae* revealed that the co-factor glutathione does not covalently bind to the substrate but appears to be involved in the initial deprotonation and enolization of the OPDA. The transformation resembles that of a mammalian GST-catalyzed isomerization of Δ^5^-3-ketosteroids to Δ^4^-3-ketosteroids or the conversion of prostaglandin A_1 to the biologically inactive prostaglandin B_1.

The glutathione transferases (GSTs) are generally associated with cellular defense against electrophiles; in this capacity, they catalyze the conjugation of the tripeptide glutathione (GSH) with an electrophilic species. These reactions are meant both to decrease the reactivity of electrophiles and to increase their polarity so that they can be either further metabolized or readily excreted (1). In addition to this primary function, the GSTs have been repeatedly shown to catalyze several other reaction types, including isomerizations. An important common feature of all these reactions is the apparent lack of GSH consumption, which correlates with the absence of stable GSH conjugates. The most thoroughly studied example is the *cis*-trans isomerization of maleylacetoacetate to fumarylacetoacetate (2). Here, the conversion proceeds, presumably via GSH conjugation, to the α,β-un saturated system, rotating via the resulting single bond and then eliminating the GSH (3). Another class of GST-catalyzed isomerization reactions can be found in prostaglandin biosynthesis, where prostaglandin D_2 results from the GSH-dependent transformation of prostaglandin H_2 (4). Although GSH is not being consumed in this transformation, it is postulated to act as a covalent nucleophilic catalyst and a general base catalyst in the conversion of the endoperoxide (prostaglandin H_2) to the β-hydroxy ketone (prostaglandin D_2) (5). The tautomeration of 2-hydroxymethyfuran to mintlactones constitutes yet another instance of a GST-mediated isomerization reaction; in these, the thiolate form of GSH is proposed to deprotonate the substrate and in this way initiate the reaction (6). The best known example, however, of positional isomerization of double bonds is the conversion of Δ^5^-3-ketosteroids to Δ^4^-3-ketosteroids, which has been shown to be catalyzed among others by mammalian GSTs (7, 8). This case bears the highest formal resemblance to the isomerization of the plant-signaling molecule *cis*- (+) -12-oxophytodienoic acid, which we found to be catalyzed by insect GSTs (Fig. 1A) (9). Quite interestingly, an almost identical reaction, also similar to ketosteroid isomerization, has been previously described for prostaglandins of the A series (Fig. 1B) (10). However, the relevant enzyme, prostaglandin A_1, Δ-isomerase, has never been fully purified or correlated with GST activity (11–13).

Δ^5^-3-Ketosteroid isomerase, which catalyzes the allylic isomerization of variety of Δ^5^-3-ketosteroids to Δ^4^-3-ketosteroids, was first isolated from *Pseudomonas testosteroni* and is one of the most efficient enzymes known to date (14). The mechanism of the isomerase action, which was confirmed by studies with the deuterated substrate (15) as well as by analysis of the crystal structure of the enzyme (16), implies a stereospecific intramolecular transfer of the labile 4β-proton to the 6β-position, proceeding through a dienolate intermediate (Fig. 1C). Although the bacterial isomerase does not require co-factors for its activity, some of the mammalian enzymes exhibiting identical activity were discovered to be GSH-dependent (7); for example, the most efficient human steroid double-bond isomerase known to date is the GST A3-3 (17). In the case of the bacterial enzyme, the role of the base (Fig. 1C), which abstracts the 4-proton and simultaneously redeposits it in position 6, is played by the aspartate residue of the enzyme (Asp-38) (18), whereas in human GST-correlated isomerases, the base part is contributed to the thiolate of GSH (Fig. 1D) (19). In both of these enzymes, an important catalytic role is being played by the tyrosine residue in the active site (Fig. 1C: R1-OH), which is assumed to stabilize the dienolate intermediate by the formation of the hydrogen bond to the 3-oxo-group of the substrate. However, recent findings, based on the examination of the crystal structure of the GST A3-3, revisited the role of tyrosine in the mammalian KSI reaction (Fig. 1D) (36). According to the latest hypothesis, the tyrosine residue (Tyr-9) is hydrogen-bonded to the sulfur of GSH and in this way facilitates the delivery of the proton to C-6. Moreover, the human
GST A3-3 seems not to stabilize the intermediate dienolate by an oxyanion hole in the active site. In light of previously characterized GST-catalyzed isomerization reactions and considering certain similarities between these processes, we wanted to investigate whether the mechanism of the 12-oxophytodienoic acid transformation complies with already existing ideas. Because isomerization requires a shift of the double bond from position 10,11 to position 9,13, we have synthesized cis-9-[2H]-12-oxophytodienoic to investigate the fate of the deuterium during the transformation. Furthermore, identification of a putative OPDA2 isomerase, GST-16 (accession number FJ546089) (9), enabled us to create a homology model of this protein and conduct modeling studies. Here, we present a putative mechanism for the OPDA isomerization reaction based on combined results from experiments with specific deuterium-labeled OPDA and the results of modeling studies.

EXPERIMENTAL PROCEDURES

Synthesis of cis-9-[2H]-12-Oxophytodienoic Acid (cis-9-[2H]-OPDA) — cis-9-[2H]-OPDA has been synthesized from 9-[2H]-linolenic acid using a modified Zimmerman-Feng approach (20, 21). 9-[2H]-Linolenic acid was prepared following the procedure modified by Pohnert et al. (22, 23) from methyl 9-[2H]-oxononanoate, obtained as in Crombie et al. (24) (see Fig. 2) Details of the synthetic preparation as
Mechanistic Studies with Deuterium-labeled cis-9-[2H]-OPDA—To investigate the fate of the deuterium during the double-bond translocation, we synthesized the OPDA molecule with the deuterium at C-9. To avoid the time- and resource-consuming effort of synthesizing this structure, we decided to use a combination of synthetic and biotechnological approaches. Accordingly, cis-9-[2H]-OPDA was obtained directly from 9-[2H]-linolenic acid. The labeled linolenic acid was synthesized in five steps from azelaic acid monomethyl ester (Fig. 2).

Feeding Experiments with Larvae of Spodoptera littoralis—Artificial pinto bean-based diet (25) was soaked with the aqueous solution of cis-9-[2H]-OPDA to achieve a final concentration of ∼6 μg/g in the diet. Fourth instar S. littoralis larvae were starved for 2 h prior to the start of the experiment and placed in separate Petri dishes. Then they were allowed to feed on the cis-9-[2H]-OPDA-supplemented diet for 24 h. The feces (∼0.5 g) were collected and kept in −80 °C for further analysis, performed as described previously (21).

Homology Model of Putative OPDA Isomerase (GST-16)—For protein homology modeling, the modeling program package YASARA was used (26–28). This program automatically performs a BLASTp search in the Protein Data Bank (PDB) for most homolog proteins. Altogether, 51 templates were identified as useful for homology modeling, but the x-ray structure of an insect, ε-class glutathione S-transferase from the malaria vector Anopheles gambiae (PDB code: 2imi) appeared most similar, with a value of 6.03 × 10−44 (29). The quality of the model was evaluated by YASARA as optimal. Further checks with PROCHECK (more than 91% amino acid residues in most favored areas), PROSA2 (combined z-score = −8.80, which is in the range for an excellent folded protein containing 217 amino acids), and ERRAT (overall quality factor 98.6%) confirmed the quality of the model. During the refinement and energy optimization of the model, YASARA automatically merged glutathione from the template protein (2imi) into the protein model, resulting in an optimal position for the co-factor. Using this structure, docking studies were performed with PLANTS (30, 31). In each case, 30 different docking poses were calculated and subsequently analyzed. This program allows the inclusion of flexibility of some side chains of the active site. Included flexible protein side chains were: Leu-116, Phe-119, Val-115, Tyr-120, Lys-121, Phe-119, Leu-36, and Ser-11. Semi-empirical quantum mechanical calculations were performed with the program Spartan’02 (32) using AM1 (33, 34).

RESULTS

Mechanistic Studies with Deuterium-labeled cis-9-[2H]-OPDA—To investigate the fate of the deuterium during the double-bond translocation, we synthesized the OPDA molecule with the deuterium at C-9. To avoid the time- and resource-consuming effort of synthesizing this structure, we decided to use a combination of synthetic and biotechnological approaches. Accordingly, cis-9-[2H]-OPDA was obtained directly from 9-[2H]-linolenic acid. The labeled linolenic acid was synthesized in five steps from azelaic acid monomethyl ester (Fig. 2). Methyl 9-[2H]-oxononanoate was generated following a slightly modified procedure from Crombie et al. (24), and the subsequent crucial coupling was achieved via the bis-Wittig approach (22). The final transformation to cis-9-[2H]-OPDA was based on the enzyme blend generated from flaxseed (20) (see supplemental material for details). Importantly, a mixture of two cis enantiomers of OPDA is formed in this preparation: cis-(+)-OPDA (9R,13S)-12-oxophytodienoic acid and cis-(−)-OPDA (9S,13R)-12-oxophytodienoic acid; only the SS configuration occurs naturally in plants. Our previous studies, however, which used material obtained in the identical preparation, showed that both of these enantiomers are equally isomerized in the insect gut (9, 21). A deuteration grade of 93% for the product was found satisfactory for subsequent studies. cis-9-[2H]-OPDA was fed to the S. littoralis larvae, and the collected feces were analyzed for labeling patterns of the formed iso-OPDA (9, 21). The specific labeled substrate was incubated simultaneously with the dissected gut tissue of the larvae for 2 h. Corresponding to the results for ketosteroid isomerization, the obtained OPDA isomer showed a full retention of the deuterium after the gut-localized transformation (Fig. 3). These experiments prove that an intramolecular deuterium transfer happens during the conversion of OPDA to iso-OPDA, but they do not specify the position of the translocated deuterium atom. In the enzymatically formed iso-OPDA, there are only two possible positions to which deuterium can be shifted: C-10 or C-11. According to examples known from the field of ketosteroid isomerases (15) and implications from the prostaglandin A isomerization case (11), the C-11 position should be favored. To establish whether the deuterium has indeed been moved to this location, we incubated the product of the gut assay in MeOH with a minimal amount (10 μl) of acetyl chloride for 5 min. When deuterium occupies the C-11 position, the acid-catalyzed formation of enol should lead to its removal and subsequent substitution by a proton. As expected, the methyl ester...
of the obtained deuterated iso-OPDA measured after this experiment was fully deuterium-free. To estimate the rate and speed of a possible exchange between deuterium and a proton at this position, we performed identical trials with comparable amounts of non-deuterated iso-OPDA in MeOD. After 5 min, we could recognize incorporation of two deuterium atoms only at position C-11 (and two at C-2, next to the carboxylic function, not relevant to our studies). We were able to discriminate between these deuterated positions on the basis of the fragmentation pattern in the mass spectrum. Notably, prolonged treatment (around 30 min) eventually also resulted in deuterium being incorporated at position C-10.

Furthermore, to check the selectivity of the enzyme-dependent transformation, we incubated the mixture of trans- and cis-9-[2H]-OPDA. Assay results, in which mainly the cis-9-[2H]-OPDA was isomerized, confirmed our previous finding, supporting the specificity of the putative OPDA isomerase for this particular epimer (21).

**Homology Modeling and Docking Studies of the GST-16 Enzyme**—To propose a plausible mechanistic route of the OPDA isomerization, we had to model the active site of the putative OPDA isomerase (GST-16) together with the docked substrates: cis-OPDA (of both enantiomer S,S and enantiomer R,R, which will allow the enzymatic conversion of both, accordingly to observations mentioned above) and GSH. Due to the high homology of the GST-16 to several known GSTs, including the e-class glutathione S-transferase from the malaria vector *A. gambiae* (for which the X-ray structure has been described), it was relatively easy to start the initial modeling by generating the three-dimensional structure of the GST-16 model. In the next step, on the basis of the analogy to the known enzymes, the glutathione residue was docked in its characteristic domain, indicating the location of the active site. Next, both enantiomers of cis-OPDA, cis-(+-)OPDA-(9S,13S) and cis-(+-)OPDA-(9R,13R), were docked into the enzyme pocket. From all analyzed dockings of OPDA into the active site of GST-16, only those were considered in which the cyclopentenone ring was positioned in close proximity to the catalytically important glutathione (Fig. 4). Modeling studies have shown that both enantiomers could be "held" in the appropriate position via the strong interactions of salt bridges; in the case of the S,S enantiomer, this position was between the carboxylic moiety of the side chain with Lys-121, and for the opposite enantiomer, it was with Lys-215. Additionally, a network of stabilizing hydrogen bonds can be formed for two of them between the carbonyl function of the cis-OPDA and Ser-11 and the cysteine residue of the GSH. Interestingly, however, in the case of the S,S-configured OPDA, it is Ser-11 that forms a hydrogen bond to the carbonyl group of the substrate and thus plays the role of proton donor in the formation of the intermediate enolate (see mechanism proposal in Fig. 5), whereas the cysteine of the GSH serves as the alpha-proton (at C-13) acceptor. On the other hand, in the R,R-configured substrate, both these residues seem to be exchanged with regard to recognition and function. Furthermore, as the carboxyl terminus of the GSH seems not to be involved in any salt bridge with either lysine or arginine residues, a counter ion in the form of a hydronium ion is likely to be located close to this terminus. In both docked enantiomers, the hydronium ion is stabilized by hydrogen bonds to the carboxyl terminus of the GSH and to the phenolic hydroxyl group of the Tyr-120. Refinements of the protein complex, including GSH, OPDAs, and the hydronium ion, were performed by a molecular dynamics simulation using YASARA; these refinements include a periodic boundary water box surrounding the protein for 100 ps with subsequent energy optimization. The docking arrangement of all ligands stayed almost unchanged. The final structure of the active site is shown in Fig. 4.

From the catalytic point of view, the close (~2.2 Å) proximity of one hydrogen atom of the hydronium ion to the C-10 atom of the double bond of the cyclopentenone ring, as well as the similar distance (~2.8 Å) between the sulfur atom of the cysteine of GSH to the hydrogen atom at C-13, appears to be significant. The highly hydrophobic binding site consisting of Phe-108, Leu-112, Val-115, Leu-116, Phe-119, Leu-205, and Phe-206 is common for the specific docking arrangement of both substrates.

**Possible Reaction Mechanism for the GST-catalyzed OPDA Isomerization**—To reduce the computational effort required to study a multitude of alternative catalytic mechanisms, we omitted the time-consuming quantum mechanics/molecular mechanics calculation, which would have necessitated including the whole protein. Instead, starting from the above described arrangements, systematic semi-empirical quantum mechanical calculations with AM1 were performed. During all calculations except for those involving the final structure, two distance constraints reflecting the constraints of the enzyme
were applied; the distance between the oxygen atom of the hydronium ion to the sulfur atom of cysteine (GSH) was fixed at a distance of 6.0 Å, and the distance between the sulfur atom to the C-13 atom of the substrate was fixed at a distance of 3 Å. For the model reaction mechanism studies, the substrate was truncated to the fragment shown in Fig. 5. Starting from the docking arrangement in the enzyme complex, the energy of the system was optimized, and for each step, the corresponding heats of formation were calculated. Subsequently, according to Fig. 5, reaction coordinates in 10 steps, one for each changing bond, were calculated (full calculation results are shown in supplemental Table S1). From these calculations, the transition state energies were estimated (indicated in Fig. 5 by #). Considering only the thermodynamic stability (heat of formation) of OPDA, the calculations indicate an energy gain of 9.6 kcal/mol for the formation of the isomerized product. This energy gain is likely caused by slightly reduced repulsive interactions between the first carbon atoms of each side chain, which is in the adduct $\sim 2.9$ Å but in the product 3.1 Å.

Because the thermodynamics of the conversion are favored by the kinetics, the level of energy barriers for each step of the reaction mechanism will be crucial. Furthermore, the experimentally proven deuterium transfer has to be reflected in the mechanism. To derive a mechanism consistent with the experiments and showing low transition state energies, several alternative mechanisms were investigated in detail. These alternative mechanisms include the formation of dienolates and nucleophilic attack of the sulfur (anion) atom of cysteine of GSH at the double bond of OPDA, along with different intermediate reaction steps. All of those, except the one described in the following paragraph, exhibited energy barriers of more than 60 kcal/mol in any step of the reaction mechanism and can therefore be ruled out.

The favored reaction likely starts (Fig. 5A) with the enolization of the carbonyl group of the substrate via Ser-11, which is simultaneously acting as the acceptor of the GSH proton leading to formation of the thiolate. Abstraction of proton from the C-13 position by the activated thiolate sulfur and a proton transfer from the hydronium ion to the C-10 position proceed synchronously; together these result in the formation of the cyclopentenyl cation (Fig. 5B). According to calculations, this reaction step is highly favored by an energy gain of $-76.6$ kcal/mol with a very low activation barrier of 1.6 kcal/mol. Alternatively, the protonation of the double bond without simultaneous enolization would require about 7 kcal/mol and is therefore less favored. In the next step (Fig. 5, B and C), a formal 1,3-hydride shift of the C-9 deuterium to the C-11 position is supported by an energy gain of $-15.8$ kcal/mol and a quite low estimated activation barrier of 17.3 kcal/mol. Finally, a deprotonation of the enol oxygen atom by the sulfur atom of cysteine (energy gain of $-11.9$ kcal/mol) results in the formation of the product. Due to the lack of an energy barrier for this step, it can also proceed synchronously with the preceding deuterium shift.

From this reaction mechanism, an overall energy gain of $-104.6$ kcal/mol, which can be attributed to the higher proton affinity of the thiol function of cysteine in comparison with the
one of water and about 10 kcal/mol, favored the heat of formation of the product in comparison with the substrate.

**DISCUSSION**

Experiments with cis-9-[2H]-OPDA clearly confirmed that an intramolecular transfer of the proton from position C-9 to C-11 takes place during the enzyme-catalyzed isomerization of the OPDA in the insect gut (Fig. 3). These results, combined with modeling and docking studies, enabled a plausible mechanistic hypothesis to be designed (Fig. 5). The proposed mechanism takes into account formal similarities between the GST-catalyzed OPDA and steroid transformations and so resembles the ketosteroid isomerization process only partially. In the first reaction step of both cases, glutathione is proposed to act as a base, abstracting the acidic proton: in the OPDA from position C-13 and in 3-keto steroids from position 4β. On this stage, the enzyme residues also play an important role: in the case of the GST-16, the Ser-11, by protonating the carbonyl function (thus increasing the acidity of the C-13 proton and making it more susceptible for abstraction by the GSH) (Fig. 5), and in the case of human GST A3-3, the Tyr-9, by stabilizing the formed enolate structure via the hydrogen bond to the oxygen (Fig. 1D) (17). Nevertheless, although the formal intermediate resulting from this initial reaction stage for the OPDA is the allyl cation (additional protonation at position C-10 by the hydronium cation), for the ketosteroids, it is a dienolate intermediate. In the next step, mechanistic postulates for the ketosteroid isomerases suggest that the proton abstracted by the thiolate of the glutathione is being “carried over” to position 6β, where it is then redepotted. For the non-GSH-dependent bacterial enzyme, the transporter function has been attributed to the aspartate residue (Asp-38). The possibility of the proton exchange with protons of the medium has been dismissed on account of the hydrophobicity of the active center of the enzyme and the high speed of the reprotonation step. Although this explanation seems to be supported by the crystal structure of the bacterial ketosteroid isomerases (16, 18), an analogous aspect for the mammalian GSTs with steroid isomerase activity has yet to be clarified. The 1,3-hydride shift, which we propose in the second step of the OPDA isomerization (Fig. 5B), constitutes an elegant explanation for the absence of a detectable deuterium loss (on the basis of the comparison of the deuteration degree, read from mass spectra, between the product and the substrate) during the transformation. Without more detailed studies, it is difficult to differentiate between a 1,3-hydride shift or two subsequent 1,2-hydride shifts (from C-9 to C-10 and then to C-11). Inspection of the signs of the highest occupied molecular orbitals as well as the lowest occupied molecular orbitals clearly indicates a thermodynamically allowed suprafacial hydride transfer due to equal signs for the allylic orbital at C-11 and of the C-9-H bond (supplemental Fig. S1). On the other hand, relevant studies for the cyclopentenyl cation have suggested that although the direct 1,3-hydride shift does not occur (35), two consecutive 1,2-hydride shifts do. Furthermore, considering the relatively high hydrophobicity of the active site, the modeling and docking studies of the GST-16 revealed that neither the GSH nor other enzyme residues are so conveniently located as to act as the “carriers” for proton 9. Obviously, without a crystal structure of the enzyme, our studies alone cannot be a basis for dismissing entirely the possibility of the proton transfer by the GSH, but with the evidence suggested by modeling studies, we find the 1,3-hydride shift far more credible.

Notably, the mechanistic proposal is thoroughly consistent with all of our previous observations (9). It provides an explanation for the lack of the glutathione-OPDA conjugate, which we failed to detect in substantial amounts in insect gut and feces, as well as for the low isomerization rate of the synthetically prepared GS-OPDA (9). Instead, the formation of small amounts of iso-OPDA from the GST-catalyzed transformation of the conjugate could be attributed to the spontaneous conjugate cleavage and subsequent isomerization of the free OPDA. Moreover, the failure to detect the postulated β,γ-unsaturated intermediate could be understood in the light of allyl cation formation. Interestingly, this mechanistic scheme is formally highly similar to the alternative mechanistic route we proposed previously (9), which was based on the initial enolization of the carbonyl function. Additionally, overall energy calculations for the hypothetic mechanistic flow are highly favorable (summed energy gain of 104.6 kcal/mol); each step proceeds with a distinct energy gain and low energy barriers.

Remarkably the modeling and docking studies are also able to explain the observed similar isomerization of both cis-OPDA enantiomers (Fig. 4). Although the mechanistic process described above (Fig. 5) applies to the cis-(+)-OPDA naturally occurring in plants, it can also be adapted to the opposite enantiomer, in which the two critical residues, the Ser-11 and the GSH, exchange their functions as a result of a somewhat different spatial positioning of the substrate in the active center (Fig. 4B). Such uncommon enzyme plasticity toward two opposite enantiomers, which includes the exchange of the functions of different enzymatic residues, is interesting, but its significance for the process is not yet understood. Furthermore, because the trans-OPDA could not be satisfactorily positioned in the active center of the enzyme (supplemental Fig. S2A), the lack of isomerization for this stereoisomer is explained. Finally, modeling of the structurally similar prostaglandin A₁ in the active center of the GST-16 did not result in localization, which would have allowed a conversion similar to that of cis-OPDA to take place (supplemental Figs. S2B and S3).

To understand the basis for the GST-16 specificity, we also performed the initial modeling of the active site of the GST-6, which does not exhibit OPDA isomerization activity: only the side chain epimerization at C-13 (9). Although the catalytically important Ser-11 occupies the same position, several other amino acids building the active site differ significantly; for example, Tyr-120 is exchanged by phenylalanine. This modification alone can disable the crucial stabilization of the hydroxynium ion and in turn lead to the altered catalytic characteristic.

Considering the striking similarity of the OPDA isomerization to the transformation of the prostaglandins A₁, the question about the resemblance of the relevant enzymes naturally arises. None of the previous studies on the prostaglandin A isomerase ever connected this enzyme with the class of GSTs; moreover, none of the insect GSTs we investigated were capable of this transformation, as reported previously (21). However, because
the prostaglandin A1-Δ-isomerase has only been partially purified, important data allowing the enzymes to be compared are either not fully available or inconsistent. For example, the molecular mass of the prostaglandin A isomerase has been estimated by one group to be 110 kDa (12), whereas another study states 28 kDa (13). One possible explanation for this apparently contradictory information is that, as in the case of ketosteroid isomerases, there are different enzyme classes that have “learned” to catalyze identical transformations. In light of the well known flexibility, robustness, and high diversity of the GSTs, they should be considered one of several likely candidates.

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