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Double duty isomerases: a case study of isomerization-coupled enzymatic catalysis

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Abstract

Enzymes can usually be unambiguously assigned to one of seven classes that specifies the basic chemistry of their catalyzed reactions. Less frequently, two or more reaction classes are catalyzed by a single enzyme within one active site. Two examples are an isomerohydrolase and an isomerooxygenase that catalyze isomerization-coupled reactions crucial for production of vision-supporting 11-*cis*-retinoids. In these enzymes, isomerization is obligately paired and mechanistically intertwined with a second reaction class. A handful of other enzymes carrying out similarly-coupled isomerization reactions have been described, some of which have been subjected to detailed structure-function analyses. Here, we review these rarefied enzymes focusing on the mechanistic and structural basis of their reaction coupling with the goal of revealing catalytic commonalities.

Keywords

enzymology; catalysis; isomerization; retinoids; isoprenoid chemistry; alkene bond transformations

Main Text

Combo catalytic activities

Enzymes are life-sustaining catalysts that enable much of the diverse biological chemistry we observe on earth. As such, it is unsurprising that enzyme-encoding genes occupy sizable fractions of genomes from all domains of life [1]. Considering that there are millions or

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Declarations of Interest

None are declared.

possibly billions of extant species on earth [2], the number of enzymes found in nature is truly immense. Despite the breadth of the enzyme universe, it was found that most enzymes can be assigned to one of seven classes based on the overall reaction they catalyze [3]. This taxonomy, developed by the International Commission on Enzymes (EC) and adopted by the International Union of Biochemistry and Molecular Biology (IUBMB), represents the most widely used system for reaction chemistry-based enzyme categorization.

It is known, however, that not all enzymes can be unambiguously categorized into a single enzyme class within the **EC system** (Box 1). Indeed, there exist single polypeptide enzymes with two or more active sites, catalyzing separate reactions (*i.e.*, multifunctional enzymes such as fatty acid synthase [2.3.1.86] [4] and tryptophan synthase [EC 4.2.1.20] [5]). Others perform distinct chemistries within a single active site, independently of each other (*e.g*, RubisCO, [EC 4.1.1.39] [6]). Additionally, there are enzymes that showcase an exceptional capacity to orchestrate two chemistries occurring together, in some cases in an integrated fashion, within the same active site. This review focuses on the latter category of enzymes. Of particular interest to us are single active site isomerases that link isomerization activity with a second activity from a different reaction class to enable mechanistically or thermodynamically challenging isomerization reactions to occur. We have identified several examples of such ***in situ*-coupled reactions** involving substrate isomerization, some of which have been subjected to detailed structure-function analysis (Table 1). Here, we focus on select cases that illustrate mechanistic and teleological principles underlying these unusual isomerization reactions, which are involved in diverse biological processes such as vision, photosynthetic light harvesting, lipid and amino acid biosynthesis, and terpene degradation.

Isomerases involved in visual chromophore synthesis

We begin with two enzymes involved in metazoan vision, vertebrate retinoid isomerohydrolase (RPE65, EC 3.1.1.64) and invertebrate isomerooxygenase (NinaB, EC 1.13.11.65), which are part of biosynthetic pathways leading to the production of the **visual chromophore**, 11-*cis*-retinaldehyde [7]. This chromophore binds to visual opsins and undergoes **photoisomerization** to the all-*trans* configuration, which is the first step of visual perception [8]. The 11-*cis* configuration of the retinoid backbone is thermodynamically unfavorable [9]. Consequently, energy input is required to drive the production of 11-*cis*-retinoids from all-*trans*-retinoid precursors. For both RPE65 and NinaB, the energy release associated with the *in situ*-coupled reaction is used to drive the uphill *trans-cis* isomerization reaction. Moreover, the *in situ*-coupled reaction involves temporary electron withdrawal from the polyene, providing a mechanism to achieve single-bond character at the site of *trans-cis* isomerization.

RPE65 and NinaB belong to the carotenoid cleavage dioxygenase (CCD) superfamily of enzymes that employ a non-heme iron(II) cofactor for catalysis [10-12]. In the case of NinaB, the role of iron is understood to be in the activation of dioxygen for cleavage of the C15-C15’ bond of carotene/xanthophyll substrates [13]. Because RPE65 is not known to catalyze oxidation reactions, the function of its bound iron in the isomerohydrolase reaction was not obvious [14]. The structure of RPE65 in complex with a retinoid mimetic

and palmitate revealed a coordination complex between iron and the palmitate carboxylate moiety, suggesting that iron promotes *O*-alkyl cleavage and consequent formation of a retinyl cation intermediate believed to be critical for isomerization [15, 16] (Figure 1A). The structure revealed features of the retinoid-binding site likely relevant for the isomerization process, including an overall anionic electrostatic potential and the presence of cation-stabilizing aromatic and polar residues that are important for isomerization selectivity [17, 18], as well as a curved geometry that promotes *trans-cis* isomerization [19].

The crystal structure of NinaB was recently determined, providing an opportunity to compare how CCDs function as *trans-cis* isomerases [20]. NinaB cleaves carotenes and xanthophylls symmetrically while isomerizing one-half of the substrate to form a ~1:1 mixture of all-*trans*- and 11-*cis*-retinal isomers [21] (Figure 1B). Despite the long divergence time of the NinaB and RPE65 enzyme lineages [22], the similarity of their active sites suggested that they may employ comparable mechanisms of retinoid isomerization [13]. To test this hypothesis, the active site of NinaB was systematically mutagenized to identify site substitutions selectivity impacting its isomerase activity [20]. This screen identified multiple mutations that significantly altered the ratio of 11-*cis* to all-*trans* isomers being formed. Among these, a W17L mutant exhibited a profound loss of 11-*cis*-retinoid production despite complete preservation of the canonical oxygenase activity of the enzyme, demonstrating that the two components of the *in situ*-coupled reaction catalyzed by NinaB are separable. All residues selectively affecting the isomerase activity of NinaB were found within the innermost (distal) region of the active site, whereas the site of isomerization is within the membrane-proximal region of the RPE65 active site [20]. The differing loci of isomerase activity in these enzymes demonstrates that the ability of animals to produce visual chromophore under dark conditions was acquired by functionally convergent evolution within the CCD superfamily.

Tautomer-enabled geometric isomerization in a dehydrogenase-isomerase

2-aminomuconate-6-semialdehyde dehydrogenase (AMSDH, EC 1.2.1.32) provides another example of an enzyme catalyzing an *in situ*-coupled **geometric isomerization,** but it uses an isomerization mechanism distinct from that of RPE65 and NinaB [23]. This enzyme, which has been characterized from mammals and bacteria, combines aldehyde dehydrogenase chemistry with *E-Z* isomerization as part of the kynurenine pathway for tryptophan catabolism. ASMDH, and the kynurenine pathway in general, have gained notoriety due to their involvement in neurological, immunological, and metabolic physiology and diseases [24, 25]. AMSDH oxidizes 2-aminomuconate-6-semialdehyde (2-AMS) to 2-aminomuconate (2-AM) in an NAD^+-dependent manner [23]. Interestingly, this enzyme also displays retinal dehydrogenase activity towards 9-*cis*-retinal [26].

Studies on AMSDH using a substrate analog, 3-hydroxymuconate-6-semialdehyde (3-HMS), demonstrated an unexpected substrate isomerization that precedes the NAD^+-dependent oxidation step of the pathway (Figure 2A). This discovery was made *via* **kinetic crystallography** experiments in which AMSDH crystals precomplexed with NAD^+ were soaked in a solution containing the 3-HMS substrate for various periods [23]. After 10 min, the substrate was observed in the *2E, 4E* configuration with the carboxylic group forming an

intramolecular interaction with the terminal aldehyde moiety, which positions the aldehyde away from the catalytic Cys302 thiol (Figure 2B). Two Arg side chains interact with the α-hydroxy carboxyl group of the substrate. After 40 min of incubation, the aldehyde moiety reacted with the catalytic cysteine to form a thioacyl adduct. Electron density at the other end of the substrate revealed that an *E-Z* isomerization at the C2-C3 alkene bond had taken place concurrently. It was speculated that this geometric transformation was enabled by the formation of a keto/enol tautomer or imine/enol tautomer in the case of the physiological substrate [13] (Figure 2C).

Evidence for the tautomerized intermediate was serendipitously obtained in a follow-up study using an N169D mutant of AMSDH [27]. Like the wild-type enzyme, N169D ASMDH readily formed a binary complex with NAD^+ and could be trapped in the 2-HMS thioacyl intermediate form. A shorter (5 min) soak with 2-HMS revealed electron density consistent with a non-planar C3-C4-C5-C6 dihedral angle and its conversion to an extended conformation, placing the aldehyde moiety in proximity to the catalytic Cys residue. Based on these data, the authors proposed a 'pitcher and catcher' mechanism for the *E-Z* isomerization where protonation of, or strong hydrogen bonding to, the aldehyde moiety results in tautomerization of the substrate and its isomerization to the Z configuration [14] (Figure 2C). This *E-Z* isomerization occurs spontaneously once the barrier to bond rotation is relieved *via* tautomerization owing to the 4.2 kcal/mol lower free energy of the *Z* isomer [23]. The enzyme could capture this free energy release to promote the subsequent dehydrogenase reaction. Additionally, the isomerization produces a configuration of the substrate whereby the aldehyde is closely positioned to the catalytic Cys residue enabling facile formation of the thioacyl intermediate, which is obligatory for the NAD^+-dependent oxidation step of the reaction [27].

Photosynthetic chromophore synthesis by bilin lyase-isomerases

The physiological tuning of light capture by cyanobacteria for photosynthesis is another process that involves modification of the light-absorbing properties of organic chromophores through *in situ*-coupled isomerization [28, 29]. **Phycobiliproteins** (PBPs) are light-harvesting proteins found in cyanobacteria that contain covalently linked linear tetrapyrrole chromophores, known as bilins [30]. These proteins are key components of the phycobilisome antennae that absorb radiant energy for transmission to the chlorophyll *a* molecules of photosystem II [31]. Marine cyanobacteria reside at different depths of water, which alters the spectral distribution of light to which they are exposed. Cyanobacteria can adapt to this varying light exposure through type IV chromatic acclimation (CA4) in which the ratio of the blue light-absorbing phycourobilin (PUB) to the green light-absorbing phycoerythrobilin (PEB) in phycobilisomes is modified to match the environmental light spectrum [32]. This transformation is achieved by altering the expression of bilin lyases needed to link bilin chromophores to apo-phycobiliproteins [33]. Some of these enzymes function purely as lyases while others are lyase-isomerases that alter the chromophore structure during the conjugation reaction thereby changing its absorbance spectrum. The differential expression of these two types of enzymes underlies the CA4 adaptive process [33, 34]. MpeQ is a single chain E/F-type lyase-isomerase that links and isomerizes phycoerythrobilin (PEB) with the PBP MpeA to form the phycourobilin (PUB)-

MpeA holoprotein [34]. Another E/F type lyase-isomerase is PecE/F, which performs an analogous reaction involving phycocyanobilin (PCB) attachment to PecA with concurrent isomerization to form phycoviolobilin (PVB)-conjugated holoprotein [35]. PecE/F is a heterodimer, whereas MpeQ is a single chain protein having the two subunits fused. Despite the homology of these enzymes, they may employ distinct mechanisms of isomerization as discussed further below.

The crystal structure of MpeQ was determined allowing the molecular basis of its dual lyase-isomerase activity to be studied [36] (Figure 3A). The N-terminal (E) and C-terminal (F) domains of MpeQ adopt a “question mark” architecture that provides active site accessibility from two different sides, enabling the binding of bilin and apo-MpeA substrates. The catalytic function of putative active site residues was probed through mutagenesis studies. Lyase activity was completely abolished by mutation of a conserved Tyr318 residue, indicating an important catalytic role. Additionally, mutation of cationic residues lining the chamber interior abolished activity likely due to their role in binding the anionic bilin substrate. Analysis of several MpeQ-like proteins from marine *Synechococcus* strains showed that all possess a Val residue at position 319 which neighbors the catalytic Tyr318 mentioned above. Mutation of site 319 to Gly, as it is found in non-isomerizing E/F-type bilin lyases, resulted in the formation of significant amounts of non-isomerized, PEB-conjugated PBP. Additional substitutions at sites with differentially conserved residues in isomerizing and non-isomerizing resulted in a further decrease in the isomerase activity despite preservation of lyase activity [36]. Interestingly, the residues thought to underlie isomerase activity in PecE/F [35] are not conserved in MpeQ, suggesting that multiple pathways to the isomerization functionality were found within this enzyme family.

Docking of the bilin substrate into the active site pocket of MpeQ showed a potential mechanism for the isomerization-promoting function of the Val319 side chain [36] (Figure 3A). In this model, the Val side chain sterically clashes with A-ring methyl group of the substrate. During the lyase reaction, this clash deforms the methyl towards a planar conformation, promoting the rearrangement needed to form the PUB chromophore. In the non-isomerizing bilin lyases, the corresponding Gly residue lacks the requisite steric bulk, resulting in conjugation of the non-isomerized PEB. Bilin isomerization was suggested to occur prior to its conjugation with MpeA based on the apparent formation of PUB with C81A (*i.e.*, conjugation incompetent) MpeA [36]. However, consideration of the chemistry of the attachment process indicates that isomerization destroys the alkene bond involved in the conjugation reaction. A possible coupled reaction could involve proton transfer from the sulfhydryl group to the C4-C5 alkene generating a tertiary carbocation intermediate that undergoes 1,3-hydride transfer in concert with sulfhydryl attack on $C3^1$ (Figure 3A). The double bond shift produces a thermodynamically favorable product with the nascent tetrasubstituted alkene bond in conjugation with the amide carbonyl.

Dehydratase–isomerases involved in anaerobic lipid metabolism

Unsaturated fatty acids are indispensable for most forms of life. While eukaryotes utilize dioxygen to desaturate fatty acids, prokaryotic organisms living in anaerobic conditions evolved a dioxygen-independent biosynthetic pathway to convert saturated fatty acids into

unsaturated ones [37]. This anaerobic pathway employs fatty acid desaturase enzymes, known as β-hydroxydecanoyl thiol ester dehydratases (EC 4.2.1.59), that introduce *cis* alkene bonds into the acyl chain by dehydration [38] (Figure 3B). These enzymes are symmetric dimers adopting a "hot dog" fold featuring a long α-helix (the "hot dog") surrounded by β-strands (the "bun") as well as a tunnel-shaped active site pocket made up of residues from both chains of the dimer [39]. The catalytic His residue (His70 in *E. coli* FabA) is localized roughly at the midpoint of the tunnel. Interestingly, some members of this protein family are endowed with dehydratase-isomerase activity whereas others display only dehydratasee activity. For example, the FabN (dehydratase-isomerase) and FabZ (dehydratase) proteins from *Enterococcus faecalis* exhibit differing catalytic activities despite their relatively high sequence identity (57.4%) including at sites constituting the catalytic center [40]. This led to the hypothesis that differences outside of the primary catalytic sphere could determine the specific activity exhibited by these enzymes. To probe this idea, FabN/FabZ chimeric proteins were constructed and tested for hydratase and isomerase activity [40]. Consistent with the hypothesis, introduction of β-strands 3 and 4 from FabN in place of the corresponding regions of FabZ transformed this enzyme into a dehydratase-isomerase. Conversely, no direct active site substitutions led to the same transformation. Structurally, the newly acquired isomerase activity was explained by a change in the conformation of the central α-helix exerted by the β-strands. This change altered the shape of the active site tunnel placing the *trans*-2 substrate in an appropriate conformation for isomerization to occur [41]. Hence, like bilin lyase-isomerase, active site steric factors play key roles in triggering allylic rearrangement in the bacterial fatty acid dehydratases.

Anaerobic degradation of terpenes by bacteria is another process involving an enzyme exhibiting *in situ*-coupled isomerase activity. **Terpenes** are ubiquitous lipidic compounds that, among many other roles, serve as carbon sources for bacterial growth. However, their breakdown under anaerobic conditions is challenging as many catabolic pathways rely on the use of dioxygen to initiate the conversion of terpenes to more polar metabolites [42, 43]. Linalool dehydratase-isomerase (LinD, EC 4.2.1.127) overcomes this limitation by using water instead of dioxygen for monoterpene functionalization. This pentameric enzyme interconverts three different terpenes known as geraniol, *S*-linalool, and β-myrcene (Figure 3C) using an active site embedded within an $(\alpha,\alpha)_6$-barrel fold [44, 45]. Although LinD shares little or no sequence similarity with any known superfamilies, its overall structure resembles that of the β-domain of class II terpene synthases [44, 45]. The LinD active site features a mixture of polar and apolar side chains together with a pair of Cys residues that are capable of undergoing disulfide bond formation (Figure 3D) [46]. The roles of these active site residues were investigated through site-directed mutagenesis studies as well as computational chemistry approaches, leading to the proposal of two different catalytic mechanisms for the conversion of *S*-linalool into geraniol or β-myrcene. In the first, the dissociation of the C3 hydroxyl group is accompanied by the attack of the Cys180 thiolate on the terminal alkene moiety giving rise to a covalent thioether intermediate that decomposes either by water attack or proton abstraction to give geraniol or β-myrcene, respectively [44]. In the second, dissociation of the tertiary hydroxyl group produces a carbocation that is quenched by proton shuffling or attack of water on C1 to give geraniol or

β-myrcene, respectively [44]. Subsequent data that failed to show a covalent intermediate as well as the high computed activation energy for the formation of the thioether intermediate, suggest that the carbocation-based mechanism is more plausible [47]. Despite extensive mutagenesis of LinD active site residues, no substitutions have been found that selectively abolish one or the other activity. However, these studies uncovered a reaction pathway leading directly to β-myrcene from geraniol without the *S*-linalool intermediate, revealing a non-linear mode of LinD catalysis [44, 47].

Enzymes catalyzing oxidoreductase chemistry together with structural isomerization.

We noted a group of phylogenetically unrelated enzymes that catalyze a type of structural isomerization known as a semipinacol rearrangement [48] coupled with oxidoreductase chemistry. These reactions all involve a substrate or intermediate possessing a tertiary alcohol adjacent to an electrophilic carbon center (Figure 4A). Ketol-acid reductoisomerase (KARI, E.C. 1.1.1.86), is an enzyme involved in the biosynthesis of valine, leucine, and isoleucine that requires NADPH and divalent cation cofactors to support its dual catalytic activity [49, 50]. The importance of this enzyme for branched chain amino acid synthesis in plants, bacteria, and fungi have made it a target for antimicrobial and herbicidal agent development [51]. Crystal structures and biochemical studies have established that KARI possesses a binuclear metal center participating in catalysis by direct coordination of its substrates [50, 52], either α-acetolactate or α-aceto-α-hydroxybutyrate, and by providing bound solvent for acid-base chemistry [53, 54]. In the first reaction step, a Mg^{2+} at metal site 2 coordinates the hydroxyl-keto functionality, enhancing the electropositivity of the carbonyl carbon and facilitating deprotonation of the hydroxyl group, both of which trigger the 1,2-alkyl rearrangement step of the reaction. In the second step, polarization of the newly formed keto group by the divalent cation at metal site 1 facilitates hydride transfer from the reduced dinucleotide cofactor to the carbonyl carbon. It is established that the first reaction has a selective requirement for a Mg^{2+} cofactor, while the subsequent reduction step can be catalyzed with Mg^{2+}, Mn^{2+}, or Co^{2+} serving as effective cofactors [49]. Notably, the hypothetical isomerized intermediate could not be isolated in free form during the enzyme-catalyzed reaction [55], which suggests that the normal reaction sequence involves a tightly coupled isomerase-reduction reaction. However, external addition of the isomerized intermediate, 3-hydroxy-3-methyl-2-oxobutyrate, to KARI results in the correct final product, α,β-dihydroxy-β-methylvalerate [49], indicating that the isomerized intermediate could exist as a discrete, albeit transient, intermediate. The overall reductoisomerase reaction is reversible; however, determination of the *isomerization* equilibrium constant showed a strong thermodynamic preference for the α-acetolactate starting substrate [56]. These data indicate that coupling of the isomerization and reduction reactions is necessary to ensure productive formation of the final dihydroxy acid products. Indeed, active site substitutions that preserve reduction chemistry but abolish isomerization have been documented but not vice versa, indicating that oxidoreductase activity drives isomerization [56].

A second enzyme operating via a semipinacol rearrangement is the flavin adenine dinucleotide (FAD)-dependent monooxygenase, AuaG, which is involved in the biosynthesis of a class of prenylated quinoline alkaloid antibiotics known as **aurachins** [57]. These compounds exhibit antimicrobial activity due to their respiratory chain inhibitory activity

[58]. AuaG oxidizes and rearranges aurachin C *via* a type-3 semipinacol rearrangement mechanism. This involves monooxygenation of an alkene functionality producing an epoxide intermediate that then breaks down with an accompanying 1,2-shift of the prenyl group to form the product (Figure 4B). Details about the active site features that promote the isomerization remain to be revealed. However, it is known that the isomerized product is not the favored state of the rearrangement equilibrium [57] suggesting that the energy of epoxidation and specialized active site features are used to drive the uphill isomerization. Moreover, formation of the final product of the pathway, aurachin B, requires immediate processing of the product of AuaG catalysis due to its unstable nature [57].

Another enzyme catalyzing a reaction resembling that of KARI is 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase (DXR, EC 1.1.1.267) [59].This enzyme catalyzes the rate-limiting step of the non-mevalonate **isoprenoid pathway** *via* a mechanism that involves isomerization followed by reduction of its substrate (DXP) thereby producing 2-*C*-methyl-D-erythritol 4-phosphate (Figure 4C) [59]. Owing to the key role for this pathway in some pathological organisms and its absence in humans, inhibition of DXP reductoisomerase has been heavily pursued as a strategy in the development of novel antibacterial and anti-malarial agents [60]. Like KARI, DXR uses divalent cation and NAPDH cofactors to catalyze the reaction. Unlike KARI, DXR contains a single metal-binding site that can productively use Mg^{2+}, Mn^{2+}, and Co^{2+} as cofactors [61-63]. Although several potential catalytic mechanisms for DXR have been proposed, including a semipinacol arrangement, isotope labeling experiments revealed that the rearrangement proceeds through a retro aldol-aldol type mechanism [64] with DXP likely coordinating the metal cofactor through its C3 and C4 hydroxyl groups [65]. Reminiscently of KARI, the isomerized intermediate (2-*C*-methyl-D-erythrose 4-phosphate) does not detectably accumulate during catalysis, although it is a competent substrate for the reduction reaction when added exogenously [66]. This suggests that the two reactions are tightly coupled. The DXR-catalyzed reaction, like that of KARI, is reversible. Although the 2-*C*-methyl-D-erythritol 4-phosphate product is strongly favored thermodynamically, the equilibrium of the isomerization half-reaction lies heavily on the reactant side of the equation, suggesting that the reductive reaction coupling is employed to make the overall reaction thermodynamically favorable [66].

Concluding remarks.

In this article, we aimed to bring together biochemical and structural information on a diverse set of enzymes that all catalyze isomerization combined with a second reaction type within a single active site. In doing so, we sought to reveal mechanistic principles that can be applied to yet-to-be characterized enzymes catalyzing related transformations. We found that these enzymes participate in diverse biological processes but are frequently involved in terpenoid-related biochemistry and often catalyze alkene bond transformations, likely due to the ease of alkene bond shuffling that can occur in these conjugated terpenoid compounds. In many cases the coupled reaction provides a pathway to overcome kinetic or thermodynamic barriers to substrate isomerization, which can occur before, after, or coincident with the coupled reaction. Additionally, we noted examples in which similar isomerization-linked reaction pathways have been achieved through distinct evolutionary routes. Structural studies in combination with site-directed mutagenesis experiments have,

in some cases, been successful in providing a molecular understanding of the specific active site regions responsible for the distinct reaction chemistry. These studies have shown that active site steric factors are frequently pivotal in driving isomerization chemistry. Taken together, structural biology approaches together with classical enzymology and computational chemistry have provided a comprehensive and detailed understanding of the "hows and whys" of some of these coupled reactions. However, there are several reactions in Table 1 that remain to be fully characterized, indicating that there is much more work to be done in this area (see Outstanding Questions). Beyond isomerases, there are other examples of enzymes catalyzing *in situ*-coupled reactions, some of which are familiar and mechanistically well understood. These include malate dehydrogenase (EC 1.1.1.38, oxidoreductase and lyase activities) [67], phosphoenolpyruvate carboxykinase (EC 4.1.1.32, lyase and transferase activities) [68], glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, oxidoreductase and transferase activities) [69], methionine adenosyltransferase (EC 2.5.1.6, transferase and hydrolase activities) [70], and firefly luciferase (EC 1.13.12.7, oxidoreductase, lyase, and hydrolase activities) [71], which illustrate complexities of enzyme functionality that challenge singular reaction-based classification. We expect that a comparative enzymology analysis, like the one performed in this article, might be fruitfully employed to gain a deeper mechanistic comprehension of enzymes catalyzing other types of *in situ*-coupled reactions.

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Glossary

Enzyme Commission (EC) system:
A classification system for enzymes based on the chemical reactions they catalyze. Enzymes are categorized according to a four-part numerical code. The first digit specifies the main reaction class while the other digits further specify the reaction properties such as substrates involved, cofactor or co-substrate usage, and reaction stoichiometry.

***In situ*-coupled reaction:**
A term we use to define an enzyme-catalyzed reaction involving two or more EC reaction classes occurring in a joint or integrated fashion within a single active site. "*In situ*" is included to distinguish this usage from the many other meanings of "coupled reaction" used in enzymology.

Photoisomerization:
A process in which a photon induces structural rearrangement of a molecule, resulting in the formation of a different isomer.

Geometric isomerization:

A type of isomerization in which there is a change in the spatial arrangement of atoms at bonds with restricted rotatability (*i.e.*, a double bond or single bonds within a ring structure). Also known as *cis-trans* or *E-Z* isomerization.

Kinetic crystallography:
A time-resolved crystallography method that studies dynamic processes, such as enzyme catalysis, occurring within the crystalline state.

Isoprenoid pathway:
Biosynthetic pathways for production of the isoprenoid (terpenoid) building blocks, isopentenyl pyrophosphate and dimethylallyl pyrophosphate. There are two distinct pathways: the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) or non-mevalonate pathway.

Phycobiliproteins:
Protein-linear tetrapyrrole complexes found in certain photosynthetic organisms that function within auxiliary photosynthetic complexes called phycobilisomes to capture light energy and transfer it to chlorophyll for photosynthesis.

Terpenes:
A large group of natural products produced by enzymatic polymerization of isoprene (C_5H_8) units. They are further classified based on the number of isoprene units that they contain. Also known as isoprenoids.

Aurachins:
Bioactive prenylated quinoline alkaloids produced by myxobacteria that act as antimicrobials.

Visual chromophore:
11-*cis*-retinaldehyde or one of its 3-hydroxy, 4-hydroxy, or 3,4-dehydro derivatives. These molecules are conjugated to an opsin molecule forming what is known as a visual pigment, which is used for light perception.

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Box 1:

Enzyme classification: How to best accommodate multi-catalytic enzymes?

The Enzyme Commission (EC) classifies enzymes according to the general type of *net* reaction they catalyze. Seven reaction classes have been defined to date: Class 1: oxidoreductases, Class 2: transferases, Class 3: hydrolases, Class 4: lyases, Class 5: isomerases, Class 6: ligases, and Class 7: translocases. The existence of enzymes that act on a given substrate in more than one way sequentially or simultaneously, was recognized even in the earliest efforts to classify enzymes [72]. Long-known examples include malate dehydrogenase (EC 1.1.1.38), and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). More recently recognized dual-acting isomerases (EC 5.6.x.x) are molecular machines that hydrolyze ATP to drive the “isomerization” of polypeptide or nucleic acid structures into new conformations.

The multi-catalytic nature of these enzymes leads to an ambiguity in how they should be named and classified. This problem was recognized early on by Hoffmann-Ostenhof, who suggested that the enzyme name reflect all of the operative activities [72]. This convention has been frequently adopted in assigning trivial names to enzymes (*e.g.*, reductoisomerase). IUBMB systematic naming does not permit such combined names. Instead, other activities are then specified in parentheses at the end of the systematic name, *e.g.*, glyceraldehyde-3-phosphate dehydrogenase (phosphorylating). Thus, a decision needs to be made to which main EC class the enzyme should be placed. There are at least two different approaches in use. The first, which represents the recommendation of the IUBMB, is that *“classification and naming of the enzyme should be based on the first enzyme-catalyzed step that is essential to the subsequent transformations.”* A second approach is to place the enzyme in the main EC category that best reflects its “primary” function. In the case of the ATP-driven machines, this led to their reclassification as isomerases (EC 5) even though ATP hydrolysis (EC 3) is the essential first step of the catalytic cycle. A difficulty with the latter approach to enzyme nomenclature is that multiple activities could all have equal functional primacy. Although neither classification and nomenclature system is completely unambiguous, the original system defined by the IUBMB may be the clearest and mechanistically most informative. The nomenclature and classification of multi-catalytic enzymes will undoubtedly continue to evolve with the discovery of new enzymes and changing researcher priorities.

Highlights

- The biosynthesis of 11-*cis*-retinoids by the isomerohydrolase RPE65 and isomerooxygenase NinaB has recently been described in molecular detail revealing an unexpected functional convergence of their isomerase activities
- Recent structure-function studies have elucidated the mechanisms of other enzymatically catalyzed reactions that involve isomerization coupled with a second reaction type occurring within a single active site.
- Through comparative enzymology analysis, we find that enzymes catalyzing these *in situ*-coupled reactions participate in diverse biochemical pathways but are often involved in terpenoid metabolism and frequently catalyze alkene bond transformations such as *cis-trans* isomerization or allylic rearrangements. Active-site steric factors are often pivotal in driving the isomerization reaction.
- We draw attention to issues surrounding classification and nomenclature of multi-acting enzymes.

Outstanding Questions

- What is the mechanism and structural basis for reaction coupling in other coupled isomerases that have not yet been subjected to detailed structure-function studies?
- How prevalent are other types of in *situ*-coupled reactions, and are there unifying mechanistic principles operative in the enzymes catalyzing such reactions?
- What is the best approach for naming multi-catalytic enzymes within the framework of the EC system?

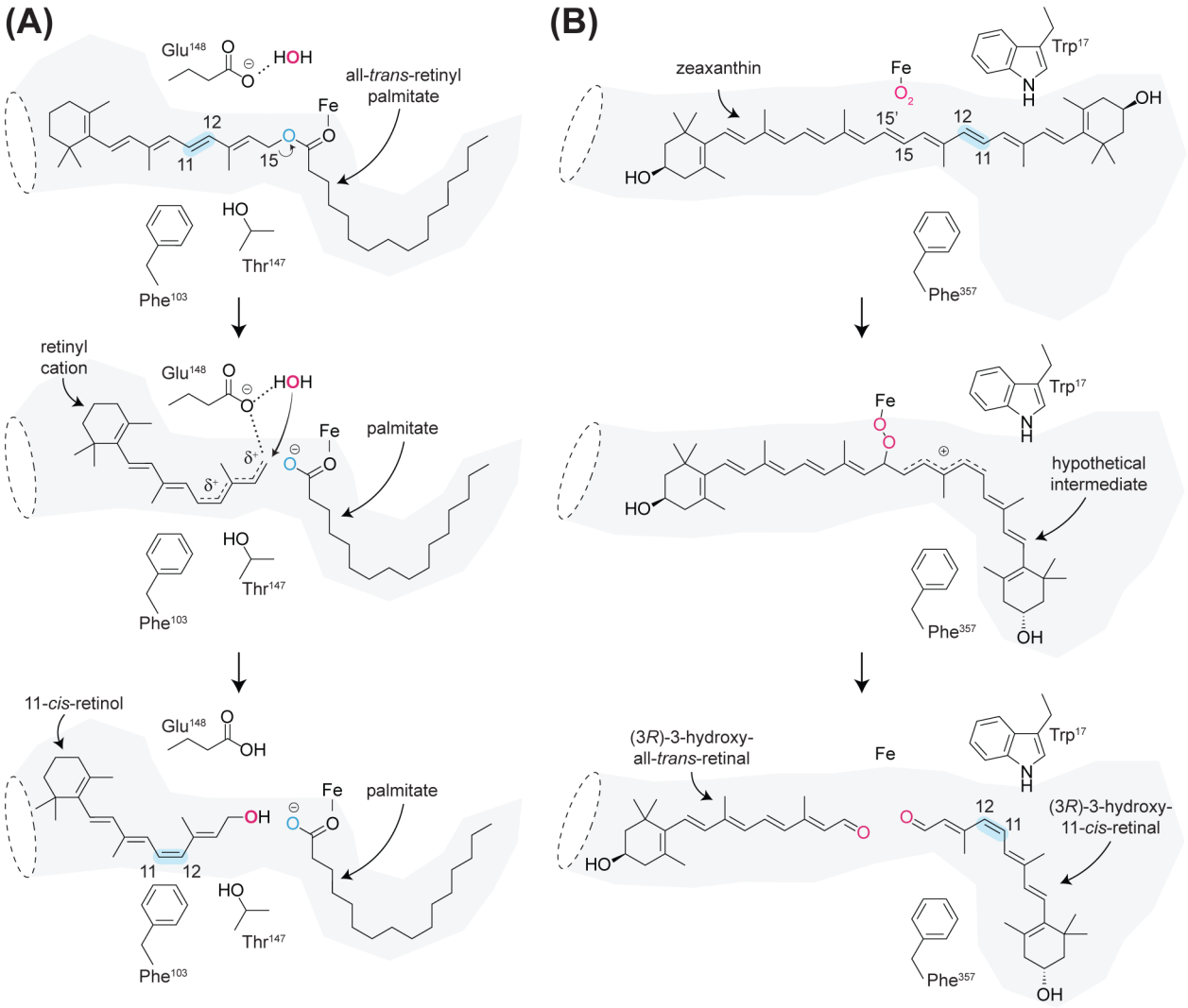

Figure 1.

Structure-based reaction mechanisms for 11-*cis*-retinoid-forming CCDs. (**A**) Mechanism proposed for RPE65 showing polarization for the ester group by the iron center (top), cleavage of the C15-O bond and formation of a retinyl cation intermediate that allows *trans-cis* isomerization to occur (middle), and attack of water on C15 to produce the 11-*cis*-retinol product (bottom). Phe103, Thr147, and Glu148 participate in the reaction through stabilization of the carbocation intermediate. (**B**) Mechanism proposed for NinaB in which attack of dioxygen on the C15-C15' alkene bond of zeaxanthin (top) produces a carbocation (or radical cation) intermediate (middle) that enables *trans-cis* isomerization to take place. Completion of the alkene oxidation reaction produces the all-*trans*- and 11-*cis*-retinal products (bottom). Trp17 and Phe357 are key residues enabling the *trans-cis* isomerization activity in NinaB. Note the differing sites of *trans-cis* isomerization in these homologous enzymes.

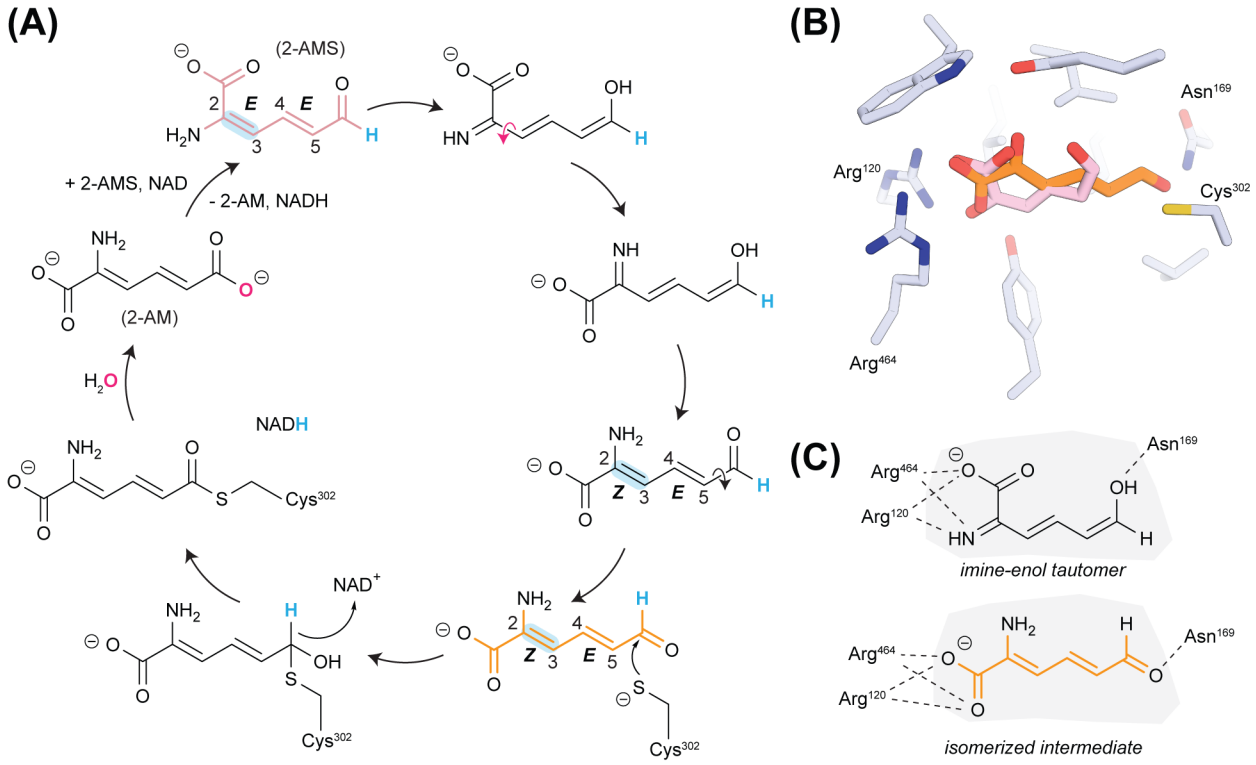

Figure 2.

Structure-based mechanism for dehydrogenase-isomerase, AMSDH. (**A**) Catalytic cycle of AMSDH starting at the top left with the 2E, 4E isomer of 2-AMS (pink lines). A tautomerization-associated double bond shift allows rotation of the C2-C3 bond to a Z-like conformation (illustrated with a magenta arrow). Return of the original double bond structure allows rotation of the C5-C6 bond (illustrated with a black arrow), positioning the aldehyde (structure shown in orange sticks) for attack by the Cys302 thiol moiety and NAD+-dependent oxidation to form the final (2*Z*,4*E*)-2-aminomuconate product. For clarity, NAD^+ is omitted from the steps in which it is not involved. (**B**) Crystal structures of AMSDH in complex with the 2-AMS substrate shown as pink sticks (wild-type enzyme, PDB accession code: 4I2R) as well as the isomerized substrate (orange sticks) obtained following a 5 min soak of 2-AMS with crystals of N169D mutant protein (PDB accession code: 5KLL). Key residues involved in acid-base and redox chemistry are shown as sticks. (**C**) Schematic showing the proton donor/acceptor groups that act via a ‘pitcher and catcher’ mechanism to tautomerize the substrate allowing *E-Z* isomerization.

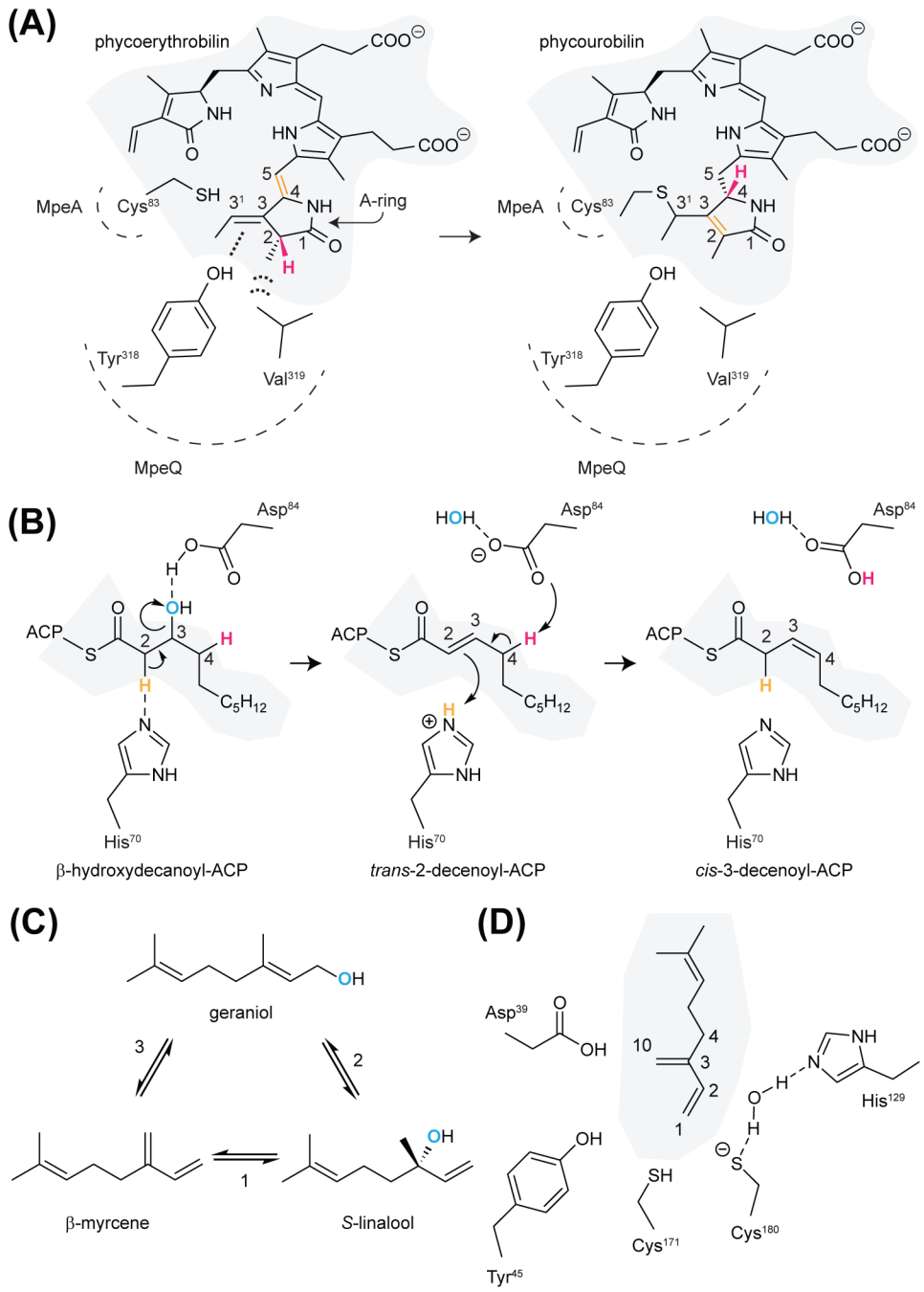

Figure 3.

Structure-based mechanisms for lyase-isomerases. (**A**) Mechanism proposed for MpeQ where the attack of MpeA-Cys83 on the reactive alkene group of phycoerythrobilin (facilitated by MpeQ-Tyr318-mediated polarization) is accompanied by a shift in the location of the A-ring chiral center from C2 to C4. This shift is triggered in part by the steric influence of the Val319 side chain on the A-ring methyl group. This change in double bond conjugation alters the absorbance properties of the bilin chromophore. (**B**) Mechanism proposed for the dehydration and alkene bond shift catalyzed by β-hydroxydecanoyl

thioester dehydratase-isomerase. The dehydration is accomplished through acid-base chemistry involving His70 and Asp84 side chains. The subsequent allylic rearrangement is promoted by a curved structure of the active site in vicinity of the C3-C4 bond with proton reshuffling involving the same His and Asp residues. (**C**) Interconversion of the isoprenoid compounds β-myrcene, *S*-linalool, and geraniol is catalyzed by the (de)hydratase-isomerase, LinD. (**D**) A depiction of the orientation of β-myrcene with respect to catalytically-relevant residues in the LinD active site. With reference to the scheme in panel C, reaction 1 involves protonation of C10 by Asp39, generating a tertiary carbocation intermediate that is quenched by attack of water on C3. Reaction 2 involves acid-base-catalyzed isomerization of the hydroxyl group from C3 to C1. Reaction 3 is a dehydration reaction accompanied by acid-base chemistry to generate β-myrcene. Geraniol is believed to be the physiological product of LinD that is subject to downstream catabolism.

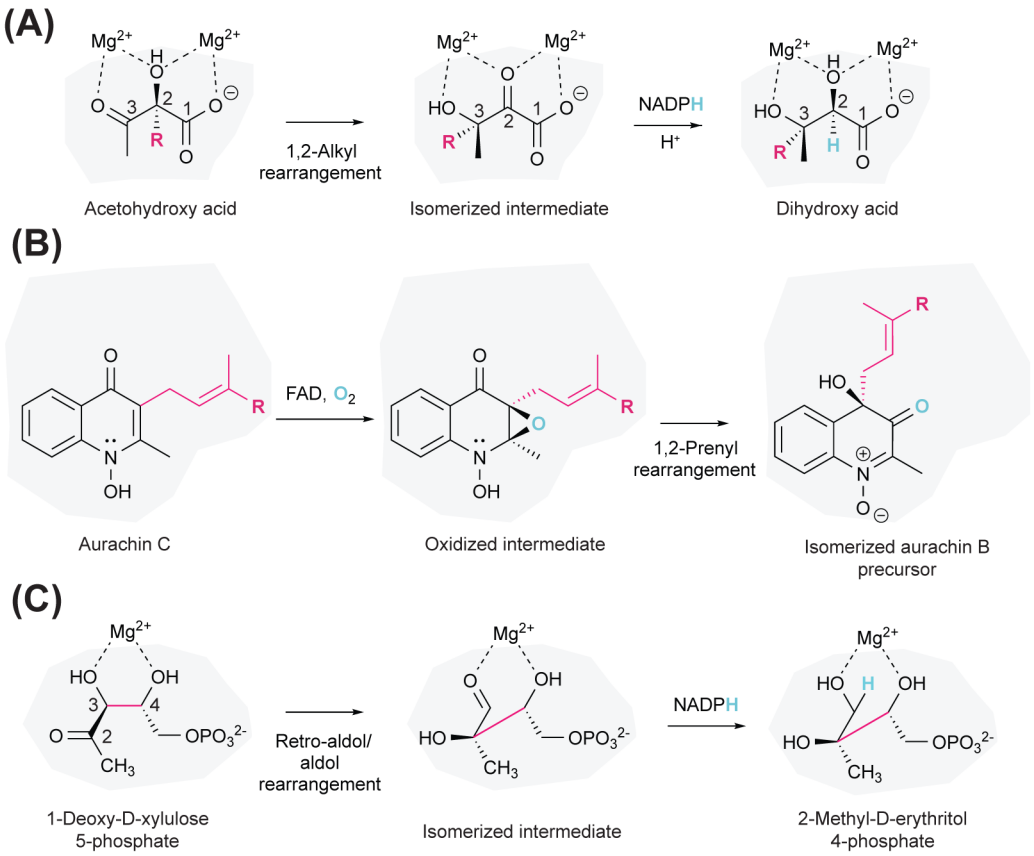

Figure 4.

Alkyl migration reactions coupled with oxidoreductase chemistry. (**A**) Reaction mechanism proposed for KARI involving a Mg^{2+}-catalyzed alkyl rearrangement followed by NADPH-dependent reduction to form the final product. R – CH_3 or CH_2CH_3. (**B**) Reaction mechanism proposed for AuaG involving FAD-dependent alkene oxidation of aurachin C followed by 1,2-migration of the prenyl moiety. R = CH_2-geranyl. The reactions in (A) and (B) are both types of semipinacol rearrangements. (**C**) Reaction mechanism proposed for DXR involving a retro-aldol/aldol rearrangement of 1-deoxy-D-xylulose 5-phosphate followed by NADPH-dependent reduction to generate the product, 2-methyl-D-erythritol 4-phosphate.

Table 1:

Examples of enzymes classified outside of EC group 5 that catalyze isomerization as part of their overall reactions.[†]

EC number	Accepted name	Biochemical pathway	Type of isomerization	Cofactors involved	Reaction sequence>*	Representative structure (PDB code)	Key references
1.1.1.86	ketol-acid reductoisomerase (KARI); *aka*, acetohydroxy acid isomeroreductase	Branched chain amino acid biosynthesis	Structural (methyl shift, semipinacol rearrangement)	NADP(H), binuclear Mg2+ (Mn2+, Co2+)	Isomerization + Reduction	1YVE	[49, 50, 52, 56]
1.1.1.145	3β-hydroxy-Δ5-steroid dehydrogenase	Sex hormone biosynthesis	Structural (C-C double bond shift)	NAD(H)	Oxidation → Isomerization	N/A	[73, 74]
1.1.1.267	1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)	Isoprenoid biosynthesis	Structural (retro aldol-aldol rearrangement	Mononuclear Mg2+ (Mn2+, Co2+)	Isomerization + Reduction	1Q0Q	[59, 61-63]
1.1.1.421	D-apionate oxidoisomerase	Sugar metabolism	Structural (unknown mechanism)	NAD(H) Zn^{2+}/Ca^{2+} ?	Unknown	N/A	[75]
1.2.1.32	2-aminomuconate-6-semialdehyde dehydrogenase (AMSDH)	Kynurenine pathway	Geometric (*E-Z*)	NAD(H)	Isomerization → Oxidation	5KLL	[23, 27]
1.13.11.65	carotenoid isomerooxygenase (NinaB)	Retinal/11-cis-retinal biosynthesis	Geometric (trans-cis)	Mononuclear Fe^{2+}	Oxidation + Isomerization	8FTY	[20, 21]
1.14.13.222	Aurachin C monooxygenase/ isomerase (AuaG)	Aurachin biosynthesis	Structural (semipinacol rearrangement)	FAD(H_2) NAD(H)	Oxidation → Isomerization	N/A	[57]
2.4.99.17	S-adenosylmethionine:tRNA ribosyltransferase-isomerase (QueA)	Queuosine biosynthesis pathway	Structural (sugar ring rearrangement)	None known	Unknown	1VKY	[76, 77]
3.1.1.64	retinoid isomerohydrolase (RPE65)	11-*cis*-retinal biosynthesis (visual cycle)	Geometric (trans-cis)	Mononuclear Fe^{2+}	Hydrolysis + Isomerization	4RSE	[15, 78, 79]
3.1.3.77	acireductone synthase	Methionine salvage pathway	Structural (enolization)	Mononuclear Mg^{2+}	Unknown	1ZS9	[80]
3.5.99.6	glucosamine-6-phosphate deaminase (NagB)	Glucosamine metabolism	Structural (sugar rearrangement)	None	Isomerization → deamination (reversible)	2RI0	[81, 82]
4.2.1.59	β-hydroxydecanoyl thiol ester dehydratase (FabA)	Unsaturated fatty acid biosynthesis	Structural (allylic rearrangement)	None	Dehydration → Isomerization	1MKA	[39, 40]
4.2.1.127	linalool dehydratase (LinD)	Anaerobic isoprenoid catabolism	Structural (allylic rearrangement and hydroxyl migration)	None	Hydration → Isomerization (reversible)	5G1U	[44, 46, 47]
4.2.3.6 (others within 4.2.3.X)	trichodiene synthase (class II terpene synthases)	Terpenoid biosynthesis	Geometric (*trans-cis*) and structural (rearrangements)	Trinuclear Mg^{2+} (Mn^{2+})	PP_idissociation → isomerization → cyclization	1JFA	[83, 84]
4.4.1.31	phycoerythrocyanin α-cysteine-84	Phycobiliprotein synthesis	Structural (C-C double bond shift)	None	Conjugation + Isomerization	7MC4	[36]

EC number	Accepted name	Biochemical pathway	Type of isomerization	Cofactors involved	Reaction sequence>*	Representative structure (PDB code)	Key references
	phycoviolobilin lyase/ isomerase (MpeQ)				(not fully resolved)		

† Search terms used within ExplorEnz [85] to identify dual-activity isomerases: “isomeroreductase”, “reductoisomerase”, “/isomerase”, “ATP-hydrolyzing”, “ATP-hydrolysing”, “Isomero”, “lyase/isomerase”, “isomerizing”. Additional examples were identified through primary literature searches.

* The order of transformations is given in the known or presumed physiological direction. An arrow indicates that the reactions occur in sequence, whereas as “+” sign indicates the reactions are believed to be tightly coupled or concerted. Underlined transformations enable or drive the coupled transformation in the case of irreversible or weakly reversible reactions.