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Celine Eidenschenk
Genentech

Lionel Cheruzel
San Jose State University, lionel.cheruzel@sjsu.edu

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Review article

Ru(II)-diimine complexes and cytochrome P450 working hand-in-hand

Celine Eidenschenk[a], Lionel Cheruzel[b,*]

[a] Department Biochemical and Cellular Pharmacology, Genentech, One DNA Way, South San Francisco, CA 94080, USA
[b] San José State University, Department of Chemistry, One Washington Square, San José, CA 95192-0101, USA

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ABSTRACT

With a growing interest in utilizing visible light to drive biocatalytic processes, several light-harvesting units and approaches have been employed to harness the synthetic potential of heme monooxygenases and carry out selective oxyfunctionalization of a wide range of substrates. While the fields of cytochrome P450 and Ru(II) photochemistry have separately been prolific, it is not until the turn of the 21st century that they converged. Non-covalent and subsequently covalently attached Ru(II) complexes were used to promote rapid intramolecular electron transfer in bacterial P450 enzymes. Photocatalytic activity with Ru(II)-modified P450 enzymes was achieved under reductive conditions with a judicious choice of a sacrificial electron donor. The initial concept of Ru(II)-modified P450 enzymes was further improved using protein engineering, photosensitizer functionalization and was successfully applied to other P450 enzymes. In this review, we wish to present the recent contributions from our group and others in utilizing Ru(II) complexes coupled with P450 enzymes in the broad context of photobiocatalysis, protein assemblies and chemoenzymatic reactions. The merging of chemical catalysts with the synthetic potential of P450 enzymes has led to the development of several chemoenzymatic approaches. Moreover, strained Ru(II) compounds have been shown to selectively inhibit P450 enzymes by releasing aromatic heterocycle containing molecules upon visible light excitation taking advantage of the rapid ligand loss feature in those complexes.

1. Introduction

The heme-thiolate cytochrome P450 enzymes have attracted great attention due to their unique ability to activate molecular dioxygen to carry out selective oxyfunctionalization of a wide range of substrates [1]. They have emerged as valuable biocatalysts [2,3] for the synthesis of pharmaceuticals [4], the late stage diversification of natural products [5] and lead compounds in important biomedical [6], biotechnological [7] and industrial [8] applications. Moreover, several P450 enzymes are amenable to protein engineering using rational design or directed evolution approaches [9–11] as to confer unique properties and broad substrate scope and recently to expand their chemical reaction space towards abiological reactions [12].

The delivery of the necessary reducing equivalents has been a focal point as it often involves diverse redox partners [13] complementing the rich superfamily of P450 enzymes. These partners play a crucial role in the catalytic mechanism and the delivery of electrons, one at a time, towards a productive pathway. Misuse of the electrons leads to uncoupled pathways and release of reactive oxygen species detrimental to the overall process [14,15]. The archetypical system is the P450 BM3 holoenzyme from *Bacillus megaterium* [16] where the redox partner is fused to the heme domain in a single polypeptide resulting in a highly coupled system and the highest catalytic rate observed in the selective oxidation of long chain fatty acids. In order to address some of the limitations associated with electron delivery, redox-partners or cofactor dependence in many P450 enzymes, a myriad of alternative approaches have encompassed chemical [17,18] and electrochemical [19] reductions, cofactor regeneration [20,21], design of fusion proteins [22], use of peroxides via the peroxide shunt [23] as well as recently the use of light-harvesting units to activate various P450 enzymes [24].

Harnessing visible light to activate biocatalytic processes is currently gathering a lot of interest [25,26] and several light harvesting units have been employed to activate heme monooxygenases for the selective oxyfunctionalization of substrate C—H bonds. The focus herein is on the use of the inorganic Ru(II)-diimine complexes in conjunction with P450 enzymes. These metal complexes have been extensively investigated due to the unique nature of the excited state and the ability to initiate various single electron transfer events [27–29]. This review starts with a brief introduction on cytochrome P450 enzymes, the diverse photosensitizers used to activate monooxygenases or peroxygenases for selective C—H oxyfunctionalization, and the photochemistry of Ru(II) complexes. A description will follow on strategies to

* Corresponding author.
E-mail address: lionel.cheruzel@sjsu.edu (L. Cheruzel).

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rapidly inject electrons into P450 active site using bound or covalently attached complexes leading to efficient photocatalysis in the Ru(II)-modified P450 BM3 heme domain mutants under reductive conditions. The next section is dedicated to the existing P450 crystal structures complexed with their natural or artificial redox partners.

The concept of Ru(II) complexes coupled with P450 enzymes has been expanded in several directions which will be discussed in the context of current work on photobiocatalysis, protein assemblies, chemoenzymatic reactions and P450 inhibitions. Specifically, we would like to focus on five areas: 1) protein engineering and tuning of the Ru (II) photophysical properties for the development of the next generation of hybrid P450 enzymes; 2) introduction of various groups on the ancillary ligands to promote covalent dimerization and heterogeneous P450 biocatalysts; 3) determination of the coupling efficiency in the hybrid enzymes and a complimentary approach in the light-activation of P450 enzymes with peroxygenase activity; 4) merging of chemical catalysis with the unique synthetic potential and evolvability of P450 biocatalysts and 5) strained Ru(II) complexes that selectively inhibit P450 enzymes utilizing a rapid ligand photorelease feature.

2. Background

2.1. Cytochrome P450 enzymes

The heme thiolate enzymes have attracted the interest of the scientific community since their discovery in the early 1960s and the characterization of the red pigment in liver microsomes [30,31]. The following decades have seen a surge of attention from mechanistic, pharmaceutical, biophysical, protein engineering and biocatalytic applications. The reader is directed to some of the most recent reviews on those topics with a special emphasis on the unique P450 synthetic potential [2–7].

The reducing equivalents necessary for the dioxygen activation are provided by several redox partners organized in various classes using mostly NAD(*P*)H as cofactor [13]. The redox active centers in the electron transfer machinery comprise flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) flavins in P450 reductases and/or iron-sulfur clusters in various ferredoxins. The redox partners can either be soluble or membrane bound. The consensus mechanism includes well-characterized intermediates illustrated in Fig. 1 leading to the formation of the highly oxidative Compound I, a ferryl species with a porphyrin radical [32]. Disruption of the electron delivery leads to the formation of reactive oxygen species, such as superoxide or hydrogen peroxide, detrimental to the enzymes [14,15]. A small class of P450 enzymes is known to utilize hydrogen peroxide via the peroxide shunt pathway to sustain catalysis (Fig. 1) [23,33].

Due to their unique regio- and stereoselectivity, P450 enzymes have been of particular interest for biocatalytic applications. Protein engineering of highly mutation tolerant P450 enzymes has provided opportunities to expand their reaction space and substrate scope from small alkanes to large hydrophobic molecules as well as to diversify natural products and produce valuable drug metabolites. Beyond site-directed mutations and rational design, directed evolution has conferred unique properties to these enzymes [9–11] and recently enabled some abiological reactions [12,34]. Very innovative approaches have also emerged to complement protein engineering. Decoy molecules developed by Watanabe and Shoji are used to functionalize small substrates by tricking the P450 BM3 holoenzyme in a conformation primed for catalysis [35]. Engineering of substrate anchoring or directing group has been successfully employed to alter the regioselectivity of enzymatic hydroxylation [36–38]. Alternatively, Gillam and coworkers pioneered ancestor reconstruction methodology in a vertebrate CYP3 family to obtain mutants with enhanced thermal stability and broad substrate promiscuity [39]. The recent pursuit of light-harvesting units has enabled the photoactivation of several members of the P450 superfamily.

2.2. Light-harvesting units employed to power various heme monooxygenases

Over the last two decades, efforts to convert light energy into biochemical transformations have led to the use of various light-harvesting units, covering the visible range, to activate P450 enzymes or unspecific peroxygenases (UPOs) for the selective oxyfunctionalization of substrate C−H bonds. The structure and absorption maxima of the various complexes ranging from organic to inorganic complexes as well as biological, nanostructures and semi-conductors are summarized in Fig. 2.

To photoactivate P450 enzymes, three approaches have emerged [24]. First, the photosensitizer can interact with molecular dioxygen to generate reactive oxygen species that can be utilized for peroxygenase activity. The excitation of CdS quantum dots (Q.D.) has enabled the activation of the $P450_{BS\beta}$ peroxygenase immobilized on the nanostructures [40]. Urlacher and coworkers utilized various flavin mononucleotide (FMN) derivatives to activate P450 members of the CYP152A family [41]. Hollmann recently leveraged several acridine derivatives covering the visible range to activate an evolved unspecific peroxygenase from *Agrocybe aegerita* [42]. The same group also reported high total turnover numbers (> 60,000) in the light-driven oxyfunctionalization of ethylbenzene using a graphitic carbon nitride ($g\text{-}C_3N_4$) as photosensitizer and by physically separating the UPO enzyme [43]. Second, the reducing equivalents can be provided to the redox partners and, utilizing intrinsic electron transfer pathways, activate P450 heme domains. Deazaflavin, known to slowly react with molecular dioxygen in the reduced state, supported photocatalysis with the P450 BM3 holoenzyme but not with the heme domain [44]. Biological components, such as Photosystem I or II, have emerged as promising avenues [45]. Photosystem I in conjunction with a membrane-bound CYP79A1 fused with ferredoxin was shown to produce the desirable cyanogenic glucoside, dhurrin, upon visible light excitation [46]. The Bibby group repurposed the wasted electrons from water oxidation in an engineered cyanobateria to activate an heterologous CYP1A1 enzyme [47,48]. The third approach has consisted in supplying the electrons directly to the heme domain circumventing the need of redox partners or cofactors. By confining the CYP3A4 enzyme in a microporous ordered silica and with a 2,9,16,23-tetraaminophthalocyanine cobalt (CoTAPc) stacked onto reduced graphene oxide nanosheets, modest photocatalytic activity was achieved in the *O*-demethylation of 7-ethoxytrifluoromethyl coumarin [49]. In a whole cell approach, Eosin Y has been employed with P450 heme domain mutants to access several drug metabolites [50]. Our group has focused on the use of Ru(II)-diimine complexes which exhibit a strong, broad absorbance in the visible range to sustain P450 photocatalysis [51].

2.3. Ru(II)-diimine complexes

The d^6 $[Ru(LL)_3]^{2+}$ complexes with LL = polypyridyl ligands have been the most deeply investigated class of metal complexes due to their unique combination of chemical stability, excited-state reactivity, luminescence emission and excited-state lifetime (Fig. 3A) [27–29]. The long-lived excited state, $*[Ru(LL)_3]^{2+}$ can also encounter other solute molecules, quenchers, and participate in bimolecular processes such as energy transfer (1) and both reductive (2) and oxidative (3) electron transfers as shown in Fig. 3B. Thus, photoactivation of this complex has most commonly been used to drive redox processes.

These complexes have been extensively used in triggering fast electron transfers in metalloenzymes [52] and more recently in initiating radical processes in photoredox catalysis [53–55] and biocatalysis [56] to enable unique organic transformations. These complexes have also found important biological applications [57,58] in photodynamic therapy [59] with the local generation of singlet oxygen or by taking advantage of the rapid ligand dissociation via populating a thermally accessible metal-centered ([3]MC) excited state (Fig. 3A)

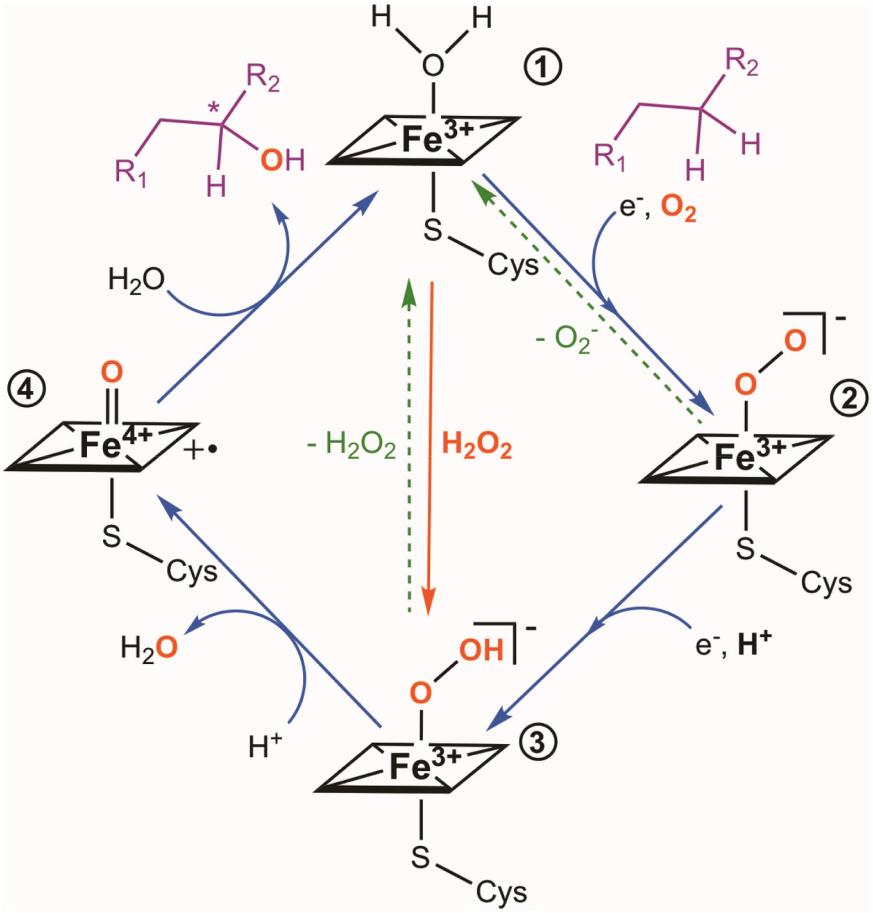

Fig. 1. Abbreviated P450 mechanism with well-characterized intermediates (**2–4**) in the oxyfunctionalization of unactivated C—H bonds, uncoupling reactions producing reactive oxygen species (green dashed lines) and peroxide shunt pathway (red arrow).

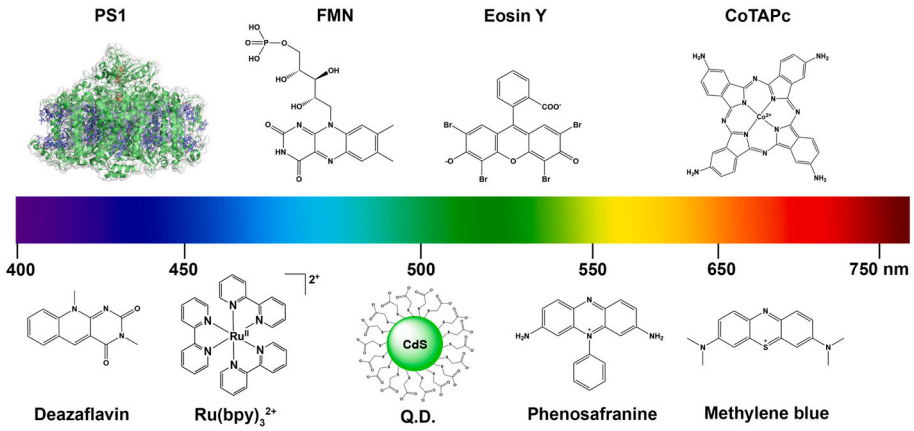

Fig. 2. The various light-harvesting units used to power heme enzymes for selective C—H oxyfunctionalization ranging from biological, photosystem I (PSI) to organic (flavin, deazaflavin, phenosafranine and methylene blue) to inorganic complexes (Ru $(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine) and 2,9,16,23-tetraaminophthalocyanine cobalt, CoTAPc) and nanostructures (quantum dots, Q.D.).

[60,61].

3. Hybrid P450 enzymes featuring covalently attached Ru(II) complexes

The field of P450 enzymes and Ru(II) converged around the turn of the century with the initial development of Ru(II) molecular wires designed to bind to the substrate access channel of cytochrome P450 and several heme-thiolate enzymes [62]. Notably, rapid nanosecond reduction of the ferric resting state was achieved in the P450cam heme domain [63]. However, other elusive intermediates couldn't be detected and photobiocatalysis couldn't be achieved until the Ru(II) complex was covalently attached to the heme domain of P450 BM3 mutants [64,65]. The covalent attachment was accomplished with sulfhydryl specific Ru (II) complexes bearing iodoacetamido or epoxy moieties on the ancillary ligands [66].

The Ru(II)-diimine functionalized P450 BM3 enzymes have been designed to use the photophysical properties of the Ru(II) excited state and stemmed from the vast study on intramolecular electron transfers in metalloenzymes [52]. The proof-of-concept establishing electronic communication between the photosensitizer and the light-harvesting unit came with the K97C-Ru_1 hybrid enzyme where the Ru_1 photosensitizer, bis(2,2'-bipyridine)(5-acetamidophenanthroline)Ru(II), Ru $(bpy)_2$PhenA (Ru1), was covalently attached to a non-native single

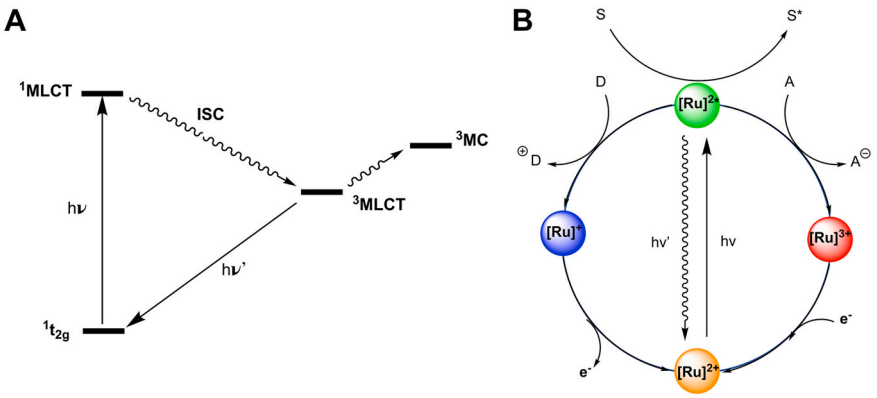

Fig. 3. A) Jablonski diagram and B) excited state properties of a typical Ru(II)-diimine complex.

cysteine residue (K97C) of a P450 BM3 heme domain mutant. Under flash quench oxidative conditions (Fig. 3B), rapid generation of a highly oxidative Ru(III) species led to the oxidation of the porphyrin ring followed by intramolecular reorganization to the elusive Compound II species, a Fe(IV)-OH species [64]. The presence of the tryptophan at position 96 is proposed to mediate and enhance the oxidative electron transfer steps through a hopping mechanism [67,68].

Building on these initial results, our group utilized the hybrid P450 enzymes in a reductive quenching approach to inject the necessary electrons, one electron at a time, into the heme active site and sustain photocatalysis (see Fig. 4) [51,69]. To this end, we screened a range of known natural reductive quenchers that would be suitable for photocatalytic activity in aerated aqueous buffer.

The simple anion, diethydithiocarbamate (DTC) met all the necessary requirements and allowed the production of hydroxylated products with the hybrid enzymes under flash quench reductive conditions. Modest photocatalytic activity was initially observed with the K97C-Ru_1 and Q397C-Ru_1 hybrid P450 BM3 enzymes [65]. We consequently explored various non-native single cysteine positions on the proximal side of the heme domain in the C and L helices region where the reductase is binding [69]. The position 407 in a bowl-like cavity enabled close access to the heme active site and rapid electron injections. The sL407C-Ru_1 mutant displayed exquisite photocatalytic activity surpassing any of the known artificial systems at the time with close to

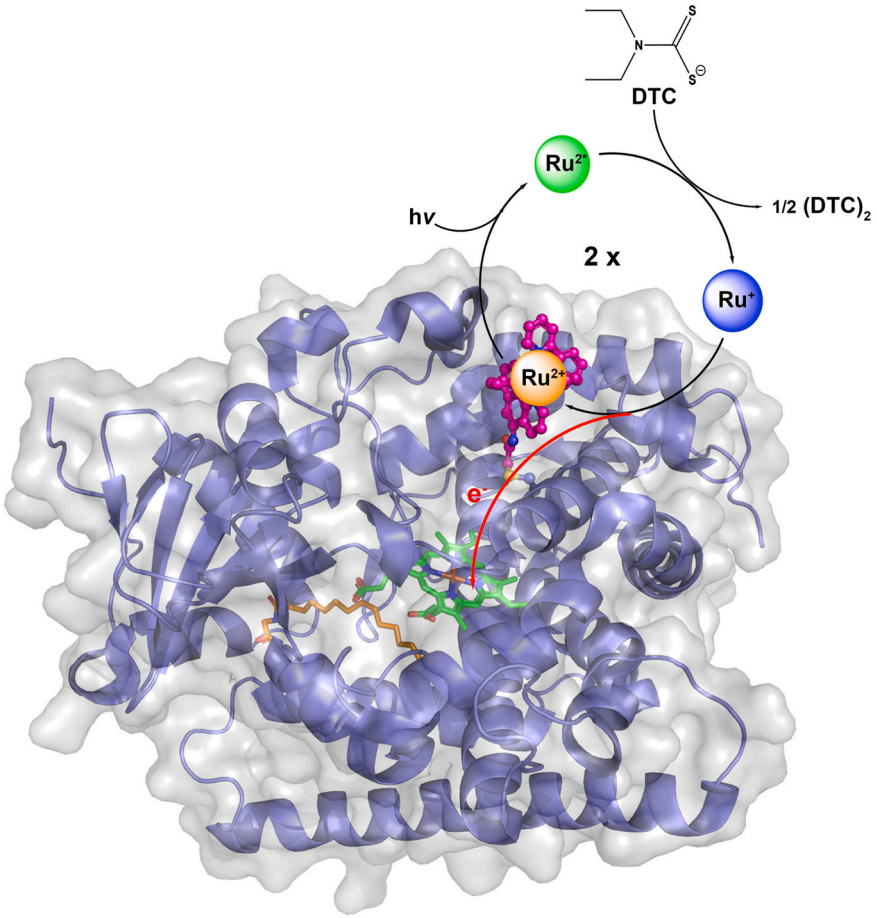

Fig. 4. Representation of the hybrid P450 BM3 enzyme (PDB ID: 5JTD [70]) featuring a Ru(II) complex covalently attached to activate P450 enzyme activity upon visible light excitation using the sacrificial electron donor, diethyldithiocarbamate (DTC).

1000 total turnover numbers in the hydroxylation of the natural substrate lauric acid [51]. Using transient absorption measurement, the kinetics for the electron transfer could be mapped out revealing electron transfer rate constant orders of magnitude faster than with natural redox partner enzymes [70].

4. Crystal Structures of P450 heme domains with their redox partners

Crystal structures of P450 heme domains interacting with their natural or artificial electron transfer partners have been highly pursued in order to gain insights into the proteins interface, their conformations and the key role of amino acids involved in the electron transfer pathway. The first such crystal structure was solved in 1999 by Poulos disclosing a proteolysed FMN domain complexed with the P450 BM3 heme domain [71]. Fourteen years later, a 1,6-bismaleimidohexane linker was successfully used, by the same group, to crosslink the redox partner with a P450cam heme domain revealing the crystal structure of a functional complex and details of the effector role of the putidaredoxin [72]. Later on, NMR [73] and double electron-electron resonance (DEER) studies [74,75] corroborated similar structures in solution. Meanwhile, two crystal structures of the Ru(II)-modified enzymes were solved showing the unique location of the photosensitizer on the proximal heme side (see Figs. 4 and 5). The distances between the two metal centers of 18 and 24 Å for the L407C-Ru_1 and K97C-Ru_1, respectively are well within single electron transfer distances [64,70]. In the L407C-Ru_1 structure, the electron transfer pathway was proposed to involve two highly conserved residues (Q403 and F393) and could be further enhanced by stacking aromatic residues as shown in the Q403W mutant [70]. Noteworthy, in most crystal structures in Fig. 5, a redox active tryptophan residue (i.e. W574 interacting with the flavin ring, W106 at the putidaredoxin/P450cam interface, W96 and Q403W in the hybrid enzyme structures) is present in close proximity to each redox center and likely promotes electron transfer. In 2018, the crystal structure of a Co(III) sepulchrate in electrostatic interaction with the P450 BM3 M7 heme domain variant was reported. The mediator compound was located at the entrance of the substrate channel [76] and the observed distance of 35 Å was prohibitively long for electron transfer. The location in the crystal structure was in contrast with previous molecular dynamic simulations, which predicted alternative binding sites [77] in support of the high activity observed with this system [17,18]. While the crystal structure provided insights into the role of mutated residues, further studies are needed to identify the molecular mediator-protein interactions.

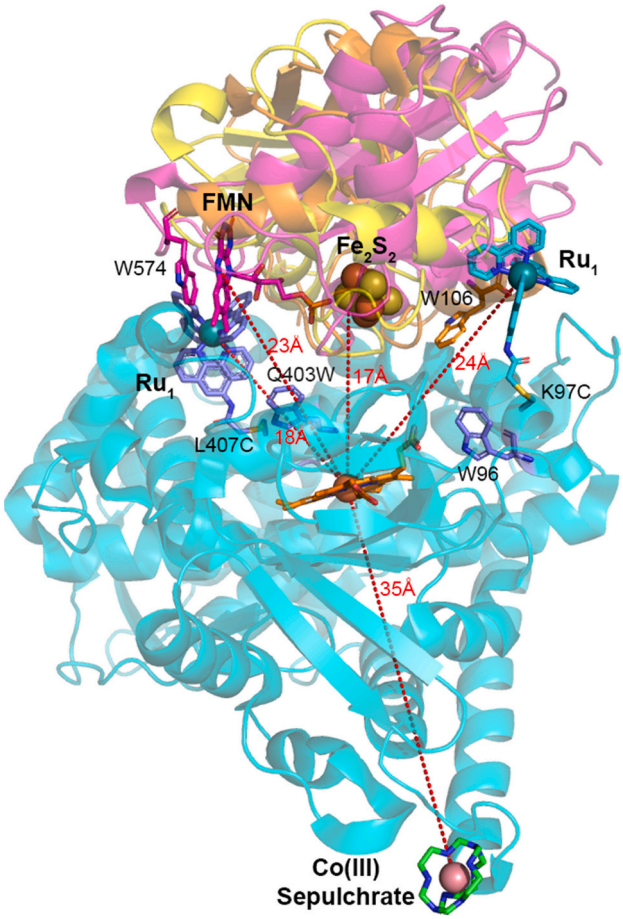

Fig. 5. X-ray crystal structures of P450 heme domain (blue) with the natural and artificial redox partners: FMN domain (magenta, PDB ID: 1BVY) [71], Fe_2S_2 cluster of putidaredoxin (yellow, PDB ID: 5GXG) [72] and ferredoxin (orange) [75], Ru(II) photosensitizers (blue, PDB ID: 3NPL [64] and 5JTD [70]) and Co (III) sepulchrate (green, PDB ID: 5E78) [76].

5. Expanding on the Ru(II) complexes and P450 applications

In this section, we would like to highlight and put into context some of the recent advances from our group (see Fig. 6) and others in combining Ru(II)-diimine complexes and P450 biocatalysis. A special emphasis will be on 1) the development of the next generation of hybrid enzymes and the application of the photosensitizer covalent attachment to other P450 heme domains taking advantage of the highly conserved P450 tertiary fold; 2) the determination of coupling efficiency in the hybrid enzymes by indirect quantification of reactive oxygen species and the development of a complimentary light-driven bimolecular approach to activate P450 peroxygenases; 3) the functionalization of the Ruthenium complexes to enable the formation of dimers and protein aggregates; and 4) the development of a selective light-driven chemoenzymatic approach. We will also introduce recent work by the groups of Turro and Glazer on strained Ru(II) complexes able to selectively photorelease P450 inhibitors.

5.1. Next generation of hybrid enzymes and application to other P450 heme domains

The development of colorimetric and fluorimetric assays has facilitated the rapid and high-throughput screening of enzymatic activity. In the case of P450 BM3, chromogenic substrates containing a nitrophenoxy moiety were initially developed to mimic the natural long chain fatty acids [78] and thus enabled the rapid generation of mutants with exquisite catalytic activity. The assay was also compatible with the light-driven reaction conditions and thus enabled to rapidly probe the substrate scope and reactivity of the hybrid enzymes [79]. A wide range of compounds bearing the nitrophenoxy moiety could be synthesized and was tested with a panel of hybrid enzymes. With an interest in benzylic hydroxylation, we initially focused on the 1-benzyloxy-4-nitrobenzene derivative and implemented a directed evolution program to evolve the hybrid enzymes. The P450 BM3 D52-Ru_1 mutant harboring 4 mutations was identified with three to ten-fold activity enhancement compared to the sL407C-Ru_1 parent [80].

Because of the large superfamily of P450 enzymes and the vast redox partners library, considerable efforts have been dedicated to develop versatile redox partners [81,82]. The highly conserved tertiary fold in the cytochrome P450 enzymes prompted us to implement the light-driven approach to other P450 enzymes keeping the point of covalent attachment at the same location corresponding to the L407C mutation. As early attempts with the P450cam were promising [83], we recently turned our attention to the thermophile CYP119 from *Sulfolobus acidocaldarius*. This CYP119 was an attractive candidate as it is considered an orphan cytochrome with no dedicated redox partner. The hybrid CYP119-Ru_1 enzyme displayed high photocatalytic activity in

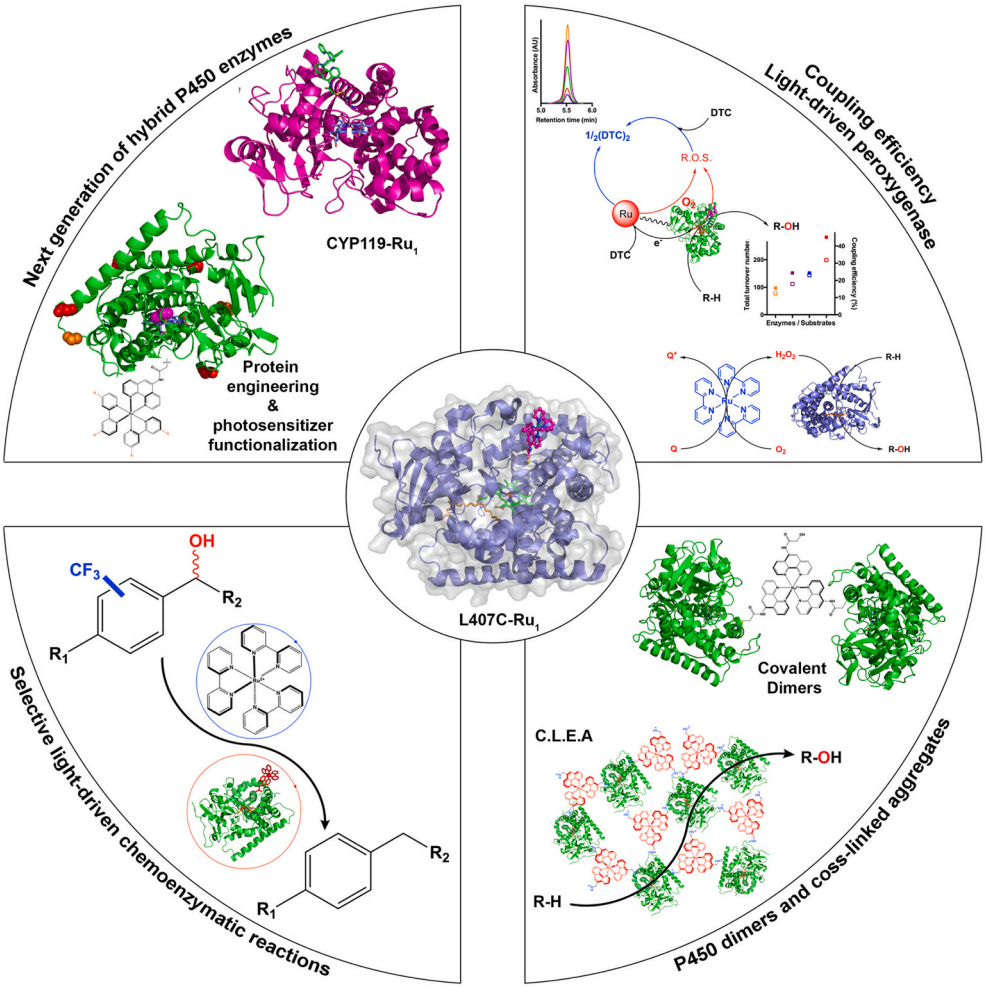

Fig. 6. Recent advances encompassing Ru(II) photochemistry and P450 biocatalysis.

the hydroxylation of the chromogenic substrate, 11-nitrophenoxyundecanoic acid at elevated temperature (~50 °C). The determined k_{cat} was the highest among reported CYP119 systems using natural redox partners [84]. Those findings establish the versatility of the covalently attached Ru(II)-diimine approach to activate various P450 enzymes and the potential of directed evolution to improve the efficiency of the hybrid enzymes and various artificial metalloenzymes [85,86].

5.2. Coupling efficiency and light-driven peroxygenase

One of the premier concerns in P450 biocatalysis is the economical use of the reducing equivalents towards productive pathway, i.e. oxygenated product, as opposed to the leakage of detrimental reactive oxygen species (ROS) either as superoxide or hydrogen peroxide (see Fig. 1) [14,15]. In addition, excess holes generated at the heme active site are thought to be funneled away to the surface by chains of stacked aromatic residues [87]. Typically, those unproductive pathways are reflected in the coupling efficiency, which varies from few percents in human P450 enzymes [88–90] to 100% for the P450 BM3 holoenzyme. The formation of ROS species is usually surmounted with the use of radical scavengers or enzymes, such as superoxide dismutase or horseradish peroxidase. An elegant approach by Reetz has recently combined a P450 enzyme with a peroxygenase as to use the side product from one enzyme to initiate the second biocatalytic transformation [91].

The coupling efficiency is primarily determined from the rate of NAD(*P*)H, dioxygen or electrons consumption versus the rate of product formation. Moreover, simultaneous quantification of hydrogen peroxide produced has led to a more accurate determination of coupling efficiency [92].

For the light-driven hybrid enzymes, we recently took advantage of the dual properties of the sacrificial electron donor, DTC [84]. In addition of being a suitable quencher of the Ru(II) excited state, this soluble anion displays unique ROS scavenging properties leading to the formation of a dimeric oxidized species, DTC_2, known as tetraethylthiuram disulfide. The dimer formation can be quantified concomitantly with hydroxylated product using HPLC. We proposed that the DTC_2 dimer formation can be used to quantify the formation of ROS generated over the course of the photoreaction and hence contribute to the determination of the coupling efficiency in the various light-driven hybrid enzymes [84]. As expected, an increase in the coupling efficiency correlates with a gain in total turnover numbers. For example, the directly evolved mutant, D52-Ru_1, displayed low turnover numbers and coupling efficiency (9%) in the photocatalytic hydroxylation of a long chain acid substrate mimic. However a 2.8-fold increase in turnover numbers was observed in the non-native substrate hydroxylation consistent with a higher coupling efficiency (32%) [84].

While working with dioxygen under reductive conditions has its own challenges [93], peroxygenases, that utilize directly hydrogen peroxide, have gathered increasing attention as valuable biocatalysts [94,95]. In particular, the recent identification of a class of fungal unspecific peroxygenases (UPO) with a broad substrate scope is particularly attractive albeit protein expression still being a major bottleneck [96,97].

A small class of P450 enzymes and several P450 BM3 mutants have displayed unique peroxygenase activity [23,33]. Notably, a promising candidate for biofuel application is the OleT enzyme that decarboxylates long chain fatty acids to the corresponding C-1 alkene. Recent work by Makris established that this enzyme is going through a Compound I intermediate but the rebound mechanism is disfavored for the decarboxylation pathway [98,99]. In addition, clear evidence from the same group established that this enzyme is best suited to utilize hydrogen peroxide as it is sluggish to activate molecular dioxygen due to rapid autooxidation [100].

Utilizing directed evolution, Cirino and Arnold engineered a lineage of P450 BM3 heme domain enzymes with peroxygenase activity and enhanced thermostability [101,102]. Initially, a 21B3 mutant was evolved to display high peroxygenase activity with 10 mM H_2O_2 in the hydroxylation of long chain fatty acids [101].

As mentioned previously, several light-driven approaches have already been employed to activate peroxygenases. Urlacher and coworkers utilized FMN derivatives to activate two members of CYP152A family [41] while Hollmann pioneered several light-driven strategies to activate unspecific peroxygenase [42,43,103].

As part of expanding the hybrid enzyme library, we initially generated the hybrid 21B3—Ru_1 enzyme where the photosensitizer Ru_1 was attached to the L407C mutant of the 21B3 variant. No detectable activity was noted in this hybrid enzyme compared to the efficient L407C-Ru_1 mutant (see Fig. 7).

However, a light-initiated bimolecular approach using $Ru(bpy)_3^{2+}$ in solution and the sacrificial triethanolamine (TEAO) quencher producing in-situ hydrogen peroxide unveils high total turnover numbers with the 21B3 variant rivaling those obtained with addition of 10 mM hydrogen peroxide (see Fig. 7). Noteworthy, in this bimolecular approach, no activity is observed with the hydrogen peroxide sensitive L407C mutant or with the ROS scavenging DTC quencher. These findings strongly support that efficient electron delivery is achieved in the hybrid L40C-Ru_1 enzyme and photocatalysis is enabled by the dual role of the sacrificial electron donor. No H_2O_2 is available to initiate the peroxygenase activity of the 21B3 mutant when DTC is used as the sacrificial electron donor. Also, the inability to activate molecular dioxygen in the 21B3—Ru_1 enzyme is consistent with rapid autooxidation as observed in the OleT enzyme [100]. These results confirmed key observations about the hybrid enzyme system and provide a complementary strategy for Ru(II)-diimine photoinduced P450 catalytic activity.

5.3. Functionalization of the Ru(II)-diimine photosensitizers

This section focuses on the selective functionalization of the ancillary ligands to tune the complex photophysical properties and on the introduction of reactive groups to promote formation of protein assemblies (see Fig. 8). In a series of seven hybrid enzymes, we investigated the effect of altering the redox properties of the covalently attached photosensitizer by varying the para substituents on the bipyridine ancillary ligands, from chloro to dimethylamino [104]. A three-fold increase in photocatalytic activity was observed for the *tert*-butyl derivative compared to the chloro substituent while the more electron donating methoxy and dimethylamino substituents resulted in a decreased photocatalytic activity. A concave Hammett plot was indicative of a change in rate limiting steps, presumably from the electron injection to the quenching step with the sacrificial electron donor [104].

Furthermore, introduction of reactive groups such as carboxaldehyde or iodoacetamide to the homoleptic Ru(II) complex led to the formation of P450 cross-linked aggregates and dimers. The aldehyde groups on the tris(1,10-phenanthroline)Ru(II) complex could react with surface exposed lysine residues to yield cross-linked P450 enzyme aggregates. Like in a classical chemical process, working with heterogeneous biocatalysts enables their recovery and re-use, and in the case of the cross-linked enzyme technology has often resulted in a catalytic efficiency enhancement [105,106]. In a quest to determine suitable cross-linkers, we recently demonstrated that Ru(II)-diimine compounds bearing aldehyde functionalities surpass currently available organic cross-linkers leading to P450 enzyme aggregates with greater activity recovery than the enzyme in solution and reusable for several rounds of reaction [107]. Meanwhile, the sulfhydryl specific iodoacetamido groups on the homoleptic phenanthroline Ru(II) complex reacted with surface-exposed non-native single cysteines to yield P450 heme domain homodimers. Protein gel electrophoresis and mass spectrometry confirmed the formation of dimers, that could be further separated from the monomer by size exclusion chromatography [108]. Overall, these examples illustrate the potential of those complexes to not only act as photosensitizers but also as building blocks to promote various protein assemblies [109] towards the development of heterogeneous photobiocatalysts.

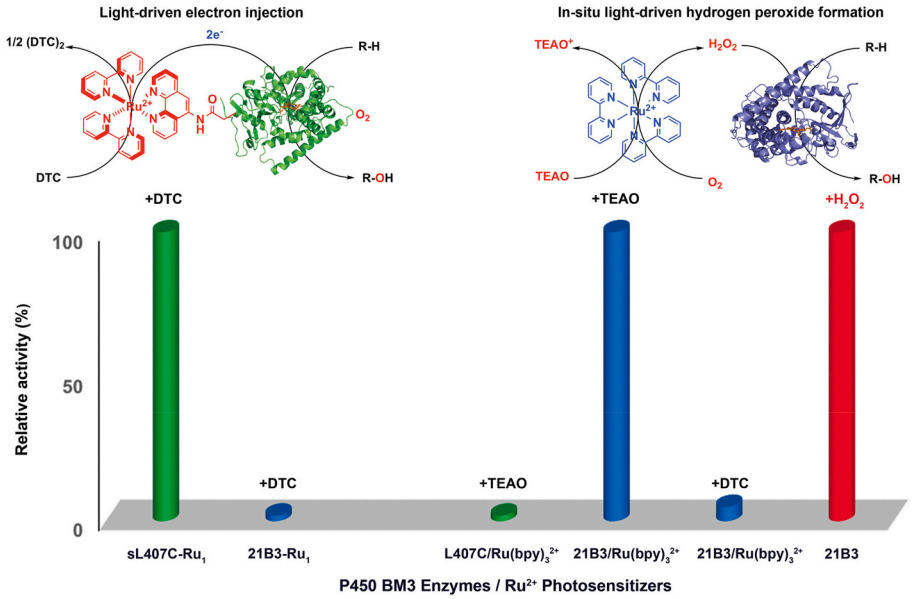

Fig. 7. Comparison of photocatalytic activity between the L407C and 21B3 variants using the electron injection approach with covalently attach Ru_1 complex (left) versus the bimolecular peroxygenase system using $Ru(bpy)_3^{2+}$ in solution (right).

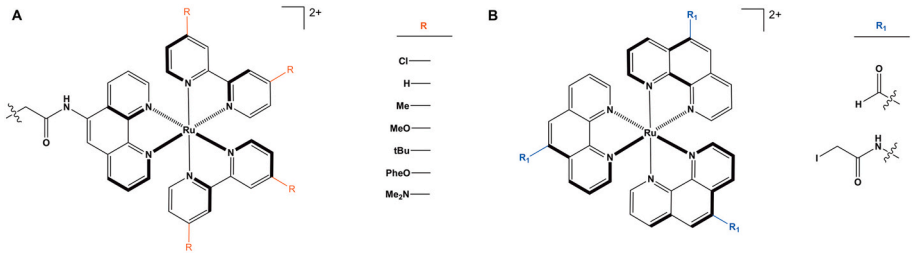

Fig. 8. Introduction of electron donating substituents (A) or reactive groups (B) on the Ru(II) complexes to alter their photophysical properties or promote various protein assemblies, respectively.

5.4. Chemoenzymatic strategies with P450 enzymes

The synthetic potential of P450 enzymes has been widely recognized in the myriads of reactions and non-natural substrates that they are able to functionalize [3–5,7]. Several strategies have thus been put forth to employ monooxygenases in enzymatic cascades [110] as well as in chemoenzymatic reactions [111]. However, challenges remain in finding optimal reaction conditions (buffer, pH, substrate loading) for enzymatic and chemical compatibility [112]. While great advances have been made in enzymatic cascade reactions, especially in one-pot and whole cell biocatalysis [113], we wish to focus on the lesser investigated chemoenzymatic approaches marrying the advantages of the P450 biocatalysts with several chemical catalysts.

Fasan and Arnold first demonstrated the use of P450 BM3 mutants combined with the selective fluorinating agent, diethylaminosulfur trifluoride or DAST, to access singly fluorinated compounds via deoxyfluorination of the enzymatically oxidized products (Fig. 9A) [114]. The groups of Zhao and Hartwig use P450 BM3 mutants to selectively epoxide the cross-methasesis products from various alkenes starting materials (Fig. 9B) [115,116]. The Reetz laboratory relied on Pd catalysis for selective amination of the P450 BM3 allylic hydroxylated cyclohexene-1-carboxylic acid methyl ester [117] and for Suzuki-Miyaura C—C coupling of an iodo intermediate to access several monooxygenated aryl compounds (Fig. 9C–D) [118]. The Flitsch group recently exploited a unique CYP166 variant to selectively hydroxylate long chain fatty acids at the 5th position and upon tosylic acid catalyzed cyclization produced chiral lactones (Fig. 9E) [119]. Macrolactonization was also employed for the synthesis of the natural product putaminoxins using the chiral allylic alcohol intermediates obtained with a P450 BM3 mutant [120]. Since Ruthenium(II) complexes have recently enjoyed a renaissance in photoredox catalysis, our group combined the hybrid P450 enzymes with photoredox trifluoromethylation (Fig. 9F) in a light-driven chemoenzymatic approach. This strategy led to the selective trifluoromethylation and oxyfunctionalization of several substituted arenes taking advantage of the unique selectivity of P450 BM3 mutants [80].

5.5. Ruthenium complexes with photolabile P450 inhibitors

Due to a thermally-accessible [3]MC from the [3]MLCT excited state (Fig. 3A), several strained Ruthenium complexes display loss of a chelating ligand. This feature has been advantageous in newly developed

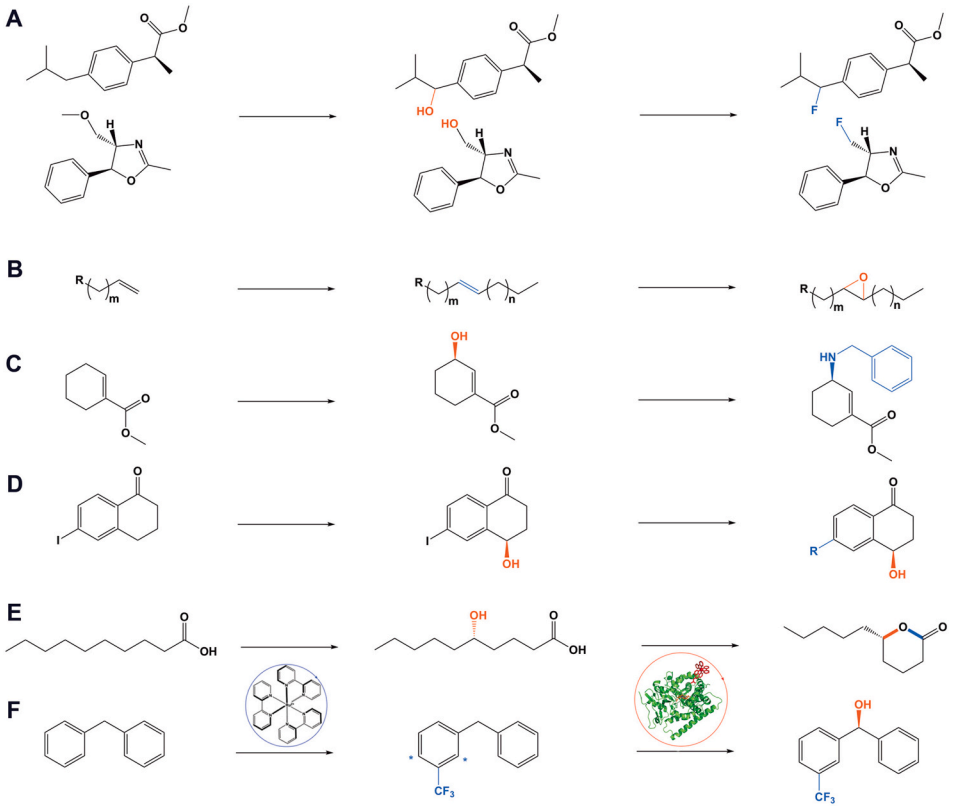

Fig. 9. Various chemoenzymatic approaches marrying the advantages of chemical catalysis and P450 biocatalysis.

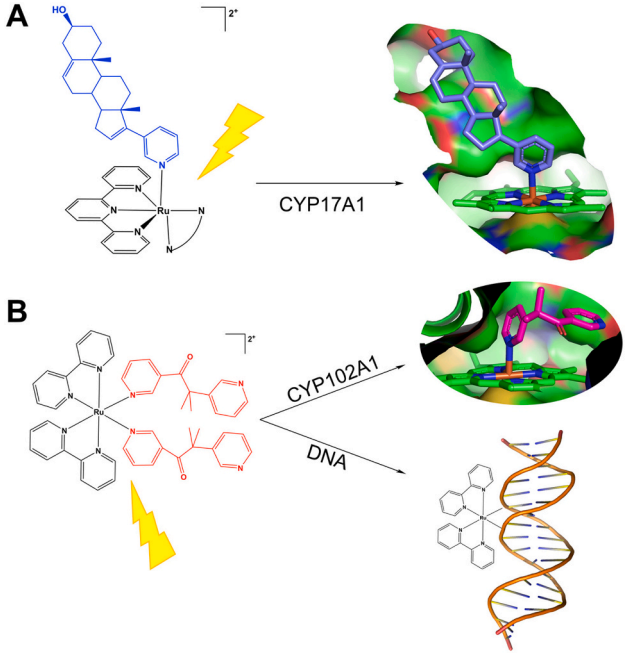

Fig. 10. Photocaged Ru(II) complexes to deliver P450 inhibitors upon visible light excitation.

strategies to deliver drugs [61], target DNA [121] and more recently to inhibit several P450 enzymes [60].

The groups of Turro and Glazer independently reported on Ruthenium complexes containing chelated P450 aromatic heterocycle inhibitors (Fig. 10). Photoexcitation of Turro's complex releases one molecule of the steroidal abiraterone to selectively inhibit the cytochrome CYP17A1 (Fig. 10A) [122]. The Glazer group demonstrated a dual modality with the Ru(II) complex shown in Fig. 10B where the photoreleased molecule binds to cytochrome P450 BM3 and the resulting bis-chelated complex covalently bind to DNA [123]. Current work [124,125] is focusing on tuning the photophysical properties of the complexes. The photocaging approach offers unique advantages in the spatial and temporal control of biological activity, selective inhibition of a target of interest and drug release in selected tissues in vivo.

6. Conclusion and perspectives

Albeit major advances in the field of light-driven P450 enzymes, additional efforts are still needed to rival the well-established P450 systems displaying high catalytic activity and coupling efficiency as well as the benefits of the regeneration strategies [20,21]. Valuable insights can be gained from this mature field to improve the efficiency of the light-driven processes. Ongoing work in various laboratories including ours are centered around improving the electronic coupling between the photosensitizer and P450 heme domains and directing the electrons towards the enzyme active sites using various protein engineering strategies [80,126].

Taking into account some of the current shortcomings limiting the light-driven P450 biocatalysis (i.e. generation of reactive oxygen, high levels of uncoupling…), there are several under explored areas where the use of Ru(II) photosensitizers could provide substantive advantages with their tunability, functionalization and importance in initiating various light-driven redox processes. For example, the recent utilization of Ru(II) complexes in biological applications [57] could be beneficial in whole cell light-driven biocatalysis following on the pioneering work with Eosin Y as photosensitizer [50]. In addition, to mirror some of the recent development in heterogeneous photocatalysis [127,128], heterogeneous photobiocatalysts could be an area of interest. Regarding P450 peroxygenases, expanding their substrate scope and stability would provide opportunities for *in situ* light-driven generation of hydrogen peroxide in a control manner and at desirable excitation wavelength as well as opportunity to expand the scope of chemoenzymatic reactions. Alternatively, the sacrificial electron donor in the light-driven system could be regenerated or even circumvented by coupling the reductive electron transfers to various oxidation processes. Some examples are starting to emerge using a water oxidation scheme with a light-driven peroxygenase [103] or chemical oxidation as proposed in a Ru(II)-modified laccase system [129]. The recent development of abiological reactions necessitating inert atmosphere [130] offers opportunities for the controlled delivery of the reductive equivalents using various photosensitizers with tunable redox potential.

In conclusion, while biocatalysis is gaining increasing importance in biotechnological and industrial applications, significant headways towards functional light-driven biocatalysts have been made in the last two decades. The use of visible light offers unique advantages to trigger biocatalytic processes with spatial and temporal controls. As highlighted in this review, Ru(II)-diimine complexes have been one of many valuable light-harvesting units employed to initiate light-driven P450 biocatalysis. Their unique properties and tunability have also enabled the development of P450 assemblies, selective photocage inhibitor deliveries and their use in photoredox catalysis and biocatalysis thus expanding their application beyond photosensitization. The continuous optimization of biocatalysts with a recent influx from machine learning [131,132] combined with an ever-growing photosensitizer library [133] hold great promises for a bright future in light-initiated P450 catalysis and photobiocatalysis in general.

Abbreviations

^{3}MC and ^{3}MLCT	triplet metal centered and metal to ligand charge transferrespectively
CYP119-Ru$_1$	singly mutated (R324C) CYP119 heme domain labeled with photosensitizer Ru$_1$
DAST	diethylaminosulfur trifluoride
DTC	diethyldithiocarbamate
(DTC)$_2$	tetraethylthiuram disulfide
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
NAD(*P*)H	Nicotinamide adenine dinucleotide (phosphate)
Q403W-Ru$_1$	doubly mutated (L407C/Q403W) P450 BM3 heme domain labeled with photosensitizer Ru$_1$
ROS	reactive oxygen species
Ru(bpy)$_2$PhenA (Ru$_1$)	bis(2,2′-bipyridine)(5-acetamidophenanthroline)Ru(II)
sL407C-Ru$_1$	singly mutated (L407C) P450 BM3 heme domain labeled with photosensitizer Ru$_1$
UPO	unspecific peroxygenase

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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