Handbook of Flavoproteins

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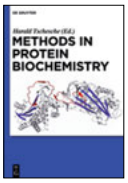

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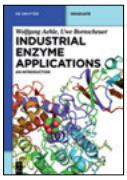

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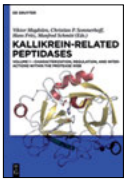

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Complex Flavoproteins, Dehydrogenases and Physical Methods

Volume 2

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Preface

Since the discovery of what was initially called the Yellow Enzyme by Otto Warburg in 1933 (and subsequently renamed Old Yellow Enzyme, a name the protein still retains) flavoproteins have been identified and characterized from virtually every organism known. More recent investigations have demonstrated that, in addition to their now well-established roles in catalyzing oxidation-reduction reactions of all sorts, flavoproteins also play important roles in a broad range of biological processes, including signal transduction and photochemistry. In addition, biotechnological applications of flavoproteins have progressed significantly since the development of blood glucose tests based on the action of glucose oxidase. Finally, flavoproteins have provided the testing ground for some of the newest and most cutting edge experimental methodologies in biochemistry and biophysics, and have provided critical insight into, for example, the short-time molecular dynamics of flavoproteins and the manner in which flavoproteins activate triplet molecular oxygen for reaction with singlet molecules.

Given the significant progress in our understanding of both the diversity of processes in which flavoproteins are involved and the specific manner in which they carry out their roles within (or without) the cell, it seems both appropriate and timely to provide a survey of the field in this and the accompanying volume. We have attempted, within the constraints of space and availability of the material, to compile a comprehensive pair of volumes that together accurately reflect our present understanding of how a broad spectrum of flavoproteins work. It is our hope that these volumes will prove to be useful reference sources both for workers in the field and for instructors at both the undergraduate and graduate level not only in biochemistry and biophysics, but also pharmacology, medicine and bioengineering.

We wish to thank our contributors who have prepared such lucid accounts of their respective areas. We are also particularly indebted to three individuals at Walter de Gruyter: Dr. Stephanie Dawson for her enthusiasm and support for this project from the outset, Ms. Julia Lauterbach for her excellent editorial efforts that made publication of these volumes a reality, and to Ms. Sabina Dabrowski for coordinating technical production. Finally, we are of course grateful to our spouses (Kim Hille, Walter Moos and Kim Palfey) for their support and patience throughout our careers generally, and during the preparation of these volumes specifically. One of us (RH) would also like to thank the Alexander von Humboldt Foundation of Germany for its support during the editing of these volumes.

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Table of contents

1 The reaction mechanisms of Groups A and B flavoprotein monooxygenases

David P. Ballou and Barrie Entsch

Abstract

Flavoprotein monooxygenases, found in species ranging from microorganisms to mammals, transfer one oxygen atom derived from O_2 to a substrate, oxidizing it. In this chapter, we review the enzymes in Groups A and B, which accomplish all their chemistry with just one protein. The catalytic cycles of both groups are roughly similar. NAD(P)H reduces the enzyme-bound flavin, which then reacts with oxygen to form a flavin C4a-(hydro)peroxide – the key oxygenating intermediate. The terminal oxygen of the (hydro)peroxide is transferred to the substrate, leaving the hydroxyflavin, which eliminates water to form oxidized enzyme. Catalysis in both groups is strictly regulated, but in very different ways, to limit NAD(P)H oxidase activity. Group A enzymes only allow the fast reaction of NAD(P)H when the substrate to be oxygenated is bound. In contrast, Group B monooxygenases do not require substrate to be present for rapid flavin reduction, but after the flavin is reduced, NAD(P) remains bound, stabilizing the flavin (hydro) peroxide until it encounters the substrate to be oxygenated. The enzymes in Group A are aromatic hydroxylases; they add oxygen to an activated aromatic ring by electrophilic substitution. The most studied flavoprotein monooxygenase, *p*-hydroxybenzoate hydroxylase, belongs to this group and will be discussed in detail. The enzymes in Group B catalyze nucleophilic and electrophilic oxygenations. Their substrates include aldehydes, ketones, amines, thiols, boronates, selenides, and thioethers. Conformational changes are important for controlling catalysis in both Group A and Group B monooxygenases.

1.1 Introduction

Over the past twenty years, a great many flavoprotein monooxygenases from species as diverse as microorganisms and mammals have been reported. These monooxygenases have been classified into six logical groups (A–F) based upon sequence, structure, and function by van Berkel *et al.* [1]. Most of the enzymes fall within Enzyme Commission classification [2] EC 1.14.13 and EC 1.14.14, which are enzymes that require an external reducing substrate (either NADH or NADPH) to reduce the flavin cofactor as part of catalysis. There are two basic structural themes found with flavin-dependent monooxygenases. In one, both the reaction with the external reductant and that with oxygen and the substrate are carried out by a single protein in which the FAD or FMN cofactor is tightly bound, whereas in the second group, reduction of the flavin (FMN or FAD) is catalyzed by a reductase, and the reduced flavin is delivered to a separate

oxygenase that catalyzes the oxygenation reaction. These two-component oxygenases constitute groups C through F in the van Berkel *et al.* classification [1] and are reviewed in Chapters by Ellis and by Tu. In this Chapter, we review the enzymes in Groups A and B [1], which achieve reduction and oxygenation with just one protein. Nearly all of the enzymes in Group A are aromatic hydroxylases that are mostly found in bacteria and fungi, and they add oxygen by electrophilic substitution to an activated aromatic ring to form phenolic products. These enzymes are all related in general structure. The most studied flavoprotein monooxygenase belongs to this group – *p*-hydroxybenzoate hydroxylase (PHBH), EC 1.14.13.2. The enzymes in Group B add oxygen to a wide variety of aliphatic substrates, as well as to heteroatoms such as found in amines, thiols, boronates, selenides, and thioethers. In many cases the reactions involve nucleophilic substitutions carried out by proteins with structural features quite distinct from those enzymes in Group A. In recent years, there have been other reviews of the Group A enzymes; those reviews provide some alternative perspectives on this story [1,3,4].

1.2 Enzymes acting upon aromatic substrates – Group A

1.2.1 Reactions catalyzed

In all of the aromatic hydroxylases, the flavin (usually FAD) remains tightly, but non-covalently, bound to the protein as a prosthetic group throughout catalysis, and thus is considered part of the protein structure. In catalysis, the flavin is reduced by NAD(P)H to initiate the reaction. Most of these enzymes require the aromatic substrate to be bound before efficient reduction can occur. The reduced flavin reacts with O_2 to form a transient flavin hydroperoxide, which is the reactive species that oxygenates the aromatic ring of the substrate to form the hydroxylated product and water. The known wide range of compounds oxygenated by this class of enzymes can be found under EC 1.14.13.n [2], by referring to enzymes utilizing a flavin prosthetic group. There are roughly forty different reactions identified at present, most of which are from bacteria, but there are certainly many more, because very little research has been done upon the potential enzymes in fungi. The substrates for Group A are all aromatic compounds that are activated for electrophilic aromatic substitution by the presence of *ortho*- or *para*-hydroxyl or amino substituents.

The reactions catalyzed by these enzymes are most often involved in microbial pathways for the degradation of aromatic compounds (especially those derived from lignin) to provide carbon for metabolism. For example, PHBH is a component of the β-ketoadipate pathway in bacteria [5]. The substrate, *p*-hydroxybenzoate (pOHB), along with a small number of other compounds, induces expression of the enzymes of the pathway, and because of this induction, pOHB can be the sole carbon source for growth. In addition to degradative pathways, a small number of Group A enzymes have recently been found to participate in the biosynthesis of complex metabolites with specialized functions. For example, PhzS is integral to the formation of pyocyanin in *Pseudomonas aeruginosa* [6]. PhzS is particularly interesting because of suggestions (needing research) that the oxygenation reaction carried out may be mechanistically different from other Group A enzymes. Recently there have been reports of human PHBH-like structures – for example, MICALs are multidomain proteins involved in cytoskeletal rearrangements

[7]. Within the protein sequence is a structure like that of PHBH, and work is underway in several laboratories to find its function.

1.2.2 Protein structures

The 3-D structures of at least seven different enzymes from Group A [1] have been deposited in structural databases [8]. Most of these structures are considered variations upon that of PHBH, which was the first published structure of this family; PHBH has been extensively investigated. Therefore, we use PHBH to illustrate the common structural features of this class of enzymes. In the SCOP database [9], PHBH is classified in the Family of FAD-linked reductases, N-terminal domain, within the broad class of α- and β-proteins (with parallel β-sheet). PHBH is a homodimer, with a monomeric length of 394 amino acids.

The 3-D fold of the enzyme is complex (▶Fig. 1.1); the N-terminal domain binds FAD, while parts of the C-terminal helical domain form the interface between the monomers. The core central domain incorporates a partly buried active site in which the isoalloxazine ring of FAD resides above pOHB (▶Fig. 1.1). Strands of the polypeptide interconnect the domains into an integrated structure. A striking feature of the structure is the absence of a separate domain for binding NAD(P)H.

Variations on the structure of PHBH have been reported for phenol hydroxylase from a yeast [10] and for 3-hydroxybenzoate 4-hydroxylase [11]. Phenol hydroxylase has an extensive addition to the C-terminus, resulting in a polypeptide of 665 amino acids, about 270 amino acids longer than PHBH. This added polypeptide forms a fourth domain with a thioredoxin-like fold. The thioredoxin structure appears to be

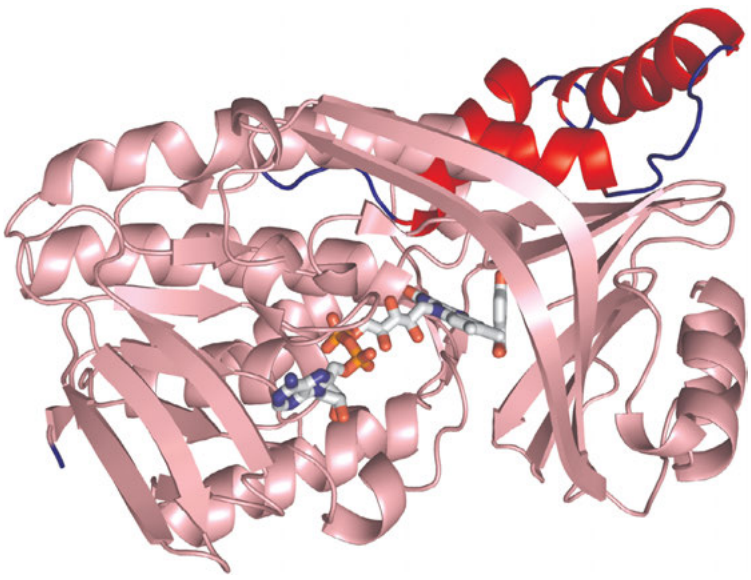

Fig. 1.1: The 3-D folding of a monomer of PHBH. The structure was adapted from 1PBE [8]. The N-terminus is on the left side and develops a domain that binds the FAD (shown as a stick model) in the middle of the structure with an extended conformation. The isoalloxazine ring is in the middle-right with pOHB bound under it. The β-sheet faced with a helix on the right binds the substrate, pOHB. The back of the structure consists of a series of α-helices towards the C-terminus. The red color represents the primary area of interaction between monomers in the dimer structure.

involved in dimer interactions in phenol hydroxylase; these interactions are completely different from those in the dimer interface of PHBH. Aside from dimer formation, no biological significance has been found for the extra domain in phenol hydroxylase and related structures. Importantly, most of the fold of the known catalytic portion of phenol hydroxylase is extremely similar to PHBH.

1.2.3 Detailed mechanism of PHBH

Since 1970, a great deal has been published about PHBH, and because there are some common features across all of the Class A flavin monooxygenases (according to the classification of van Berkel *et al.* [1]), PHBH is a good model for Group A enzymes and for flavoprotein oxygenases in general. In practical terms, PHBH is particularly suitable as a model (note that PHBH from both *P. aeruginosa* and *P. fluorescens* are discussed; their structures differ by only two insignificant amino acid residues, and their properties are nearly identical) because PHBH is stable, easily purified, and amenable to experimental investigation. Moreover, the aromatic substrate and many substrate analogues are also stable and readily available. PHBH from *P. fluorescens* was one of the early examples for which high quality 3-D structures from X-ray crystallography were obtained [12,13], and PHBH from *P. aeruginosa* was an early candidate for site-directed mutagenesis studies of the mechanism [14].

The first comprehensive steady-state and rapid kinetics studies of PHBH [15,16] gave important clues to mechanistic features of the enzyme. For example, even though it is a favorable exergonic reaction, the FAD in PHBH and other enzymes in Group A are only reduced very slowly unless the aromatic substrate that is to be oxygenated is present. Upon addition of pOHB and O_2, the reaction proceeds rapidly and quantitatively to form product (3,4-dihydroxybenzoate [3,4DOHB]) until pOHB (if it is the limiting substrate) is totally consumed. Importantly, the 3,4DOHB product is not further oxygenated even though a second oxygenation (to form 3,4,5-trihydroxybenzoate) is thermodynamically favorable. Another property of the Class A monooxygenases that was demonstrated from the kinetics studies was that the reaction with oxygen did not occur until NADP was released from the enzyme. Thus, catalysis occurs in two parts; first, NADPH reduced the FAD rapidly, but only with pOHB bound to the enzyme; second, after NADP dissociates, the bound $FADH^-$ reacts with oxygen in the presence of bound pOHB to form product, oxidized FAD, and water. If NADPH were able to facilely reduce the FAD in the absence of substrate, the $FADH^-$ would react rapidly with O_2 to form H_2O_2 (a toxic reactive oxygen species) and consume NADPH wastefully. Because this reaction with oxygen is even more energetically favorable than is flavin reduction, regulation of the reduction of PHBH is particularly important. Thus, PHBH has evolved to prevent the consumption of NADPH and oxygen unless pOHB is bound to the enzyme. We note that other flavoprotein oxygenases not in Group A employ a variety of strategies to limit the unwanted reaction with oxygen. Later in this Chapter is described a different strategy used by enzymes of Group B to avoid wasteful use of NAD(P)H.

PHBH has a turnover number of ~45 s^{-1} under optimum conditions at 25ºC, implying that the rates of formation and decay of intermediates are considerably >45 s^{-1}. Thus, to successfully resolve and characterize transient events by stopped-flow techniques,

PHBH had to be studied at 3 to 4ºC. PHBH was an excellent candidate for detailed transient kinetic analysis [17,18], and it was one of the first multiple-step enzymes for which kinetics and spectral properties of intermediates were characterized in great detail. Its organic substrates (NADPH and pOHB) change absorbance during catalysis, and FAD, as the core catalytic factor, undergoes unique absorbance and fluorescence changes. The catalytic cycle was easily divided into two separate events. In the absence of oxygen, the processes of control and catalysis of the reduction of FAD could be studied with the enzyme, NADPH, and pOHB [17]. Then, by using enzyme in complex with pOHB, reducing it anaerobically with dithionite, and then mixing it with a solution of oxygen, the oxygenation process and its control could be studied [18]. A striking feature of the overall enzymatic catalysis under optimum conditions (pH 7.5 to 8.0) is the rate-determining step as illustrated in ►Fig. 1.2.

During the reductive half-reaction, a charge-transfer complex between $FADH^-$ and NADP is observed beyond 700 nm (in the infrared) before NADP is released from the enzyme [16]. ►Fig. 1.2 shows an enzyme-monitored turnover experiment with measurement at 790 nm. Very rapidly during the reductive half-reaction (pre-steady state conditions) a charge-transfer complex forms. The reaction settles into steady-state, and it can be seen that a substantial portion of the enzyme continues to exhibit the charge-transfer interaction described above. If the reaction is monitored at 450 nm (data not shown), the absorbance is weak, indicating that most of the flavin is reduced during turnover. Thus, the process that releases NADP from the reduced enzyme is the rate-determining step. This slow step does not involve a chemical reaction, and thus likely involves a conformational change in the enzyme (more later).

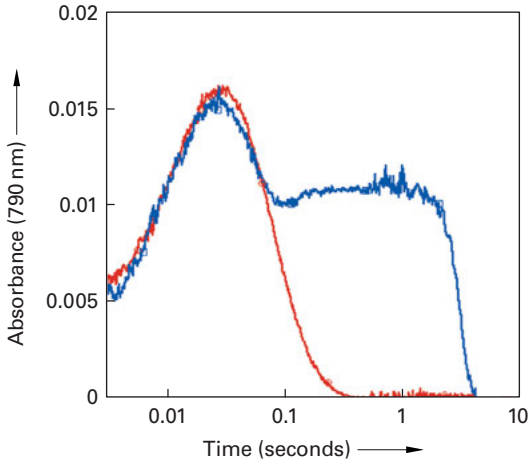

Fig. 1.2: The rate-determining step in catalysis by PHBH. The traces shown were collected in an enzyme-monitored turnover experiment conducted at pH 8 and 4°C. Absorbance at 790 nm is characteristic of the charge-transfer complex between $FADH^-$ and $NADP^+$. The red trace shows the change upon reduction of PHBH by NADPH in the presence of pOHB (no oxygen). The blue trace shows the same enzyme concentration in turnover with oxygen as the limiting substrate. Approximately 65% of the enzyme is in the charge-transfer complex during steady-state (the flat trace between 0.1 and 3 s).

1.2.3.1 Reductive half-reaction

Many flavoproteins exhibit the property of pyridine nucleotide reductases, but only a small number have a strict control mechanism (like PHBH) over this exergonic process. Also, PHBH and similar enzymes have the unusual property of having no classical binding domain for NADPH. It is these features of PHBH that have been partly responsible for much research attention. Remarkably, in PHBH, the rate constant for reduction of FAD by NADPH in the presence of pOHB is more than 10^5-fold greater than that in the absence of pOHB [16], yet the redox potential of FAD is almost the same with or without pOHB bound to the protein [19]. Thus, the enzyme must control the approach and orientation of NADPH to FAD through the binding of pOHB, but how? Early in the study of PHBH, it was found that several structural analogues of pOHB bound in the active site, and some stimulated the reductive half-reaction and some did not [15]. Critical to stimulating the reduction of the flavin by NADPH was the presence of a dissociable proton at the 4-position of the aromatic ring. The outstanding exception was the product (3,4DOHB), which has such a dissociable proton, but stimulated reduction only about 100-fold. This stimulation of reduction that depends on having a dissociable proton on the substrate or substrate analog is frequently observed, even when no hydroxylation is involved. Such compounds are often called effectors. If substrate analogues that stimulated reduction but did not become oxygenated [15] were present in high concentrations, the enzyme would be stimulated to catalyze the rapid formation of H_2O_2 and consume NAD(P)H unproductively, thus creating a lethal condition. This dual control on the use of NAD(P)H (substrate binding combined with mobile substrate proton signal) has not been demonstrated in other Group A enzymes.

An explanation of the control of reduction in PHBH was realized via a combination of X-ray crystallography and site-directed mutagenesis. The high-resolution structure of wild type PHBH [13] with pOHB bound to the enzyme gave no clue to how the reductive half-reaction was catalyzed because there was no place to pack NADPH near FAD; the FAD was fully buried in the active site in a conformation we refer to as “in”.

Then, in 1994, the structure of PHBH with 2,4-dihydroxybenzoate (2,4DOHB, an alternative substrate) bound in the place of pOHB showed that the enzyme could adopt a conformation with the isoalloxazine ring rotated about the ribityl side chain so that the N5 position (site of hydride transfer from NADPH) moved ~7 Å and was exposed at the surface of the protein (►Fig. 1.3) [20,21]. This conformation (termed the “out” conformation) could provide a way for NADPH to approach FAD. Site directed mutagenesis studies indicated surface residues potentially involved in binding NADPH [22,23]. It was not possible to obtain a structure of wild type PHBH with NADPH bound but without pOHB, because it did not crystallize in a single, stable conformation, suggesting that it is in a dynamic equilibrium. Finally, in 2002, a structure of the R220Q variant of PHBH with NADPH bound was obtained in the absence of substrate so that virtually no reduction of the FAD occurred [24]. The R220Q variant freezes an open conformation of the enzyme that can bind NADPH and likely is involved in substrate binding. In this conformation, two domains separate and the isoalloxazine swings out slightly from the active site to open up a solvent channel for substrate (or product) to access the active site. This third conformation has been referred to as “open”. NADPH was bound in an elongated state along a cleft on the surface of PHBH without any

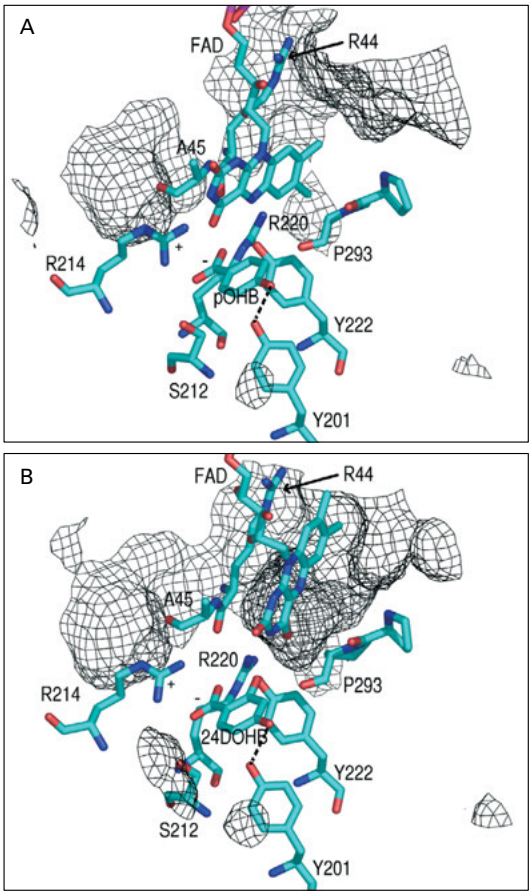

Fig. 1.3: Selected groups in the active site of PHBH that illustrate the in and out conformations. (A) The active site formed by the complex with pOHB – the in conformation (from 1PBE [8]). The orientation shows the *re* face of the isoalloxazine and the hatched shading shows the solvent accessible areas. (B) The active site formed by the complex with 2,4DOHB – the out conformation (from 1DOD [8]). The orientation is identical to A, and the hatched shading again shows the solvent accessible area. The principal difference is the position of the isoalloxazine ring.

canonical nucleotide domain fold being present. This conformation did not allow the nicotinamide ring to approach FAD in this "open" conformation, but modeling FAD into the structure in the "out" conformation (see above), where it is exposed to solvent, showed that a small rotation of the nicotinamide ring allowed it to meet the isoalloxazine in an orientation that was suitable for hydride transfer [24]. Thus, we have a rational explanation of how at least three different protein conformations ("in", "out", and "open") control the spatial arrangements of NADPH and FAD required to regulate the initiation of reduction. The crystal structures illustrate the complex dynamics that are necessary for the function of the enzyme, but do not help us understand what makes them occur.

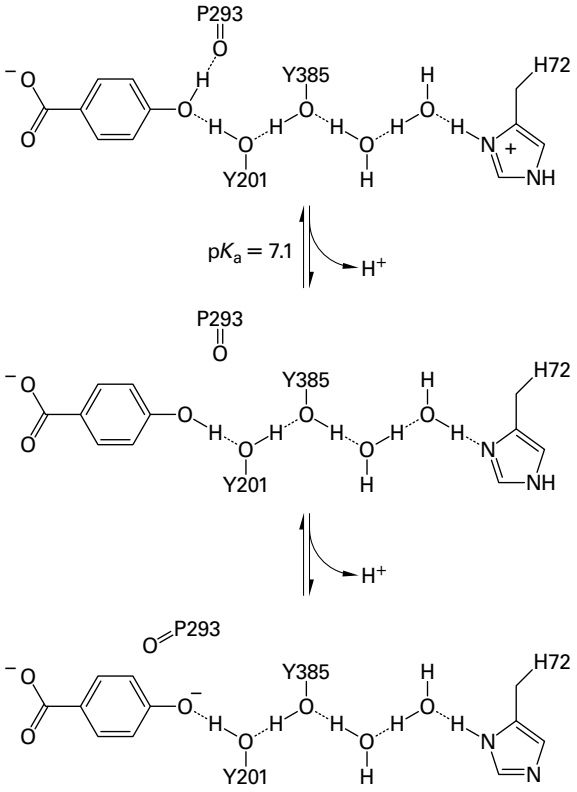

Fig. 1.4: Diagrammatic representation of the H-bond network linking pOHB to the surface in PHBH. The arrangement of structures to form a connection of H-bonds that can exchange protons with solvent is described in detail in [46]. The figure shows how the pKa of H72 can control the ability of the network to control the interaction of the H at position 4 of pOHB. This mechanism is an integral component of both the reductive and oxidative half-reactions of PHBH. This figure is reprinted with permission from [43]. Copyright 2004 American Chemical Society.

The high-resolution structure containing pOHB (the "in" conformation) [13] showed that the 4-hydroxyl of the substrate was H-bonded in a potential network of H-bonds that connected to H72 at the surface of the protein, as illustrated diagrammatically in ▶Fig. 1.4.

The function of these H-bond interactions was tested by specific variants: Y201F, Y385F, and H72N, in combination with structural and kinetic analysis [19,25,26]. Structural analysis showed that only the network of H-bonds was disrupted in the variants. Detailed kinetics studies of the reduction reactions (including using NADPD) showed that each substitution disrupted enzyme reduction to a different degree. Other important evidence came from disruptions to the mobility of the ribityl side chain of FAD [27,28]. When the evidence was integrated, the following model for the reductive half-reaction emerged [25,29].

When NADPH and pOHB bind to the enzyme, pOHB is transiently and rapidly deprotonated by the H-bond network (▶Fig. 1.4). The phenolate form of pOHB triggers a

conformational rearrangement in the protein through the highly conserved loop behind the isoalloxazine ring, resulting in movement of the isoalloxazine from the "in" position to the "out" conformation. The full details of the protein conformational changes are not yet understood. The nicotinamide ring of NADPH bound on the surface then rotates to meet the isoalloxazine ring, forming a charge-transfer interaction (observed by transient absorbance at long wavelengths) that is competent for reduction. Hydride transfer occurs from the *pro*R position of NADPH to the *re* face of the isoalloxazine ring [30]. After hydride transfer, the order of events is not clear. However, the $FADH^-$ rotates back into the "in" conformation, probably under the influence of the very positive electrostatic field around the interior position [31], pOHB is re-protonated from the H-bond network and the NADP dissociates from the enzyme. Note that the conformational changes occurring after reduction of FAD make up the rate-determining step in catalysis (►Fig. 1.2). This control over FAD reduction by the presence of a mobile proton on pOHB is probably unique to PHBH. The structures of other related enzymes (e.g., phenol hydroxylase) do not show similar H-bond networks. We have suggested [15,25] that this feature of PHBH is an important evolutionary adaptation of the enzyme to discriminate between pOHB and *p*-aminobenzoate, a molecule that is essential for cellular function (for synthesis of folate). Because the aminobenzoate has no easily dissociable proton at the 4-position, it does not stimulate reduction of the FAD in PHBH and therefore, the subsequent oxygenation of this substrate to 3-hydroxy 4-aminobenzoate, a cytotoxic molecule, is avoided.

The final (and perhaps most significant) feature of the control by the enzyme of the reduction of FAD is the dramatic change in the kinetics of binding of pOHB. At 4ºC, the rate constant for binding pOHB to oxidized enzyme is 5×10^6 $M^{-1}s^{-1}$, and the corresponding dissociation rate constant is 60 s^{-1}. For the reduced enzyme (with almost the same K_d for pOHB) the corresponding values are only 1.6×10^2 $M^{-1}s^{-1}$ and 0.0034 s^{-1} [18]. Thus, dissociation of pOHB from the reduced enzyme does not occur during a catalytic cycle, and this feature prevents uncoupling and oxidase activity. The reduced structure with pOHB kinetically stabilizes the "in" conformation. This means that pOHB is locked in the active site for the reactions with oxygen. This active site trap appears to be a unique property of the Group A hydroxylases in general. With almost no structural changes between the oxidized and reduced enzyme forms [32], this trap must be due to the negative charge on the reduced isoalloxazine ring positioned in the "in" conformation within the strong positive electrostatic potential of the active site [31].

1.2.3.2 Oxidative half-reaction

The fundamental function of hydroxylases involves the reactions with oxygen. With PHBH and related enzymes, there are at least three consecutive chemical processes during the oxidative half-reaction: reaction with oxygen to form a flavin hydroperoxide, transfer of one atom of oxygen to a substrate, leaving the hydroxyflavin, and finally, the elimination of water from the hydroxyflavin to form its starting oxidized state. This mechanism, accepted universally today, was a complete mystery in 1970. Most of our knowledge about the mechanism of the oxygen reactions of flavin-dependent hydroxylases comes from work on PHBH.

A fundamental chemical property of flavins is their reactivity with oxygen [33], which can be attributed to their ability to form free radicals. Oxygen has a radical ground

state structure, so chemical interactions involve radical species to preserve electron spin (Vol. I Chapter 10 by Jorns). The initial reaction is always the transfer of one electron from the anionic reduced flavin to oxygen to form a radical pair. The radical pair can exchange a second electron to form H_2O_2 and oxidized flavin, the reaction pathway channeled by flavoprotein oxidases, or the radical pair can diffuse apart, the common outcome of the accidental reaction of flavoprotein reductases with oxygen to form $O_2^{-\bullet}$ and flavin semiquinone. Finally, the radical pair can combine and form a covalent bond, thus creating flavin peroxide, the pathway channeled by flavoprotein hydroxylases [33]. The final pathway was only speculation in 1970, as was the location of the oxygen adduct on the isoalloxazine structure. In aqueous solutions, free flavin reactions with oxygen are even more complicated because of the development of autocatalytic kinetics due to radical recombinations [34].

The first indication of a new chemical species between flavin and oxygen in enzymatic reactions was published in 1972 [17], but it took extensive experimental work over the following years to fully resolve the kinetics and the properties of transient oxygen derivatives of FAD in the function of PHBH [18]. Subsequently, in the Massey laboratory, the same types of oxygen derivatives were also found for both melilotate hydroxylase [35] and phenol hydroxylase [36].

A typical example of the characteristic absorption spectra of flavin species in these reactions is shown in ►Fig. 1.5; these spectra were deduced from stopped-flow studies of the reaction of O_2 with PHBH that had been reduced in the presence of the

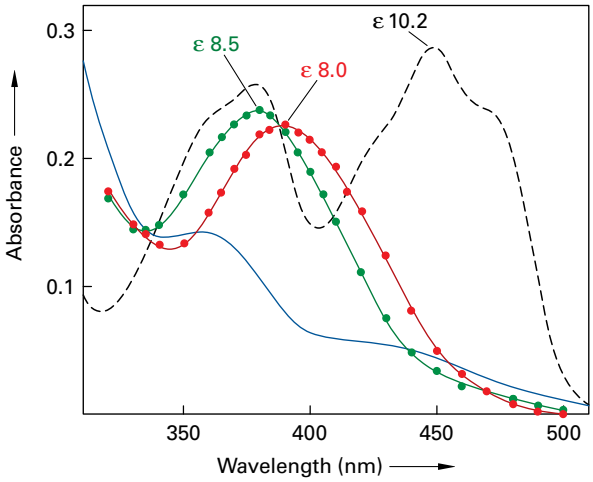

Fig. 1.5: Absorption spectra of FAD obtained when reduced PHBH in complex with pOHB was reacted with oxygen. The experiment was carried out at pH 6.55 and 2°C. The solid blue curve is of reduced enzyme and the dashed black curve of oxidized enzyme. The spectrum marked by the red circles was calculated from reaction traces recorded at multiple wavelengths as described in [37] and represents the flavin C4a-hydroperoxide formed from oxygen. The calculated spectrum marked by the green circles represents the flavin C4a-hydroxide formed from the hydroperoxide following oxygenation of pOHB. The extinction values in the previously published figure are incorrect. The correct extinction values at the spectral peaks (ε on the figure) were obtained by multiplying the original figure values by 0.9. This figure is reprinted by licence from [37].

substrate, pOHB. The first stable oxygen derivative of a flavin was the C4a-peroxyflavin demonstrated for bacterial luciferase ([38] and Vol. I Chapter 5 by Tu). The chemical structure of the flavin derivatives with oxygen was inferred from extensive model chemistry of flavin derivatives [39], but it was many years later that direct proof was obtained from NMR studies on the stable derivative in luciferase that the oxygen adduct of the flavin was actually a C4a-(hydro)peroxyflavin [40]. Over the last 40 years, all experimentally observed adducts between flavin and oxygen in proteins have been C4a-peroxides and C4a-hydroxides, as shown in the oxygen reactions of PHBH (►Fig. 1.6).

To experimentally study the oxygen half-reaction of PHBH and similar enzymes, the multi-step process was studied directly by stopped-flow spectrophotometry and fluorimetry, combined with low temperatures, pH shifts, changes in solvent conditions, and mutations. Combinations of these conditions have been used to modulate the kinetics of this exergonic series of consecutive reactions, and thus define the chemistry. Spectra like those in ►Fig. 1.5 were often calculated from individual kinetic traces by hand using integrated rate equations for consecutive reactions [18,35]. Today, computer software is available to rapidly deconvolute the component spectra from data consisting of rapidly scanned spectra with time. However, although these tools are convenient and impressive, it is important to use them with caution, since incorrect assumptions about the mechanistic models used will lead to incorrect outputs.

In the absence of pOHB bound in the active site, reduced PHBH behaves like an oxidase, forming H_2O_2 in a second-order reaction with oxygen without any observed flavin derivatives [18]. However, by selecting appropriate solvent conditions, especially by including anions such as azide, the kinetics can be manipulated to better resolve intermediates. By these means, it was found that even the reaction without substrate present actually proceeded through a transient C4a-hydroperoxide [18]. It has recently been found that some oxidases also react via this pathway ([41] and Vol. I Chapter 9 by Wongnate and Chaiyen). Note that the first electron transfer from flavin to oxygen is always found to determine the rate of formation of the flavin peroxide. The reaction of oxygen with reduced PHBH is 10^3-fold faster than that with free flavin [42], and some hydroxylases react even faster. With pOHB present, the reaction of reduced PHBH with oxygen also forms quantitatively the same C4a-hydroperoxide, but 10-fold faster. In specific protein environments, the kinetic barriers to reaction with oxygen are dramatically lowered (see Vol. I Chapter 10 by Jorns). With PHBH, the second-order rate constant for the reaction of reduced PHBH with oxygen is independent of pH, consistent with the anionic reduced flavin being present in the active site at all practical pH values [43]. Group A enzymes like PHBH are different from many other hydroxylases that react preferentially with oxygen without substrate to form a flavin (hydro)peroxide that has a half-life of many seconds to minutes – such as the enzymes in Group B described below. In PHBH, pOHB in the complex with reduced enzyme helps to prevent access to solvent [13] and this barrier is essential for extending the half-life for the flavin hydroperoxide to ~1 s, which is adequate for the hydroxylation reaction [19].

From several pieces of indirect evidence, it is clear that in PHBH, the flavin C4a-peroxide initially formed in the reaction with oxygen is immediately protonated to form the flavin C4a-hydroperoxide, which is an electrophile, and this process is common to Group A enzymes. The proton probably comes into the active site from a solvent channel into the *re* side of the flavin [44]. Only with the K297M variant of PHBH (located

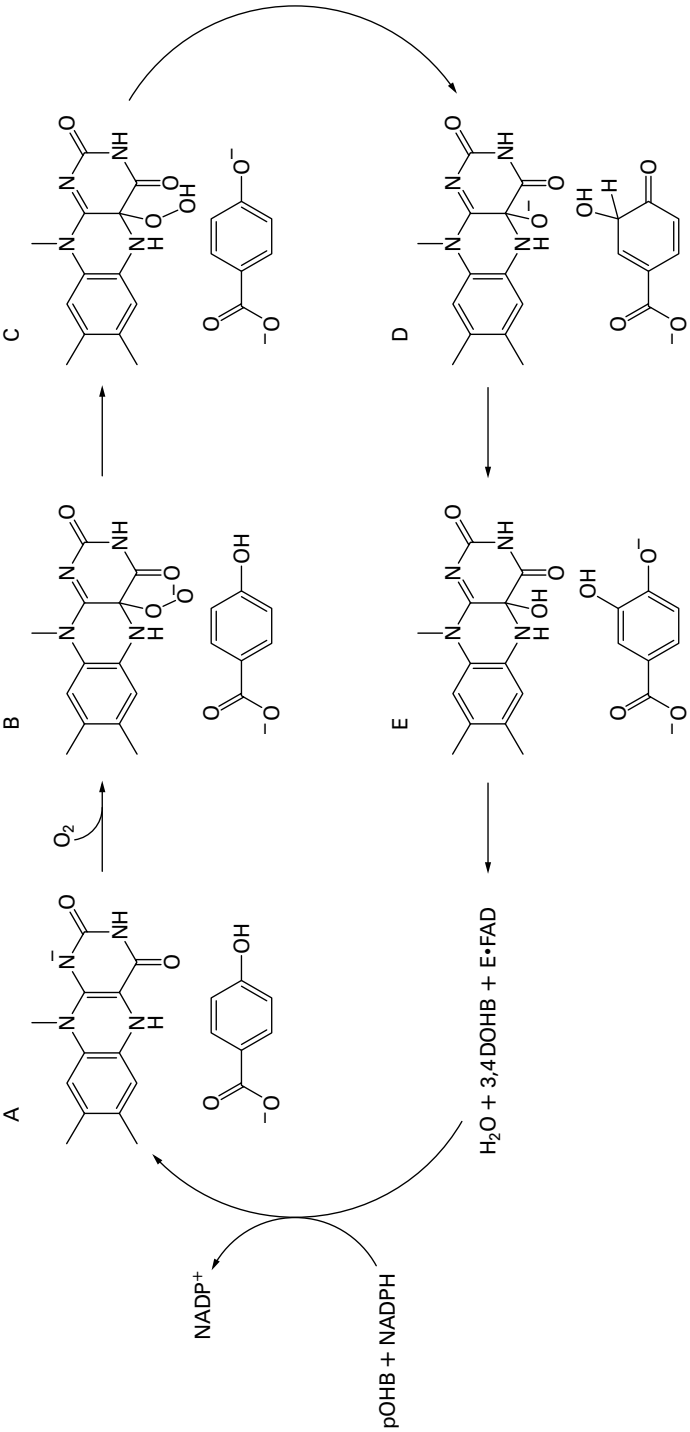

Fig. 1.6: Structural representation of the catalytic cycle of PHBH. A, Reduced flavin; B, C4a-peroxyflavin; C, C4a-flavin hydroperoxide, D, C4a-flavin alkoxide with dienone form of the product; E, C4a-flavin hydroxide. In experiments with pOHB bound to PHBH, the non-aromatic dienone intermediate is not detected because the rate constant for decomposition to 3,4DOHB is much larger than that of formation. Note that the oxygen derivatives of FAD are bound to the bridging C4a-carbon. Oxygenations of electrophilic substrates such as in Baeyer-Villiger reactions, involve the nucleophilic flavin peroxide, Species B.

where it can disrupt the proposed solvent channel) has some indication been found of a protonation step slow enough that it could be measured by stopped-flow fluorescence [43]. By contrast, some enzymes in Group B specifically inhibit protonation of the peroxide [45], since, unlike the aromatic hydroxylases, the nucleophilic peroxide is the catalytically active species.

The next phase in the oxygen reactions is the transfer of the distal oxygen atom of the hydroperoxide to the substrate. To understand PHBH, it is essential to consider this process with respect to the H-bond network (►Fig. 1.4). As mentioned above, after the isoalloxazine has been reduced, the enzyme-bound $FADH^-$ is in the "in" conformation with pOHB bound with its 4-hydroxyl protonated, and in juxtaposition to the isoalloxazine ring for the ensuing hydroxylation reaction. After reaction of the bound $FADH^-$ with O_2 to initially form the flavin C4a-peroxide, proton exchanges occur, largely facilitated by the hydrogen bond network. This leads to a deprotonation of the more acidic substrate to its nucleophilic phenolate form and protonation of the basic flavin peroxide to yield its electrophilic hydroperoxy form. The positive electrostatic field in the active site appears to be able to support approximately one negative charge, so that the overall exchange of protons preserves charge balance while setting up the active oxygen species and the substrate for the hydroxylation step. These processes are practical because the rate of proton exchange is much faster than other chemical processes occurring [46]. Measurements of the rate constants for hydroxylation as a function of pH showed a pKa of 7.1, which we have attributed to H72 at the surface of the protein at the end of the hydrogen bond network (►Fig. 1.4 and [43]). A limiting rate constant at low pH for hydroxylation at ~18 s^{-1} contrasted with a limiting value of ~120 s^{-1} at high pH. Several lines of evidence are consistent with the hydroxylation occurring by electrophilic substitution. For example, the disruption of the H-bond network in the Y201F variant (Y201 directly bonds to pOHB, see ►Fig. 1.4) decreases the rate of hydroxylation by 1000-fold [19], such that the enzyme becomes an oxidase that primarily forms H_2O_2. In addition, PHBH does not effectively hydroxylate *p*-aminobenzoate, which does not have a dissociable proton at the *para* position. Hydroxylation of *p*-aminobenzoate occurs with a rate constant of only 5 s^{-1}, which is independent of pH [43]. At higher pH in the *p*-aminobenzoate reaction, the enzyme becomes more of an oxidase because these conditions cause loss of the proton at N5 of the isoalloxazine ring, promoting elimination of H_2O_2 [41]. These examples illustrate how hydroxylation of substrate is a competition between oxygen transfer and peroxide elimination from the flavin. PHBH may be unique in being nearly 100% coupled with its native substrate. Thus, for each mole of NADPH oxidized, one mole of 3,4DOHB is produced and one mole of O_2 is consumed. If a substrate analogue that is not susceptible to electrophilic attack by the hydroperoxide is used with PHBH (such as 5-hydroxypicolinate or 6-hydroxynicotinate), then the enzyme becomes a pure NADPH oxidase [18] as mentioned earlier.

With pOHB as substrate, the hydroxylation step is experimentally detected as a first-order reaction that transforms the flavin hydroperoxide into the closely related flavin C4a-hydroxide [18], the logical product of oxygen transfer to the substrate. The spectrum of this species is similar to that of the hydroperoxide, but the absorbance maximum is slightly blue-shifted (►Fig. 1.5). A useful tool for studying the hydroxylation reaction has been fluorescence, because in most cases with PHBH (as well as many other flavin-dependent

hydroxylases), the flavin hydroperoxide lacks fluorescence, but the flavin hydroxide is highly fluorescent. Thus, the appearance of fluorescence helps to identify the phase of the reaction that is due to oxygen transfer. We note that the observed single phase for hydroxylation is actually composed of two chemical processes – the measured rate of oxygen transfer to form a non-aromatic dienone product, and the very fast tautomerization to the aromatic product (►Fig. 1.6), probably with the assistance of the H-bond network [43]. When 2,4-dihydroxybenzoate is substrate for PHBH, we know that the dienone intermediate is involved, because a spectral intermediate with a high extinction can be resolved [18]; after a long period of research, it was concluded that this spectral species is actually due to contributions from both the flavin C4a-hydroxide and the dienone form of the product (►Fig. 1.6 and [47]). This dienone does not rapidly rearrange to the aromatic product in the active site of the enzyme. Similar kinetically stabilized dienone-like intermediates have also been observed with *p*-aminobenzoate as substrate for PHBH (in this case, it has extreme stability [18]) and with resorcinol as substrate for phenol hydroxylase [48]. Observation of such intermediates provides strong evidence for the chemistry of hydroxylation being an electrophilic aromatic substitution.

The final tasks for flavoprotein hydroxylases are to eliminate water from the flavin and release the aromatic product into solution. For many enzymes, release of the final product is the rate-determining step in catalysis. However, for PHBH under optimum conditions (►Fig. 1.2), release of NADP from the reduced enzyme flavin limits the rate of catalysis, although at pH values below 7, the release of 3,4DOHB limits turnover [43]. It was noted in early studies of PHBH that at high concentrations, substrate inhibits catalysis by forming a dead-end complex with the flavin still as the C4a-hydroxide [15]. This suggested that product is usually released from the enzyme prior to water elimination, and that after product dissociates, high concentrations of substrate can quickly replace it, thereby isolating the flavin from solvent before H_2O could be released from the C4a-hydroxyflavin; the latter step is normally fast when solvent has access to the flavin [39]. Binding of substrate and release of product likely involve similar changes between the "in" and "open" conformations (see above). However, when pOHB is bound in the active site, it likely favors the "in" conformation, which prevents access of solvent and the consequent rapid dehydration of the flavin hydroxide. When reaction kinetics are studied under conditions that avoid high substrate inhibition, the rate constant for product release is dependent upon pH [43], with a pKa of 7.1, indicating again a role for the H-bond network in this phase. The rate of product release is substantially greater at high pH, implying that the 3,4DOHB phenolate is an important trigger for this process. H-bond network mutants lose this pH-dependent trigger [43]. The structure of the product complex of PHBH shows that the 3-hydroxyl of the product is H-bonded to the backbone carbonyl of P293, causing a slightly different orientation from that of the substrate complex [51]. This interaction is important because it prevents reorientation of the product in the active site and the consequent stimulation of the reduction of FAD and hydroxylation of the product. The particular interaction of product with P293 (an essential residue that interacts with substrate to promote the "out" conformation necessary for reduction of the FAD [50]) probably does not favor the "out" conformation. Instead, we surmise that the subtle changes in the protein caused by interaction with the product promote the "open" conformation, which facilitates product release. Although the crystal structure of PHBH with 3,4DOHB bound shows that the "in" conformation is formed in the crystal [51], in solution, evidence from pH dependence (above) suggests

that mobilization of the 4-hydroxyl proton of 3,4DOHB through the H-bond network provides a trigger to promote formation of the "open" conformation and thus completion of catalysis [43]. Single molecule studies have shown that, in the absence of substrate, the protein structure is in rapid equilibrium between "open" and "in" conformations [52], and these dynamics are probably the reason substrate-free PHBH does not form crystals suitable for high-resolution structures.

1.2.3.3 Hydroxylation chemistry

It is remarkable that PHBH and other enzymes in Group A can oxygenate aromatic rings with a flavin hydroperoxide. In the section above, some evidence is presented for a mechanism of electrophilic attack of the terminal oxygen of the flavin hydroperoxide upon the activated aromatic ring of the substrate. Perhaps the strongest evidence for this electrophilic substitution reaction comes from a study of fluorinated substrates of PHBH that are converted into orthoquinones [53] and from a Hammett relationship study using 8-substituted-FADs [54].

PHBH at 4°C and pH 9 hydroxylates pOHB with a rate constant of 120 s^{-1}. We have described above how manipulation of the phenolic proton of pOHB in the H-bond network of PHBH can contribute an acceleration of up to 10^3. This contribution is not available to the same extent in other Group A enzymes such as phenol hydroxylase [55], which has no H-bond network, exhibits no pH dependence of hydroxylation, and is at least one order of magnitude less active than PHBH in the hydroxylation reaction. There must be other chemical contributions to lowering the activation energy for the hydroxylation reaction in other Group A monooxygenases, and some answers have come from studies with PHBH.

The role of the strong positive electrostatic potential around the active site of PHBH in the largely solvent-free environment in the "in" conformation has been studied [31]. A series of highly conserved basic residues surround the active site with positive charges, and we have studied the effects of two mutations, K297M [31] and E49Q [49] that are not integral to substrate interactions with enzyme. The removal of a positive charge (K297M) decreased the rate constant for hydroxylation at pH 9 from 120 s^{-1} for wild-type to 5 s^{-1} for the mutant enzyme. Increasing the positive potential by removing a conserved negative charge (E49Q) had the opposite effect. The rate constant for hydroxylation became larger than that for the preceding reaction with oxygen, even at the highest concentrations of oxygen obtainable, and could not be measured [49]. It was proposed that the positive electrostatic potential stabilizes the flavin C4a-alkoxide leaving group formed during hydroxylation [43], resulting in faster oxygen transfer. Thus, it is clear that the positive electrostatic potential in the active site is a major contributor to catalysis.

A number of studies of PHBH with FAD analogues chemically modified in the isoalloxazine ring have also drawn attention to the contribution of the isoalloxazine structure. For example, PHBH with 1-carba-1-deaza-FAD [56] converted the enzyme into an NADPH oxidase, even though during catalysis, substantial amounts of flavin C4a-hydroperoxide were formed. The results obtained could be due to a combination of a less stable hydroperoxide and less active oxygen on the modified isoalloxazine. Analysis of a series of 8-substituted FADs bound to PHBH [54] showed that hydroxylation rates increased with more electron-withdrawing substituents, presumably by stabilization of the flavin C4a-alkoxide leaving group in hydroxylation. This work demonstrated an

important role for the electronic properties of the isoalloxazine in hydroxylation and fully supported an electrophilic aromatic substitution mechanism.

It is clear that a combination of susceptible substrate, substrate activation, protein environment (positive electrostatic field and alignment of catalytic groups, etc.), and flavin chemistry combine to produce high rates of hydroxylation in PHBH, making it an effective enzyme.

1.2.3.4 Summary

PHBH (and similar enzymes) function to efficiently achieve several consecutive and diverse chemical reactions in turnover within a single polypeptide. For PHBH to be successful, we have found that it has to integrate at least four rapid conformational changes on the fly in catalysis over two active sites. Two of these conformational changes are rate determining (above). The importance of the delicate balance between conformations has been demonstrated in several studies of carefully chosen single mutations in the structure that can disrupt the dynamic properties of the protein so that its catalytic function is substantially impaired [57,58].

1.3 Enzymes acting upon non-aromatic substrates – Group B

1.3.1 Reactions catalyzed and subclasses

Flavin-dependent oxygenases of Group B [1] are involved in a wide variety of both electrophilic and nucleophilic oxygenations. There are four general classes of enzymes within Group B: the Baeyer-Villiger monooxygenases (BVMOs), the flavin-containing monooxygenases (FMOs), the microbial *N*-hydroxylating monooxygenases (NMOs), and the YUCCA enzymes that are found in plants. By-and-large, all of the enzymes in this group use NADPH as the physiological reductant.

A characteristic that distinguishes Group B flavin-dependent monooxygenases from those in Group A is that in most cases, reduction of the enzyme-bound flavin of Group B enzymes does not require substrate to be present. Thus, control of catalysis does not occur by down-regulating reduction of the flavin in the absence of substrate, as was discussed above for the enzymes in Group A. Instead, the enzyme-bound FAD readily becomes reduced by NADPH and then reacts with O_2 to form a quasi-stable C4a-FAD (hydro)peroxide. Like the aromatic hydroxylases in Group A, these enzymes also utilize C4a-flavin (hydro)peroxides as activated forms of oxygen to carry out their oxygenation reactions. However, their kinetic mechanisms differ from those of Group A. For effective catalysis, after the FAD is reduced, the NADP product remains bound to the enzyme throughout the remainder of the catalytic cycle, whereas with Group A enzymes the NADP is released after the flavin is reduced and before the $FADH^-$ reacts with O_2. With Group B enzymes, control of catalysis is manifest after the reaction with O_2 forms the C4a-(hydro)peroxy-FAD. The bound NADP is critical for helping to maintain a quasi-stable flavin (hydro)peroxide, a general theme for Group B flavin-dependent monooxygenases; in fact, in the presence of NADP, many of the C4a-(hydro)peroxides of Group B enzymes have half-lives of several minutes at 25ºC and 2 hours at 4ºC. Without substrates present, it is these long half-lives for the C4a-FAD-(hydro)peroxides

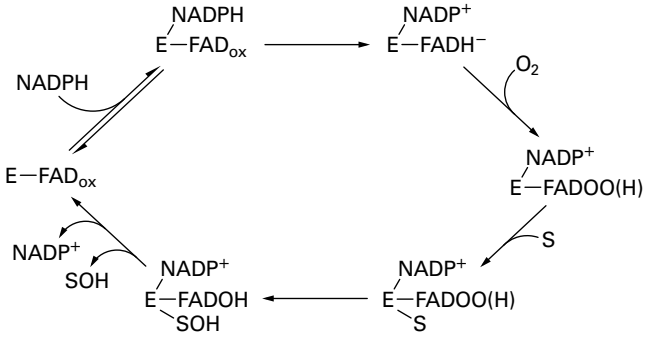

Fig. 1.7: Schematic representation of the kinetic scheme for the Group B flavin-dependent monooxygenases. This has been called the bold mechanism in which the activated oxygen is formed before the substrate binds [4].

that prevent rapid turnover and consequent oxidase activity. This same tactic is also used by several of the two-component flavin-dependent oxygenases that are described in Chapter 11 (Ellis *et al.*) and Vol. I Chapter 5 by Tu.

A schematic of the general mechanism for Group B oxygenases is shown in ▶Fig. 1.7. This has been called a bold mechanism [4] because the activated form of oxygen, the C4a-flavin (hydro)peroxide, is formed before the enzyme encounters the substrate to be oxygenated. Thus, the C4-adduct is (as Henry Kamin has suggested) like a "cocked gun" ready to go off as soon as substrate appears.

1.3.1.1 BVMOs

The BVMOs catalyze the conversion of a ketone to an ester by inserting an oxygen atom between the carbonyl and the adjacent methylene group as shown in ▶Fig. 1.8 for cyclohexanone.

This process involves a nucleophilic C4a-FAD-peroxide attack on an electrophilic carbonyl functional group to produce a tetrahedral Criegee adduct that rearranges to form the ester (or lactone) product, as shown for the cyclohexanone monooxygenase (CHMO) reaction [45,59]. This mechanism is similar to that of the classic Baeyer-

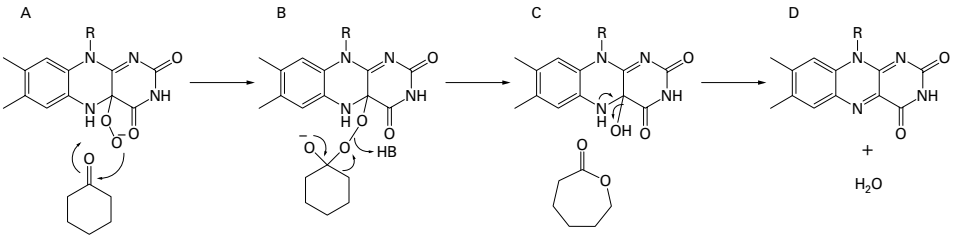

Fig. 1.8: Structural representation of the monooxygenation reaction brought about by the BVMO, cyclohexanone monooxygenase. (A) C(4a)-FAD peroxide attacking the electrophilic cyclohexanone, (B) Criegee intermediate that rearranges to form the 7-membered lactone, (C) C(4a)-FAD hydroxide, (D) oxidized FAD.

Villiger reactions used in organic synthesis [60]. In 1976 CHMO from *Acinetobacter* NCIMB 9871 was the first BVMO to be isolated and partly characterized [61]. The first mechanistic studies were carried out in 1982 [59], the enzyme was cloned and expressed in 1988 [62], and more detailed mechanistic studies followed in 2001 [45]. These studies have formed the basis of our current knowledge about the mechanism until the present day. As elaborated below, there is active research in determining crystal structures and diverse uses of BVMOs.

BVMOs are found in a wide variety of bacteria and fungi, but none have yet been found in animals or plants [63]. The BVMOs generally have broad substrate tolerances, yet they often carry out reactions with remarkable regio-selectivity and stereoselectivity. In organisms, they are involved in degradation pathways for cyclic and linear ketones, and in the biosynthesis of steroids, antibiotics, pheromones, and other chiral molecules. Many Baeyer-Villiger reactions are very useful in a wide variety of important synthetic schemes to produce precious compounds, especially for the pharmaceutical industry [63–65]. In addition to carrying out the classical Baeyer-Villiger reaction, BVMOs can also carry out oxygenations of phenyl boronic acids to yield phenols, aldehydes to acids, tertiary amines to N-oxides, and sulfur containing molecules such as thiols (often stereospecifically), sulfides, and even disulfides and dithiolanes to sulfenic acids and sulfones [64–66]. There have even been cases where BVMOs carry out epoxidations of double bonds [65]. Curiously, BVMOs that function with aromatic ketones usually do not function effectively with aliphatic ketones, and vice versa. This great diversity of reactions suggests that BVMOs are basically "vehicles" that provide reactive (hydro) peroxides to carry out both nucleophilic or electrophilic oxygenations. In contrast to the classical Baeyer-Villiger reactions, when BVMOs use sulfur-containing substrates, the C4a-adduct is most likely reacting as a hydroperoxide rather than a peroxide.

Because of the great diversity and usefulness of reactions catalyzed by BVMOs, in the past 20 years there has been extensive research into using BVMOs for green biocatalytic purposes; this research has been recently reviewed extensively [63–65]. The classical Baeyer-Villiger reaction requires the use of toxic and unstable reagents such as *m*-chloroperbenzoic acid and frequently, halogenated solvents. Thereby producing more waste than product. The use of BVMOs avoids such reagents and makes these biocatalytic processes considerably greener, often using NADPH regenerating enzymes to also avoid the need to supply costly reagents. Considerable efforts to improve the specificity, stability, and product yields, using mutagenesis, coupled reaction schemes, hybrid enzymes, incorporation of BVMOs into the metabolic schemes of microorganisms, etc. are currently in progress [63–65]. The recognition of a common sequence motif for the enzymes [67] has encouraged data-mining that has resulted in the identification of a great many BVMOs from a wide variety of species.

CHMO from *Acinetobacter* ATCC 9871 and phenylacetone monooxygenase (PAMO), a thermostable enzyme from *Thermobifida fusca,* are the BVMOs that have received the most intense mechanistic study [45,59,67–70]. The two enzymes have substantially the same overall mechanisms (►Figs. 1.7 and 1.8), so the following points about CHMO have relevance to PAMO as well. Kinetic studies [45] at 4ºC have shown that NADPH binds tightly and reduces CHMO at 22 s^{-1} at saturating concentrations of NADPH. This rate is the same between pH 7 and 9. The resulting reduced CHMO-NADP complex reacts with O_2 with a second-order rate constant of $\geq 5 \times 10^6$ $M^{-1}s^{-1}$, so fast that

accurate measurement was not possible by standard stopped-flow methods. The first intermediate, with an absorbance peak at 366 nm, has been attributed to a C4a-peroxy-FAD, as indicated in ►Fig. 1.8, species A. This species reacts rapidly (110 s^{-1}) with cyclohexanone to form the ε–caprolactone product and oxidized flavin, but with NADP still bound to the enzyme. The hydroxyflavin (C in ►Fig. 1.8) is generally not observed because the rate of dehydration to form oxidized FAD prevents its accumulation. Release of NADP at ~2 s^{-1} is the rate determining step of the catalysis under most conditions [45] and seems to involve two steps, one a conformational change that could be related to the protein dynamics described below. If the species absorbing at 366 nm is incubated at pH 7.2, it converts at ~3 s^{-1} to a species absorbing at 383 nm, and this species was shown to be essentially unreactive with cyclohexanone. When the apparently protonated species absorbing at 383 nm was shifted back to pH 9 (using double-mixing stopped-flow methods), it reverted to a 366 nm species that was reactive with cyclohexanone. It was concluded that these changes in absorbance and reactivity properties were due to protonation/deprotonation events (with an apparent pKa of 8.4), with the unprotonated species being the flavin peroxide and the protonated species being the hydroperoxide. This notion is consistent with the reactive peroxide being the nucleophile to form the Criegee intermediate in the Baeyer-Villiger reaction. Although there is definitely a protonation event with an apparent pKa of 8.4 that is important for the Baeyer-Villiger reaction, it is not totally clear that this event involves protonation of the peroxide. Preliminary studies show that the 383 nm species, which is not competent for oxygenating cyclohexanone, is also not competent for oxygenating nucleophilic sulfides, contrary to what might be expected if it were a hydroperoxide (unpublished data, E. Rees and DP Ballou). Yet the 366 nm species is competent to oxygenate sulfides. Thus, this protonation event with a pKa of 8.4 might involve a protein conformational change that prevents oxygenation from occurring rather than being due to the conversion of a peroxide to a hydroperoxide. To account for the C4a-intermediate carrying out both nucleophilic and electrophilic reactions from the 366 nm species, we assume that the requisite protonation/deprotonation events are rapid.

1.3.1.2 FMOs

The FMOs are reviewed in more detail in Chapter 3 by van Berkel in this volume. The first documented oxygenase of Group B was the FMO from liver microsomes discovered by Ziegler and colleagues [71] (at that time it was called a mixed function oxidase). Early detailed characterizations of FMOs from mammals (pig liver) were carried out largely by the groups of Ziegler and Ballou [72–75]. FMOs use an enzyme-bound electrophilic C4a-FAD hydroperoxide to oxygenate an incredibly broad range of carbon-bound nucleophilic nitrogens, sulfurs, or halides [76,77]. An early study characterized the differences in the mechanisms of oxygenation of sulfides by cytochrome P450s and by FMO, showing that P450 reactions are initiated by a single electron abstraction from the sulfide to the activated heme-oxygen, whereas with FMO the nucleophilic sulfide attacked the electrophilic C4a-flavin hydroperoxide [78]. FMOs can also oxygenate the highly electrophilic phenylboronic and butylboronic acids [75], suggesting that like organic peracids, FMOs (and BVMOs) are vehicles of both peroxides and hydroperoxides. FMOs functions in mammals (largely in conjunction with cytochrome

P450s) are to participate in the metabolism of food products, including trimethylamine, as well as to detoxify xenobiotic compounds such as drugs and other substances, roles to which they are well suited due to their very broad substrate tolerances. Genome sequencing has shown that FMOs are found in all kingdoms of life, with most species having several FMOs that are often specific for given tissues and substrates [76]. Humans have five FMOs. The isozyme FMO3, which is the principle FMO for metabolizing drugs and other xenobiotics, is particularly important for oxygenating trimethylamine; mutations in its gene often lead to trimethylaminuria, the fish-odor syndrome [79–81]. Bacterial, yeast, and plant FMOs have also been isolated. The yeast enzyme is thought to mainly oxygenate thiols to their sulfenic acids, which eliminate H_2O to form disulfides. This process could aid in forming disulfide bonds in proteins in the endoplasmic reticulum [82].

The general kinetic mechanism of FMOs is described in ▶Fig. 1.7. Curiously, the k_{cat} for most substrates is nearly the same. This is due to the fact that the rate-determining step is usually the release of NADP from the enzyme with the concomitant dehydration of the C4a-flavin hydroxide (E–FADOH in ▶Fig. 1.7). In the absence of substrate, the C4a-flavin hydroperoxide species is very stable. At 4ºC and pH 7.2 it takes 2 hours for it to lose H_2O_2 and form FAD [74]; at higher pH values, the rate of H_2O_2 loss is faster, and this characteristic is in agreement with findings by Sucharitakul *et al.* [41] for flavoprotein oxidases. This stability made it possible to form the C4a-hydroperoxide from reduced enzyme and O_2, and then react it with a series of substrates [75]. When *N,N*-dimethylaniline (DMA) was the substrate, the C4a-hydroperoxide peak at 377 nm shifted to the blue to 370 nm at a rate that was second order with respect to DMA concentration. The decay of this intermediate to the spectrum of oxidized flavin was independent of the substrate concentration, but was faster at higher pH, similar to the effects of pH on the decay of the C4a-hydroperoxide in the absence of substrate (the oxidase reaction) [75]. Similar results were observed with other substrates, but with different rates of forming the 370 nm species. With thiobenzamide and iodide, the oxygenation step caused spectral changes that could be followed spectrophotometrically. The rates of oxygenation of these two substrates corresponded to the rates of the blue shift of the C4a-spectrum. Thus, it was concluded that this blue shift was due to the formation of the C4a-FAD hydroxide, analogous to the reactions described above for PHBH. In this same study [75], reduced FMO was also incubated with various oxidized pyridine nucleotides and then reacted with O_2. In a few cases, some C4a-hydroperoxide intermediate was formed. However, none of the analogues provided the extent of stability bestowed by NADP. NHDP, which replaces the adenine of NADP with hypoxanthine, provided ~55% of the stability of NADP, whereas NAD only gave about 17% of the stability.

1.3.1.3 NMOs

NMOs are found in bacteria and fungi and are reviewed in more detail in Chapter 2 of this volume. The NMOs catalyze the hydroxylation of soft nucleophiles, such as the amines in ornithine and lysine, using mechanisms similar to that of FMOs. These enzymes are involved in biosynthetic pathways, especially those for the hydroxamate-based siderophores. The scavenging of iron by bacteria is crucial

to pathogenic bacterial virulence, and therefore, enzymes involved in siderophore biosynthesis are potential drug targets.

In contrast to the broad substrate tolerance of FMOs and BVMOs, NMOs usually are very specific [83,84]. For example, ornithine monooxygenases are not capable of oxygenating lysine or arginine and lysine monooxygenase cannot oxygenate ornithine. The most thoroughly characterized NMOs are the ornithine monooxygenases from *Aspergillus fumigatus* (called SidA or Af-OMO) [83,85,86] and from *Pseudomonas aeruginosa* (PvdA) [87–89]. One of the problems with studying the OMOs is that they do not bind FAD very tightly. Therefore, only a fraction of the enzyme is populated with FAD. Af-OMO binds FAD considerably more tightly than does PvdA [88].

Reduction rates of Af-OMO or of PvdA by NADPH are about the same either in the presence or absence of ornithine, similar to the BVMOs and FMOs described above. However, in contrast to the BVMOs and FMOs, formation of the C4a-FAD hydroperoxy is faster in the presence of ornithine. The rate with Af-OMO is ~15-fold faster with ornithine present [90], while that for PvdA is ~80-fold faster [87]. Because formation of the C4a-hydroperoxide is not the rate-determining step of catalysis, this stimulation does not increase the overall rate of turnover. Thus, its activating function on the reaction with O_2 is not clear. In the case of Af-OMO, arginine is an effector for Af-OMO and stimulates oxidase turnover, completely uncoupling the reaction from oxygenation and simply releasing H_2O_2 [90]. Although lysine is not oxygenated, it is also an effector for Af-OMO, causing NADPH turnover at about the same rate as does ornithine; it does not stimulate the reactions to the same extent as does arginine. The rate of reduction of Af-OMO is 1.46 s^{-1} with ornithine and 20.3 s^{-1} with arginine present. The rate of reaction with O_2 (125 μM) to form its C4a-hydroperoxide is 1.28 s^{-1} without substrate, 19.8 s^{-1} with ornithine, and 144 s^{-1} with arginine present [90]. In the absence of substrate, the C4a-FAD hydroperoxides of the ornithine hydroxylases are quite stable. That for Af-OMO has a half-life at pH 8, 25ºC, of 33 min. Although overall the reaction mechanisms are very similar to those of FMOs, it is clear that in contrast to FMOs, ornithine stimulates the reaction of the reduced FAD with O_2.

1.3.1.4 YUCCAs

The fourth subclass of the Group B monooxygenases contains the YUCCA enzymes, which are found in all plants and are involved in the biosynthesis of auxin (2-(1H-indol-3-yl)acetic acid, IAA), the primary growth hormone and developmental regulator of plants [91,92]. Auxin is synthesized from tryptophan. There are 11 YUCCAs in the *Arabidopsis thaliana* genome, and they have functions in different developmental stages as well as in different parts of the plant [91,92]. The YUCCA enzymes are related by sequence to the FMOs and, by analogy, were previously thought to catalyze the *N*-oxygenation of tryptamine; it was postulated that its hydroxylated product was further oxidized to auxin [93]. Recently, however, it has been shown that YUCCA2 (and probably most or all YUCCAs) decarboxylate indole pyruvic acid (IPA) to form auxin (►Fig. 1.9) [94].

The IPA substrate derives from a transamination of tryptophan by a family of amino acid transaminases [94]. The mechanism of YUCCA enzymes likely involves a nucleophilic attack of the C4a-FAD peroxide on the keto function of IPA to form a Criegee intermediate, analogously to the BVMOs. Thus, in contrast to the related FMOs, YUCCAs most likely

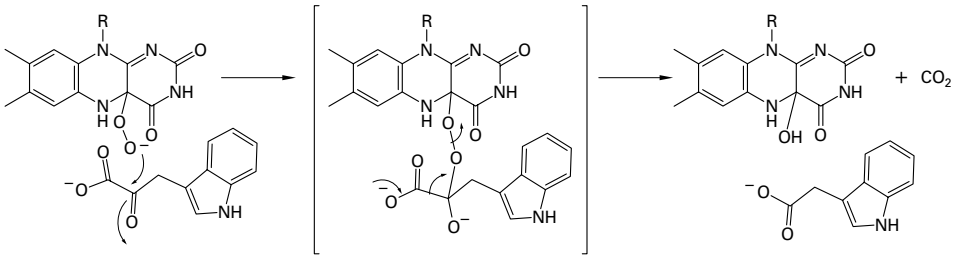

Fig. 1.9: Structural representation of the formation of auxin (indole acetic acid) by YUCCA. The flavin C4a-peroxide attacks the carbonyl of the indole pyruvate to form a Criegee intermediate (brackets). This intermediate decarboxylates, leaving the indole acetic acid and the C4a-flavin hydroxide, which loses H_2O to form oxidized flavin.

use a C(4a)-FAD peroxide. Mechanistic work on YUCCAs has been sparse because of difficulties in handling these unstable enzymes. However, newly developed preparations and conditions have permitted rapid kinetics and steady-state kinetics studies in the Zhao, Ballou, and DuBois laboratories demonstrating that a C4a-FAD peroxide forms and reacts with IPA to form auxin, as shown in ▶Fig. 1.9 [95].

1.3.2 Structural features

All of the enzymes in Group B that have been studied by X-ray crystallography have similar structures. There are published structures for two FMOs: from *Schizosaccharomyces pombe* [96], and from *Methylophaga sp*. SK1 [97,98]. However, no structures are available for the mammalian FMOs. There are structures for four BVMOs: PAMO, which was the first available structure for a Group B monooxygenase [68,99], CHMO [69,70], 2-oxo-Δ(3)-4,5,5-trimethylcyclopentenylacetyl-coenzyme A monooxygenase (OTEMO) of *Pseudomonas putida* ATCC 17453 [100], and mithramycin monooxygenase (MtmOIV) [101]. The last structure is not similar to the other BVMOs, and it falls better into Group A. Little is known about this enzyme, and it will not be discussed. OTEMO is unusual in that it uses FMN rather than FAD, but still has many similar characteristics to other BVMOs. There are also published structures for one NMO: PvdA, the OMO from *P. aeruginosa* [88,89].

There are many common features to the structures of Group B monooxygenases. They all have two Rossmann folds, one largely at the N-terminus for binding FAD and the other for binding NADP(H), whereas Group A enzymes only have a single Rossmann fold for the FAD. Therefore, the Group B monooxygenase structures are more similar to the flavoprotein disulfide reductases, such as glutathione reductase, than they are to the Group A monooxygenases. This second Rossmann fold in Group B enzymes accounts for their ability to retain the pyridine nucleotide throughout catalysis. The active site of the BVMOs and NMOs is in a cleft at the domain interface of the two Rossmann folds; in contrast, FMOs, as expected, given the wide range of substrates they can oxygenate, do not have a well-defined substrate-binding site. The Baeyer-Villiger sequence motif (FXGXXXHXXXW(P/D)), which is also nearly the same in the FMOs [67], is found to be quite distant from the active site, but part of a surface loop that connects the two

Rossmann fold domains. This linker is important for various protein dynamics required for efficient catalysis (see below). The FAD binding domains are all α/β folds like those of the glutathione oxidoreductase family. In general, the FADs are buried within the protein, although with PvdA, the FAD is more exposed, which probably accounts for its weak binding in this NMO.

The BVMOs have a conserved arginine (Arg337 in PAMO) that likely is important for stabilizing the Criegee intermediate during catalysis, most likely by interacting with the carbonyl to be attacked by the C4a-peroxide [68,69]. Common to most of the Group B enzyme structures is the finding that more than one conformation exists. This undoubtedly reflects the plasticity and the protein dynamics that are important for catalysis and crucial to accommodating such a wide variety of substrates, both large and small. For example, PAMO exists in "out" and "in" forms in which the nicotinamide takes on two conformations [68], and CHMO has analogous "open" and "closed" conformations with NADP binding in two distinct conformations [70]. In one conformation of PAMO, the nicotinamide, before substrate binds, helps O_2 to approach the front of the flavin to form the C4a-adduct, and it forms important H-bonds with the N5-hydrogen and the C4-carbonyl of the flavin to stabilize the C4a-peroxide adduct. In CHMO it was shown that "sliding" of the nicotinamide away from the flavin opened up a well-defined substrate-binding pocket [69]. This helps to explain the kinetic mechanism in which substrate binds after forming the C4a-peroxide [45,59]. Recently, it was shown that OTEMO also exhibits similar plasticity in its structure [100]. Yachnin *et al.* [69] have shown in CHMO how a major rotation of the nicotinamide away from the isoalloxazine ring permits the substrate to be positioned correctly to react with the C4a-flavin peroxide to form the Criegee intermediate. These motions are facilitated by the linking loop containing the signature motif (above); mutations in this loop impair activity.

The structural plasticity of BVMOs and other Group B monooxygenases helps to explain how such complicated monooxygenation reactions can be catalyzed by a single enzyme. The movement of the nicotinamide of NADP is reminiscent of the movement of the isoalloxazine in PHBH that was described above. Different conformations help develop different characteristics to promote the diverse segments of the catalysis. It was postulated that CHMO, for example, employs a series of conformations that allow reduction of the flavin, formation and stabilization of the C4a-FAD peroxide by the NADP, binding of substrate, formation of the Criegee intermediate, and stereochemical selection of a given Baeyer-Villiger rearrangement [69]. A picture emerges that extensive conformational dynamics are important for many of these Group B enzymes. Future studies will undoubtedly show in greater detail how the proteins enable such a complex catalytic dance.

1.4 References

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2 Flavin-dependent monooxygenases in siderophore biosynthesis

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Abstract

Microbial *N*-hydroxylating monooxygenases (NMOs) are a class of flavin-dependent enzymes involved in the biosynthesis of hydroxamate-containing siderophores. These flavoenzymes catalyze the NADPH- and oxygen-dependent *N*-hydroxylation of a narrow group of substrates that include L-ornithine, L-lysine, and primary aliphatic diamines. Siderophores are essential for microbial pathogenesis and the activities of NMOs are essential for their biosynthesis and function. To date, only a small number of NMOs have been characterized. While the biochemical properties of NMOs vary, two common characteristics have been observed. These include a high degree of substrate specificity and the stabilization of a long-lived C4a-hydroperoxyflavin intermediate. These properties ensure efficient hydroxylation of the appropriate substrate and minimal release of hydrogen peroxide, respectively. Recent mechanistic and structural studies have provided insight into the molecular mechanisms of these two traits. The high substrate specificity has been attributed to a unique "molecular ruler mechanism," and stabilization of the C4a-hydroperoxyflavin is achieved by specific interactions with $NADP^+$. In addition, a high-throughput screening assay that allows for identification of NMO inhibitors from small molecule libraries has been developed. This, compounded with rational drug design, holds promise for the identification of inhibitors to combat increasingly virulent microbes.

2.1 Iron, an essential but scarce nutrient

Iron is an essential element required by most living organisms [1]. The need for this metal originates from its requirement as a cofactor in a wide range of biological reactions. The functional diversity of iron results from its inherent nature as a reductant or an oxidant, depending on whether it is present in the ferrous (Fe^{II}) or ferric state (Fe^{III}), respectively. Furthermore, while these two oxidation states dominate in biological systems, when iron is bound to certain proteins the reaction between ferrous iron and molecular oxygen can lead to the formation of high-valent iron-oxo species, which catalyze a number of oxygenation reactions.

One important role of iron is demonstrated by the oxygen transport protein hemoglobin [2]. Here, iron is complexed with a porphyrin ring, which allows oxygen to be transported to different tissues of the body for use in many oxygen dependent reactions. In plants, iron plays an essential role in photosynthesis. Chloroplast ferredoxin is an iron-containing protein that acts as a carrier of electrons that are produced from

absorbed sunlight [3]. In microorganisms, perhaps the most important iron-containing reaction is observed with nitrogenase, which catalyzes the reduction of dinitrogen to ammonia [4]. The resulting "fixed nitrogen" can be utilized in the nitrogen cycle where it is used for the biosynthesis of amino and nucleic acids, as well as other nitrogen-containing biomolecules.

In vivo, free iron is toxic [5]. The reaction of iron in its ferrous or ferric form with hydrogen peroxide, termed the Fenton reaction, can lead to the generation of reactive oxygen species such as superoxide and hydroxyl radicals [6]. Effects of these oxidative by-products include lipid peroxidation, protein denaturation, and DNA strand breaks, all of which are deleterious to the cell [7].

Mammals have evolved several mechanisms to lower the concentration of free iron to minimize the level of toxic oxidative by-products. Iron-binding proteins such as transferrin, lactoferrin, and ferritin store excess iron and make it available during periods of iron limitation [8,9]. Iron sequestration in mammals also has antimicrobial activity as it limits the availability of this essential nutrient in serum [10]. This, compounded with the fact that free ferric iron forms insoluble iron-hydroxide complexes (10^{-18} M at pH 7), represents a fundamental problem of iron deficiency that pathogenic microbes must overcome [11]. In response, pathogens have evolved mechanisms to scavenge iron from mammalian hosts by synthesizing and secreting low-molecular weight iron chelators termed siderophores (Greek for "iron carrier") [12].

2.2 Siderophores

Siderophores provide a unique mechanism for pathogens to acquire iron where they can utilize it for their own metabolic needs in a non-toxic fashion. Siderophores competitively acquire iron from the bacterial host due to their remarkably high affinity for ferric iron. For example, the catechol siderophore enterobactin produced by the bacterium *Escherichia coli* possesses a K_d value for Fe^{3+}on the order of 10^{-49} M [13]. This high affinity allows pathogens to proliferate by scavenging iron from hosts. Siderophores vary in their structures and denticity for iron, ranging from bi- to hexadentate [14]. Siderophores can contain a number of functional groups that chelate ferric iron, including catechols, phenols, hydroxamates, and carboxylates [15]. Examples of siderophores from different organisms are shown in ►Fig. 2.1, and a list of siderophores along with their respective affinity constants for Fe^{III} is given in ►Tab. 2.1.

2.2.1 Siderophores are important virulence factors

Siderophores have been shown to be linked to virulence in many human pathogens. For instance, deletion of the gene cluster involved in the biosynthesis of the siderophore anthrachelin in *Bacillus anthracis* resulted in both attenuated growth and virulence in macrophages and mice [25]. A similar effect was demonstrated in *Pseudomonas aeruginosa* where pyoverdin- and pyochelin-deficient mutants grew poorly in immunosuppressed mice. Lethality in mice was reduced from 100% in wild-type to 0% in the double mutant 48 hours post inoculation [26]. Effects on virulence were also observed in *Aspergillus fumigatus* and *Burkholderia cepacia* upon deletion of genes involved in siderophore biosynthesis [27–29]. In *A. fumigatus*, disruption of the biosynthesis of the