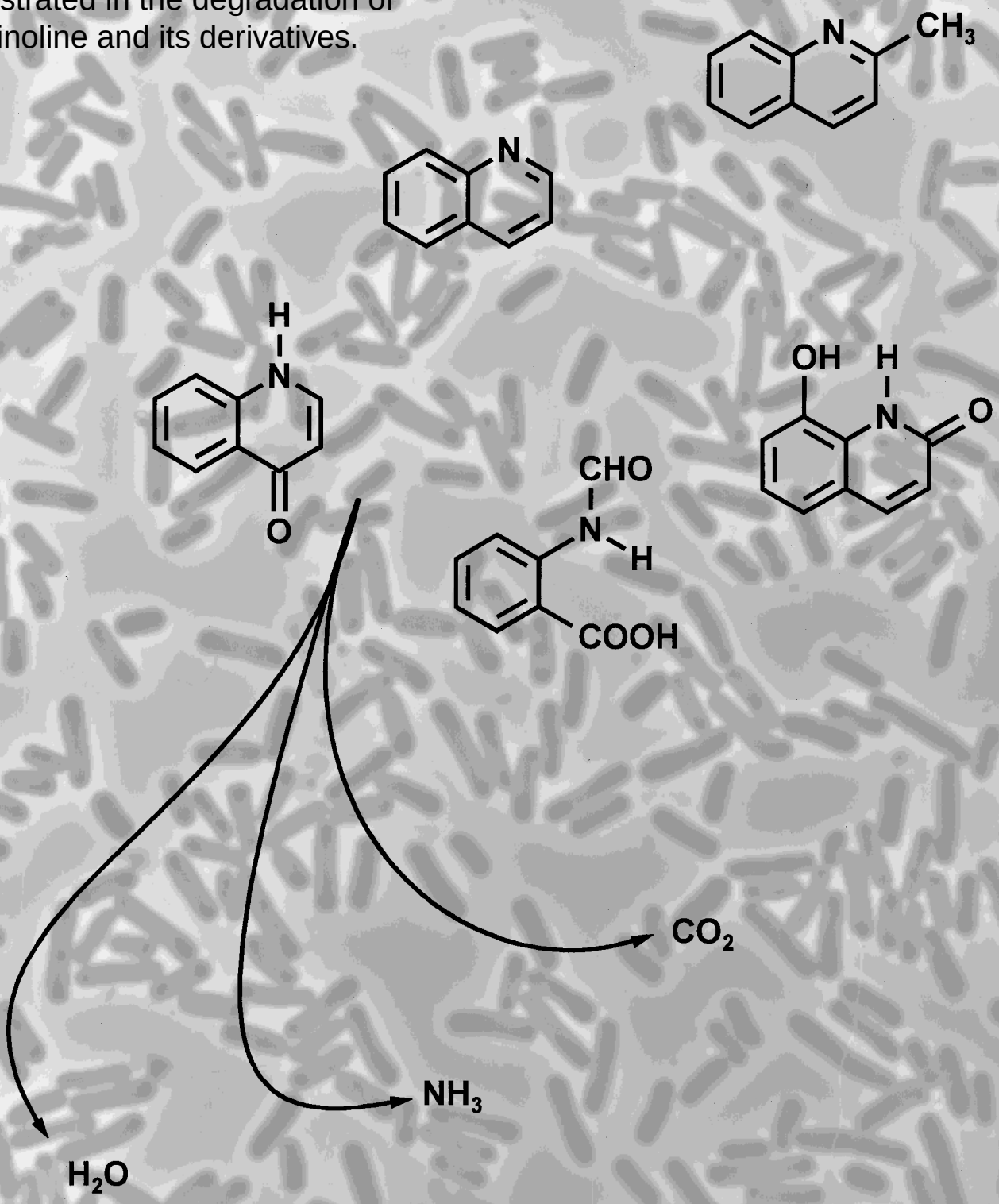
The versatility and diversity of bacterial metabolism is well illustrated in the degradation of quinoline and its derivatives.



Various aerobic bacteria utilize these compounds as sole source of carbon and energy, leading to mineralization.

Bacterial Degradation of Quinoline and Derivatives—Pathways and Their Biocatalysts

Susanne Fetzner,* Barbara Tshisuaka, Franz Lingens, Reinhard Kappl, and Jürgen Hüttermann

In memory of Adolf Butenandt and Heinrich Hellmann

As a consequence of the continuous selection pressure exerted by the abiotic and biotic world, bacteria have evolved an immensely diverse potential to transform and even mineralize numerous organic compounds of both natural and xenobiotic origin. Quinoline (2,3-benzopyridine) derivatives were chosen as model compounds for studying the bacterial catabolism of heteroaromatic substances. The investigation of even this fairly limited spectrum of organic growth substrates yielded several catabolic pathways, which demonstrated the diversity of bacterial metabolism. However, common features of these aerobic pathways are enzyme-catalyzed hydroxylation reactions that prepare the substrate for the attack by ring-cleavage enzymes, and dioxygenolytic cleavage of the aromatic or heterocyclic ring. To develop a comprehensive understanding of enzyme-catalyzed reactions, various disciplines such as microbiology, biochemistry, biophysics, and molecular genetics need to contribute. The ultimate goals are to understand the mechanism of action of such biocatalysts, to get a view of the three-dimensional structure of the enzyme proteins, and to elucidate the molecular basis of reactivity. Such knowledge will allow us to understand metabolic processes on the molecular level, and it may also enable us to evaluate the potential of biocatalysts for use in industrial applications.

Keywords: enzymes · enzyme catalysis · heterocycles · metabolism · quinoline

1. Introduction

Quinolines and related N-heterocyclic compounds are ubiquitous environmental contaminants, typically released in association with coal gasification, fossil fuel processing activities, coal tar wastes, and creosote wood preservation. Compared with other azarenes occurring in coal tar and crude oil, quinolines have a relatively good solubility in water. The environmental persistence of these N-heterocyclic aromatic compounds is apparent from their detection in the tissues of fish,[1, 2] in sediments,[1, 3, 4] in urban air,[1, 4] in soils, and in groundwater, especially near landfills containing azarene-contaminated solid wastes, and near coal tar distillation and creosote wood preservation facilities.[1, 5]

N-heterocyclic aromatic compounds are of particular concern, because many are biologically more active than their homocyclic analogues. Quinoline is a hepatocarcinogen in mice and rats,[6] and quinoline and several derivatives have been shown to be indirect mutagens with the Ames assay.[4, 7] N-Heterocyclic amines, isolated from cooked food, pyrolysates of amino acids and protein, and heated mixtures of creatine, amino acids, and sugars exhibit mutagenic and carcinogenic activity towards bacteria and mammals.[8] Furthermore, potentially neurotoxic effects of halogen-substituted oxyquinolines were reported. Subacute myelo-optico neuropathy (SMON), which in the 1960s in Japan reached epidemic proportions, was suspected to be elicited by oxyquinoline derivatives under the names Entero-Vioform or Mexaform frequently used to treat enteric disorders.[9]

Nevertheless, quinoline derivatives are indispensable for various medicinal purposes. Cinchona bark, which contains quinine alkaloids, has been known for its therapeutic properties for more than three centuries.[10] Till today, quinine, primaquine, and resoquine are used as antimalarial agents. 8-Hydroxyquinolines are prescribed to treat infections of skin and intestine. Fluoro-substituted 4-quinolones are widely used due to their bactericidal effect against a number of gram-negative and some gram-positive pathogens.[11, 12] Continuous efforts to develop new fluoroquinolone compounds now allow a multiplicity of applications (for instance, in

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treatment of leprosy and other mycobacterioses,[13] typhoid fever, and enteric infections such as drug-resistant shigellosis).[14]

Apart from these applications as pharmaceuticals, quinoline derivatives are also used as raw material and solvent in the manufacture of agrochemicals, dyes, and paints.[15] Quinoline yellow, produced by condensation of 2-methylquinoline and phthalic acid anhydride, is a coloring agent for food licensed in Germany as E104.

However, the widespread use of these N-heterocyclic aromatic compounds in our everyday life has led to an increase in the number of persons afflicted with allergies towards quinoline derivatives.[16]

Numerous quinoline derivatives are produced naturally, mainly by higher plants. By 1987 the structures of several hundred quinoline alkaloids had been established.[10] The quinine alkaloids mentioned above are mainly produced by subtropical trees belonging to the genera *Cinchona* and *Remija*. Papaverine from *Papaver somniferum* is a well-known isoquinoline alkaloid. However, formation of quinoline derivatives also occurs in mammals and insects: Kynurenic acid (1*H*-4-oxoquinoline-2-carboxylic acid) was first

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Susanne Fetzner, born in 1963 in Calw, Germany, studied biology at the Universität Hohenheim (1982–1988). Her graduate work under the direction of Prof. Dr. F. Lingens involved studies of the bacterial degradation of haloaromatic compounds and of a bacterial enzyme system that catalyzes a dehalogenation reaction. She received her doctoral degree in 1990 from the Universität Hohenheim. Following six years of postdoctoral work on microbial enzymes that catalyze hydroxylation reactions and ring cleavage of aromatic and heterocyclic compounds, she received her venia legendi for microbiology in 1996 from the Universität Hohenheim. Since 1996, she has been deputizing for the Professor of Microbiology at the Carl-von-Ossietzky-Universität Oldenburg. Her research interests include enzymology and genetic organization of catabolic pathways.

Barbara I. Tshisuaka, born in 1951 in Rudolstadt, Thuringia (Germany), spent ten years as Medical-technical assistant before studying Biology at the Universität Hohenheim (1984–1990). She obtained her doctorate in 1992 on the enzymatic hydroxylation of quinoline under the supervision of Professor F. Lingens. Still part of this research group she concentrates on bacterial molybdenum-containing hydroxylases and pterin-molybdenum cofactors, as well as on enzymatic and genetic topics concerning the microbial degradation of quinoline and its derivatives. Since her research in Brazil in 1996 she is also studying bacterial endoinulinases.

Franz Lingens, born in 1925, received his doctoral degree and venia legendi in Tübingen, and has worked at the Universität Hohenheim in Stuttgart since 1967. His research interests are the synthesis of amino acids with Mannich bases, the asymmetric three-carbon condensation, the Amadori rearrangement, anabolic and catabolic pathways in microorganisms, the biosynthesis of amino acids, riboflavin and pyrrolnitrin, the mechanisms of mutagenesis, biological halogenation and dehalogenation, microbial degradation of aromatic and heterocyclic compounds, and multicomponent enzyme systems, their properties and genetics.

Reinhard Kappl, born in 1956, studied Physics at the Universität Regensburg and the University of Colorado from 1975 and obtained his Diplom in 1982. He completed a doctorate at the Institut für Biophysik in Regensburg in 1988. Since 1986 he has been a scientific officer at the Institut für Biophysik und Physikalische Grundlagen der Medizin at the Universität des Saarlandes and the university clinic, Homburg/Saar.

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isolated from the urine of a dog by Liebig in 1853, and kynurin (1*H*-4-oxoquinoline) was isolated from pupae of the silk-moth *Bombyx mori* by Butenandt, Karlson, and Zillig.[17] Both kynurenic acid and kynurin are formed as degradation products of the aromatic amino acid tryptophan. Some bacteria belonging to the *Pseudomonas* group produce pseudans (2-*n*-alkyl-4-hydroxyquinoline derivatives) as secondary metabolites.[10, 18]

Presumably as a consequence of the natural occurrence of a variety of quinoline derivatives, bacteria have evolved strategies for metabolizing these heteroaromatic compounds.[19] In several laboratories bacterial strains that utilize quinoline or quinoline derivatives as their sole source of carbon and energy have been isolated from soil and water samples. The facile isolation of quinoline-degrading bacteria indicates that such organisms are widespread in nature. The metabolism of aerobic bacteria that grow on quinoline (derivatives) has been studied by the authors' group. Putative intermediates of the catabolic pathways were isolated and characterized, and enzymes catalyzing important steps of the degradative pathways have been purified and investigated. Unexpectedly, a novel type of ring-fission enzymes was discovered, namely, dioxygenases that catalyze the cleavage of two C–C bonds with concomitant formation of carbon monoxide—a quite exceptional way to break open a heterocyclic compound. Another important family of enzymes involved in the catabolic pathways are the molybdenum-containing hydroxylases, which in their active centers contain distinct forms of a pterin molybdenum cofactor.

The sophisticated enzyme proteins synthesized by all living organisms are fascinating objects to the scientist who tries to understand how these biocatalysts work. From a more practical point of view, however, such biocatalysts may well be suitable tools of applied chemistry. Particularly, hydroxylation reactions are important steps of various chemical syntheses for the manufacture of agrochemicals and pharmaceuticals. Biocatalysts such as molybdenum-containing hydroxylases offer substrate- and regiospecific hydroxylations free of side products, which may be difficult and/or expensive to achieve with conventional chemistry. Thus, it may be a prospect in the near future to produce hydroxylated intermediates with bacterial strains or isolated bacterial enzyme systems for use in subsequent syntheses.

2. Pathways of Aerobic Bacterial Degradation of Quinoline and Derivatives

2.1. General Features of Bacterial Utilization of Quinoline and Derivatives

Our studies on bacterial quinoline degradation started with the isolation of 16 bacterial strains capable of utilizing quinoline as sole source of carbon, nitrogen, and energy from soil and water samples, and from activated sludge of a sewage treatment plant.[20] Two strains belonging to the Gram-negative pseudomonads and the Gram-positive *Rhodococcus* strain B1 were chosen for more detailed investigation. From metabolic intermediates found in the growth medium, two different pathways for the degradation of quinoline were proposed[21] (Sections 2.3 and 2.5). In the initial step of both pathways, quinoline is converted into 1*H*-2-oxoquinoline. Actually, the bacterial degradation of quinoline derivatives as a rule starts with a hydroxylation reaction either in 2- or 4-position to the N atom. Labeling studies with $H_2{}^{18}O$ performed later with purified hydroxylating enzymes revealed that the oxygen atom incorporated into the product in almost all cases is derived from a water molecule, not from molecular oxygen.

When growing on quinoline (or its derivatives), many bacterial strains initially converted the substrate nearly quantitatively into such an 1*H*-2-oxo- or 1*H*-4-oxo- compound, which transiently accumulated in the culture medium before degradation proceeded. As an example, Figure 1 shows the accumulation of kynurenic acid (1*H*-4-oxoquinoline-2-carboxylic acid) during growth of a *Serratia* strain on quinaldic acid (quinoline-2-carboxylic acid). In general, such a massive

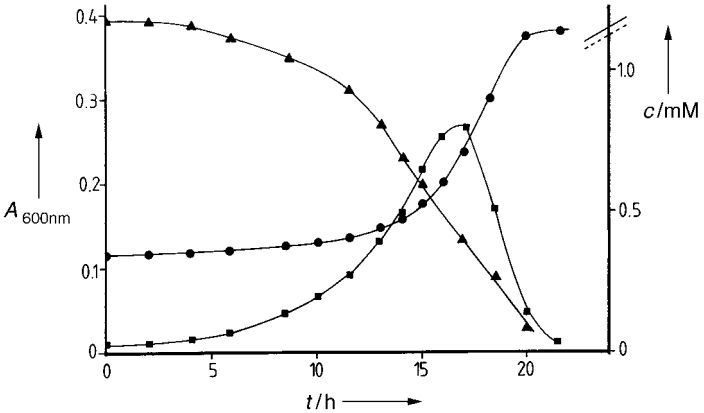


Figure 1. Growth of *Serratia marcescens* 2CC-1 on quinaldic acid. ●: Absorption (turbidity) at 600 nm (A_{600nm}) indicates the growth; ▲: *c*(quinaldic acid); ■: *c*(kynurenic acid).

accumulation of a metabolic intermediate is unusual. It may occur if the next enzyme of the catabolic pathway is a "bottleneck" due to its low catalytic activity. Another possibility is a differential regulatory mechanism such as sequential induction of distinct sets of catabolic genes, each set regulated by a specific effector (substrate), which activates gene expression when reaching a certain threshold concentration. Up to now, we do not know the reason for the observed accumulation of the 1*H*-2-oxo- or 1*H*-4-oxo-compounds. However, rapid initial hydroxylation may well be a vital function for instantaneous detoxification of quinoline (or derivatives), since the oxo compounds were found to be considerably less toxic for the bacteria than the unhydroxylated substrate.

Transient excretion into the culture broth of a catabolic intermediate is, of course, very helpful in establishing the reactions of a degradation pathway. Putative intermediates also can be obtained by the production and selection of mutants blocked in distinct catalytic steps of the pathway or by the use of antimetabolites, which are structural mimics of natural metabolites. Both methods may lead to accumulation of the metabolite prior to the block, or, less favorably, to the

accumulation of a transformation or decomposition product of the true intermediate. In contrast to such secondary products, true catabolic intermediates should disappear and support growth when fed to the bacteria. However, even a true intermediate may not be consumed if the bacterial cells fail to take up the exogenously supplied metabolite.

Nevertheless, purification of putative metabolic intermediates and structural determination with methods such as UV/Vis spectroscopy, mass spectrometry, ^{1}H and ^{13}C NMR spectroscopy and infrared spectroscopy finally may allow the formulation of a hypothetical reaction sequence. Currently four quinoline catabolic pathways of aerobic bacteria are known, which are presented in the following: the anthranilate pathway (Section 2.2), the 5,6-dihydroxy-1*H*-2-oxoquinoline pathway (Section 2.3), the 7,8-dihydroxy-1*H*-2-oxoquinoline pathway (Section 2.4), and the 8-hydroxycoumarin pathway (Section 2.5).

2.2. Anthranilate Pathway

The degradation of quinaldine (2-methylquinoline **1**) by *Arthrobacter* sp. Rü61a (Scheme 1a) is initiated by a hydroxylation at C4 of the substrate. The second step, a monooxygenation of the product **2** at C3, is followed by 2,4-dioxygenolytic cleavage of 1*H*-3-hydroxy-4-oxoquinaldine (**3**). This rather unique ring cleavage reaction (cf. Section 3) yields carbon monoxide and *N*-acetylanthranilate (**4**). The hydrolysis product anthranilate **5** is further degraded via catechol **6** and the well-known 2-oxoadipate pathway into Krebs cycle intermediates.[22] An analogous pathway of 1*H*-4-oxoquinoline (**7**) degradation via the hydroxylated intermediates **8** and **9** to anthranilate **5** has been found in *Pseudomonas putida* 33/1 (Scheme 1a).[23] Thus, in the anthranilate pathway, cleavage of the N-heterocyclic ring occurs prior to benzene ring cleavage.

2.3. 5,6-Dihydroxy-1*H*-2-oxoquinoline Pathway

Comamonas testosteroni 63 degrades 3-methylquinoline (**11**) via 5,6-dihydroxy-1*H*-3-methyl-2-oxoquinoline (**19**), which is proposed to undergo cleavage of the benzene ring, since 3-methyl-2,5,6-trihydroxypyridine (**21**) was isolated as N-heterocyclic intermediate (Scheme 1b).[24] 5,6-Dihydroxy-1*H*-2-oxoquinoline (**18**) presumably also is an intermediate in the degradation of quinoline (**10**) by *Comamonas testosteroni* 63,[25] *Pseudomonas putida* K1,[26] *Rhodococcus* sp. B1,[21] a *Moraxella* sp.,[27] and perhaps a *Nocardia* sp.[28] An aromatic *ortho*-dihydroxy derivative such as **18** or **19** might either derive from a dioxygenase-catalyzed formation of a dihydrodiol followed by a dehydrogenase reaction, or it might be formed in two monooxygenase-catalyzed steps. In the case of the 1*H*-(3-methyl-)2-oxoquinolines (**12** or **13**), which are converted by *Comamonas testosteroni* 63, a 5,6-dioxygenase was shown to catalyze the formation of the corresponding 5,6-dihydrodiol **14** or **15** (Scheme 1b).[25]

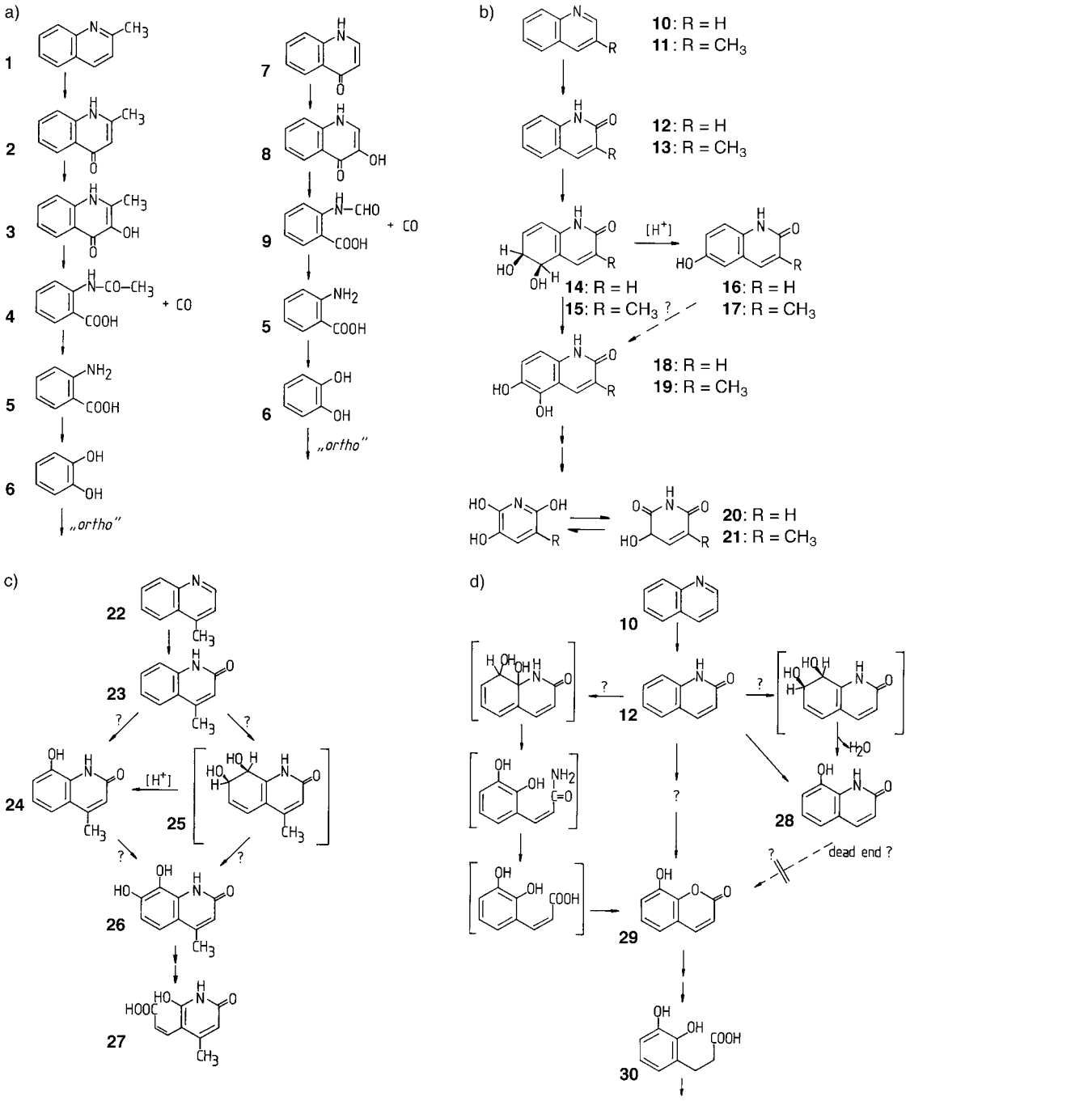
2.4. 7,8-Dihydroxy-1*H*-2-oxoquinoline Pathway

Similar to the pathway described in Section 2.3, the 7,8-dihydroxy-1*H*-2-oxoquinoline pathway also proceeds via an *ortho*-dihydroxy benzenoid compound, which probably undergoes benzene ring cleavage. 7,8-Dihydroxy-1*H*-2-oxoquinoline derivatives are key intermediates in the degradation of 4-methylquinoline (**22**) by *Pseudomonas putida* K1[26] (Scheme 1c), of 4-hydroxyquinoline by a soil pseudomonad,[29] and of kynurenic acid.[30, 31] The 8-monohydroxy derivative **24** of 1*H*-4-methyl-2-oxoquinoline (**23**) has been found as metabolite in the acidic extracts of the culture broth of the 4-methylquinoline degrader *Pseudomonas putida* K1 (Scheme 1c),[26] but this compound might be a dead-end product derived from dehydration of a hypothetical 7,8-dihydrodiol (**25**) rather than a true intermediate. The putative 7,8-dioxygenases of these pathways have not been investigated yet. However, in the degradation pathway of kynurenic acid by *Pseudomonas fluorescens*, the *cis*-7,8-dihydrodiol was detected as metabolite.[30]

2.5. 8-Hydroxycoumarin Pathway

Whereas in both pathways outlined in Scheme 1b and 1c the benzene nucleus is cleaved prior to the pyridine ring, there is a unique pathway of quinoline (**10**) degradation involving preferential cleavage of the N-heterocyclic ring and formation of 8-hydroxycoumarin (**29**, Scheme 1d). This pathway of quinoline degradation was first described by Shukla,[32] and it was also found in two *Pseudomonas* strains by our group.[21] However, the reaction sequence of quinoline degradation remains to be elucidated: Only a few metabolites have been identified as true intermediates, and the mechanism of N-heterocyclic ring cleavage per se is unknown. In *Pseudomonas putida* 86,[21] *Pseudomonas fluorescens* 3,[21] *Pseudomonas pseudoalcaligenes*, and *Pseudomonas stutzeri*,[32] 1*H*-2-oxoquinoline (**12**), 1*H*-8-hydroxy-2-oxoquinoline (**28**), 8-hydroxycoumarin (**29**), and 2,3-dihydroxyphenylpropionate (**30**) were found as metabolites. Compounds **29** and **30** appear to be true intermediates, but the mode of formation for **30** from **29** or from another hypothetical intermediate is not known yet. However, the enzymes catalyzing the first and second step of this pathway were investigated. Quinoline 2-oxidoreductase from *Pseudomonas putida* 86, which catalyzes the formation of 1*H*-2-oxoquinoline (**12**), was characterized thoroughly (Section 4.2).

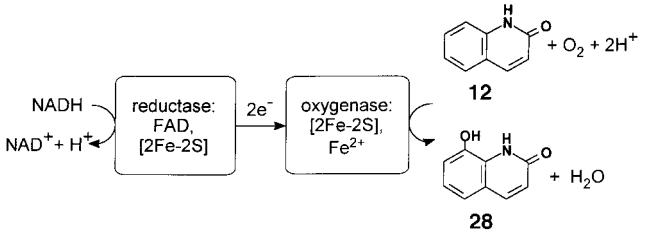
1*H*-2-Oxoquinoline 8-monooxygenase, which in the next step catalyzes the formation of 1*H*-8-hydroxy-2-oxoquinoline (**28**), consists of two inducible protein components, a "reductase" and "oxygenase" component. These protein components comprise a short electron transfer chain from the exogenous electron donor NADH via the redox centers of the reductase component (FAD and [2Fe-2S] ferredoxin) to the redox centers of the oxygenase (Rieske-type [2Fe2S] clusters), and finally to a proposed mononuclear Fe^{2+} center of the oxygenase component (Scheme 2). The latter is believed to activate molecular oxygen, and is thus the site of substrate oxygenation.[33] The subsequent steps of the degradation



Scheme 1. Pathways of the aerobic bacterial degradation of quinoline and its derivatives. a) Anthranilate pathway: degradation of quinaldine (**1**) by *Arthrobacter* sp. Rü61a[22] and degradation of 1*H*-4-oxoquinoline (**7**) by *Pseudomonas putida* 33/1.[23] b) 5,6-Dihydroxy-1*H*-2-oxoquinoline pathway: degradation of quinoline (**10**) and 3-methylquinoline (**11**) by *Comamonas testosteroni* 63.[24] c) 7,8-Dihydroxy-1*H*-2-oxoquinoline pathway: degradation of 4-methylquinoline by *Pseudomonas putida* K1.[26] d) 8-Hydroxycoumarin pathway: degradation of quinoline (**10**) by *Pseudomonas putida* 86,[21] *Pseudomonas fluorescens* 3,[21] *Pseudomonas stutzeri*, and *Pseudomonas pseudoalcaligenes*.[32]

pathway are speculative (Scheme 1d), since the failure to detect further proposed enzymes so far has precluded the detailed elucidation of the 8-hydroxycoumarin pathway.

Despite our current knowledge of a number of catabolic pathways, the metabolic route for the degradation of a quinoline derivative by a bacterial strain cannot be predicted. In *Comamonas testosteroni* 63, quinoline (**10**) and 3-methylquinoline (**11**) both are metabolized along the same pathway (Section 2.3), presumably by the same set of catabolic enzymes.[25] In contrast, *Pseudomonas* sp. K1 utilizes 4-methylquinoline (**22**) in the 7,8-dihydroxy-1*H*-2-oxoquinoline pathway (Section 2.4), but presumably degrades quinoline (**10**) along the 5,6-dihydroxy-1*H*-2-oxoquinoline pathway (Section 2.3); thus with this strain, each substrate induces the expression of a separate set of catabolic enzymes.[26] Fundamentally, the unsubstituted quinoline was degradable along three different pathways (those of Sections 2.3, 2.4, and 2.5), depending on the bacterial strain.



Scheme 2. Reaction catalyzed by 1*H*-2-oxoquinoline 8-monooxygenase, a two-component enzyme system from *Pseudomonas putida* 86.[33]

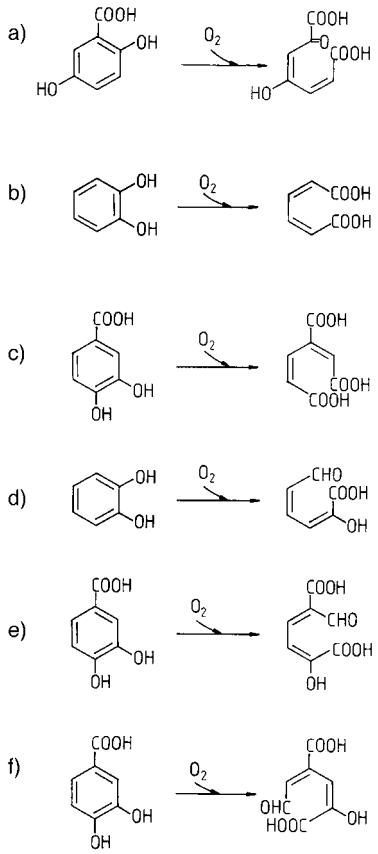
The catabolic pathways, deduced in early studies mainly from the isolation and characterization of metabolites, should be considered as tentative hypotheses, which are to be substantiated by the detection and characterization of the enzymes catalyzing each individual step of the proposed pathway. To confirm the conversions proposed, and, furthermore, to get an insight into the molecular mechanism of enzyme-catalyzed reactions, our further studies concentrated on the characterization of some catabolic enzymes. The structural and catalytic properties of the enzyme proteins were investigated, and in some cases the nucleotide sequences of the genes encoding degradative enzymes were determined and subjected to comparative analyses.

3. Dioxygenases Catalyzing the Ring-Opening Step in the Bacterial Degradation of Heterocyclic Compounds

Ring cleavage is the crucial step in the microbial degradation of aromatic and heterocyclic compounds. The aerobic biodegradation of benzenoid compounds generally proceeds with the introduction of two hydroxy groups *ortho* or *para* to each other, followed by cleavage by a dioxygenase to generate aliphatic compounds as intermediates (Scheme 3). Gentisate 1,2-dioxygenase, and the "intradiol" and "extradiol" enzymes that catalyze *ortho* and *meta* cleavage, respectively, of catecholic substrates, are well-characterized nonheme iron dioxygenases.[34, 35] In contrast, most enzymes that catalyze the cleavage of heterocyclic compounds are less understood.

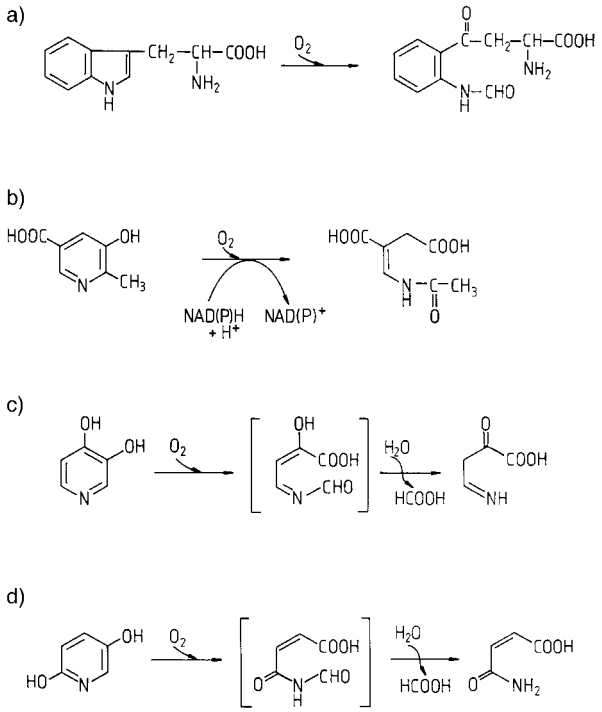
Different types of enzymes are involved in various heterocyclic ring cleavage reactions: L-Tryptophan 2,3-dioxygenases (Scheme 4a) from several bacterial strains are heme-containing enzymes.[34] 2-Methyl-3-hydroxypyridine-5-carboxylate oxygenase (Scheme 4b) is an NAD(P)H-dependent FAD-containing dioxygenase.[36] 3,4-Dihydroxypyridine dioxygenase from *Agrobacterium* sp. probably catalyzes an extradiol cleavage reaction, forming 3-(N-formyl)-formiminopyruvate, which upon hydrolysis would yield the observed products formate and 3-formiminopyruvate (Scheme 4c).[37] In an analogous reaction catalyzed by 2,5-dihydroxypyridine 5,6-dioxygenases from various strains (Scheme 4d), the hydrolysis products maleamate and formate were identified.[38] The latter enzymes require Fe^{2+} and a thiol donor such as dithiothreitol, cysteine, or glutathion for activity. However, the lability of these enzymes precluded extensive characterization.

In the anthranilate pathway of quinaldine (**1**) and 1*H*-4-oxoquinoline (**7**) degradation, we found an unusual way to break open the N-heterocyclic ring, involving cleavage of two C–C bonds with concomitant release of carbon monoxide (Section 2.2, Scheme 1a). Carbon monoxide forming enzymes are very rare. Known for some time is the mammalian microsomal heme monooxygenase system, which in the presence of molecular oxygen and NADPH forms CO and biliverdin IXα from iron protoporphyrin IX.[39] However, two enzymes are known which, in a manner analogous to that of the prokaryotic 2,4-dioxygenases active in the anthranilate pathway, catalyze an oxidative cleavage of two C–C bonds with concomitant CO release, namely, the eukaryotic flavonol 2,4-dioxygenase produced by *Aspergillus flavus*,[40, 41] and the prokaryotic aci-reductone oxidase (CO-forming) from *Klebsiella pneumoniae*.[42, 43] The latter is operative in the methionine salvage pathway, catalyzing 1,3-dioxygenolytic cleavage of the 1,2-dihydroxy-3-keto-*S*-methylthiopentene anion **32** to *S*-methylthiopropionic acid, CO, and formate. The substrates of all these dioxygenases show common structural features, since they all possess a double bond between C2 and C3 (C1 and C2), a hydroxyl group at C3 (C2), and a carbonyl group at C4 (C3) (Scheme 5).

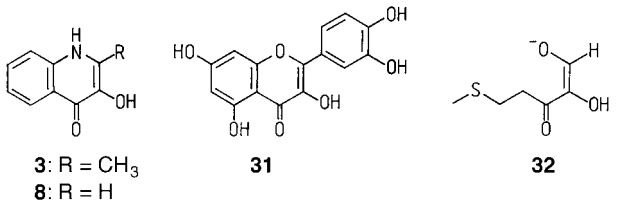


Scheme 3. Reactions catalyzed by aromatic ring cleavage dioxygenases: b, c) "*ortho*" cleavage; d)–f): "*meta*" cleavage. a) Gentisate 1,2-dioxygenase, b) catechol 1,2-dioxygenase, c) protocatechuate 3,4-dioxygenase, d) catechol 2,3-dioxygenase, e) protocatechuate 2,3-dioxygenase, f) protocatechuate 4,5-dioxygenase.

The bacterial CO-forming dioxygenases that catalyze N-heterocyclic ring cleavage reactions in the degradation of quinaldine and 1*H*-4-oxoquinoline are small monomeric proteins, which cleave their respective substrate in the presence of dioxygen, without any need of additional



Scheme 4. Reactions catalyzed by N-heterocyclic ring cleavage dioxygenases. Catalysis by a) tryptophan 2,3-dioxygenase, b) 2-methyl-3-hydroxypyridine-5-carboxylate oxygenase, c) 3,4-dihydroxypyridine dioxygenase, d) 2,5-dihydroxypyridine dioxygenase.



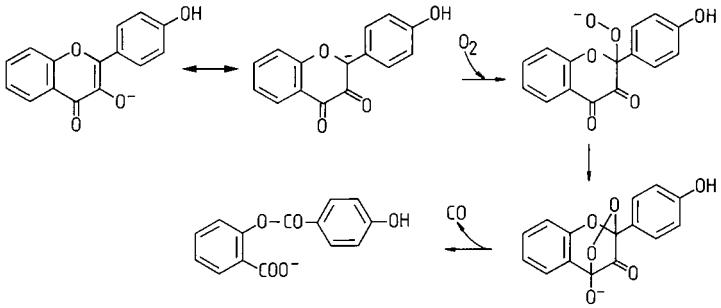
3: R = CH_3 **31** **32**
8: R = H

Scheme 5. Organic substrates of dioxygenases that catalyze cleavage of two C–C bonds with concomitant CO formation. **3** and **8**: substrates of 1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase and 1*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase, respectively.[44, 45] **31**: substrate of flavonol 2,4-dioxygenase.[40, 41] **32**: substrate of aci-reductone oxidase.[42, 43]

cosubstrates or metal ions. In oxygen isotope incorporation studies with an atmosphere of 50% $^{18}O_2$ and 50% $^{16}O_2$, two oxygen atoms of either $^{18}O_2$ or $^{16}O_2$ were incorporated at C2 and C4 of the respective substrates, indicating that these unusual enzymes indeed are 2,4-dioxygenases.[44, 45]

Flavonol 2,4-dioxygenase ("quercetinase"), which catalyzes an analogous 2,4-dioxygenolytic CO release from the O-heterocyclic flavonol quercetin **31**, was reported to contain two atoms of Cu^{II} per molecule.[40] Chemically, flavonols are known to form stable Cu^{II} chelates, presumably by coordination at the 3-hydroxy and 4-carbonyl group. Based on the model reaction of Cu^{II}-flavonolate-catalyzed oxygenation of flavonols, which exclusively gives quercetinase-like products in good yields, the mononuclear Cu^{II} cofactor was proposed to activate the flavonol substrate for dioxygen attack by formation of a Cu^{II} complex.[46]

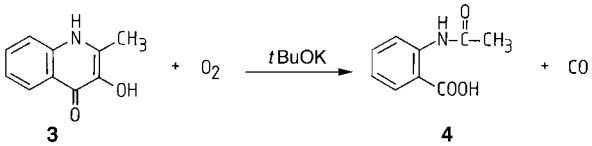
However, base-catalyzed autoxidation of flavonols was found to provide a nonenzymatic model for the reaction of flavonol 2,4-dioxygenase: when the 3-hydroxy group is deprotonated and the carbonyl character at C4 is retained, 3-hydroxyflavones undergo base-catalyzed oxygenolysis to give the corresponding depsides (phenolic carboxylic acid esters) and CO in excellent yields.[47] O_2 appears to be incorporated into the deprotonated form of the flavonol, yielding a peroxy intermediate, which upon intramolecular nucleophilic attack forms a five-membered cyclic peroxide (Scheme 6). Corresponding depside formations occur on use



Scheme 6. Proposed mechanism of base-catalyzed dioxygenolytic cleavage of the anion of **31** with concomitant CO formation.[47]

of bis(salicylidene)ethylenediaminatocobalt(II) ([Co^{II}(salen)]) as catalyst (salen = *N*,*N*′-bis(salicylidene)ethylene).[48] Catalytically active cobalt–oxygen complexes are considered to act as a base rather than as an oxidizing agent. Based on base-catalyzed model reactions, a direct ionic process was postulated for the flavonol 2,4-dioxygenase reaction.[47, 48]

Base-catalyzed chemical dioxygenation provides an analogous nonenzymatic model for the reaction of the 2,4-dioxygenases that catalyze N-heterocyclic ring cleavage: 1*H*-3-Hydroxy-4-oxoquinaldine (**3**) and 1*H*-3-hydroxy-4-oxoquinoline (**8**) were reactive towards molecular oxygen in the presence of the base catalyst potassium *tert*-butoxide in the aprotic solvent *N*,*N*-dimethylformamide. CO, *N*-acetylanthranilate (**4**), and anthranilate (**5**, resulting from amide hydrolysis) were formed from 1*H*-3-hydroxy-4-oxoquinaldine (**3**; Scheme 7). Analogously, **5** was identified as the hydrolysis



3 **4**

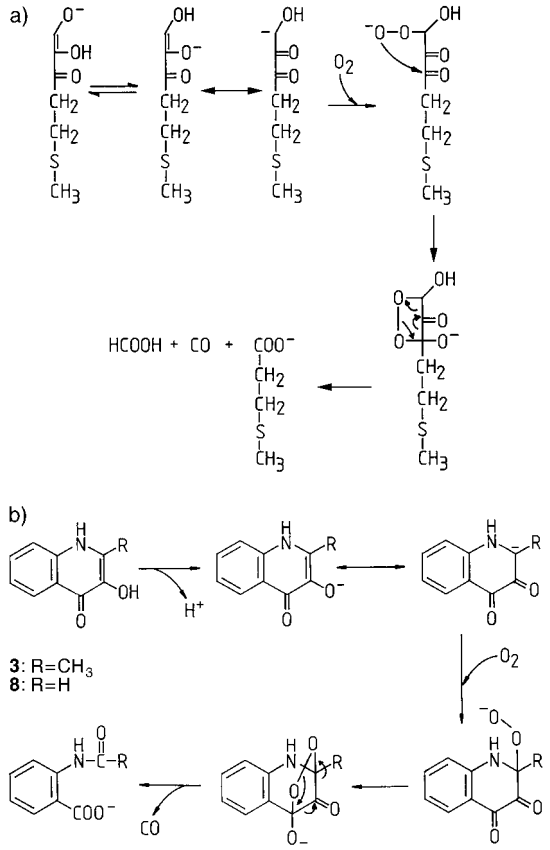
Scheme 7. Base-catalyzed dioxygenolytic cleavage of 1*H*-3-hydroxy-4-oxoquinaldine (**3**).[45]

product after base-catalyzed dioxygenolytic decarbonylation of **8**. When the assay was performed under anaerobic conditions, both **3** and **8** were completely recovered after neutralization of the samples. Thus, base catalysis mimics the enzyme-catalyzed 2,4-dioxygenolytic decarbonylation of the N-heterocyclic compounds **3** and **8**.[45]

In contrast to quercetinase, where Cu^{II} may act as a base, both 1*H*-3-hydroxy-4-oxoquinoline- and 1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase do not contain any metal or other cofactor. Thus, a functional group of an amino acid residue may act as a base catalyst. However, the active site residue(s)

proposed to activate the N-heterocyclic substrates for dioxygen attack remain to be identified.[45]

Recently, the properties of the 1,3-dioxygenolytic aci-reductone oxidase from *Klebsiella pneumoniae* were reported.[43] Analogous to the 2,4-dioxygenases involved in the anthranilate pathway of quinaldine (**1**) and 1*H*-4-oxoquinoline (**7**) degradation, this 1,3-dioxygenolytic enzyme also appears to contain neither a chromophoric nor a metallic cofactor. The authors also propose a quercetinase-like reaction mechanism, involving formation of a substrate carbanion and addition of dioxygen at C1 of the carbanion tautomer to form a hydroperoxy anion, which in a nucleophilic attack may react with the C3 carbonyl, yielding a five-membered cyclic peroxide intermediate, which then decomposes (Scheme 8a). It is not yet known whether dioxygenase-catalyzed oxygen incorporation into the substrate carbanion involves formation of radical anions as intermediates, as described for the oxygenation of various carbanions.[49] Wray and Abeles[43] suggested a stepwise single-electron transfer from the carbanion to molecular oxygen for the formation of the peroxy anion. However, as discussed by Nishinaga et al. for base-catalyzed oxygenations,[47, 50] a direct one-step ionic mechanism, not involving a free-radical-chain process, may also be possible for the reaction of the carbanion with molecular oxygen. Scheme 8b summarizes the tentative hypothesis on the mechanism of 2,4-dioxygenolytic CO formation from **3** and **8**. Further studies are necessary to elucidate the molecular mechanism of dioxygenase-catalyzed oxidative cleavage of two C–C bonds with concomitant release of CO.



Scheme 8. Proposed mechanisms of dioxygenase-catalyzed formation of CO for the a) aci-reductone oxidase from *Klebsiella pneumoniae*,[43] b) 1*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase from *Pseudomonas putida* (R = H) and 1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase from *Arthrobacter* sp. Rü 61 a (R = CH_3).[45]

4. Molybdenum-Containing Enzymes

4.1. The Pterin Molybdenum Cofactors

Bacterial utilization of quinoline and its derivatives as a rule depends on the availability of traces of molybdate in the culture medium. In contrast, growth of the bacterial strains on the first intermediate of each catabolic pathway, namely, the 1*H*-2-oxo or 1*H*-4-oxo derivatives of the quinoline compound (Scheme 1a–d), was not affected by the availability of molybdate. This observation indicated a possible role of the trace element molybdenum in the initial hydroxylation at C2 or C4 of the quinoline derivatives.

Molybdenum plays an essential role in the biogeochemical cycles of nitrogen, sulfur, and carbon. In enzymes molybdenum occurs as part of redox-active cofactors. The molybdenum-dependent nitrogenases, synthesized exclusively by prokaryotes, are well known. The nitrogenase "MoFe-protein" contains an iron–molybdenum cofactor (FeMoCo), in which molybdenum is part of an unusual iron–molybdenum–sulfur cluster.[51] At the iron–molybdenum cofactor, nature performs its own "Haber-Bosch process", reducing the atmospheric nitrogen to ammonium. Apart from nitrogenase, all other molybdoenzymes described contain a pterin molybdenum cofactor.

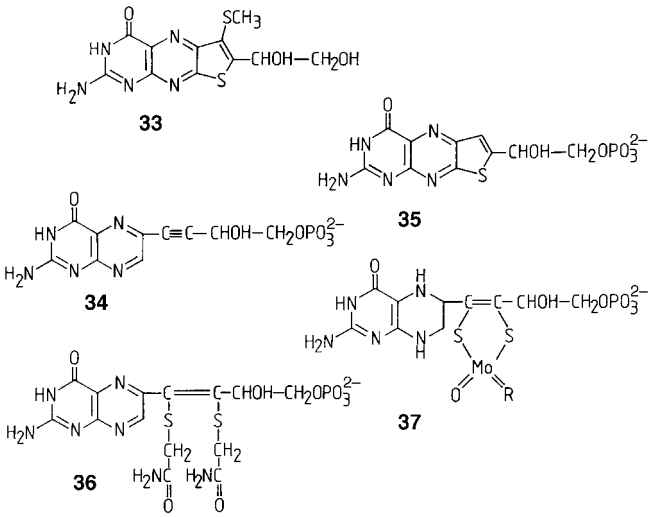
The reactions catalyzed by the enzymes dependent upon the pterin molybdenum cofactor all involve the transfer of an oxygen atom to or from a substrate molecule in a two-electron redox reaction. This large family of enzymes comprises, for example, nitrate reductase, sulfite oxidase, formate dehydrogenase, carbon monoxide dehydrogenase, arsenite oxidase, and various molybdenum-containing hydroxylases from pro- and eukaryotic sources such as aldehyde oxidase (EC 1.2.3.1) and various aldehyde dehydrogenases, purine hydroxylase, and xanthine dehydrogenase (EC 1.1.1.204)/xanthine oxidase (EC 1.1.3.22).[52–55] A number of bacterial enzymes that catalyze hydroxylations of pyridine carboxylic acids, quinoline derivatives and other N-heterocyclic compounds (see Section 4.2) also are molybdenum-containing hydroxylases.

Hydroxylation reactions are ubiquitous in the metabolic pathways of all living organisms. The majority of known hydroxylating enzymes, namely, the mono- and dioxygenases, utilize dioxygen as the source of the oxygen atom incorporated into the substrate. In this reaction reducing equivalents are consumed. In contrast, the molybdenum-containing hydroxylases utilize water as the ultimate source of the oxygen atom, and they generate rather than consume reducing equivalents according to the overall stoichiometry in Equation (1).

$$RH + H_2O \rightarrow ROH + 2\,e^- + 2\,H^+ \quad (1)$$

In the pterin molybdenum cofactor of molybdenum-containing hydroxylases, molybdenum is coordinated to molybdopterin or to a molybdopterin dinucleotide. Unlike many other organic cofactors known, these pterin cofactors in their native form are not amenable to isolation. Thus, the classical cofactor assays of reassociation with concomitant reconstitution of enzymatic activity are not feasible. The structural determination of the pterin molybdenum cofactors was severely hampered by the fact that these cofactors, once separated from the enzyme protein, immediately decayed into a multitude of fluorescent oxidation products.

In 1940, Koschara isolated tiny amounts of a sulfur-containing compound out of 1000 L of human urine.[56] He called it urothione, but at that time he naturally did not perceive that he had discovered the metabolic excretion product of the pterin molybdenum cofactor. The laborious isolation was repeated nearly 30 years later by Goto et al.,[57] who determined the structure of urothione (**33**). Its origin, however, still remained an enigma until 1982, when Johnson and Rajagopalan realized the metabolic relationship between the pterin molybdenum cofactor (**37**) and urothione.[58] From the known structures of urothione and of two fluorescent degradation products of the cofactor, called "form A" (**34**) and "form B" (**35**), a structural model of the pterin molybdenum cofactor was deduced. The model was confirmed by the characterization of an alkylated derivative of the pterin molybdenum cofactor, bis(carboxamidomethyl)-molybdopterin (camMPT **36**; Scheme 9).[58, 59]

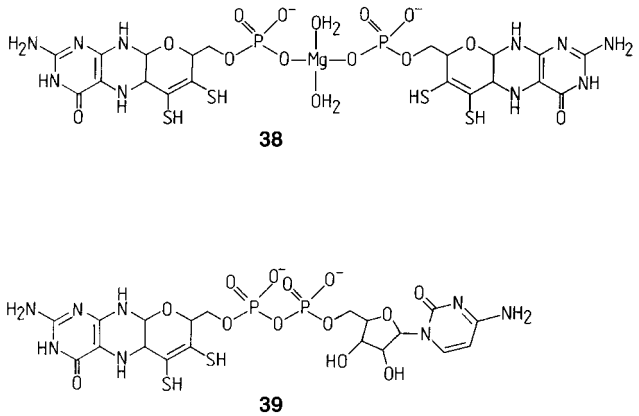


Scheme 9. Structures of pterin molybdenum cofactors and related compounds.[59] R = O, S, Se.

The generation of alkylated derivatives of the pterin molybdenum cofactor was an important accomplishment, since it allowed the discovery of the molybdopterin dinucleotides. The latter previously had escaped detection because of the common practice of demonstrating the presence of a pterin molybdenum cofactor in an enzyme by its conversion into **34** or **35**. The conditions required for these transformations are so harsh that pyrophosphate linkages are cleaved. Thus, **34** or **35** were found, irrespective of whether molybdopterin or a molybdopterin dinucleotide was primarily present. Direct evidence for the existence of a dinucleotide form of the pterin molybdenum cofactor was obtained in 1990, when an alkylated cofactor derivative was isolated from dimethylsulfoxide reductase from *Rhodobacter sphaeroides*, which upon treatment with nucleotide pyrophosphatase yielded camMPT (**36**) and GMP, thus establishing molybdopterin guanine dinucleotide (MGD) as organic part of the pterin molybdenum cofactor.[60] Shortly thereafter, molybdopterin cytosine dinucleotide (MCD) was found in carbon monoxide dehydrogenase from *Pseudomonas carboxydoflava*.[61] Since then, MGD and MCD have been found in a number of molybdenum-containing enzymes. Whereas enzymes depending on the pterin molybdenum cofactor as a rule specifically harbor either molybdopterin, MCD, or MGD, formylmethanofuran dehydrogenase from *Methanobacterium thermoautotrophicum* utilizes MGD, molybdopterin adenine dinucleotide, and molybdopterin hypoxanthine dinucleotide, that is, several purine dinucleotides of the pterin molybdenum cofactor.[62]

Major steps towards the elucidation of the structural features of the pterin molybdenum cofactor(s) were the analyses by X-ray diffraction methods of the crystal structures of aldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus* (a hyperthermophilic archaeon),[63] of aldehyde oxidoreductase from the sulfate reducer *Desulfovibrio gigas*,[64] and of dimethylsulfoxide reductase from the phototrophic bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*.[65]

Aldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus* consists of two identical subunits, each containing a [4Fe-4S] cluster and a tungsten cofactor that is analogous to the pterin molybdenum cofactor. Each subunit of aldehyde ferredoxin oxidoreductase contains two pterin molecules, linked through their phosphate groups that coordinate axial sites of one magnesium ion. The two pterin molecules coordinate one tungsten atom through four sulfur donor atoms. No protein ligands were coordinated to the tungsten atom. The pterin system was modified to contain a third ring formed by closure of the side-chain hydroxyl with the pterin ring at C7 (Scheme 10). This revised bis(molybdopterin) structure (**38**) led to the proposal that in the dinucleotide forms of the pterin molybdenum cofactor, a single molecule of



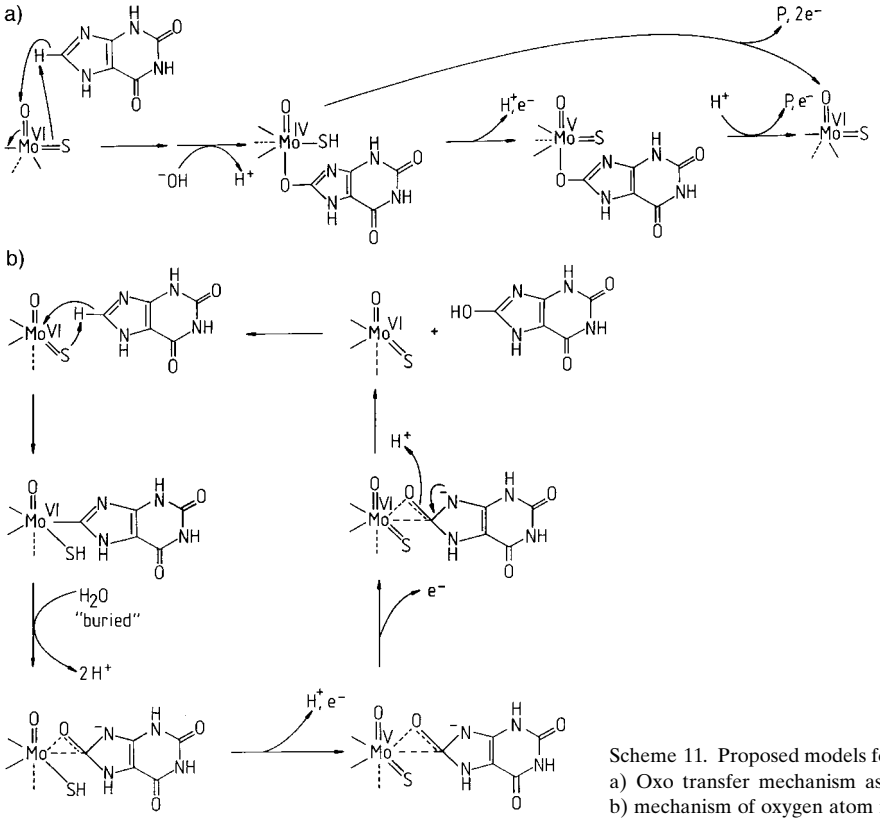
Scheme 10. Structural models for variants of the pterin molybdenum cofactor according to Chan et al.[63]

the pterin dinucleotide could possibly coordinate the molybdenum center, and that the second pterin of the bis(molybdopterin) may be replaced by the nucleotide, if the sugar hydroxyl oxygen atoms are utilized for metal ligation instead of the second dithiolene group.[63]

Aldehyde oxidoreductase from *Desulfovibrio gigas* is a homodimeric enzyme, in which each subunit contains two different [2Fe2S] clusters and one MCD as dinucleotide form of the pterin molybdenum cofactor. The amino acid sequence of this aldehyde oxidoreductase shares no homology to the tungstoprotein aldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus* described above. However, analogous to the bis(molybdopterin) cofactor of aldehyde ferredoxin oxidoreductase, the molybdopterin forms a tricyclic system in which the pterin ring is annealed to a pyran ring (**39**, Scheme 10). The Mo^{VI} center present in the oxidized form of the enzyme is pentacoordinated by two dithiolene sulfur and three oxygen ligands, which were identified as two oxo (Mo=O) and one water ligand ($Mo-OH_2$).[64]. The structure, however, is at variance with the suggestion by Chan et al.[63] of an involvement of the sugar hydroxyl groups in molybdenum binding.

Characterization of the crystal structure of aldehyde oxidoreductase from *Desulfovibrio gigas* also provided insight into some mechanistic features of aldehyde oxidation and internal electron transfer. The oxygen transferred to the substrate may either derive from the water molecule nearest to the molybdenum, or it is one of the molybdenum's ligands. The transfer of the oxo ligand of the molybdenum was suggested earlier for the xanthine oxidase reaction by Hille and co-workers.[55] According to this model, the catalytically labile oxygen of the Mo=O group is transferred to substrate with each catalytic cycle. Since water is the ultimate source of the oxygen incorporated into product, the oxo group of the molybdenum center must be regenerated from solvent. Thus, a reaction initiated by proton abstraction followed by carbanion attack on Mo=O was proposed, as outlined in Scheme 11a.[55] However, recent ^{17}O and ^{13}C electron nuclear double resonance (ENDOR) spectroscopy and kinetic studies, which indicated a molybdenum–carbon bond formation in the catalytic action of xanthine oxidase, and structural analyses of the *Desulfovibrio gigas* aldehyde oxidoreductase led to the proposal of an alternative mechanism, not involving direct participation of the oxo ligand group but transfer of the molybdenum-bound water molecule to the substrate.[66] The catalytic model for the xanthine oxidase reaction is referred to as "oxygen atom insertion" rather than "oxo transfer" and involves formal addition of the substrate across the Mo=S bond of the catalytic center of xanthine oxidase, followed by attack of a "buried" water molecule (in the vicinity of molybdenum and perhaps a ligand of it) on the bound substrate carbon. Thus, the oxygen incorporated into the uric acid product is proposed to originate from a "buried" water rather than the oxo group. The oxo molybdenum ligand in this mechanistic model plays no active part in the reaction (Scheme 11b).[66]

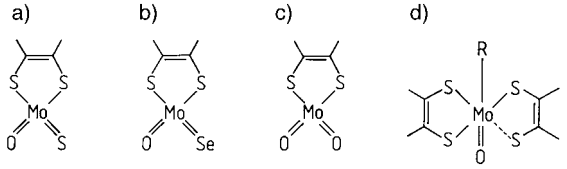
Structural data on dimethylsulfoxide reductase from *Rhodobacter sphaeroides* indicated that this protein represents a distinct group of pterin molybdenum cofactor-containing enzymes, unrelated to both the aldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus* and the aldehyde oxidoreductase from *Desulfovibrio gigas* described above. The monomeric *Rhodobacter* dimethylsulfoxide reductase harbors



Scheme 11. Proposed models for the catalytic cycle of the xanthine oxidase reaction. a) Oxo transfer mechanism as proposed by Hille and co-workers,[55] P = product; b) mechanism of oxygen atom insertion as proposed by Howes et al.[66]

two MGDs that asymmetrically coordinate the molybdenum through their dithiolene groups. A protein ligand, namely, a side chain oxygen of a serine residue, additionally coordinates the metal.[65]

Based on the type of molybdenum center, the idea emerged that three fundamentally distinct classes of enzymes depending on the pterin molybdenum cofactor may be distinguished (Scheme 12):[65] Firstly, there are the enzymes possessing a



Scheme 12. Types of molybdenum(VI) centers in enzymes depending on the pterin molybdenum cofactor.[65] a) Monooxo-monosulfido-type, b) monooxo-monoselenido-type, c) dioxo-type, d) monooxo-type; R = serine, cysteine, selenocysteine.

monooxo-monosulfido-type molybdenum center (with xanthine oxidase as prototype). The *Desulfovibrio gigas* aldehyde oxidoreductase also belongs to this group of enzymes frequently referred to as the "xanthine oxidase family". Xanthine dehydrogenase and nicotinic acid dehydrogenase of certain anaerobic bacteria form a subgroup, since they apparently possess a monooxo-monoselenido-type molybdenum center, as demonstrated for nicotinic acid dehydrogenase (hydroxylase) from *Clostridium barkeri*.[67] The second group of enzymes, represented by sulfite oxidase, harbors a dioxo-type molybdenum center. The third group of enzymes can at best be described as possessing a monooxo-type molybdenum center. This group, with dimethylsulfoxide reductase as representative, is further characterized by a protein ligand at the molybdenum center. This ligand may be either a serine, as in dimethylsulfoxide reductase from *Rhodobacter sphaeroides* described above, a cysteine, or a selenocysteine residue. The latter was identified as molybdenum ligand in *Escherichia coli* formate dehydrogenase H.[68]

The molybdenum-containing hydroxylases that catalyze a hydroxylation either adjacent to the heteroatom or at C4 of the quinoline ring belong to the first group of enzymes, the xanthine oxidase family. All the enzymes characterized in greater detail by our group contained molybdopterin cytosine dinucleotide (MCD) with a monooxo-monosulfido-type molybdenum center.[69–75]

4.2. Molybdenum-Containing Hydroxylases Involved in the Bacterial Degradation of Quinoline and Derivatives

4.2.1. Biochemistry

4.2.1.1. Structural Features

Early studies on quinoline degradation by *Pseudomonas putida* 86 indicated that molybdenum was required for the biotransformation of quinoline (**10**) to 1*H*-2-oxoquinoline (**12**; cf. Section 4.1).[76] Since the molybdenum-dependent eukaryotic xanthine oxidases were known to exhibit a rather broad substrate specificity, catalyzing the hydroxylation of a number of N-heterocyclic compounds,[77] our observation of molybdenum-dependent quinoline hydroxylation at first led us tentatively to assume that a bacterial xanthine dehydrogenase might be involved in quinoline hydroxylation. This hypothesis, however, was disproved by the finding in *Pseudomonas putida* 86 of two distinct molybdenum-containing hydroxylases: the xanthine dehydrogenase purified from this strain was composed of two nonidentical subunits arranged in an $\alpha_4\beta_4$ structure, whereas the quinoline 2-oxidoreductase from the same strain was found to be a heterotrimeric protein ($\alpha_2\beta_2\gamma_2$). Both molybdenum-containing hydroxylases contain FAD and [2Fe2S] centers, but differ in their pterin molybdenum cofactor: the bacterial xanthine dehydrogenase possesses molybdopterin (MPT), wheras quinoline 2-oxidoreductase harbors molybdopterin cytosine dinucleotide (MCD) as organic part of the pterin molybdenum cofactor.[69, 78, 79] The enzymes show distinct substrate specificities. Neither is quinoline converted by xanthine dehydrogenase, nor are xanthine and hypoxanthine transformed by quinoline 2-oxidoreductase. Quinazoline (1,3-benzodiazine, **46**; see Scheme 14) was the only substrate found to be hydroxylated by both quinoline 2-oxidoreductase and xanthine dehydrogenase.

Quinoline 2-oxidoreductases, which catalyze the conversion of quinoline (**10**) into 1*H*-2-oxoquinoline (**12**), were purified not only from *Pseudomonas putida* 86, but also from *Rhodococcus* sp. B1 and *Comamonas testosteroni* 63.[25, 70, 76, 79] All three enzymes were found to be structurally complex molybdo iron–sulfur flavoproteins. The in vitro reaction requires the presence of an artificial electron acceptor. The physiological electron acceptor of the quinoline 2-oxidoreductases has not been identified yet.

Unlike the situation for quinoline, there are comparatively few reports on the bacterial catabolism of isoquinoline. Several bacterial strains capable of degrading isoquinoline as the sole source of carbon and energy were described.[80] All these strains appear to initially transform isoquinoline to 2*H*-1-oxoisoquinoline. The complete catabolic pathway(s), which in *Brevundimonas diminuta* 7 may involve degradation via phthalate and protocatechuate,[81] remain(s) to be elucidated.

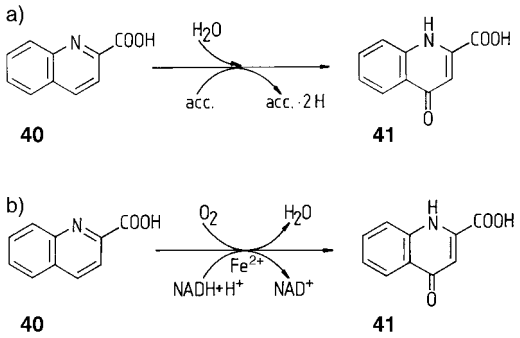
Isoquinoline 1-oxidoreductase, which catalyzes the hydroxylation at C1 of isoquinoline with concomitant reduction of a suitable electron acceptor, was purified from *Brevundimonas diminuta* 7.[75] The native enzyme is a heterodimeric molybdo iron–sulfur protein, lacking the flavin cofactor.

Quinaldine 4-oxidase from the Gram-positive bacterium *Arthrobacter* sp. Rü 61 a catalyzes the initial step in quinaldine (**1**) degradation[22] (Section 2.2, Scheme 1a): quinaldine is converted into 1*H*-4-oxoquinaldine (**2**) with concomitant reduction of an electron acceptor. The enzyme is able to function as oxidase or dehydrogenase, and molecular oxygen is proposed as physiological electron acceptor.[72] Hydroxylations in *para* position to the N-heteroatom were also described for two quinaldic acid 4-oxidoreductases.[74, 82]

Many bacterial molybdenum-containing hydroxylases that catalyze the nucleophilic attack of an N-heterocyclic compound either adjacent or *para* to the N-heteroatom are molybdo iron–sulfur flavoproteins, namely, xanthine dehydrogenases,[78, 83] nicotinate and 6-hydroxynicotinate dehydro-

genase from *Bacillus niacini*,[84] isonicotinate dehydrogenase from *Mycobacterium* sp. INA1,[85] nicotine dehydrogenase from *Arthrobacter oxidans*,[86] several quinoline 2-oxidoreductases,[25, 70, 76, 79] quinaldine 4-oxidase from *Arthrobacter* sp. Rü 61 a,[71, 72] quinoline 4-carboxylate 2-oxidoreductase from *Agrobacterium* sp. 1B,[73] and quinaldic acid 4-oxidoreductase from *Pseudomonas* sp. AK-2.[74] These prokaryotic enzymes, which contain a pterin molybdenum cofactor, iron–sulfur centers, and FAD, appear to be related to the eukaryotic molybdenum-containing hydroxylases aldehyde oxidase and xanthine oxidase. However, there are also prokaryotic molybdenum-containing hydroxylases without a flavin cofactor, such as the heterodimeric enzymes quinaldic acid 4-oxidoreductase from *Serratia marcescens* 2CC-1 and isoquinoline 1-oxidoreductase from *Brevundimonas diminuta* 7 mentioned already.[82, 75] The homodimeric aldehyde oxidoreductase from *Desulfovibrio gigas* discussed in Section 4.1 also belongs to this subgroup of molybdo iron–sulfur proteins.

As shown in Table 1, two quinaldic acid 4-oxidoreductases have been described, which differ in structure and in their set of redox-active centers. Both enzymes, however, catalyze a nucleophilic attack at C4 of quinaldic acid (**40**), incorporating the oxygen atom of a water molecule into the substrate. It is interesting to note that the very same conversion, that is, the formation of kynurenic acid (**41**) from **40**, in an *Alcaligenes* strain was catalyzed by a monooxygenase, which in an electrophilic attack incorporates an oxygen atom derived from molecular oxygen into the substrate (Scheme 13).[87]



Scheme 13. Enzyme-catalyzed conversion of quinaldic acid (**40**) to kynurenic acid (**41**).[87] a) Quinaldic acid 4-oxidoreductases from *Serratia marcescens* 2CC-1 and *Pseudomonas* sp. AK-2, b) quinaldic acid 4-monooxygenase from *Alcaligenes* sp. F2. acc. = electron acceptor.

Thus, the same reaction in different bacteria is accomplished by enzymes belonging to separate classes, differing fundamentally in their catalytic mechanism.

4.2.1.2. Substrate Specificity and Aldehyde Oxidation

Investigation of the substrate specificity of quinaldine 4-oxidase revealed that in addition to quinaldine, it converted 8-chloroquinaldine, quinoline, 2- and 8-monochloroquinoline, isoquinoline, and the benzodiazines cinnoline (1,2-benzodiazine), quinazoline (1,3-benzodiazine, **46**), and phthalazine (2,3-benzodiazine, **44**). Surprisingly, all these substrates (except **44**) elicited higher catalytic activities than quinaldine itself. Thus, the enzyme nomenclature is rather difficult: Despite its name, originally deduced from its role in quinaldine utilization by *Arthrobacter* sp. Rü 61 a (Scheme 1a),[22] the enzyme converts a number of N-heterocyclic substrates with a significantly higher conversion rate than quinaldine.[72]

To test whether other molybdenum-containing hydroxylases involved in the bacterial degradation of N-heteroaromatic compounds also convert such a broad range of substrates, the substrate specificities of quinaldine 4-oxidase, quinoline 2-oxidoreductase, and isoquinoline 1-oxidoreductase were compared (Table 2). Quinoline (**10**) was accepted as substrate by both quinaldine 4-oxidase and quinoline 2-oxidoreductase, yielding 1*H*-4-oxoquinoline and 1*H*-2-oxoquinoline, respectively. So, quinaldine 4-oxidase catalyzes the regiospecific attack in position 4, even if the carbon atom adjacent to the N-heteroatom is not blocked by a substituent. Isoquinoline and

Table 2. Enzyme-catalyzed hydroxylation of quinolines and benzodiazines.

Enzyme	Substrate	Product
quinaldine 4-oxidase	quinoline (**10**)	1*H*-4-oxoquinoline (**7**)
	quinaldine (**1**)	1*H*-4-oxoquinaldine (**2**)
	isoquinoline	2*H*-1-oxoisoquinoline
	phthalazine (**44**)	2*H*-1-oxophthalazine (**45**)
	quinazoline (**46**)	3*H*-4-oxoquinazoline (**48**)
	cinnoline	1*H*-4-oxocinnoline
quinoline 2-oxidoreductase	quinoline (**10**)	1*H*-2-oxoquinoline (**12**)
	quinazoline (**46**)	1*H*-2-oxoquinazoline
	quinoxaline (**42**)	1*H*-2-oxoquinoxaline (**43**)
isoquinoline 1-oxidoreductase	isoquinoline	2*H*-1-oxoisoquinoline
	phthalazine (**44**)	2*H*-1-oxophthalazine (**45**)
	quinazoline (**46**)	3*H*-4-oxoquinazoline (**48**)

Table 1. Molybdenum-containing hydroxylases involved in the bacterial degradation of quinoline derivatives.

Enzyme (organism)	*M* [kDa]	*M* [kDa] (subunits)	Structure	Moco[a, b]	FeS[b]	Flavin[b]
quinoline 2-oxidoreductase (*Pseudomonas putida* 86)	300	85, 30, 18	$\alpha_2\beta_2\gamma_2$	(2)MCD[c]	(2)[2Fe-2S]I (2)[2Fe-2S]II[e]	(2)FAD
quinaldine 4-oxidase (*Arthrobacter* sp. Rü 61 a)	340	82, 35, 22	$\alpha_2\beta_2\gamma_2$	(2)MCD[c]	(2)[2Fe-2S]I (2)[2Fe-2S]II[e]	(2)FAD
quinaldic acid 4-oxidoreductase (*Pseudomonas* sp. AK-2)	300	90, 34, 20	$\alpha_2\beta_2\gamma_2$	(2)MCD[c]	(8)Fe, (8)S	(2)FAD
quinaldic acid 4-oxidoreductase (*Serratia marcescens* 2CC-1)	100	80, 18	$\alpha\beta$	– [d]	(3–4)Fe	–
isoquinoline 1-oxidoreductase (*Brevundimonas diminuta* 7)	95	80, 16	$\alpha\beta$	(1)MCD[c]	(1)[2Fe-2S]I (1)[2Fe-2S]II[e]	–

[a] Pterin molybdenum cofactor. [b] In parentheses: number of cofactors per enzyme molecule. [c] Molybdopterin cytosine dinucleotide. [d] Not determined. [e] Deduced from EPR spectroscopic data.

phthalazine (**44**) are substrates not only for isoquinoline 1-oxidoreductase but for quinaldine 4-oxidase as well. Identical products were formed by the two enzymes, namely 2*H*-1-oxoisoquinoline and 2*H*-1-oxophthalazine (**45**), respectively. Quinazoline (**46**) is the only substrate accepted by all three enzymes (see Scheme 14). With quinaldine 4-oxidase and isoquinoline 1-oxidoreductase, 3*H*-4-oxoquinazoline (**48**) was produced, whereas quinoline 2-oxidoreductase hydroxylated at C2, that is, adjacent to both N1 and N3. Apparently, quinoline 2-oxidoreductase and isoquinoline 1-oxidoreductase hydroxylate their various substrates exclusively adjacent to an N-heteroatom. Quinaldine 4-oxidase is more versatile: whether the regiospecific nucleophilic attack is in *ortho* or in *para* position to an N-heteroatom depends on the substrate (Table 2).[72]

Apart from these prokaryotic enzymes, which are all involved in the bacterial degradation of quinoline and its derivatives, the eukaryotic molybdenum-containing hydroxylases xanthine oxidase from cow's milk and aldehyde oxidase from rabbit liver catalyze the oxidation of N-heterocycles, too.[77, 88] The latter two enzymes also convert aldehydes into the corresponding carboxylic acids.[89]

Since comparison of the amino acid sequence of isoquinoline 1-oxidoreductase with other molybdenum-containing hydroxylases revealed significant similarities with two aldehyde oxidoreductases, one from the industrial vinegar producer *Acetobacter polyoxogenes* and the other from the sulfate reducer *Desulfovibrio gigas* (Section 4.2.3),[90] substrate specificities of isoquinoline 1-oxidoreductase, quinoline 2-oxidoreductase, and quinaldine 4-oxidase towards various aldehydes were investigated. Surprisingly, quinaldine 4-oxidase and isoquinoline 1-oxidoreductase both catalyzed the oxidation of aromatic aldehydes. Benzaldehyde and salicylaldehyde (2-hydroxybenzaldehyde) turned out to be accepted best by quinaldine 4-oxidase. Cinnamaldehyde (3-phenyl-2-propenal) and vanillin (4-hydroxy-3-methoxybenzaldehyde) were also converted into the corresponding carboxylic acids, although at significantly lower rates, whereas short-chain aliphatic aldehydes were not oxidized. Isoquinoline 1-oxidoreductase markedly resembled quinaldine 4-oxidase in its substrate spectrum; salicylaldehyde and benzaldehyde were favorites. The aldehydes, however, were suicide substrates for isoquinoline 1-oxidoreductase. Quinaldine 4-oxidase and isoquinoline 1-oxidoreductase were more active towards aromatic than towards aliphatic aldehydes. This feature apparently does not apply for the prokaryotic aldehyde oxidoreductases described, which all convert short-chain aliphatic aldehydes far better than quinaldine 4-oxidase and isoquinoline 1-oxidoreductase do.[72] However, eukaryotic aldehyde oxidase, a molybdenum-containing enzyme well-characterized with respect to N-heterocyclic and aldehyde substrate specificity, appears to be similar to quinaldine 4-oxidase and isoquinoline 1-oxidoreductase in so far as it catalyzes the hydroxylation of N-heterocycles (such as quinoline, quinaldine, isoquinoline, cinnoline, quinazoline) and the oxidation of aromatic aldehydes with roughly comparable rates.[89]

In conclusion, molybdenum-containing hydroxylases show rather variable specificities, and even the distinction between aldehyde oxidoreductases and N-heterocycles hydroxylating enzymes remains a matter of discussion.[72] However, aldehydes are cytotoxic compounds due to their reactivity, and the molybdenum-containing hydroxylases active towards aldehydes may function as important detoxification enzymes for both the prokaryotic and the eukaryotic cell.

4.2.2. Biophysics

The internal electron transport chain of quinoline 2-oxidoreductase from *Pseudomonas putida* 86 is composed of four redox active centers (see also Table 1, Section 4.2.1.1)—FAD, two distinct [2Fe-2S] centers, and molybdenum-MCD. Each of these centers adopts a paramagnetic state during the redox cycle of the enzyme and can then be investigated by electron paramagnetic resonance (EPR) spectroscopy.[79]

Reduction of quinoline 2-oxidoreductase with dithionite or with quinoline led to the formation of an organic radical with 1.9 mT line width typical for a neutral (blue) flavin radical species and similar to eukaryotic xanthine oxidase (from cow's milk).[91] Two iron–sulfur centers were discernible by their EPR spectra: In the temperature range below 60 K distinct rhombic EPR signals were resolved. By comparison with xanthine oxidase, and according to the convention in literature, the rhombic iron–sulfur signals with the lower *g* anisotropy were designated FeS I and those with the larger *g* anisotropy FeS II, respectively (Figure 2a–c).[92, 93] The *g*

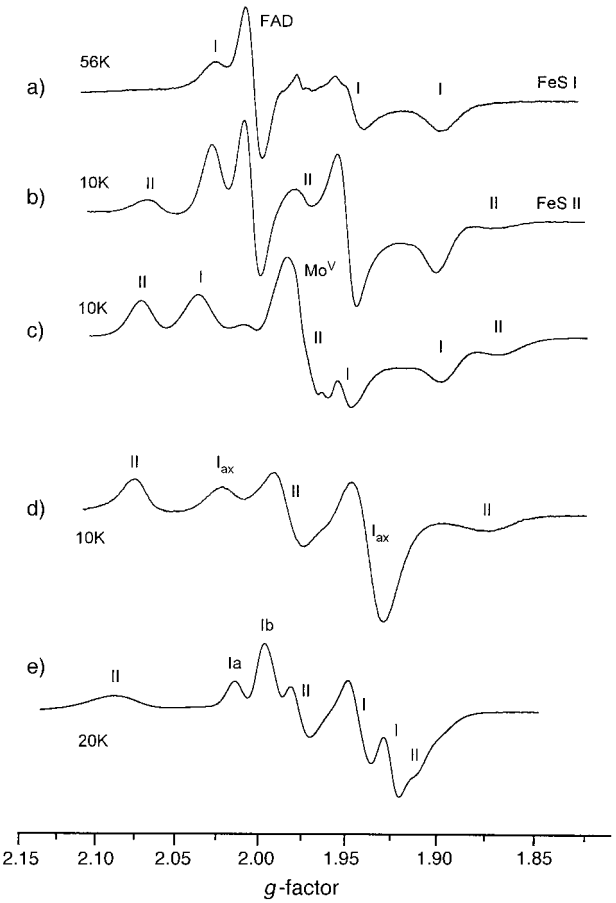


Figure 2. Comparison of the EPR signals of the iron–sulfur clusters. a)–c) Quinoline 2-oxidoreductase, a) reduced with dithionite, recorded at 56 K and b) at 10 K; c) reduced with quinoline, recorded at 10 K. The positions of the FAD radical signal as well as of a broadened Mo^V signal are indicated. d) Quinaldine 4-oxidase, reduced with dithionite, recorded at 10 K. e) Isoquinoline 1-oxidoreductase, reduced with dithionite, recorded at 20 K.

values of FeS I showed only little variation from those of the F–S centers in other proteins, implying similar stereochemical and electronic structures of the signal-generating FeS I species in the various pro- and eukaryotic enzymes. In the *g* factors for the FeS II signals, however, there were significant differences among the molybdenum-containing hydroxylases, indicative of a more pronounced variability of this signal-generating species.

The oxidation–reduction potentials, determined by titration with sodium dithionite solutions in the presence of various mediators, yielded values of -155 mV for FeS I and -195 mV for FeS II. These represent the highest redox potentials measured so far for [2Fe-2S] clusters of molybdenum-containing hydroxylases.

The molybdenum center is of fundamental interest as the putative site of substrate binding and catalysis. The EPR spectrum in Figure 3a of the oxidized quinoline 2-oxidoreductase (as isolated) recorded at 77 K resembles the Mo^V "resting" spectrum known from xanthine oxidase, aldehyde oxidase, and carbon monoxide oxidase.[79] It represents an inactive Mo^V species always present in low concentrations

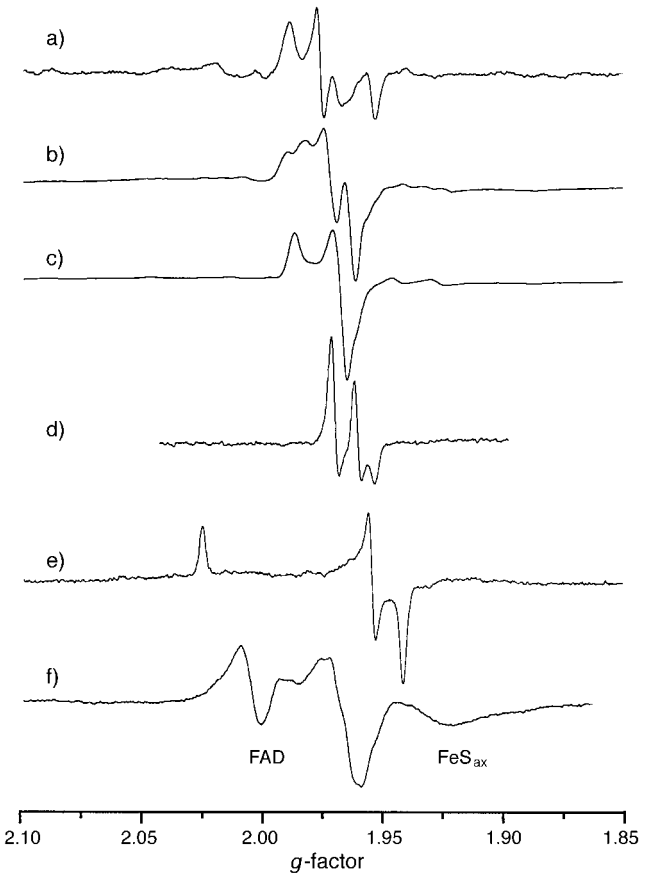


Figure 3. EPR signals of Mo^V species. a)–c): Quinoline 2-oxidoreductase. a) "Resting Q"; b) "rapid Q" in H_2O buffer; c) "rapid Q" in D_2O buffer; the hyperfine lines due to the thiol proton are no longer observed. d) "Slow Q" of the cyanide treated desulfo form. e) Isoquinoline 1-oxidoreductase: "very rapid I"; the signal was obtained by subtraction of spectra at different incubation times. f) Quinaldine 4-oxidase, reduced under anaerobic conditions with the substrate quinaldine (**1**). All spectra were recorded at 77 K.

(<5% of total Mo concentration) in the preparations. Reduction of the enzyme with its substrate or dithionite elicited an intense EPR spectrum of another Mo^V species (Figure 3b), designated "rapid Q" to characterize its origin and its similarity with the "rapid" signal of bovine xanthine oxidase.[92] Its prominent feature is a proton coupling of the sulfhydryl group, which is easily exchangeable in D_2O buffer in analogy to xanthine oxidase (Figure 3c). Small differences in the hyperfine coupling values obtained for the substrate-reduced and dithionite-reduced species reflect the influence of the substrate (or the product) in the vicinity of the Mo^V center, but direct spectral evidence for complex formation is not found by EPR. In ^{95}Mo-enriched quinoline 2-oxidoreductase hyperfine interaction of the metal nucleus with $I = 5/2$ leads to sixfold splitting of lines. Simulation of these hyperfine patterns points to a coincidence of one principal axis of the *g* and hyperfine tensor, which is typical for the presence of a complex with C_2 symmetry. The catalytically relevant "very rapid" Mo^V species, which is observed in xanthine oxidase in rapid freeze experiments on the millisecond time scale, could be isolated only in small amounts in quinoline 2-oxidoreductase by competitive binding of quinoline and the nonsubstrate quinaldine.

Quinoline 2-oxidoreductase from *Pseudomonas putida* 86 possesses a monooxo-monosulfido-type molybdenum center (see also Scheme 12) as shown by inactivation of the enzyme with cyanide with concomitant formation of thiocyanate, and reactivation of inactive enzyme by treatment with sulfide. Cyanide thus abstracts the so-called cyanolysable sulfur from the molybdenum center. In this inactive desulfo form of the enzyme the molybdenum center is thought to be of the dioxo type. In cyanide inactivation/sulfide reactivation experiments quinoline 2-oxidoreductase exhibits the same behavior as many other molybdo iron–sulfur flavoproteins. By analogy to the desulfo form of xanthine oxidase the Mo^V signal "slow Q" arising from the inactive desulfo quinoline 2-oxidoreductase was detected, which is characterized by a slightly rhombic *g* tensor and a proton interaction exchangeable in D_2O (Figure 3d).

In accordance with biochemical and genetic data, no free radical signal of a flavin-centered radical species was observed in EPR-spectroscopic analyses of isoquinoline 1-oxidoreductase from *Brevundimonas diminuta* 7. Two distinct rhombic EPR signals of iron–sulfur centers were discernible after reduction of the enzyme with dithionite or the substrate isoquinoline. At temperatures below 30 K a magnetic interaction between the iron–sulfur centers resulted in a pronounced splitting of the g_1 component of the FeS I signal (Ia, Ib in Figure 2e), indicating a spatial proximity of both clusters. While the FeS II signal occurred only in the temperature range below 40 K, FeS I signals were detectable already at 77 K, which is an unusual feature for a [2Fe-2S] center.

Upon reduction with its substrate isoquinoline the Mo^V species "rapid I" was readily observed, which again exhibited a strongly coupled proton exchangeable in D_2O, similar to the "rapid Q" signal of quinoline 2-oxidoreductase. A further Mo^V signal with nearly identical magnetic parameters to the "very rapid" species in quinoline 2-oxidoreductase and xanthine oxidase was detected in isoquinoline 1-oxidoreduc-

tase and called "very rapid I" (Figure 3e). Despite its name this signal persisted for at least 45 minutes, in contrast to its behavior as a short-lived intermediate such as in xanthine oxidase. It seems possible that a rather stable (partially inhibitory) complex between isoquinoline and the Mo^V center is formed in isoquinoline 1-oxidoreductase in the presence of an excess of substrate.

Quinaldine 4-oxidase and quinoline 2-oxidoreductase have a similar molecular mass and the same subunit and cofactor composition, whereas isoquinoline 1-oxidoreductase differs in these respects as indicated in Table 1 (Section 4.2.1.1). In order to compare these molybdenum-containing hydroxylases involved in bacterial catabolism of quinoline derivatives, the redox active centers of quinaldine 4-oxidase were additionally investigated by EPR spectroscopy.

Quinaldine 4-oxidase in the oxidized state (as isolated, aerobic conditions) did not elicit any EPR signal. Under anaerobic conditions, without substrate or reducing agent, an EPR signal of an organic radical species was observed. Upon addition of quinaldine to the enzyme this signal at $g = 2.0040$ was enhanced in intensity (Figure 3f). Its line width of 1.3 mT is characteristic for the anionic (red) flavin radical.[91] In contrast, for quinoline 2-oxidoreductase and eukaryotic xanthine oxidase the presence of a neutral (blue) radical species was deduced.

In dithionite reduced quinaldine 4-oxidase a distinct axial EPR signal of an iron–sulfur center (FeS I_{ax}) appeared at 77 K, which gained intensity at lower temperatures (Figure 2d and Figure 3f). In addition a second iron–sulfur center with rhombic symmetry was detectable below 60 K. This is at variance with the findings for quinoline 2-oxidoreductase and isoquinoline 1-oxidoreductase for which all centers provided rhombic symmetry. The pronounced axiality of the FeS I_{ax} center in quinaldine 4-oxidase should be associated with a more symmetrical ligand arrangement of the iron–sulfur cluster in this enzyme than in the other enzymes.

The generation of the "rapid" Mo^V species by addition of quinaldine to anaerobically prepared samples proved to be very sensitive to residual traces of oxygen. The corresponding axial "rapid" signal in Figure 3f exhibited a much lower spectral resolution than observed for the other enzymes, but still showed a decrease in the overall line width upon exchange of the buffer with D_2O. This is indicative for the presence of exchangeable protons at the molybdenum center, one of which probably is the sulfhydryl proton. In contrast to quinoline 2-oxidoreductase and isoquinoline 1-oxidoreductase neither the "resting" nor the "very rapid" type species could so far be detected spectroscopically.

The significant differences in EPR spectra of the three studied enzymes refer to the flavin radical, the iron–sulfur clusters, and the Mo^V signal-generating species. In particular, for Mo^V centers some spectral characteristics could be related to structural features. A more detailed understanding of the functional significance of the spectral observations for the various enzymes will be obtained from X-ray crystallographic data, which may allow for a correlation of electronic and electrochemical properties to the stereochemical arrangement of the redox active centers. In this context an important contribution is expected from the EPR-based high-resolution techniques electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) spectroscopy.

4.2.3. Genetics

In order to understand the binding of the redox-active centers by distinct peptide domains of the molybdenum-containing hydroxylases and to investigate the phylogenetic relationship of enzymes belonging to the xanthine oxidase family, the genes encoding isoquinoline 1-oxidoreductase and quinoline 2-oxidoreductase were cloned, sequenced, and subjected to comparative sequence analyses.[90, 94] The amino acid sequences of the small (IorA) and large (IorB) subunit, deduced from the nucleotide sequence of the *iorA* and *iorB* structural genes of the heterodimeric isoquinoline 1-oxidoreductase (cf. Table 1), indeed showed homologies 1) to the small (γ) and large (α) subunits of complex molybdenum-containing hydroxylases ($\alpha\beta\gamma/\alpha_2\beta_2\gamma_2$) possessing a pterin molybdenum cofactor with a monooxo–monosulfido-type molybdenum center, 2) to the N- and C-terminal regions of aldehyde oxidoreductase from *Desulfovibrio gigas*, and 3) to the N- and C-terminal domains of eukaryotic xanthine dehydrogenases. None of the regions of IorAB corresponded to the FAD-bearing middle domain of eukaryotic xanthine dehydrogenases, and there was no open reading frame putatively encoding an FAD-bearing peptide near the *iorAB* structural genes.[90]

The *qor* genes encoding quinoline 2-oxidoreductase from *Pseudomonas putida* 86 were found to be clustered in the transcriptional order 5'-*qorM-S-L*-3', corresponding to all other described structural genes of molybdenum-containing hydroxylases with $\alpha\beta\gamma$ or $\alpha_2\beta_2\gamma_2$ structure. We presume that both [2Fe-2S] centers are localized on the small, the FAD on the medium, and the pterin molybdenum cofactor as well as the substrate binding site on the large subunit, as discussed for the subunits or corresponding domains of other molybdenum-containing hydroxylases.[53, 90, 94–98]

An amino acid sequence alignment of the corresponding small subunits or domains of several pro- and eukaryotic enzymes belonging to the xanthine oxidase family with the small subunits of quinoline 2-oxidoreductase (QorS) and isoquinoline 1-oxidoreductase (IorA) revealed eight conserved cysteines, which are assumed to coordinate the two different [2Fe-2S] centers detected by EPR studies (cf. Section 4.2.2).[79, 92, 93, 99] The binding motif of the N-terminal [2Fe-2S] center (Figure 4a) is homologous to the signature sequence of bacterial and chloroplast-type [2Fe-2S]-ferredoxins, $[CX_4CX_2CX_nC]$ where X is any amino acid and n equals 11 for prokaryotic molybdenum-containing hydroxylases,[53, 98] 21 for eukaryotic molybdenum hydroxylases, and 29 or, in exception cases, 27 to 32 for bacterial and chloroplast-type ferredoxins. In contrast, the binding motif for the internal [2Fe-2S] cluster of the aligned small subunits has not been described in any other iron–sulfur protein and apparently is a distinct feature of the molybdenum-containing hydroxylases of pro- and eukaryotic organisms, where it is an absolutely conserved motif $[CX_2CX_{31}CXC]$ (Figure 4b).

a)

	★	↓					↓	★		↓					↓	
MenS	G	C	G	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[42 - 73]
MauS	G	C	G	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[45 - 76]
RatS	G	C	G	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[41 - 72]
MelS	G	C	A	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[46 - 77]
ObsS	G	C	A	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[42 - 73]
BomS	G	D	G	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[52 - 83]
HuhS	G	C	G	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[46 - 77]
CalS	G	C	G	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[55 - 86]
AspS	G	C	A	E	G	G	C	G	A	C	T	V	X_{18}	A	C	[72 - 102]
MopS	G	C	E	Q	G	Q	C	G	A	C	S	V	X_8	A	C	[39 - 60]
IorS	G	C	G	L	G	L	C	G	A	C	T	V	X_8	S	C	[38 - 59]
CsoS	G	C	E	T	G	I	C	G	A	C	S	V	X_8	S	C	[41 - 62]
CopS	G	C	D	T	S	H	C	G	A	C	T	V	X_8	S	C	[41 - 62]
NdhS	G	C	E	H	G	V	C	G	A	C	T	I	X_8	S	C	[47 - 68]
CooS	G	C	D	T	S	H	C	G	A	C	T	V	X_8	S	C	[41 - 62]
QorS	G	C	E	Q	G	V	C	G	S	C	T	I	X_8	S	C	[47 - 62]

b)

	★	↓	★		↓			★		★					★	+	↓	★	↓	+	+	★	
MenS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[112 - 153]
MauS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[114 - 155]
RatS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[110 - 151]
MelS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[116 - 157]
ObsS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[112 - 153]
BomS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[122 - 163]
HuhS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[116 - 157]
CalS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[125 - 166]
AspS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[141 - 182]
MopS	Q	C	G	F	C	S	P	G	X_8	L	X_{17}	H	R		N	A	C	R	C	T	G	Y	[99 - 142]
IorS	Q	C	G	Y	C	Q	S	G	X_8	L	X_{17}	-	-		N	L	C	R	C	G	T	Y	[96 - 137]
CsoS	Q	C	G	Y	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[100 - 141]
CopS	Q	C	G	F	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[100 - 141]
NdhS	Q	C	G	F	C	T	A	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[105 - 146]
CooS	Q	C	G	Y	C	T	P	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[101 - 142]
QorS	Q	C	G	F	C	T	A	G	X_8	L	X_{17}	-	-		N	L	C	R	C	T	G	Y	[106 - 147]

Figure 4. Alignment of amino acid sequences involved in binding of a) the N-terminal, ferredoxin-type [2Fe-2S] center and b) the internal [2Fe-2S] center of molybdenum-containing hydroxylases.[94] Numbers at the end of each line correspond to the amino acid number of the respective protein subunit or domain. Men = xanthine dehydrogenase of *Homo sapiens*; Mau = xanthine dehydrogenase of *Mus musculus*; Rat = xanthine dehydrogenase of *Rattus norvegicus*; Mel = xanthine dehydrogenase of *Drosophila melanogaster*; Obs = xanthine dehydrogenase of *Drosophila pseudoobscura*; Bom = xanthine dehydrogenase of *Bombyx mori*; Huh = xanthine dehydrogenase of *Gallus gallus*; Cal = xanthine dehydrogenase of *Calliphora vicina*; Asp = xanthine dehydrogenase of *Aspergillus nidulans*; Mop = aldehyde oxidoreductase of *Desulfovibrio gigas*; Ior = isoquinoline 1-oxidoreductase of *Brevundimonas diminuta* 7; Cso = quinaldic acid 4-oxidoreductase of *Pseudomonas* sp. AK-2; Cop = carbon monoxide dehydrogenase of *Pseudomonas thermocarboxydovorans* C2; Ndh = nicotine dehydrogenase of *Arthrobacter nicotinovorans*; Coo = carbon monoxide dehydrogenase of *Oligotropha carboxidovorans*; Qor = quinoline 2-oxidoreductase of *Pseudomonas putida* 86. S is small subunit or corresponding domain; ↓: conserved cysteine residues; ★: consistently conserved amino acid residues; + amino acid residues conserved in most sequences.

Since structural data on FAD binding by prokaryotic molybdo iron–sulfur flavoproteins are lacking, the mode of FAD binding in this subclass of enzymes belonging to the xanthine oxidase family is still unknown.

The large subunits of quinoline 2-oxidoreductase (QorL) and isoquinoline 1-oxidoreductase (IorB) probably contain the molybdopterin cytosine dinucleotide cofactor. QorL, IorB, and other large subunits or corresponding domains of a number of molybdenum-containing enzymes show significant homology to the C-terminal domain of aldehyde oxidoreductase from *Desulfovibrio gigas* (cf. Section 4.1). In this domain of the *Desulfovibrio gigas* enzyme, Romão et al. detected three molybdopterin binding amino acid segments and two dinucleotide binding segments.[64] Closely related signature sequences indeed are detectable in other molybdenum-containing enzymes belonging to the xanthine oxidase family: Figure 5 shows the sequence alignment of the molybdopterin binding and dinucleotide binding segments found in aldehyde oxidoreductase with putatively corresponding sequences of other molybdenum-containing hydroxylases. The first molybdopterin-contacting segment is the most conserved sequence of the five motifs. In aldehyde oxidoreductase from *Desulfovibrio gigas*, it contacts molybdopterin through F421 and G422, which are absolutely conserved in all aligned sequences (Figure 5).

However, the elucidation of structure and function of distinct conserved peptide domains of the molybdenum-containing hydroxylases must await further studies, such as investigation of the role of individual amino acid residues by site-directed mutagenesis, and, most significantly, X-ray structural analyses of crystallized enzyme proteins.

In analogy of the medium domain or subunit of other molybdenum-containing hydroxylases described, the medium subunit of quinoline 2-oxidoreductase (QorM) should contain the binding site for FAD.[53, 95, 96, 98, 100, 101] Several different dinucleotide (FAD, NAD/NADP, or ADP) binding motifs have been described.[102] All the FAD binding motifs comprise G-rich sequences, such as $[GXGX_2GX_3A]$ or $[GXGX_2GX_3G]$. A similar motif, $[GXGX_2AX_3A]$, is a site for NADP binding. None of these or other known motifs were detected in QorM or in the aligned sequences of nine eukaryotic xanthine dehydrogenases and five prokaryotic molybdenum-containing hydroxylases,[94] and the peptide domain proposed to bind the flavin cofactor of eukaryotic aldehyde oxidases also lacks such a consensus sequence.[103]

5. Future Prospects: Biotransformations Utilizing Molybdenum-Containing Hydroxylases?

For the manufacture of valuable intermediates for the synthesis of agrochemicals and pharmaceuticals, biotransformations with microbial strains or biocatalysts could provide versatile and economic alternatives to conventional chemical techniques. Chemical syntheses like hydroxylations of aromatic and heteroaromatic compounds usually require a high energy input. Furthermore, side-products that may be ex-

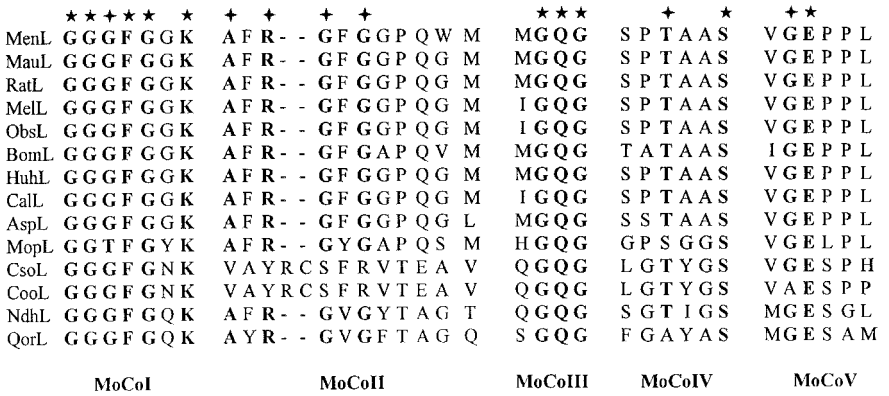


Figure 5. Alignment of amino acid sequences presumed to be involved in binding the pterin molybdenum cofactor of molybdenum-containing hydroxylases.[64, 94] MoCo I, MoCo II, MoCo III: putative molybdopterin binding segments; MoCo IV, MoCo V: putative dinucleotide binding segments.[64] The other abbreviations and the symbols are given in Figure 4. L is large subunit or corresponding domain.

pensive to separate and to dispose of may be formed, and the yields may be unsatisfactory. As a rule, however, biocatalysts possess substrate- and regiospecificities that are difficult to achieve by conventional chemistry, enabling the formation of defined products.

A number of hydroxylated quinolines, isoquinolines, and benzodiazines are important for the production of pharmaceuticals and agrochemicals:

1) Isocarbostyrils and carbostyrils: 2*H*-1-Oxoisoquinoline (isocarbostyril) is prepared chemically by treating isoquinoline in a KOH/NaOH or KOH/Na_2O melt.[104] Another possibility for the preparation of 2*H*-1-oxoisoquinolines is the Pd-catalyzed substitution of 2-bromobenzamides with ethylene and subsequent cyclization of the 2-ethenylbenzamide[105] or its preparation from *N*-propenyl-2-methylbenzamide.[106] Derivatives of 2*H*-1-oxoisoquinoline are widely used as drugs, fragrances, pharmaceuticals, and agrochemicals. In analogy to 2*H*-1-oxoisoquinoline, 1*H*-2-oxoquinoline (carbostyril, **12**) is prepared, for example, from quinoline (**10**) in a KOH/Na_2O or KOH/NaOH melt.[104] Other methods of chemical syntheses have been described. Carbostyril derivatives are useful as blood platelet aggregation inhibitors,[107] cardiotonic agents,[108] and neuroleptic agents.[109] Several 6-substituted carbostyrils were shown to inhibit vascular smooth muscle phosphodiesterases that hydrolyze cAMP and cGMP and thus may find application as therapeutic agents, for example, in cardiovascular disease.[110] Procaterol-HCl (5-[1-hydroxy-2-isopropylaminobutyl]-8-hydroxycarbostyril hydrochloride hemihydrate) is a sympathomimetic amine used as bronchodilator.[111]
2) Quinolones: "Quinolones" are generally defined as 1-substituted 1,4-dihydro-4-oxo-3-pyridinecarboxylic acids that have an additional ring fused at the 5,6-position. Quinolones, especially the 6-fluoroquinolones, have received considerable attention, and thousands of fluoroquinolone analogues have been disclosed. Fluoroquinolones are bactericidal agents against gram-negative and some gram-positive pathogens, and against mycobacteria and other intracellular bacteria, but they also exhibit promising activity against some categories of protozoa, such as *Leishmania*, *Trypanosoma*, and *Plasmodium* species, and thus may be useful in antimalaria therapy.[11–13, 112]
3) Hydroxylated benzodiazines: Quinoxalones, phthalazones, quinazolones: There are numerous patents for various methods to chemically synthesize 1*H*-2-oxoquinoxaline (**43**), which is manufactured as an intermediate for the production of agrochemicals, drugs, and dyes. The organophosphorus pesticide quinalphos (*O*,*O*-diethyl-*O*-quinoxaline-2-yl phosphorothioate) is used extensively for treatment of important crops in the tropical, subtropical, and temperate zones. For instance, lemon trees are sprayed with quinalphos for the control of the spherical mealybug *Nipaecoccus vastor*.[113] As pharmaceuticals, several 1*H*-2-oxoquinoxaline derivatives are useful in treating inflammatory and allergic diseases. 1,3-Dimethyl-1*H*-2-oxoquinoxaline, for instance, is a bronchodilator for asthma treatment.[114] For 2*H*-1-oxophthalazine **45**, too, there are numerous patents on different chemical syntheses. Substituted (2-biphenylyl)-2*H*-1-oxophthalazines are antipsychotics and antidepressants, and they are also used for the treatment of various inflammatory diseases.[115] Some 2*H*-1-oxophthalazine derivatives are vasoactive compounds and inhibit platelet aggregation.[116] Another important application of phthalazinones is for the preparation of photothermographic films.[117] Derivatives of quinazoline, especially the 3*H*-4-oxoquinazoline (**48**), also are used for the manufacture of pharmaceuticals such as antihypertensives, bronchodilators, anti-inflammatory agents, and central nervous system depressants.[118] A survey on biologically active quinazolones is given by Sinha and Srivastava, discussing their anthelmintic, central nervous system depressant, anti-inflammatory, cardiovascular, antimicrobial, and antitumor activities.[119]

In conclusion, carbostyril-, phthalazone-, quinazolone- and quinoxalone-based drugs are widely used as anti-inflammatory and cardiovascular agents, for the treatment of allergies, and as central nervous system depressants. Fluoroquinolones comprise a large class of antibacterial drugs, which are promising also for further therapeutic uses.

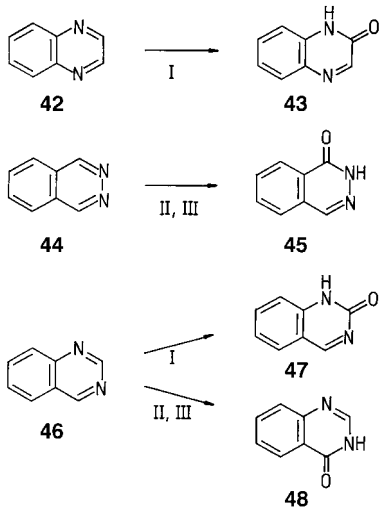
Up to now, these hydroxylated N-heterocyclic compounds almost exclusively are manufactured by chemical syntheses. The use of a bioconversion process, as exemplified in a Japanese patent, utilizing a *Pseudomonas* strain for the production of carbostyril (**12**) and/or 6-hydroxycarbostyril is rather exceptional.[120] However, hydroxylated quinolines, isoquinolines, and benzodiazines, useful as intermediates for further syntheses, alternatively might be produced by biotransformations, since biocatalysts enable substrate- and regiospecific hydroxylation free of side-products.

The molybdenum-containing hydroxylases may be utilized for the transformation of organic compounds such as quinolines and benzodiazines. Due to the observed accumulation of

the oxo compounds in the fermentation broth during growth of most bacterial strains on a quinoline derivative (cf. Section 1), such biotransformations even may be performed in a whole-cell system with the appropriate strain that degrades the N-heterocyclic compound in question. Cells of *Pseudomonas putida* 86, for instance, produce 1*H*-2-oxoquinoline (**12**) when growing on quinoline. High concentrations of quinoline prevent further degradation of **12**. Thus, it is even possible to harvest the product **12** from the culture broth in a crystalline state by regulating the concentration of the substrate quinoline during fermentation, first keeping it low in order to allow complete substrate utilization to obtain biomass, then increasing the quinoline concentration and maintaining it at a high level in order to accumulate **12**, which finally precipitates.

An efficient method to generate hydroxylated quinolines and benzodiazines, however, is biotransformation with a cometabolic process: The quinaldine-degrading strain *Arthrobacter* sp. Rü61a, for instance, does not grow on quinoline, but due to the broad substrate specificity of quinaldine 4-oxidase, cells of strain Rü61a—pregrown on quinaldine as carbon source in order to generate biomass and to induce the 4-hydroxylase—produce 1*H*-4-oxoquinoline from quinoline in excellent yields and free of side-products.

On the other hand, the isolated enzymes may be used as catalysts. The molybdenum-containing hydroxylases involved in the bacterial degradation of various N-heteroaromatic compounds offer several potential applications: Apart from the quinolines, some benzodiazines are accepted as substrates by isoquinoline 1-oxidoreductase, quinoline 2-oxidoreductase, and quinaldine 4-oxidase, yielding different hydroxylation products (Scheme 14):[72] Quinoxaline (**42**) is converted



Scheme 14. Hydroxylation of benzodiazines, catalyzed by various molybdenum-containing hydroxylases.[72] I: quinoline 2-oxidoreductase from *Pseudomonas putida* 86. II: isoquinoline 1-oxidoreductase from *Brevundimonas diminuta* 7. III: quinaldine 4-oxidase from *Arthrobacter* sp. Rü61a.

into 1*H*-2-oxoquinoxaline (**43**) by quinoline 2-oxidoreductase from *Pseudomonas putida* 86, and phthalazine (**44**) is converted into 2*H*-1-oxophthalazine (**45**) by both isoquinoline 1-oxidoreductase from *Brevundimonas diminuta* 7 and quinaldine 4-oxidase from *Arthrobacter* sp. Rü61a. The third benzodiazine, quinazoline (**46**), is hydroxylated at C4 by isoquinoline 1-oxidoreductase and quinaldine 4-oxidase, whereas quinoline 2-oxidoreductase catalyzes hydroxylation at C2 (Scheme 14).[72]

However, because biological systems have to be handled, the organic chemist would probably hesitate to consider a biochemical solution for a synthetic problem. At best, however, chemistry will supply economic precursors and enable efficient product isolation, whilst the critical step is catalyzed by an enzyme (or mixture of enzymes), or by whole cells. When using whole cells, growth and maintenance of a microbial strain in a fermentation system has to be set up, the conditions for biotransformation have to be optimized, and the biotransformation product must be purified from the culture broth. On the other hand, isolated enzymes, which may be obtained either in a crude or purified form, in a soluble or immobilized state, can be handled like a chemical catalyst. A number of applications, however, successfully use whole cells for bioconversion, such as LONZA's process to produce 6-hydroxynicotinic acid by regiospecific hydroxylation of nicotinic acid with a wild-type nicotinic acid degrading bacterial strain.[121] The bacterial enzyme involved in nicotinic acid hydroxylation also is a molybdenum-containing hydroxylase.

The more traditional biotransformation methods using whole cell systems or isolated biocatalysts and, additionally, modern techniques of molecular biology, such as cloning and (heterologous) expression of the hydroxylase genes and overproduction of the corresponding enzyme, will likely enable biotechnology to cooperate with chemistry.

6. Summary and Outlook

Microorganisms display enormous metabolic diversity and are primarily responsible for the cycling of organic compounds in the biosphere.

The pathways bacteria use to metabolize different classes of organic compounds are being studied extensively by many research groups. Biological attack on organic substances may lead to degradation, resulting ultimately in mineralization, or to transformation in which the main structure of the compound remains essentially unaltered. Mineralization of an organic compound generates carbon and energy for microbial growth. On the other hand, cometabolism, a process by which microorganisms in the obligatory presence of a growth substrate transform a non-growth substrate, is thought to play a significant role in the biotransformation and degradation of organic matter in the environment. Cometabolic conversions of N-heteroaromatic compounds may well take place in nature. However, the bacterial strains mentioned in this review article all were enriched and isolated from soil and water samples by offering quinoline (or a derivative) as sole source of carbon and energy, thus selecting for strains capable to grow on these compounds.

On investigating these bacterial isolates, four different catabolic pathways of aerobic utilization of quinoline derivatives were found. In some cases, minor amounts of side-

products (possible dead-end products) were formed in the culture broth, but degradation generally resulted in complete mineralization. Thus, bacteria have developed diverse and efficient strategies for metabolizing these N-heteroaromatic compounds. However, a detailed understanding of many degradative pathways is still lacking.

All the biochemical reactions taking place in an organism are governed by enzymes. The majority of these enzymes is highly selective, fundamentally exhibiting (depending on the reaction catalyzed) chemoselectivity, regioselectivity, and diastereoselectivity, and enantioselectivity. Detailed studies on the reactions involved in the bacterial degradation of quinoline and its derivatives have indeed revealed a number of interesting enzymes, such as the molybdenum-containing hydroxylases that catalyze regiospecific hydroxylations of their respective N-heteroaromatic substrate(s). Furthermore, unusual 2,4-dioxygenases that catalyze the cleavage of two C – C bonds with concomitant formation of carbon monoxide were discovered. However, although much is already known about the catalysts of aerobic degradation of N-heterocyclic compounds, there is still a great deal to be learned about the molecular mechanisms of enzyme-catalyzed reactions. Thus, study of bacterial enzymes progresses from physiological and biochemical investigations to a biophysical approach in order to understand how redox-active centers and cofactors work, to efforts to crystallize enzyme proteins in order to obtain structural data by X-ray diffraction methods, and to analysis of the genes encoding catabolic enzymes. Future research should also engage in the investigation of the regulatory mechanisms governing these catabolic pathways. These approaches may enable us to understand catalytic processes on the molecular level, and it may allow insight into the evolution of "enzyme families". From a practical point of view, the detailed investigation of the catalytic cycle and the study of the regulatory mechanisms of synthesis and the activity of an enzyme may help us to evaluate the potential of biocatalysts—either as whole cell systems or as isolated enzymes—for use in biotechnological processes.

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