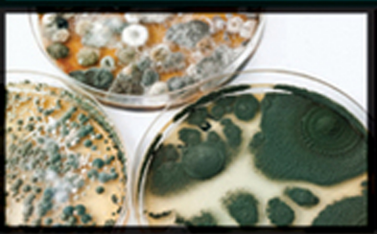
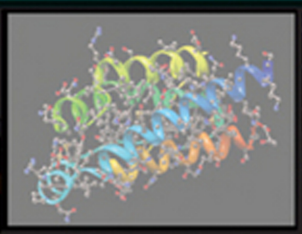
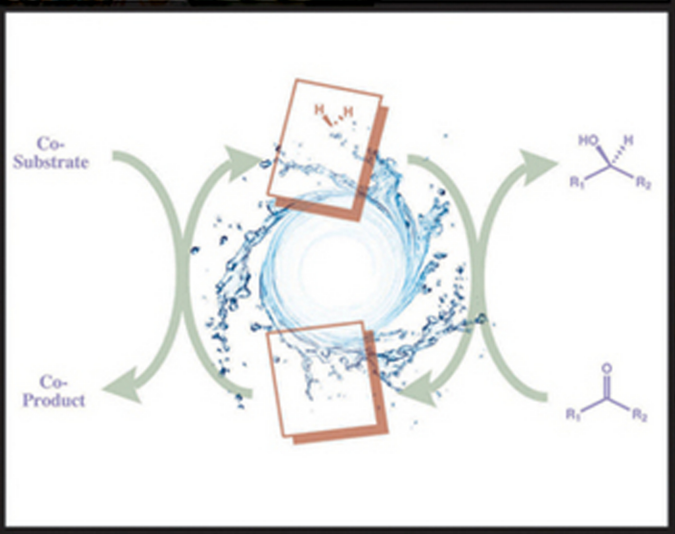
Redox Biocatalysis

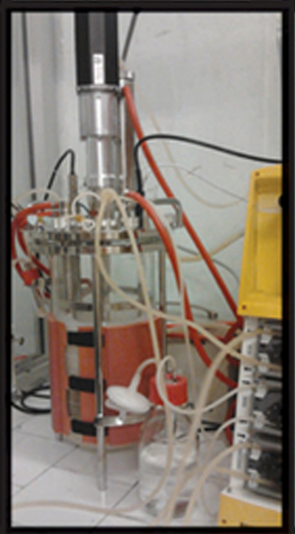
Fundamentals and Applications

Daniela Gamenara, Gustavo A. Seoane, Patricia Saenz-Méndez, and Pablo Domínguez de María









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PREFACE

The use of enzymes for redox processes has gained an increasing interest in the last decades, becoming in many cases "the first choice" for scouting novel industrial synthetic routes. This has been realized by solving issues related to cofactor regeneration, often needed for these enzymes, together with the developments in molecular biology areas that have enabled the provision of enzymes in large and reproducible scale in a fermentative sustainable manner. The development of environmentally sound synthetic protocols is mandatory in this century and, in this regard, oxidoreductases are ideally suited to the task, providing efficient and green alternatives to conventional synthetic procedures. This is particularly remarkable in oxidative processes, where oxidases and oxygenases perform clean and selective oxidations by activation of molecular oxygen with no need of heavy metals or expensive chemocatalysts. On the reductive side, these enzymes find ample application in the industry and academia for the generation of enantioenriched compounds.

This book provides a comprehensive and updated overview on the use of redox enzymes and enzyme-mediated oxidative processes. Chapters 1 and 2 provide an introduction on biochemical features of redox enzymes, together with aspects related to cofactors, and cofactor regeneration methods. Chapters 3–5 describe in detail the biocatalytic applications of different redox enzymes, namely, dehydrogenases (Chapter 3), oxygenases (Chapter 4), and oxidases and peroxidases (Chapter 5). Enzyme-mediated oxidative processes based on biocatalytic promiscuity (e.g., of hydrolases) are covered in Chapter 6. Chapter 7 focuses on the necessary steps starting from the discovery of a certain enzyme with a catalytic activity to a robust industrial process (e.g., directed evolution, high-throughput-screening methods, and medium engineering). Last but not least, Chapter 8 provides an overview on industrial cases using oxidoreductases, already commercialized or close to, showing that academic research is ending up with successful cases at the industrial arena. Overall, we believe that our contribution may well serve as a complete and first approach to academic and industrial research groups in the field of redox biocatalysis. It is our hope that readers will find this book an attractive and useful tool.

Finally, we would like to acknowledge Ms. Anita Lekhwani, Senior Acquisitions Editor at John Wiley & Sons, as well as the whole editorial team for the trust, hard work, interest, and patience that they have put into this project.

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CHAPTER 1

Enzymes Involved in Redox Reactions: Natural Sources and Mechanistic Overview

1.1 MOTIVATION: GREEN CHEMISTRY AND BIOCATALYSIS

Current environmental concerns are pressuring Chemical and Pharmaceutical industries to develop novel synthetic approaches that may operate under more benign conditions. This trend has paramounted the appearance of the "Green Chemistry" as a core discipline, with an increasing importance both in academia and industry. In a nutshell, Green Chemistry—as well as Green Engineering—has been compiled under several principles, as stated by Anastas and Zimmerman, and Tang and coworkers [1,2]. From the Green Chemistry approach, these principles are gathered in the acronym "PRODUCTIVELY":

- Prevent wastes
- Renewable materials
- Omit derivatization steps
- Degradable chemical products
- Use safe synthetic methods
- Catalytic reagents
- Temperature, ambient pressure
- In-process monitoring
- Very few auxiliary substances
- E-factor, maximize feed in product
- Low toxicity of chemical products
- Yet, it's safe

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On the other hand, from the Green Engineering perspective, those principles are grouped in the acronym "IMPROVEMENTS":

- Inherently nonhazardous and safe
- Minimize material diversity
- Prevention instead of treatment
- Renewable material and energy inputs
- Output-led design
- Very simple
- Efficient use of mass, energy, space, and time
- Meet the need
- Easy to separate by design
- Networks for exchange of local mass and energy
- Test the life cycle of the design
- Sustainability throughout product life cycle

In this respect, the use of enzymes and whole-cells as biocatalysts for synthetic purposes (White Biotechnology) is an increasingly important field that may fit, in many cases, with all or some of these Green Chemistry principles. In fact, enzymatic living processes are often conducted under extremely mild reaction conditions, for example, neutral pH, or no need of high pressures or temperatures, which may provide energy savings for the overall process. Albeit biocatalytic processes are not always in line with all Green Chemistry principles (e.g., wastewater generation after downstream processing), they often provide advantages when compared to other chemical approaches. These assets have triggered the development of biocatalysis, reaching today the status of established technology, and occupying a prominent role as "synthetic organic chemistry tool" [3–16].

1.2 SOURCES OF BIOCATALYSTS

In the early stages of biocatalysis, plant tissues, and animal organs were the most important sources of enzymes, representing in the 1960s about 70% of the biocatalysts used for synthetic purposes [17,18]. The trend rapidly changed, and 20 years later most of industrially used enzymes were already being obtained from microbial sources. There are still some commercially available enzymes from animal origin, mostly hydrolases, accounting for approximately 10% of total of enzymes used at industrial level [19]. In this group, catalase from liver (EC 1.11.1.6), triacylglycerol lipase (EC 3.1.1.3), and trypsin from pancreas (EC 3.4.21.4) are the most relevant animal enzymes currently used, mainly in food industries [20,21]. Enzymes from vegetable origins, such as papain and cysteine proteases from papaya latex (*Carica papaya*, *Carica candamarcensis*), have industrial relevance as well, representing almost 5% of the market [17,22,23]. Other enzymes, such as invertase [24], peptidases [25], and

acid phosphatase [26] are produced *in vivo* in plant cell cultures, but their production involves highly complex and expensive processes, thus showing a limited use at industrial scale [27]. However, some glycoenzymes, such as glutamine synthetase, which are not easily produced as recombinant proteins in microbial hosts, are suitable candidates for the *in vitro* production with adequate cell lines [28].

While the origin of the biocatalysts can obviously be highly diverse, microorganisms are a rich source of enzymes, and thus their use as whole-cells, or the use of isolated microbial enzymes as biocatalysts has been vastly reported in the literature. Since the 1960s microbial enzymes have been replacing the biocatalysts from other origins, and to date, represent over 90% of the total market [17]. Currently, the exploitation of microbial diversity in the quest for new enzymes with novel activities is one of the major research goals in biocatalysis. This is complemented by the rational design of enzymes, and their production and overexpression in adequate microbial hosts through genetic engineering techniques [29]. Remarkably, the use of recombinant microorganisms was originally envisaged for the production of proteins of therapeutic interest. However, its real advantage is the reduction of production costs for a wide variety of proteins, especially when compared with the fermentation of wild-type microorganisms [30].

1.2.1 Plants and Animals as Sources of Redox Biocatalysts

As stated above, animal and plant tissues are classic sources of biocatalysts. Enzymes from higher eukaryotes have been traditionally used in food industry as food additives, in fruit processing or in wine production, as well as pharmaceutical additives. Some examples are the use of papain, lipoxygenase (LOX), or pepsin in processes already developed in the 1980s, which are still widely used [31,32]. Many hydrolases such as porcine pancreas lipase (PPL), pig liver esterase (PLE), or chymotrypsin and trypsin have been isolated from animal tissues, and have been widely used as biocatalysts [33]. Oxidoreductases (EC 1.-), hydrolases (EC 3.-), lyases (EC 4.-), and isomerases (EC 5.-) provide the vast majority of examples of higher eukaryotic enzymes for industrial applications, and no commercial processes using plant or animal enzymes from other enzyme classes have been reported [32].

Some of these animal or plant tissues can provide high amounts of enzymes (up to 1% of wet weight), for example, pancreatic enzymes or others involved in specific metabolisms (from liver or heart), or enzymes located in plant reserve organs such as seeds. However, recovering these enzymes from tissues is often cumbersome, and thus alternative sources must be found for their production at large scale for synthetic purposes. In addition, in case of pancreatic enzymes, after the discovery of pancreas as insulin-producing organ in 1921, the tissue became very expensive as a source of enzymes for biocatalysis. The enzymes that are still obtained from pancreas—trypsin and chymotrypsin—are actually by-products in insulin metabolism. Furthermore, nowadays insulin is mostly produced by recombinant microorganisms (*Escherichia coli* or yeast cells), as other enzymes originally obtained from pancreas do as well.

Higher eukaryotic enzymes usually need to be isolated and purified, or cloned and overexpressed in suitable hosts in order to obtain sufficient amounts for biocatalytic

applications. The use of synthetic host-adopted genes and codon-optimized *E. coli* strains, and the development of highly successful eukaryotic expression systems such as the yeasts *Pichia pastoris* and *Hansenula polymorpha*, have enabled the production of large quantities of eukaryotic enzymes within a short time [34].

Among redox enzymes from animal origin, horse liver alcohol dehydrogenase (HLADH, EC 1.1.1.1), or cytochrome P450 monooxygenases (CYP450, EC 1.14.-.-) are the most extensively used, both in academic and industrial settings [32]. CYP450s belong to a superfamily of hemeproteins with high catalytic versatility, which generally perform the monooxygenation of aliphatic compounds as a key step in the production of fine chemicals, and catalyze the metabolism of a wide variety of endogenous and exogenous compounds (see Section "Heme iron monooxygenases cytochromes P450"). They are involved in reactions as diverse as hydroxylations, *N*-, *O*-, and *S*-dealkylation, sulfoxidation, epoxidation, deamination, desulfuration, dehalogenation, peroxidation, and *N*-oxide reduction (see Chapter 4) [35–43]. Their substrates include fatty acids, steroids, prostaglandins, and a number of exogenous compounds such as drugs, anesthetics, pesticides, and carcinogens [35]. This diverse catalytic potential attracted researchers from different fields to study cytochrome P450 systems. Industrial applications of human P450s involve two different objectives: the production of active pharmaceutical intermediates and the simple and fast production (in mg-scale) of metabolites for drug development. Human CYP450 has been expressed in different systems such as mammalian cells, yeasts, and bacteria such as *E. coli* [32]. For this application, *E. coli* expression systems are the easiest and less expensive to operate, yielding high quantities of recombinant proteins. At present, complete sets of ready-to-use liver enzymes expressed in *E. coli* are commercially available, providing kits with all six major human liver cytochromes for simple application. With regard to HLADH, three groups of isoenzymes with different substrate specificities are known, each one containing one main form [44–47]. The enzyme is a dimer, and these three forms correspond to the possible dimeric combinations of two protein chains [48–50] that are not interconvertible, therefore having different primary structures. The three isoenzymes were crystallized [51,52], and are similar in their amino acid analysis [51], but distinguishable by immunological methods [53]. Regarding synthetic applications of HLADH, its ability for the stereoselective reduction of a broad range of carbonyl compounds is outstanding, including aromatic, open-chain, or cyclic ketones, and α- or β-ketoesters (see Chapter 3).

Apart from the above-described two main enzymes, bovine liver glutamate dehydrogenase (GluDH, EC 1.4.1.2) is another example of dehydrogenases from higher eukaryotes [32]. The enzyme reversibly catalyzes the reductive amination of α-ketoglutarate to L-glutamate using NADH as cofactor. The crystal structure of this homohexameric mitochondrial enzyme has been solved at 2.8 Å resolution and its catalytic mechanism has also been demonstrated [54].

Plant cells exhibit an ample potential for the biosynthesis of secondary metabolites [55]. Although in cell cultures the formation and accumulation of these metabolites do not occur, such cultures retain the ability to transform exogenous substrates into products of interest. Thus, plant cells have been widely used in biocatalysis, as isolated enzymes as well as whole-cells (cell cultures or crude parts such as leaves, roots, seeds,

etc.), either free or immobilized, displaying hydrolase and oxidoreductase activities [55]. When performing biotransformations mediated by "native" plant organs or tissues, the main drawback is the lack of reproducibility of the experiments. Some biochemical features of these biocatalysts can vary easily depending on several factors, such as the origin of the plant, the season of the year, or particular climatic conditions [56]. Likewise, other drawbacks are the localization and concentration of enzyme in the cell, and the presence of side reactions leading to undesired by-products, or enzymes that could degrade the desired products. In addition, another issue is the mass transfer limitations involved in the transport between the bulk medium and the enzyme. Overall, these problems contribute to the often observed low efficiency of these processes [55]. Strategies such as elicitation, permeation of the cells by the addition of dimethyl sulfoxide (DMSO) or organic alcohols to promote the substrate uptake and product release, or the variation of the pH, have been developed to improve efficiencies. Cyclodextrins were also used as additives in cases of water-insoluble or poorly soluble substrates because they are able to form inclusion complexes with a variety of apolar ligands [57]. Another major drawback of cultured cells as biocatalysts is the somaclonal variation, which may lead to unstable biochemical behavior [58]. This problem can be circumvented either by the continuous screening to maintain productive lines, or by the use of organized tissues, such as roots or root cultures, which are able to provide biotransformations with potential applicability for the production of added-value products, and even for scaling up purposes. In this context hairy roots, which are obtained by the integration of a region (T-DNA) of the Ri plasmid of the bacterial soil pathogen *Agrobacterium rhizogenes* to the plant genome [59], have been used as biocatalysts.

The use of dehydrogenases from vegetal origin has been widely exploited for the reduction of carbonyl groups to the corresponding alcohols. A broad range of structurally diverse carbonyl compounds including aromatic and aliphatic ketones, diketones, ketoesters, aldehydes, steroids, alkaloids, terpenoids, coumarins, and lignans, among others, can undergo redox reactions catalyzed by plant dehydrogenases (see Chapter 3) [55,56,60–77]. Among crude plant cells or plant cell cultures used as sources of dehydrogenases, *Daucus carota* has been one of the most widely used [60,62–71,75,76,78]. Cells of *Raphanus sativus* [56], *Passiflora edulis* [77], *Cocos nucifera* [61], celeriac (*Apium graveolens*) [62,63,70], horseradish (*Armoracia lapathifolia*) [62], legumes such as *Pisum Sativum* [73], *Phaseolus angularis* [72], and *Phaseolus aureus* [74], among others [79–82], have been also used as biocatalysts in dehydrogenase-catalyzed reductions or oxidations. Likewise, *R. sativus* hairy roots were used in the stereoselective reduction of a series of prochiral alkylaryl ketones. Most of the reactions proceeded with high yields and excellent enantioselectivities [56]. Hairy roots of *Brassica napus* [83] and *D. carota* [84] were also employed as biocatalysts for the stereoselective reduction of aromatic and aliphatic ketones, diketones, and ketoesters.

In addition to the vast group of dehydrogenases, other enzyme types of vegetal origin are of synthetic use, such as oxidases and peroxidases. Glycolate oxidase (EC 1.1.3.15) is a peroxisomal oxidase isolated either from mammals including pig liver, rats and humans, or from green plants such as spinach leaves, pea, cucumber,

and pumpkin [32]. Of all of them, spinach redox enzymes displayed the highest yields also having the best specific activity for the oxidation of glycolic acid to glyoxylic acid [85]. The biocatalytic oxidation was performed in high yields (>99%) and resulted in a few undesirable by-products, in the presence of oxygen and ethylenediamine, using both glycolate oxidase and catalase. Importantly for practical purposes, spinach glycolate oxidase is also active for the oxidation of other α-hydroxy carboxylic acids [86]. Yet, purified enzymes from spinach leaves are not suitable for stable enzyme preparations for industrial applications [32]. Therefore, its gene was cloned and overexpressed in *E. coli* [87,88], *Saccharomyces cerevisiae* [89], and *P. pastoris* [90]. This was the first example of expression of a plant gene in *P. pastoris*, and also the first engineered *P. pastoris* as a whole-cell catalyst developed for a commercial bioprocess. The protein crystal structure was determined at 2.0 Å resolution, showing a catalytically active tetramer or octamer made up of identical 40 kDa subunits, which form an eight-stranded α/β barrel [91]. Optically pure (*R*)-2-hydroxyacids were also obtained on semipreparative scale with >99% *ee* and good-to-excellent conversions by α-hydroxylation of long-chain carboxylic acids with molecular oxygen, through a reaction catalyzed by an α-oxidase from peas (*P. sativum*) [92].

Finally, the importance of peroxidases is emphasized by their wide distribution among living organisms and by their multiple physiological roles. They have been divided into two main superfamilies according to their source and mode of action: plant (nonanimal) peroxidases and animal peroxidases (see Section 1.3.4) [93]. The plant peroxidases superfamily, which contains enzymes from both prokaryotic and eukaryotic origin, can be in turn divided in three classes, based on structural similarities and in a suspected common evolutionary origin [94]: peroxidases from prokaryotic origin (Class I), fungal peroxidases (Class II), and plant peroxidases (Class III). Horseradish peroxidase (HRP, EC 1.11.1.7), peanut peroxidase (PNP) [95], soybean peroxidase (SoP) [96,97], tobacco peroxidase (TobP) [98], tomato peroxidase (TomP) [99], and barley peroxidase (BaP) [100] are examples of Class III peroxidases. They contain an *N*-terminal signal peptide for secretion, two conserved calcium ions, four conserved disulfide bridges, an extra helical region that plays a role in access to the heme edge, and a carbohydrate content between 0% and 25% [93]. Of all of them, HRP is the most intensively studied peroxidase from plant origin, catalyzing a variety of reactions such as reduction of hydroperoxides, epoxidation, sulfoxidation, halogenation, and oxidation of phenols and aromatic amines (see Chapter 5).

A general strategy to improve the biocatalytic efficiency is the biocatalyst immobilization [101]. Whole-cells as well as isolated enzymes can be immobilized in order to overcome stability problems and to enable the biocatalyst reuse for cost reduction. Immobilized whole-cells have additional advantages over freely suspended cells. General methods for immobilization of plant cells are gel entrapment by ion exchange, precipitation, polymerization, and by fixing them into preformed structures [102]. Enzymes can be adsorbed into insoluble supports by hydrogen bonding, dipole–dipole interactions, or hydrophobic interactions. Most commonly used supports are polypropene (e.g., Accurel TM), and diatomaceous earth (Celite). Immobilization of enzymes by ion exchange is possible when the optimum pH of the enzyme is not

close to its isoelectric point. Polyacrylamides are common matrixes used for covalent linking of the enzymes. High degrees of cross-linking prevent leakage and loss of the biocatalyst, but are not suitable for bulky substrates. Microencapsulation, forming a microsphere of polymeric membranes around the enzyme in solution, is another frequently used method [55].

1.2.2 Wild-Type Microorganisms

Microorganisms have been widely exploited as biocatalysts in the area of medicine, agriculture, and food industry, and their industrial applications have an increasing impact. Actually, as mentioned before, most of the enzymes currently used in industry have microbial origin. Since the beginning of biotechnology, microbial wild-type strains have been used for food and beverages production. In turn, native isolated enzymes from microbial origin, as well as recombinant proteins and microorganisms, are having an increasingly widespread use in pharmaceutical, chemical, or biofuels industries, being mainly designed through genetic engineering [103].

1.2.2.1 Yeasts The large-scale use of yeasts in enzyme-catalyzed processes dates back several centuries ago, with the production of ethanol from glucose in alcoholic beverage manufactures. *S. cerevisiae* (baker's yeast, BY) was often the microorganism of choice, mainly due to its wide availability and low cost. Moreover, BY does not need sterile growth media, and remains viable and easy to work in a nonmicrobiology-specialized laboratory. In addition to these desirable features, as biocatalyst, baker's yeasts (as well as other yeasts) can be used in chirality generation, in racemic resolutions, or in the regioselective conversion of functional groups. For such purposes, besides redox-related enzymes (mainly dehydrogenases), other biocatalytic yeast enzymatic systems include hydrolytic enzymes (lipases, epoxide hydrolases) and lyases for C–C bond formation. Clearly, *S. cerevisiae* is still to date the most widely used yeast biocatalyst [104].

Among redox enzymes, dehydrogenases are versatile and powerful biocatalysts for synthetic organic chemistry, mainly involved in the reduction of carbonyl groups—generating chiral alcohols—or in the asymmetric reduction of enones or imines. On the other hand, yeast dehydrogenase-mediated oxidations generally involve the destruction of chiral centers, and thus their practical use has been mainly guided by environmental considerations, a major advantage over conventional chemical oxidations (see Chapter 3). In the reduction of carbonyl groups—usually associated to the synthesis of (*S*)-secondary alcohols—most of the yeast dehydrogenases follow the so-called Prelog's rule [105]. For these purposes, yeast dehydrogenases can be used either as whole-cells (wild type or recombinant), or isolated enzymes with the adequate cofactor supply and a suitable recycling method [106]. As mentioned above, *S. cerevisiae* is the most widely used yeast as biocatalyst, being efficient for selectively reducing monocarbonylic compounds (aldehydes and ketones) with alkyl or aryl substituents and dicarbonylic compounds (cyclic and acyclic α- and β-diketones, α- and β-ketoesters), thus obtaining (*S*)-secondary alcohols (see Chapters 3 and 7). Sterically hindered ketones are not usually substrates for

yeast dehydrogenases except with the exception of methyl ketones (see Chapter 3). Apart from baker's yeast, a wide number of oxidoreductases from *Candida* sp. have also been characterized and used as biocatalysts [107]. These enzymes are rather diverse, enabling not only highly enantioselective reductions of carbonyl groups or deracemizations, but also some promiscuous catalytic imine reductions. In addition, some *Candida* sp. dehydrogenases have been extensively used for cofactor regeneration purposes, with the aid of sacrificial substrates.

1.2.2.2 Fungi Fungi have traditionally been one of the most studied whole-cell enzymatic systems as biocatalysts [108]. They can be identified in nature through the screening of a wide variety of environments and habitats. Bioprospecting for new biocatalysts even in extreme environments—thermal, cold, or hypersaline ecosystems—can lead to the discovery of new fungal enzymes able to catalyze a wide variety of synthetically interesting reactions. Among the most extensively explored fungal enzyme systems for biocatalytic purposes, redox enzymes can be highlighted, catalyzing oxygenase- as well as peroxidase-mediated hydroxylations, sulfoxidations, epoxidations and Baeyer–Villiger oxidations, dehydrogenase-catalyzed stereoselective reductions, oxidations or deracemizations [104,108].

Fungal dehydrogenase-catalyzed reactions, in particular of carbonyl compounds to furnish alcohols regio- and enantioselectively, are the most widely described in the literature. The broad substrate specificity of these enzymes allows for the preparation of structurally different compounds, including aromatic, cyclic, open chain aliphatic (*R*)- or (*S*)-secondary alcohols, or α- or β-hydroxyesters and hydroxyketones [108]. Dehydrogenase-catalyzed racemization or deracemizations are also efficient strategies for interconversion of enantiomers, with a number of fungal enzymes reported for these types of reactions [108].

Oxygenase-, oxidase-, and peroxidase-mediated oxidations, introducing oxygen atoms into nonactivated hydrocarbon chains of organic compounds, are useful for organic synthesis as well. These fungal enzymes catalyze Baeyer–Villiger reactions [109], epoxidations [110], sulfoxidations [111], and hydroxylations [112] using growing or resting whole-cells, as well as isolated enzymes. Likewise, fungal laccases catalyze oxidation and coupling reactions [113,114]. Although these enzymes are widely distributed in fungi, higher plants, bacteria and insects, the majority of laccases characterized so far are of fungal origin, spread in more than sixty strains belonging to various classes such as *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes* [115]. Especially, white rot *Basidiomycetes* are efficient lignin degraders, being a valuable source of laccases. Moreover, fungi are also an important source of peroxidases. Due to the catalytic versatility and stability of these enzymes, fungal peroxidases are, together with fungal laccases [114,116], of particular interest as potential biocatalysts for redox processes at industrial level [93,117–119].

1.2.2.3 Bacteria The variety of bacterial genera and species, and the different enzyme types according to their metabolism, role and environment, make these microorganisms a rich source of biocatalysts. Bacteria used in biocatalysis belong to a

wide variety of genera, including *Escherichia*, *Rhodococcus*, *Bacillus*, *Lactobacillus*, *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Corynebacterium*, and so on [30,33]. Similar to enzymes from eukaryotic cells, bacterial enzymes can be used both as isolated and as whole-cells, and either free or immobilized. Due to management requirements in the laboratory, the use of isolated enzymes is mainly restricted to extracellular and cofactor-independent-enzymes, such as hydrolases, whereas the whole-cells of microorganisms are preferred in synthetic procedures involving cofactor-dependent or intracellular enzymes. However, at industrial level examples of redox enzymes comprise both isolated enzymes and whole-cells (see Chapters 7 and 8).

Bacterial enzymes or whole-cell-catalyzed biotransformations are used in the industrial preparation of a variety of compounds [33]. The use of redox enzymes from bacterial origin for the preparation of chiral intermediates for fine chemicals is highly widespread. Dehydrogenases, involved in the reduction of carbonyl groups or the enantioselective oxidation of alcohols, as well as mono- and dioxygenases for hydroxylations of arenes and unfunctionalized alkanes, Baeyer–Villiger reactions and alkene monooxygenation, are outstanding examples [104,120]. Most of the monooxygenases used in biocatalysis are from bacterial origin, and aromatic dioxygenases have only been found in bacteria, mainly in genus *Pseudomonas*, *Sphingomonas*, and *Rhodococcus*, being intracellular enzymes. Although to date many oxygenases are known and thoroughly described, their use for synthetic purposes is still limited due to their low availability in large quantities, instability, and high cost of the required cofactors. Moreover, many of them are membrane-associated proteins, reducing their synthetic potential and hampering their use as isolated enzymes. However, efforts have been done to overcome these drawbacks, for example, using recombinant whole-cells overexpressing the desired enzymes (see Chapter 4).

1.2.3 Metagenomic Assessments

An ample range of microbial diversity, not yet accessed or explored, might be a valuable source for possible novel biotechnological applications. In this respect, only 0.1–10% of natural biodiversity can be cultured under conventional laboratory conditions [121,122]. Metagenomics, that is, the genomic reconstruction of uncultivable microorganisms that emerged in the late 1990s, refers to the extraction of the entire genetic material from all organisms present in an environmental sample (metagenome) [123,124]. It involves the culture-independent analysis of the collective microbial genomes contained in the metagenome by using two approaches: (i) the function-driven analysis and (ii) the sequence-driven analysis, to extract biological information from metagenomic libraries [125–127]. The function-driven analysis identifies the clones that express a desired feature, and then characterizes the active ones by sequencing and biochemical analysis, rapidly finding clones with potential applications. The limitation of the method is that it requires the expression of the function of interest in the host cell and the clustering of all of the genes required for the function. It also depends on the availability of an adequate assay for the function of interest that can be performed on vast libraries, because the frequency

of active clones is quite low. To overcome these limitations, improved systems for heterologous gene expression have been developed with shuttle vectors that facilitate screening of the metagenomic DNA in diverse host species and with modifications of *E. coli* cells to expand the range of gene expression. Conversely, sequence-driven analysis is based on the use of conserved DNA sequences to design hybridization probes or polymerase chain reaction (PCR) primers to screen metagenomic libraries for clones that contain sequences of interest. Significant discoveries have resulted from random sequencing of metagenomic clones [127]. Metagenomics relies on the efficiency of four main steps, which are: (i) the isolation of genetic material, (ii) its manipulation, (iii) the transfer of the genetic material into a surrogate organism to create a metagenome clone library, and (iv) the analysis of the genetic material in the metagenomic library [121,122,128]. The information about diversity and community structure of microbes is further obtained through the corresponding metagenome library sequency [129,130]. The specific activities within the metagenome can be screened for particular enzymes, either *via* DNA sequences or enzymatic functions [131,132].

Metagenomic libraries have been screened for enzymes [133], and a number of metagenomic biocatalysts displayed novel substrate ranges and high stability under extreme conditions, improving their potential for industrial applications [134]. Many oxidoreductases have been incorporated into the redox-biocatalytic toolbox through metagenomic assessment [134]. As some examples, in the quest of microorganisms accepting 4-hydroxybutyrate, five clones were found displaying novel 4-hydroxybutyrate dehydrogenase activity [135]. The genes involved in metabolism of poly-4-hydroxybutyrate were also successfully screened in environmental libraries [136]. Short-chain dehydrogenases/reductases were found with <35% similarity to known enzymes, and thus could not have been detected using hybridization-based techniques such as PCR. Likewise, alcohol dehydrogenases (ADHs) oxidizing short chain polyols were obtained from twenty-four positive clones and characterized [137,138]. Polyphenol oxidase (PPO; tyrosinase), catechol oxidase, as well as multicopper oxidases, oxygenases, laccases, and L-amino acid oxidases (LAAOs) were also isolated from marine metagenome [139,140]. Clones capable of indirubin and indigo production in *E. coli* were isolated from forest soil metagenome [141]. A novel ADH, $ADHE_{Meta}$, was isolated from a waste-water treatment metagenomic library [142]. Although this enzyme showed a reasonably high sequence identity to the well-characterized ADHs from *E. coli* (60%) and *Clostridium acetobutylicum* (61%)—which catalyze the ethanol and butanol formation in a two-step reductive process—it was not deactivated by oxygen [123]. This functional property suggests that the *in vivo* role of the enzyme was catabolic, rather than the typical anabolic role of ADHs.

1.3 OVERVIEW OF REDOX ENZYMES

Enzymes catalyzing oxidation–reduction reactions—oxidoreductases, EC 1.—always act on their substrates involving an electron transfer. These biocatalytic

redox processes have been attracting an increasing interest for synthetic organic chemistry, especially for applications in the chemical and pharmaceutical industries [143]. Besides the inherent enantioselectivity of enzymes, other challenging chemical transformations like the regioselective introduction of oxygen from O_2 into chemically inert C–H bonds can be performed. Furthermore, oxidoreductases catalyze the formation of chiral products from prochiral substrates with a theoretical 100% yield, as well as other reactions such as the oxifunctionalization of C–C double bonds and C–N bonds, Baeyer–Villiger oxidations, and the oxidation of alcohols, aldehydes, acids, and aromatic compounds, or else the reduction of ketones, aldehydes, C–C and C–N double bonds, and reductive aminations [120,144,145]. Applications of these enzymes comprise mainly asymmetric oxyfunctionalization of nonactivated hydrocarbons and enantioselective reductions of carbonyl compounds, synthesis and modification of polymers, as well as oxidative degradation of pollutants and construction of biosensors for a variety of analytical and clinical applications [146].

Presently, more than 25% of the known enzymes are oxidoreductases [147,148]. Due to their biodiversity and different activities, oxidoreductases have been classified according to the nature of the oxidizing substrate as dehydrogenases, oxygenases (monooxygenases and dioxygenases), oxidases and peroxidases (Figure 1.1). Alternatively, they can be classified according to their coenzyme requirements [149]. Peroxidases are sometimes considered as a subset in the group of oxidases [106], but taking into account the differences in terms of their catalytic mechanism, as well as their synthetic applications, in this book peroxidases will be treated as independent enzymes.

Dehydrogenases catalyze reversible redox reactions and are thus used as oxidative or reductive biocatalysts. Conversely, oxygenases, oxidases and peroxidases catalyze irreversible oxidations due to the reaction thermodynamics. This is attributed to the highly exothermic reduction of O_2 or H_2O_2, which act as electron acceptors in the case of oxygenases and oxidases, or peroxidases respectively [144]. In contrast to oxidases, oxygenases incorporate one (monooxygenases) or both (dioxygenases) oxygen atoms of O_2 into their substrates.

Redox enzymes often require cofactors, which are direct products of the primary metabolism of the cells. These requirements impose special constraints on the development of bioprocesses involving oxidoreductases. These cofactors or coenzymes, such as NAD(P)H or $NAD(P)^+$, act as electron donating or accepting molecules, being coupled to the redox metabolism within cells [14,150–153]. Therefore an efficient cofactor recycling system is required to achieve economically efficient processes (see Chapters 2 and 7–8 for applying biocatalysis at practical level) [104,154–158]. Exceptions include peroxidases, which couple the reduction of hydrogen peroxide to water with the two electron oxidation of the substrate, and oxidases, which couple the reduction of O_2 to hydrogen peroxide or water with the two- or four-electron oxidation of an activated carbon scaffold. All reactions dealing with O_2 or H_2O_2 reduction involve reactive oxygen species such as oxygen radicals or hydrogen peroxide itself, which often cause extensive damage to biomolecules, including enzymes. These reactive oxygen species and their damage can be handled by the metabolism

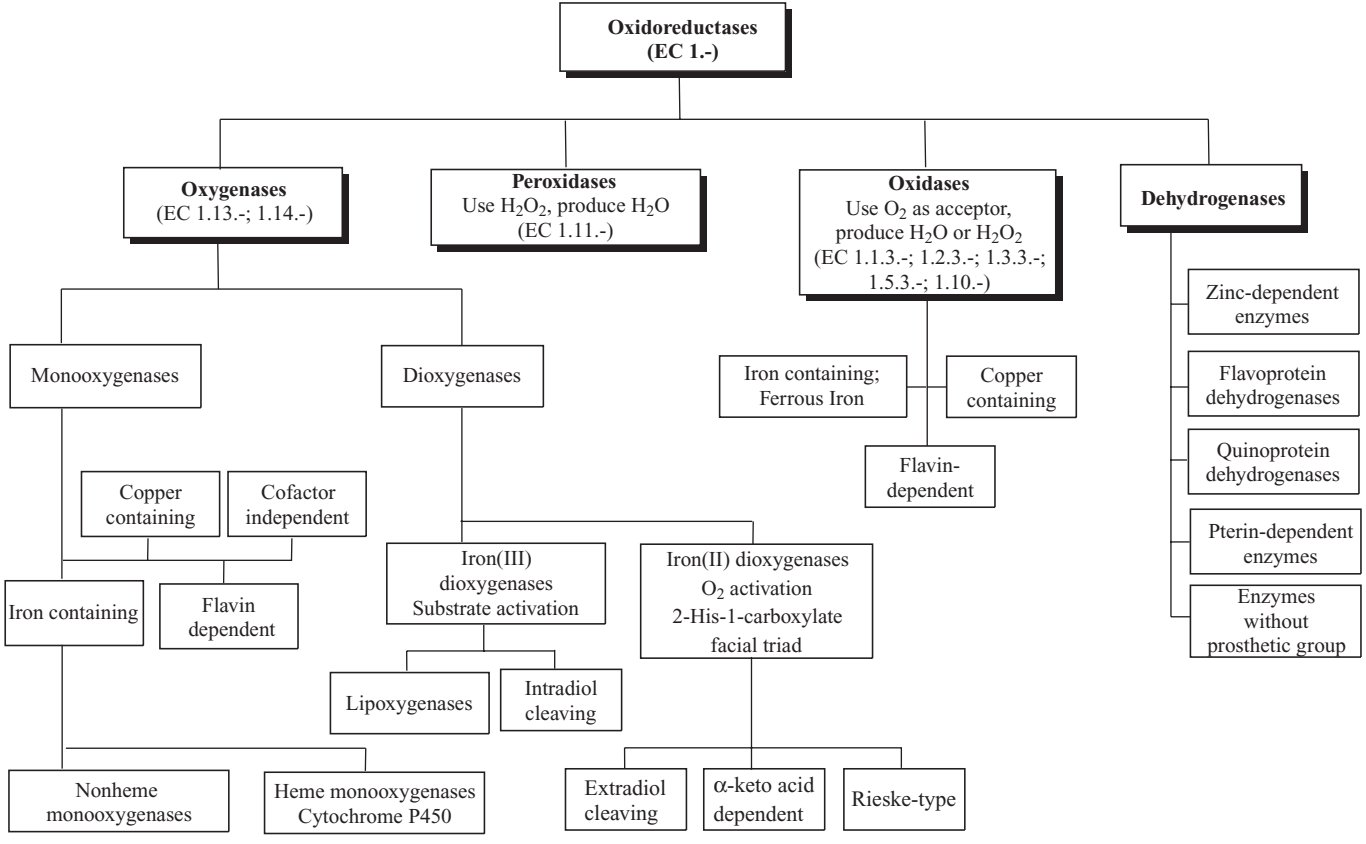

FIGURE 1.1 Classification of redox enzymes.

of resting or growing cells, an advantage that can be exploited for productive redox biocatalysis [144].

1.3.1 Dehydrogenases

Dehydrogenases (also known as reductases) are the most widely used oxidoreductases for synthetic purposes [104,143,158–160], and they are classified in groups EC 1.1.1.- (acting on the CH–OH group of donors), EC 1.2.1.- (acting on the aldehyde or oxo group of donors), or EC 1.4.1.- (acting on the CH–NH_2 group of donors). They always use NAD^+ or $NADP^+$ as cofactors [159].

Regarding substrate specificity of dehydrogenases, enantiopure alcohols, hydroxyacids, hydroxyesters, amines, and amino acids can be obtained through dehydrogenase-catalyzed reductions of the corresponding prochiral compounds [104,158,159,161]. The enzymes catalyze the reversible reaction, in which one atom of hydrogen and two electrons of the donor species are transferred to the C-4 position in the nicotinamide of the cofactor (NAD^+ or $NADP^+$). The reverse reaction involves the transfer of one atom of hydrogen and two electrons from the reduced form of the nicotinamide cofactor (NADH or NADPH) to the carbonyl (or imino) group, resulting in the reduction of the substrate, being often a highly specific reaction. The hydrogen atom transfer occurs either on the *Si* face or on the *Re* face of the carbonyl group, resulting in the corresponding (*R*)- or (*S*)-alcohol, respectively. Both, NADH and NADPH have two diastereotopic hydrogen atoms (*pro-R* and *pro-S*), which can be transferred as a hydride to an oxidized substrate (aldehyde, ketone, or imine). In turn, these substrates have two diastereotopic or enantiotopic faces (*Re*-face or *Si*-face) in the sp^2 carbon atom that will be reduced. Theoretically, any NAD(P)H-dependent redox reaction can occur stereospecifically *via* any of the four possible pathways to generate any isomer on the substrate (Figure 1.2).

In practice, most ADHs catalyze the transfer of the *pro-R* hydrogen atom at C-4 of the nicotinamide cofactor to the *Re* face of the carbonyl substrate, furnishing the corresponding (*S*)-alcohol as the main reduction product. This general behavior

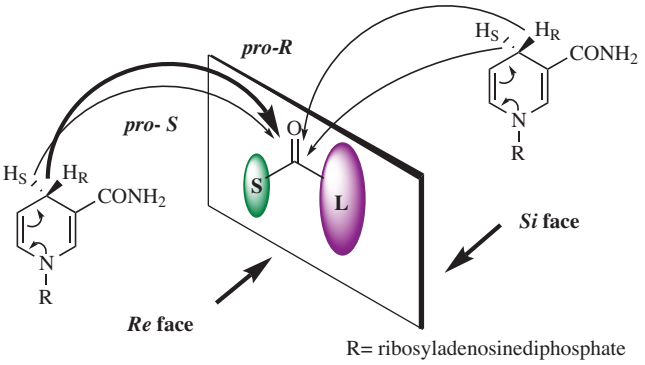

FIGURE 1.2 Stereochemical possibilities for the hydride transfer in the dehydrogenase-mediated reduction of carbonyl compounds. **L**, large substituent; **S**, small substituent.

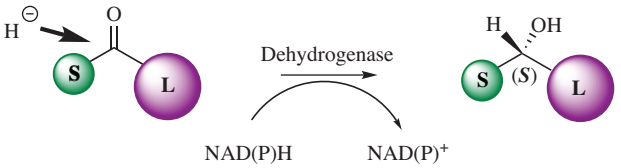

FIGURE 1.3 Stereochemical preference for the hydride transfer in the dehydrogenase-catalyzed reduction of carbonylic compounds, according to Prelog's rule. **L**, large substituent; **S**, small substituent.

is described by the Prelog's rule [105], where the stereochemical course of the reaction is predictable, and largely depends on the steric requirements of the substrate (Figure 1.3). Depending on the binding site of the cofactor in the enzyme, either the *pro-R* or *pro-S* hydrogen at C-4 of the nicotinamide is abstracted. The Prelog's rule is an empirical rule based on the stereochemistry of microbial reductions using whole-cells of *Curvularia falcata*.

Dehydrogenases can be classified into five categories depending on their prosthetic group: (i) zinc-dependent enzymes, (ii) flavoprotein dehydrogenases, (iii) pterin-dependent enzymes, (iv) quinoprotein dehydrogenases, and (v) dehydrogenases without prosthetic groups [144].

1.3.1.1 Zn-Dependent Dehydrogenases Zinc-dependent dehydrogenases comprise mainly ADHs that are grouped according to their substrate spectrum and structure into long-, medium- and short-chain ADHs [162]. These enzymes catalyze the (reversible) oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones respectively, often in a chemo-, regio-, and stereoselective way. For synthetic purposes, the most relevant enzymes of this group are HLADH and ADH from *Thermoanaerobacter brockii* (TbADH).

Structure–function studies have been undertaken for the Zn-dependent medium-chain HLADH, revealing a nucleotide binding domain and a catalytic domain, with the catalytically active zinc buried between them [163]. In HLADH, the zinc is tetra-coordinated to three conserved residues (Cys46, His67 and Cys174) of the active site, and to a water molecule [164]. There are two main mechanisms proposed for HLADH-catalyzed alcohol oxidation, which differ in the coordination of the zinc atom during the redox process. One proposes that the zinc ion stays in tetrahedrical coordination during catalysis, as the bound water molecule is replaced by the hydroxyl group of the alcohol. Then, two hydrogen atoms are removed from the substrate by a coupled process of proton abstraction and hydride ion transfer. The intermediate turns to the corresponding oxidation product, with the concomitant hydride transfer to $NAD(P)^+$ to complete the reaction. The other mechanistic proposal suggests a change in the coordination symmetry of the zinc, leading to a pentacoordinated species, by the binding of the substrate in addition to the water molecule, and followed by the same steps than in the previously proposed mechanism. Recent X-ray studies on HLADH and TbADH provided evidence for the latter mechanism, involving a pentacoordinated zinc during catalysis [165,166]. Kleifeld and coworkers proposed

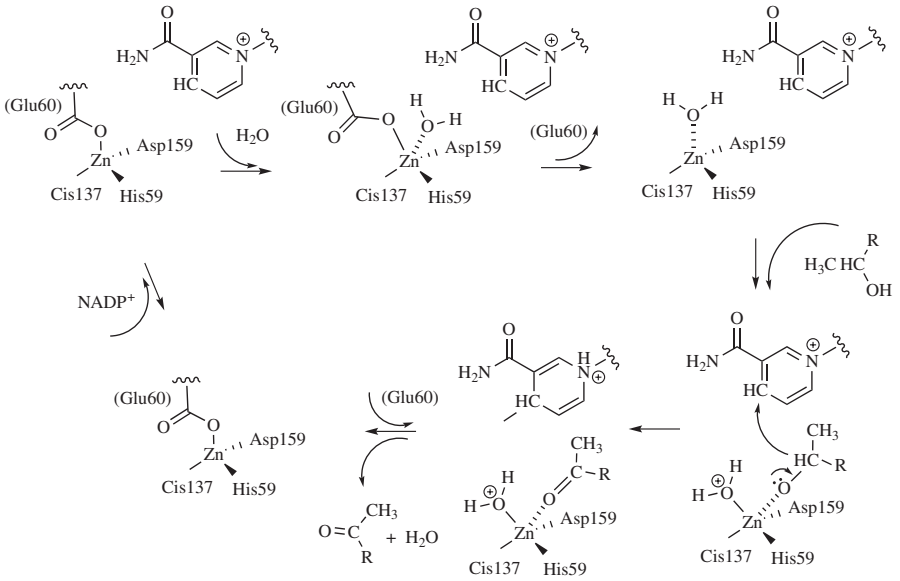

FIGURE 1.4 Proposed mechanism for the oxidation of secondary alcohols mediated by alcohol dehydrogenase from *Thermoanaerobacter brockii* (TbADH).

that TbADH differs from other ADHs, since the catalytic Zinc is bound to three conserved residues (Cys37, His59, and Asp159), and a residue of glutamic acid (Glu60) instead of water in the forth coordination site. This Glu60 is exchanged by a water molecule during the catalysis, generating a pentacoordinated intermediate (Figure 1.4).

1.3.1.2 Flavin-Dependent Dehydrogenases Enzymes containing flavin adenine dinucleotide (FAD) as prosthetic group, either covalently or noncovalently bound, known as flavoprotein dehydrogenases, are mostly involved in O_2 activation reactions. They are important enzymes in redox metabolism, yet less used for synthetic applications [144]. As in the case of other flavin-dependent enzymes such as oxidases, FAD serves as an electron acceptor during the oxidation of the substrate. Within this group, acyl-CoA dehydrogenases are a family of flavin-dependent enzymes whose members are highly conserved in sequence, structure, and function. These enzymes catalyze key steps in the mitochondrial oxidation of lysine, hydroxylysine, and tryptophan. Glutaryl-CoA dehydrogenase (GCD, EC 1.3.99.7) has a homotetrameric structure containing one noncovalently bound FAD in each monomer, and catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA [167]. The reductive half reaction generates the enzyme-bound intermediate glutaconyl-CoA and CO_2 (Figure 1.5). Upon binding the substrate glutaryl-CoA, a glutamate residue functioning as catalytic base abstracts an α-proton from the substrate, followed by hydride transfer from the C-3 position of the substrate to the flavin, yielding the two electron reduced form of FAD and the enzyme-bound desaturated

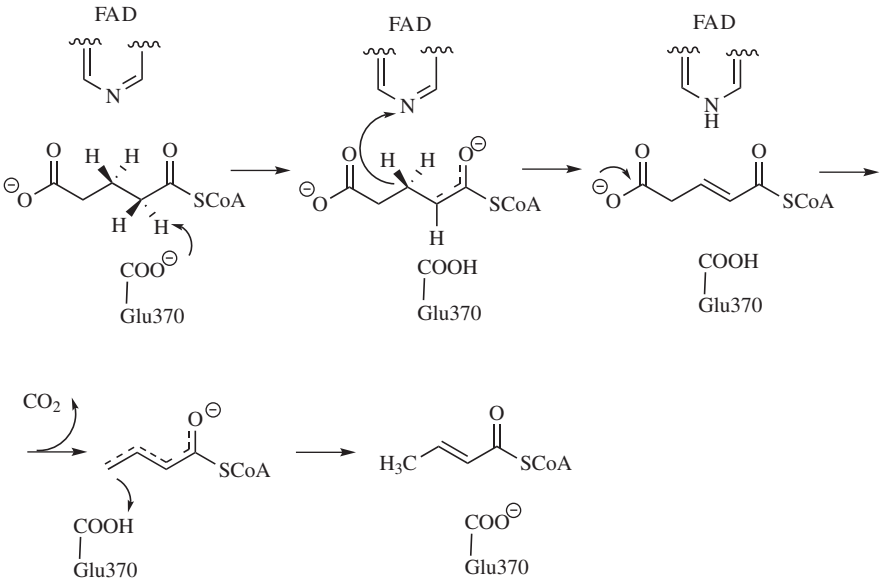

FIGURE 1.5 Proposed mechanism for the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA (human GCD numbering).

intermediate glutaconyl-CoA [168,169]. Subsequently, the latter is decarboxylated resulting in the formation of a dienolate anion and CO_2. The dienolate anion is protonated *via* proton transfer to C-4 from the catalytic base Glu370, which exchanges its proton with solvent water, giving rise to the crotonyl-CoA product (numbering of the residues is for the human GCD) [170]. The two electron reduced form of FAD is reoxidized in two consecutive steps, transferring the electrons to the respiratory chain.

Besides flavoproteins containing only the flavin in the catalytic center, there are also two-component dehydrogenases, having a covalently bound flavin in one subunit and a cytochrome in the other [171]. Examples for hydroxylating flavocytochromes include *p*-cresol dehydrogenase [172,173], and eugenol dehydrogenase [174], found in *Pseudomonas fluorescens* strains and denitrifying bacteria. After a fast electron transfer from the flavin to the cytochrome, *p*-cresol dehydrogenase transfers the electron to azurin or nitrate as physiological electron acceptors. During the reaction, a proton is removed from the hydroxyl group of the substrate *p*-cresol and a hydride ion is transferred from the methyl group to the N-5 atom of the covalently bound flavin. The formed quinone methide intermediate undergoes a nucleophilic attack of water at the methylene group, yielding *p*-hydroxybenzyl alcohol.

1.3.1.3 Pterin-Dependent Dehydrogenases Pterin-dependent dehydrogenases also belong to the group of hydroxylating enzymes, which use water as electron donor. They contain a so-called molybdopterin cofactor, where molybdenum is usually coordinated to the two thiol groups of a pyranopterin-based cofactor [144].

These enzymes can be composed of a variable number of subunits and contain different additional cofactors such as hemes, iron–sulfur clusters, and flavins, which transfer electrons from the molybdopterin cofactor to an electron acceptor. These molybdoenzymes catalyze the hydroxylation of activated carbon atoms, such as heteroaromatic ring carbons. Furthermore, selenium has been reported to participate in reactions catalyzed by xanthine dehydrogenase [175] and nicotinic acid dehydrogenases of anaerobic strains [176], for which NAD^+ and $NADP^+$ serve as electron acceptors, while nicotinic acid dehydrogenases from aerobic strains are selenium independent [177]. Molybdenum-enzymes are found in nearly all organisms, with *Saccharomyces* as a prominent eukaryotic exception [178]. The reaction mechanism has been characterized for xanthine dehydrogenase. A molybdenum center (as Mo(VI) in the resting state) with a water-derived oxo- or hydroxyl group in the ligand sphere is reduced to Mo(IV) during the substrate oxidation. Electrons derived from substrate hydroxylation are transferred through a [2Fe–2S] cluster and FAD to NAD^+ [179].

1.3.1.4 Quinoprotein Dehydrogenases Quinoproteins contain an amino acid-derived *o*-quinone as a cofactor. Different kind of quinone cofactors are known, but only pyrroloquinoline quinone (PQQ), which is not covalently bound to the enzyme, is involved in oxidoreduction reactions [180]. Quinoprotein dehydrogenases are highly interesting enzymes regarding redox metabolism, transferring reducing equivalents directly to the bacterial aerobic respiratory chain [144]. These enzymes are located in the periplasm, and are mainly involved in the dehydrogenation of primary or secondary hydroxyl groups in alcohols and sugars. These enzymes are frequently applied in biosensors [181]. Mainly two dehydrogenases of this class, in which PPQ is the only cofactor, have been extensively studied [182,183]. These are the membrane-bound quinoprotein glucose dehydrogenase (m-GDH, EC 1.1.5.2) and the soluble methanol dehydrogenase (s-MDH, EC 1.1.1.244), which catalyzes the oxidation of methanol to formaldehyde. Glucose dehydrogenase was found in a variety of bacteria including Gram-negative facultative anaerobes such as enteric bacteria and *Zymomonas*, as well as aerobic bacteria such as *Pseudomonas* and acetic acid bacteria [184]. The enzyme was originally investigated in *Acinetobacter calcoaceticus* in early 1960s by Hauge [185–187], and subsequently in late 1970s by Duine et al. [188], who reported that the enzyme was a quinoprotein. Mechanistic studies on s-MDH showed that upon substrate binding, the oxidation is initiated by an aspartate residue located in the active site, which is responsible for the abstraction of the proton from the alcohol group [189]. Then, a Ca^{2+} ion coordinated to the C-5 of the cofactor acts as a Lewis acid during substrate oxidation, stabilizing the C-5 carbonyl atom for the electrophilic attack by the hydride [144]. The proton abstraction is followed by a direct hydride transfer from the methyl group of methanol to the C-5 carbonyl of PQQ, followed by tautomerization to $PQQH_2$ [190]. The oxidized product, formaldehyde, is then released while $PQQH_2$ is reoxidized by sequential single-electron transfer to a *c*-type cytochrome in the electron transport chain.

The mechanism of the oxidation for GDH is similar to the one described for methanol oxidation by MDH. The proton in the anomeric hydroxyl group of glucose

is abstracted by an aspartate residue in the active site of the enzyme, and a hydride is transferred from the C-1 of the glucose molecule to the C-5 carbon of the PQQ cofactor, yielding gluconolactonate and $PQQH_2$. Further oxidation of $PQQH_2$ involves either ubiquinone or *c*-type cytochromes as electron acceptors in the electron transport chain. The enzyme from *E. coli* is a monomeric protein of 87 kDa [191]. The *N*-terminal domain (154 residues) has five transmembrane segments, and it is likely where the ubiquinone-binding site is contained. The remaining periplasmic region (641 residues) is similar to the α-subunit of MDH. The prosthetic group PQQ in MDH is held in place in the active site by stacking interactions between a tryptophan residue and a disulfide ring structure made up of adjacent cysteine residues [192,193]. In GDH from *E. coli*, this disulfide ring is replaced by a histidine residue (His262), which is essential for binding PQQ [191]. The entrance to the active site is more open than in MDH, and His262 is located at the entrance, together with a second histidine residue (His775). GDH from *E. coli* has two ubiquinone binding sites: one of them is located near the active site and contains a tightly bound ubiquinone that performs single electron transfer steps; the second site is in the amphiphilic segment of the membrane bound GDH and reversibly binds ubiquinone from the membrane pool [194]. In the cell, the enzyme is responsible for the direct oxidation of glucose to gluconate in the periplasm, but also showed the ability to oxidize a wide range of pentoses and hexoses [191]. Disaccharides such as maltose, lactose, trehalose, and sucrose are neither substrates for GDH, nor was the trisaccharide raffinose, or D-fructose, *myo*-inositol, methanol, or ethanol. None of these compounds inhibited the oxidation of glucose, showing that they are not able to bind at the active site. L-Hexoses were not substrates, including L-glucose, L-mannose, L-allose and L-rhamnose. The only common feature of these L-hexoses is that they have a C-6 hydroxymethyl (or methyl) group below the plane of the ring, suggesting that this prevents binding by steric hindrance. As understanding of the mechanism of catalysis and electron transfer of quinoproteins progressed, the application of these enzymes as biosensors became attractive, and thus electrochemical studies aimed at the design of specific quinoprotein-based electrodes have increased [184]. Soluble glucose dehydrogenase (s-GDH) from *A. calcoaceticus* was the first quinoprotein applied to such a biosensor, and a D-glucose sensor with s-GDH in a single-use electrochemical test strip containing ferricyanide as a mediator is already in the market [184].

1.3.1.5 Dehydrogenases without Prosthetic Group Although most redox enzymes require metal or organic prosthetic groups for the hydride transfer, several dehydrogenases have neither metal ions nor any other prosthetic group bound to their active sites. The most prominent example of such enzymes is formate dehydrogenase (FDH), well known for its use in enzyme-coupled cofactor regeneration systems (see Chapters 2 and 8). The enzyme catalyzes the oxidation of formate to CO_2, cleaving a single carbon–hydrogen bond in the substrate and concurrently forming a new one in the nicotinamide cofactor, which also accepts the electrons [195]. During catalysis, a hydride anion from the substrate formate attacks the electrophilic C-4 of the nicotinamide cofactor NAD^+. Consequently, two uncharged products, CO_2 and NADH, are formed [144,196].