Review of NAD(P)H-dependent oxidoreductases: Properties, engineering and application

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ABSTRACT

NAD(P)H-dependent oxidoreductases catalyze the reduction or oxidation of a substrate coupled to the oxidation or reduction, respectively, of a nicotinamide adenine dinucleotide cofactor NAD(P)H or NAD(P)⁺. NAD(P)H-dependent oxidoreductases catalyze a large variety of reactions and play a pivotal role in many central metabolic pathways. Due to the high activity, regiospecificity and stereospecificity with which they catalyze redox reactions, they have been used as key components in a wide range of applications, including substrate utilization, the synthesis of chemicals, biodegradation and detoxification. There is great interest in tailoring NAD(P)H-dependent oxidoreductases to make them more suitable for particular applications. Here, we review the main properties and classes of NAD(P)H-dependent oxidoreductases, the types of reactions they catalyze, some of the main protein engineering techniques used to modify their properties and some interesting examples of their modification and application.

1. Introduction

Oxidoreductases (Enzyme Commission [EC] primary class 1) catalyze the oxidation of one chemical species (a reducing agent or electron donor) with the concurrent reduction of another (an oxidizing agent or electron acceptor) in the form A⁺ + B → A⁺ + B⁻ and comprise almost one third of all enzymatic activities registered in the BRENDS [German Enzyme Database, BRENSA] (Fig. 1). Oxidoreductases can act on a wide range of both organic substrates including alcohols, amines and ketones and inorganic substrates including small anions such as sulfite, and metals such as mercury.

NAD(P)H-dependent oxidoreductases are able to oxidize a substrate by transferring a hydride (H⁻) group to a nicotinamide adenine dinucleotide cofactor (either NAD⁺ or NADP⁺), resulting in the reduced form NADH or NADPH (Fig. 2), and make up over 50% of all oxidoreductase activities registered in the BRENSA (Fig. 1). There are over 150,000 different sequences annotated as or predicted to be NAD(P)H-dependent oxidoreductases [1].

NADH/NAD⁺ and NADPH/NADP⁺ serve as pools of redox cofactors for the cell. The nicotinamide ring of NADH/NAD⁺ or NADPH/NADP⁺ is the part of the cofactor directly involved in the transfer of electrons during the reactions catalyzed by NAD(P)H-dependent oxidoreductases, while the C4 carbon atom of the nicotinamide ring acts as the acceptor/donor of a proton [2]. The addition of the phosphate to the 2'-OH group of the adenosine ribose ring in NADPH/NADP⁺ does not modify the electron transport capability as it is located far from the electron transfer region (Fig. 2). However, the phosphate group modifies the structure of the cofactor, which allows different enzymes to have different specificities for NADH/NAD⁺ and NADPH/NADP⁺, thereby allowing these to act as two equivalent but independent redox systems. This has a physiological function, allowing different redox poise (degree of reduction of the cofactor pool) to be maintained in the two systems, and independent fluxes. Typically, at least in heterotrophs, enzymes of catabolic pathways use NADH/NAD⁺, while NADPH/NADP⁺ is the preferred cofactor for anabolism [3,4]. The redox poise of the NADH/NAD⁺ pool depends upon the availability of external electron acceptors and of substrates. This variation in redox poise can be observed for example in Escherichia coli growing via aerobic respiration, anaerobic respiration, or anaerobic fermentation, where factors such as the oxygen availability when growing aerobically, or the redox potential of other electron acceptors used when growing anaerobically, affect the steady-state NADH/NAD⁺ ratio [5]. In contrast, the poise of the NADPH/NADP⁺ pool is maintained in a more reduced state in order to more effectively provide reducing power for biosynthesis [6]. Accordingly, NADH-dependence is more prevalent among oxidoreductases acting on smaller molecules, which include most substrates and products of catabolism (Fig. 3). Interestingly, substrates of low molecular weight can be metabolized by a higher number of enzymatic activities, indicating that smaller substrates have a role as central hubs of redox metabolic networks (Fig. 3b).

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also another, smaller increase of both the absolute number of oxidoreductase activities (Fig. 3a) and the number of oxidoreductase activities per substrate for substrates of molecular weight between 700 and 1100 Da, which is partially due to substrates that need to be activated by binding to coenzyme A (CoA) before the corresponding oxidoreductase can act on them (Fig. 3). Canonically the differing redox states of the two systems (NADH and NADPH) is achieved in heterotrophs using NAD<sup>+</sup>-dependent glycolysis and the NADP<sup>+</sup>-dependent pentose phosphate pathway, but there are several variations and alternatives [7], and photoautotrophs generate NADPH using the light-dependent reactions of photosynthesis. The independence of the NADH/NAD<sup>+</sup> and NADPH/NADP<sup>+</sup> pools also allows different enzymes with different redox cofactors to catalyze key steps of opposite pathways helping to prevent futile cycles [8].

NAD(P)H-dependent oxidoreductases are of great interest from an industrial point of view as they perform the critical steps in the production of many hard-to-synthesize compounds, under mild conditions. For example, even though several chemical methods have been developed to perform the oxidation of primary alcohols, they are usually laborious and can lead to the formation of toxic products [9], which can be avoided through enzymatic catalysis. Additionally, NAD(P)H-dependent oxidoreductases possess other properties which make them very attractive alternatives to organic chemical synthesis, such as stereospecificity, regiospecificity and the possibility to tailor them to have the appropriate kinetic parameters and the desired substrate specificity [10]. For these reasons, NAD(P)H-dependent oxidoreductases have been used extensively in metabolic engineering for the production of...
low, mid and high value products [11].

NAD(P)H-dependent oxidoreductases are too numerous and diverse to review exhaustively. Therefore, the reader is provided with a list of recommended papers and reviews relevant to NAD(P)H-dependent oxidoreductases (Table 1). Here we review this important group of enzymes broadly, with an applied perspective. The common functional, structural, mechanistic and physiological properties are described. The various classes of these enzymes are introduced, with emphasis placed on catalytic mechanisms and their structural basis, which can provide important information to guide protein engineering approaches. Examples of enzymes of applied interest are included, often entailing metabolic engineering or protein engineering using common techniques.

2. Specificity and promiscuity of oxidoreductases

Promiscuity of enzymes can refer to two different concepts: catalytic promiscuity, and specificity. Promiscuous enzymes and enzymes with a wide substrate range are of particular interest, since even an initially low catalytic activity towards a specific substrate can be a good starting point to generate, by means of protein engineering and directed evolution approaches, an efficient catalyst suitable for a specific reaction, which does not necessarily match the physiological reaction carried out by the original enzyme [54].

Catalytic promiscuity is the ability of an enzyme to catalyze a second reaction in addition to the main reaction for which it is physiologically specialized, with a different transition state. Hydrolyases, and in particular lipases, are some of enzymes where catalytic promiscuity has been most extensively studied, as they are able to catalyze a variety of different reactions other than hydrolysis of esters in non-aqueous media, such as transesterification alcoholysis or acidolysis [55]. However, more recently other enzymes have been described to have catalytic promiscuity, including NAD(P)H-dependent oxidoreductases. Certain alcohol dehydrogenases (in particular, alcohol dehydrogenases from horse liver, Rhodococcus ruber, Ralstonia sp. and Lactobacillus kefir) have been found to catalyze the conversion of oximes (specifically phenylacetaldoxyme) to the corresponding alcohol [56]. In another study, an NADPH-dependent ene-reductase (Δ4-3-ketosteroid-5β-reductase, which reduces a C=C double bond in Δ4-3-ketosteroids) gained the ability to reduce the carbonyl group of the substrate to a hydroxy group with a single point mutation, an activity that is usually carried out by 3α-hydroxysteroid dehydrogenase [57]. This indicates that the two enzymes probably had a common ancestor from which they diverged to catalyze consecutive steps of the same metabolic pathway. Substrate promiscuity refers to the property of an enzyme of being able to catalyze the same reaction with a range of different but related substrates [55]. Some are very specific and have just one possible substrate, such as α-hydroxyisovalerate dehydrogenase from the depsipeptide-producing fungus Fusarium sambucinum [58]. However, many NAD(P)H-dependent oxidoreductases are not strictly specific for a single substrate, and instead display a varying degree of promiscuity, being able to act on a set of substrates. The extent to which they are promiscuous differs even between enzymes of the same subclass. For example, Thermus sp. ATN1 alcohol dehydrogenase (TADH) exhibits activity towards a very large range of different alcohols with multiple different functional groups (including both aliphatic and aromatic alcohols) [59], while Zymomonas mobilis alcohol dehydrogenase 2 is only able to oxidize ethanol, 1-propanol and allyl alcohol [60].
There is great variation in the promiscuity of NAD(P)H-dependent oxidoreductases for the nicotinamide cofactor. Some are highly specific for either NADH/NAD⁺ or NADPH/NADP⁺, while others can use both with varying degrees of preference for one over the other. For example, the previously mentioned D-hydroxyisovalerate dehydrogenase of Fusarium sambucinum, as well as the soluble NADPH-dependent Fe(III) reductase from Geobacter sulfurreducens, are highly specific for NADPH, whereas many NADP⁺ and are unable to accept NADH/NAD⁺. At the opposite extreme, the E. coli aldo-keto reductase encoded by the ydG gene is highly specific for NADH/NAD⁺ and is unable to accept NADPH/NADP⁺. This enzyme is considered to be an exception among the family of aldo-keto reductases, which typically either are specific for NADPH/NADP⁺ or can accept both NADH/NAD⁺ and NADPH/NADP⁺ [62].

Many NAD(P)H-dependent oxidoreductases are described as being able to use both NADH/NAD⁺ and NADPH/NADP⁺ (Figs. 1 and 3). These include the B2FLR2 flavin-containing monoxygenase from Stenotrophomonas maltophilia, which prefers NADH/NAD⁺, and xyllose reductase of Neurospora crassa, which prefers NADPH/NADP⁺ [63,64]. There are also cases in which multiple enzymes with the same catalytic activity but different cofactor specificity are found in the same organism. This is the case in barley, which contains two nitrate reductases, regulated in different ways: one of them is specific for NADH (Nar1), while the other can use both NADH and NADPH (Nar7) [65].

3. Structural features of NAD(P)H-dependent oxidoreductases

The number of NAD(P)H-dependent oxidoreductases for which a structure is available is growing rapidly, allowing protein engineering of these enzymes to be tackled with a rational design or data-driven approach. However, it has also become clear that it is not possible to develop a unified approach that can be broadly applied, since the huge variety of substrates upon which this class of enzymes can act as a group is associated with a similarly large variety of structural motifs. In this section we describe the two main structural features of each class of NAD(P)H-dependent oxidoreductases, important conserved catalytic residues and a brief overview of how these features relate to reaction mechanisms.

3.1. Cofactor binding domain

One of the key structural motifs of NAD(P)H-dependent oxidoreductases is the nucleotide binding domain which allows them to recruit the NAD(P)H/NAD(P)⁺ redox cofactor essential for their activity. The most common fold employed by these enzymes for such purpose is the Rossmann fold, but there is a variety of other less common structural motifs which can also bind the redox cofactor, such as the TIM-barrel, the dihydroquinooate synthase-like and the FAD/NAD⁺ binding folds [66].

The Rossmann fold is a common structural motif found in many nucleotide-binding proteins, such as 3-phosphoglycerate dehydrogenase and lactate dehydrogenase [67,68]. It is named after Michael Rossmann, one of the members of the team who first identified the fold in lactate dehydrogenase [69] and later realized it was a conserved motif present in other NAD(P)H-binding enzymes [70]. Its most conserved core consists of two parallel β-strands separated by an α-helix (αβ motif) (Fig. 4). A tight loop is formed between the first β-strand and the α-helix, which establishes direct contact with the cofactor. This loop contains the consensus sequence Gly-X-Gly-X-X-Gly/Ala (with X being any amino acid) [71,72]. This glycine-rich loop is involved in binding to the pyrophosphate of dinucleotides. Computational analysis revealed that a water molecule is invariably present in a very conserved position bridging the pyrophosphate to the glycine-rich loop [73]. The cofactor binding domains of NAD(P)H-dependent oxidoreductases typically contain two Rossmann folds, one interacting with the adenine moiety and the other with the nicotinamide ring.

The core αβ can be extended in some cases with additional β-strands to form a larger β-sheet with the two core β-strands. Usually, all β-strands are parallel, although there are some cases where some of the strands are antiparallel [74]. The segments between the additional β-strands are variable, and can consist of additional α-helices, random coil regions or complex combinations of short helices and coiled segments.

3.2. Catalytic domain

The other main motif of NAD(P)H-dependent oxidoreductases is the catalytic domain, which coordinates the substrate and provides the residues essential for the redox reaction to take place. Due to the very large variety of different oxidoreductases and reactions they can catalyze, it is impossible to define common motifs for all of them (other than the frequent presence of other redox cofactors such as FAD, FMN or metallic centres). Thus, here we focus on the catalytic domains of oxidoreductases and how they are connected to the nucleotide binding domain.

The catalytic domains of oxidoreductases are more variable than the coenzyme binding domains. For example, NAD(P)H-dependent oxidoreductases of the D-stereoisomers of 2-hydroxyacids (such as D-glycero dehydrogenase or phosphoglycerate dehydrogenase) have a catalytic domain similar to their well-defined cofactor binding domain, which belongs to the family of Rossmann folds [75]. On the other hand, short-chain alcohol dehydrogenases/reductases do not have a distinct, separate catalytic domain to bind the substrate. Instead, they display a single domain consisting of a Rossmann-fold scaffold with a highly variable C-terminal extension, which acts as the substrate binding site [76].
The catalytic domains of enzymes containing Rossmann folds (most of which are oxidoreductases), can belong to seven different superfamilies of the Structural Classification of Proteins (SCOP) [77]. The different catalytic domains determine the substrate specificity and the exact catalytic mechanism employed by the enzyme. NAD(P)H-dependent oxidoreductases also differ in the way that the cofactor-binding domain and the catalytic domain are connected. Four different types of connections have been found. The Rossmann domain can be connected to the N terminus of the catalytic domain or to its C terminus; the connections have been found. The Rossmann domain can be connected in the same way to the Rossmann domain. Interestingly within each of the seven SCOP superfamilies containing Rossmann folds, the catalytic domains of each superfamily are always connected in the same way to the Rossmann domain.

4. Classes of NAD(P)H-dependent oxidoreductases and reaction mechanisms

In the following section, we aim to give an overview of the different types of reactions catalyzed by NAD(P)H-dependent oxidoreductases, classified according to the EC system. NAD(P)H-dependent oxidoreductases catalyze a very broad range of redox reactions (Table 2), and the specific catalytic mechanism differs, even within each EC class. Table 3 shows a simplified summary of the different possible paths electrons can follow during the reaction catalyzed by an NAD(P)H-dependent oxidoreductase. Although electron transfer can happen directly between the nicotinamide cofactor and the substrate, there are also many cases where one or more intermediary redox cofactors are involved. It should be noted that while enzymes can potentially catalyze redox reactions in both directions, in practice the direction in which the reaction takes place is determined by thermodynamics and the concentration of substrates, products and cofactors. Thus, the physiological direction of the reaction might not coincide with the one described here in all cases.

4.1. EC 1.1.1: alcohol dehydrogenases

Alcohol dehydrogenases constitute one of the largest and most diverse groups of NAD(P)H-dependent oxidoreductases, and they can be classified into several superfamilies according to different types of criteria. Alcohol dehydrogenases catalyze the oxidation of alcohols to the corresponding aldehydes or ketones, coupled to the reduction of NAD\(^+\) to NAD(P)H, as well as the reverse reaction. Here, we describe the three families that can be distinguished based on the size of the substrate on which they can act, with enzymes of each of the families sharing structural and mechanistic features.

Most members of the alcohol dehydrogenase family of short-chain alcohol dehydrogenases/reductases (SDRs), such as dihydropyridine dehydrogenase or 17β-hydroxysteroid dehydrogenase [78], have a key catalytic Tyr residue whose hydroxyl group donates or accepts a proton from the substrate. An adjacent Lys residue, and the nicotinamide ring of NAD(P)\(^+\), lower the pKa of the hydroxyl group of the Tyr residue to facilitate its role as a proton acceptor/donor (Fig. 5). As the Tyr residue abstracts a proton from the substrate, a hydride is transferred from the substrate to the oxidized cofactor. However, in certain SDRs able to catalyze the reduction of enoyl-thioesters, the Tyr residue is present at the active site but does not act as a proton/donor acceptor, or is not present at all (such as in human peroxisomal enoyl-CoA reductase). Instead, protons are transferred directly to or from the solvent [76].

Medium-chain alcohol dehydrogenases (MDRs) need Zn as a cofactor, and they use either a Tyr-based catalytic mechanism similar to SDRs, or alternatively a Zn-based mechanism. In the latter, the Zn\(^2+\) ion is coordinated, in the absence of the substrate, by a water molecule and three residues (two Cys and one His) adopting a tetrahedral geometry [79]. After the alcohol substrate binds to the enzyme, it displaces the water molecule from the Zn coordination shell (Fig. 6) and the alcohol substrate transfers a proton to the solvent, forming an alkoxide intermediate which is stabilized by the catalytic Zn\(^2+\) ion. The alkoxide ion transfers a hydride to the oxidized cofactor and collapses to an aldehyde/ketone [79].

Finally, long-chain alcohol dehydrogenases (LDRs, such as the mannitol 2-dehydrogenase of Pseudomonas fluorescens), constitute a heterogeneous group that differ in the catalytic mechanism and additional cofactors needed (Zn, Fe, both, or no metal at all) [80,81]. Many employ a third catalytic mechanism different to those employed by SDR and MDR members, in which a Lys residue accepts a proton from the substrate, and then donates it to a solvent molecule. The capacity of the Lys residue to act as a general base is enhanced due to a hydrogen bond facilitated its role as a proton acceptor/donor (Fig. 5). As the Tyr residue abstracts a proton from the substrate, a hydride is transferred from the substrate to the oxidized cofactor [82].

Iron-containing alcohol dehydrogenases have been less extensively studied than their Zn-containing counterparts [83]. These enzymes contain a divalent Fe\(^3+\) ion which is typically coordinated by one Asp and three His residues arranged in a tetrahedral geometry. The catalytic mechanism of Fe-dependent alcohol dehydrogenases is based on a reduction of the pKa of the hydroxyl group of the substrate upon binding.
Table 2
Types of reactions catalyzed by NAD(P)H-dependent oxidoreductases. Data was extracted from BRENDA and the recommendations of the Nomenclature Committee of the IUBMB [REF BRENDA + REF EC].

| EC Systematic name | Reaction |
|--------------------|----------|
| 1.1.1 Alcohol dehydrogenases | \( \text{R}_1 \text{R}_2 + \text{NAD(P)H} \leftrightarrow \text{R}_1 \text{R}_2 + \text{NAD(P)}^+ \) |
| 1.2.1 Aldehyde dehydrogenases | \( \text{R}_1 \text{R}_2 + \text{NAD(P)H} \leftrightarrow \text{R}_1 \text{R}_2 + \text{NAD(P)}^+ \) |
| 1.3.1 Ene reductases | \( \text{R}_1 \text{R}_2 \leftrightarrow \text{R}_1 \text{R}_2 + \text{NAD(P)}^+ \) |
| 1.4.1 Amino acid dehydrogenases | \( \text{R}_1 \text{R}_2 + \text{NAD(P)H} + \text{NH}_3 \leftrightarrow \text{R}_1 \text{R}_2 + \text{NAD(P)}^+ \) |
| 1.5.1 Oxidoreductases of CH-NH group | \( \text{R}_1 \text{R}_2 + \text{NAD(P)H} \leftrightarrow \text{R}_1 \text{R}_2 + \text{NAD(P)}^+ \) |
| 1.6.1 NAD(P)+ transhydrogenases | \( \text{NAD(P)H} + \text{NAD(P)}^+ \leftrightarrow \text{NAD(P)H} + \text{NAD(P)}^+ \) |
| 1.7.1 Oxidoreductases acting on nitrogenous compounds | \( \text{Nitrile} + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{Nitrite} + \text{NAD(P)H} + \text{H}_2\text{O} \) |
| 1.8.1 Oxidoreductases acting on sulfur | \( \text{R}_1 \text{S} \leftrightarrow \text{R}_1 \text{SH} + \text{H}_2\text{S} \) |
| 1.10.1 Oxidoreductases of diphenols and related substances donors | \( \text{O} + 2 \text{NAD(P)H} \leftrightarrow \text{O} + 2 \text{NAD(P)H} \) |
| 1.11.1 Peroxidases | \( \text{H}_2\text{O}_2 + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{NAD(P)}^+ + 2\text{H}_2\text{O} \) |
| 1.12.1 Hydrogenases | \( \text{H}^+ + \text{NAD(P)H} \leftrightarrow \text{H}_2 + \text{NAD(P)}^+ \) |
| 1.13 and 1.14 Oxygenases and monoxygenases | \( \text{RH} + \text{O}_2 + \text{NAD(P)H-hemoprotein reductase} \leftrightarrow \text{ROH} + \text{H}_2\text{O} + \text{NAD(P)}^+ \) |
| 1.16.1 Oxidoreductases of metal ions | \( \text{M}^+ + \text{NAD(P)H} \leftrightarrow \text{M} + \text{NAD(P)}^+ \) |
| 1.17.1 Oxidoreductases on CH or CH2 groups | \( \text{R}_1 \text{C} + \text{NAD(P)H} \leftrightarrow \text{R}_1 \text{C} + \text{NAD(P)}^+ \) |
| 1.18.1 Oxidoreductases of iron-sulfur proteins donor | \( \text{Oxidized FeS protein} + \text{NAD(P)H} \leftrightarrow \text{Reduced FeS protein} + \text{NAD(P)}^+ \) |
| 1.19 Flavodoxin | \( \text{Oxidized flavodoxin} + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{Reduced flavodoxin} + \text{NAD(P)}^+ \) |
| 1.20.1 Oxidoreductases of phosphorous or arsenic compounds | \( \text{O} + \text{NAD(P)H} \leftrightarrow \text{O} + \text{NAD(P)}^+ \) |
| 1.21.1 Reductive dehalogenases | \( \text{I} + \text{NAD(P)H} \leftrightarrow \text{I} + \text{NAD(P)}^+ + \text{NAD(P)}^+ \) |
| 1.23.1 Oxidoreductases reducing C-O-C group | \( \text{Pinoresinol} + \text{NADPH} + \text{H}^+ \leftrightarrow \text{Lariciresinol} + \text{NAD(P)}^+ \) |
| | \( \text{Lariciresinol} + \text{NADPH} + \text{H}^+ \leftrightarrow \text{Secoisolariciresinol} + \text{NAD(P)}^+ \) |
to the enzyme due to interaction with the F2+ ion, which facilitates the transfer of a hydride group to NAD+ [84,85]. Fe-dependent alcohol dehydrogenases have been reported to be sensitive to oxygen inactivation, unlike Zn-dependent enzymes, due to Fe-catalyzed oxidation reactions that can take place in the presence of oxygen [86,87].

### Table 3
Possible paths of electrons during electron transfer in reactions catalyzed by NAD(P)H-dependent oxidoreductases. Many NAD(P)H-dependent oxidoreductases, including alcohol dehydrogenases, transfer electrons directly between the substrate and the nicotinamide cofactor. However, many others require additional intermediate cofactors, most commonly flavins and metallic centres.

| Electron transfer path                      | EC number |
|--------------------------------------------|-----------|
| Substrate $\rightarrow$ [e$^-$] NAD(P)H/NADP$^+$ | 1.1.1     |
| Modified substrate $\rightarrow$ [e$^-$] NAD(P)H/NADP$^+$ | 1.2.1     |
| Substrate $\rightarrow$ [e$^-$] Flavin $\rightarrow$ [e$^-$] NAD(P)H/NADP$^+$ | 1.3.1, 1.11.1 |
| Substrate $\rightarrow$ [e$^-$] Metallic redox cofactor $\rightarrow$ [e$^-$] NAD(P)H/NADP$^+$ | 1.7.1, 1.8.1 |
| Substrate $\rightarrow$ [e$^-$] Metallic redox cofactor $\rightarrow$ [e$^-$] Flavin $\rightarrow$ [e$^-$] NAD(P)H/NADP$^+$ | 1.17.1 |
| Substrate $\rightarrow$ [e$^-$] Side chain of amino acids $\rightarrow$ [e$^-$] NAD(P)H/NADP$^+$ | 1.18.1, 1.19.1 |

#### 4.2. EC 1.2.1: aldehyde dehydrogenases

Aldehyde dehydrogenases catalyze the oxidation of aldehydes to carboxylic acids, or in some cases their CoA esters or acyl carrier protein (ACP) thioesters, coupled to the reduction of NAD(P)$^+$ to NAD(P)
H, as well as the reverse reaction. Enzymes of this class are sometimes referred to by other names, such as carboxylic acid reductases (CAR), depending upon their substrate specificity and/or the physiological direction of the reaction.

The key catalytic residue of aldehyde dehydrogenases is a Cys residue from whose sulfhydryl group a proton is abstracted by a Glu residue, or by a His residue in the exceptional case of *Vibrio harveyi* aldehyde dehydrogenase (Fig. 8) [88]. The thiolate can act as a nucleophile and attack the carbon of the carbonyl group. This nucleophilic attack causes the formation of a thiohemiacetal intermediate. The thiohemiacetal contains a negatively charged oxygen atom which is stabilized, at least in part, by an Asn residue which is also essential for catalysis. The thiohemiacetal oxyanion then spontaneously donates a hydride group to NAD(P)⁺. In this process, a thioester bond is formed between the aldehyde and the catalytic Cys. The thioester bond is hydrolysed by a water molecule (which is activated by a Glu residue to carry out hydrolysis), allowing the carboxylic acid to be released [89,90].

Some aldehyde dehydrogenases such as methylmalonate-semialdehyde dehydrogenase (MMSDH) yield CoA esters instead of free carboxylic acids. These CoA-dependent aldehyde dehydrogenases are noteworthy due to their important role in natural and engineered fermentation pathways which resemble the reverse of beta-oxidation of fatty acids. The *E. coli* ethanol pathway [91] and the *Clostridium* butanol pathway [92] are the best-studied natural examples, but longer-chain alcohols can also be formed in this way [93], and have been the subject of recent metabolic engineering studies [94,95]. The CoA-dependent aldehyde dehydrogenases can occur independently [96] or as part of bifunctional aldehyde-alcohol dehydrogenases (ADHE) which are present in many bacteria [91,92]. Bifunctional aldehyde-alcohol
dehydrogenases are able to catalyze the sequential reduction of a CoA ester to the aldehyde and then to the alcohol, most commonly acetyl-CoA to acetaldehyde and then to ethanol. They typically comprise two main domains, an N-terminal aldehyde dehydrogenase domain and an iron-dependent alcohol dehydrogenase C-terminal domain. The two domains are connected by a small linker [97,98]. It is unclear whether CoA-dependent aldehyde dehydrogenases gained CoA ester formation activity during evolution, or if this feature was originally present in primitive aldehyde dehydrogenases but it was later lost in most of them [99].

Carboxylic acid reductases are able to reduce fatty acids (carboxylic acids with a long aliphatic chain) to the corresponding aldehydes are of special interest due to their ability to generate alcohols and alkanes from the carboxylic acids naturally found in oils and fats. An NADPH-dependent oxidoreductase able to catalyze the reduction of a wide range of fatty acids (with an aliphatic chain containing between 6 and 18C atoms) has been identified in Mycobacterium marinum, with the peculiarity over other aldehyde dehydrogenases that the reaction requires ATP in addition to NADPH [100]. An E. coli strain able to convert fatty acids into long-chain alcohols and alkanes (which have numerous applications as fuels, detergents, food additives and many others) was engineered by introducing the gene encoding the CAR from M. marinum along with an aldehyde reductase (a synonym for alcohol dehydrogenase) to form alcohols and an aldehyde decarbonylase, a recently-discovered enzyme able to catalyze the conversion of a long-chain aldehyde to an alkane and formate [100].

Another type of aldehyde dehydrogenase, known as fatty acyl-CoA/ACP reductase, has also been investigated for the ability to produce long-chain aldehydes and alcohols. These enzymes are able to form long-chain aldehydes by reduction of fatty acids bound to acyl carrier protein (ACP) and/or to CoA, the latter case being similar to the CoA-dependent aldehyde dehydrogenases described above, but acting on long-chain substrates. Some of the enzymes of this group prefer acyl-ACP as the substrate, while others act more efficiently on acyl-ACP substrates, so will generate products from CoA-dependent fatty acid beta-oxidation or ACP-dependent fatty acid synthesis pathways, respectively, both of which are being investigated through metabolic engineering [101,102]. Interestingly, some of them are able to reduce fatty acid acyl-CoA/ACP directly to long-chain alcohols, requiring oxidation of two molecules of NAD(P)H, instead of one [103].

4.3. EC 1.3.1: ene reductases

Ene reductases catalyze the hydrogenation of activated alkenes to the corresponding alkanes. Only activated alkenes are substrates for these enzymes. For example, one of the carbons participating in the double bond should have an electron-withdrawing group (EWG) as a substituent (a functional group able to remove electron density from the double bond, making it more electrophilic). The carbon with the EWG is referred to as α, while the other carbon atom is called β. The reaction starts with the transfer of a hydride group from a reduced flavin co-factor to the β, followed by the addition of a proton from a nearby Tyr residue to α. The proton donated by the Tyr residue is replenished by a water molecule. Finally, a reduced NAD(P)H molecule reduces the oxidized flavin cofactor [104].

One of the largest groups of ene reductases are the members of the long-known family of Old Yellow Enzymes (OYE). OYE was first identified in the 1930s as a yellow enzyme able to form a complete respiratory system together with glucose-6-phosphate dehydrogenase acting on glucose-6-phosphate and using molecular oxygen as the final electron acceptor [105]. Since then, variants have been identified in yeasts, plants and bacteria [106]. When it was purified by Theorell in 1935, it was shown to contain a protein, colourless component and a yellow component essential for activity [107]. Theorell later demonstrated that the yellow component was riboflavin 5′-phosphate, also known as flavin mononucleotide (FMN) [108]. Extensive biochemical and structural studies have been carried out with OYES, which have demonstrated their ability to reduce a large variety of unsaturated compounds to their saturated counterparts. However, their physiological function has not been completely established, although proposals suggesting a conserved role in the detoxification of electrophilic compounds have been made [106].

Many of the reactions catalyzed by OYES are interesting from the perspective of an industrial application. For example, OYE can reduce nitroalkenes to nitroalkanes in a process involving the formation of a nitronate intermediate. OYE1 from Saccharomyces carlsbergensis with Y196F mutation has been shown to cause the accumulation of the nitronate intermediate, which could be then chemically alkylated to generate nitroalkanes with two chiral centers [106]. Another potential application is to use them to easily carry out small modifications in the structure of drugs to alter their pharmaceutical properties, such as morphine alkaloids. Coexpressing a morphinone reductase (an OYE)
with a morphine dehydrogenase in E. coli results in a strain able to efficiently convert morphine and codeine to hydromorphone and hydromorphone, which are effective analgesics [109].

4.4. EC 1.4.1: amino acid dehydrogenases

The general reaction catalyzed by amino acid dehydrogenases is the transfer of hydride from the Cα atom of an amino acid to NAD(P)⁺. As a consequence, the corresponding α-keto acid and ammonium are generated. Two amino acid dehydrogenases whose catalytic mechanism has been well studied are glutamate dehydrogenase and phenylalanine dehydrogenase [110–112]. In both cases, the catalytic mechanism is very similar. Initially, an Asp residue acts a general base and abstracts a proton from the α-amino group of the amino acid. Then, a hydride ion is transferred to NAD(P)⁺ from the Cα, forming an intermediate α-imino acid [111,113]. Finally, the intermediate imino acid is hydrolyzed to the corresponding α-keto acid and ammonium [111].

Amino acid dehydrogenases play a key role in the catabolism of amino acids, since they allow the removal of their amino group to take place, which is a necessary step for their incorporation into other catabolic pathways, such as the tricarboxylic acid cycle (TCA cycle). In the deamination process, an ATP molecule is generated (which can be used for energy-requiring processes), and the amino group is released as free ammonia, which can then be employed by the cell for different purposes. Additionally, some organisms use the inverse reaction catalyzed by amino acid dehydrogenases to incorporate free ammonia into carbon skeletons when the levels of free ammonia are high [20].

4.5. EC 1.5.1: oxidoreductases of CH–NH groups

This family of oxidoreductases comprises a group of enzymatic activities acting on a rather large variety of substrates, with the only common feature being that all of them contain a single or double C–N bond which donates electrons to reduce NAD(P)⁺.

Dihydropteridine reductase (EC 1.5.1.34), a key enzyme for the synthesis of folate, catalyzes the reduction of a quinonoid 6,7-dihydropteridine to the corresponding 5,6,7,8-tetrahydropteridine. NAD(P)H provides the required electrons, which are accepted by the pyrazine heteroaromatic ring. The catalytic mechanism is very similar to that of the SDR subfamily of alcohol dehydrogenases, with a Tyr residue directly participating in proton transfer from or to the substrate and a Lys residue facilitating the role of the Tyr residue by lowering the pKa of the hydroxyl group of the Tyr residue [21].

Dihydrofolate reductase (EC 1.5.1.3), which is also essential for the synthesis of folate, is another example of NAD(P)H-dependent oxidoreductase acting on CH-NH groups. However, in this case an Asp residue plays the central role in catalysis (Fig. 9). The Asp residue forms a hydrogen bond with the N3 atom of the pterin ring of the substrate and a water molecule which establishes another hydrogen bond with the O5 atom. The pKa of the N5 atom of the pterin ring is thus substantially elevated, making it able to accept a proton directly from a water molecule. Finally, after the proton has been accepted by the substrate, hydride transfer from the reduced cofactor takes place [114,115].

Dihydrofolate reductase is particularly interesting, as it is the target of different types of drugs. For example, methotrexate is an inhibitor of dihydrofolate reductase (DHFR) used as a chemotherapy agent for several types of cancer. The use of a DHFR inhibitor takes advantage of the fact that rapidly dividing cells have a very high demand for folate since it is necessary to synthesize thymine. Some antibacterial agents aim to specifically inhibit bacterial DHFR, such as trimethoprim. Trimethoprim is used in the standard therapy against the opportunistic pathogen *Pneumocystis jirovecii*. In a recent study, six variants with a single amino acid substitution of *Pneumocystis jirovecii* DHFR were found to confer resistance against trimethoprim. An experimental drug also targeting DHFR but with a higher degree of conformational flexibility than trimethoprim (known as OAAG324) was tested against these variants, and found to be effective with one of them [116].

4.6. EC 1.6.1: NAD(P)⁺ transhydrogenases

Pyridine nucleotide transhydrogenases catalyze the reversible transfer of hydride between NADH/NAD⁺ and NADPH/NADP⁺ pools. In most cases, the reaction is as follows: $NADPH + NAD⁺ \rightleftharpoons NADP⁺ + NADH$. The hydrogen atom is transferred from the C4 carbon atom of the reduced cofactor to the C4 carbon atom of the oxidized cofactor. Two groups of transhydrogenases can be distinguished: energy-linked and non-energy-linked transhydrogenases.

Energy-linked transhydrogenases are integral membrane proteins which do not require any flavin cofactor. They receive this name because they couple the transhydrogenation reaction to proton translocation across the membrane where they are located. They are found in mitochondria, some heterotrophic bacteria and photosynthesizing...
bacteria [117]. Thanks to the proton gradient generated across both the mitochondrial inner membrane and the cell membrane of bacteria, the apparent equilibrium constant of the system is changed, causing a shift towards the production of NADPH and NAD⁺ [118]. In eukaryotes, energy-linked transhydrogenase has been suggested to act as a redox buffer able to counteract both an excessive depletion of NADPH and a dissipation of the proton gradient. In prokaryotes, the physiological role is believed to be mostly the generation of NADPH for anabolism [4]. These transhydrogenases are also called Re/Si-specific transhydrogenases or AB transhydrogenases, as they transfer hydrogen 4A of the nicotinamide ring (situated on its Re face) from NADH to NADP⁺, or hydrogen 4B (situated on the Si face) from NADPH to NAD⁺.

Non energy-linked transhydrogenases are soluble flavoproteins which contain FAD. They are less widespread than energy-linked transhydrogenases and only found in some heterotrophic bacteria. Usually, only one of the two types of transhydrogenases is found in a specific organism, with the exception of Enterobacteriaceae (including Escherichia coli), which possess one energy-linked and one non energy-linked transhydrogenase encoded by the pntAB and udhA genes respectively. Non energy-linked transhydrogenases are also known as Si/Re-specific transhydrogenases or BB transhydrogenases, since they transfer hydrogen 4B of the nicotinamide ring, both from NADH to NADP⁺ and from NADPH to NAD⁺. Contrary to energy-linked transhydrogenases, it has been suggested that the physiological role of non-energy-linked transhydrogenases is to convert NADPH to NADH [4]. However, this seems to be dependent on growth conditions and organism, with some reports indicating that non energy-linked transhydrogenases can actually increase NADPH availability when the demand for NADPH is increased [119]. Interestingly, it has been found that expressing a non-energy-linked transhydrogenase in Synechococcus results in a reduced growth rate [120], perhaps reflecting the importance of the highly reduced state of the NADPH pool normally maintained in cyanobacteria.

Both types of transhydrogenases have been explored as metabolic engineering targets to improve the yield of products whose synthesis depends on cofactor availability. Results have been mixed, with some studies showing that overexpressing pntAB is sufficient to enhance NADPH regeneration [121] while others show that the overexpression of pntAB does not increase NADPH availability and can even decrease it [122]. In the latter study, it was found that overexpressing udhA increased the levels of available NADPH.

4.7. EC 1.7.1: oxidoreductases acting on other nitrogenous compounds (other than CH–NH groups)

This group includes dehydrogenases acting on a variety of different compounds containing nitrogen and which cannot be classified into any of the other categories of oxidoreductases. Substrates include both inorganic compounds (such as nitrate and nitrite) and organic compounds (such as azobenzene and nitroquinoline). The exact reaction mechanisms of many of these enzymes are still poorly understood, partly due to the lack of structural information for many of them, although for an example see [123]. However, in most of the cases NAD(P)H does not directly transfer electrons to the substrate, but instead provides the electrons to regenerate the reduced form of the other cofactor which directly participates in the reduction of the substrate.

NAD(P)H-dependent nitrate reductases (EC 1.7.1.1–3) are relatively well characterized, and they catalyze the reduction of nitrate to nitrite, with NAD(P)H providing the source of electrons for the reduction of nitrate. There are four different types of nitrate reductases (NRs): euukaryotic assimilatory NR, cytoplasmic bacterial assimilatory NR, membrane-bound bacterial respiratory NR and periplasmic bacterial dissipatory NR [124]. Only assimilatory nitrate reductases, which carry out the incorporation of nitrogen into organic molecules for growth, are directly NAD(P)H-dependent (nitrate and periplasmic nitrate reductases). All NRs contain a molybdenum cofactor (Mo-Co) with a molybdenum atom bound to a set of ligands which varies across the different NRs. Eukaryotic nitrate reductases contain molybdopterin, while in prokaryotic enzymes the Mo-Co is molybdopterine-guanine dinucleotide.

Eukaryotic NRs have been extensively studied, and their reaction mechanism is well known due to the available structural information. In eukaryotic NRs, Mo is initially in its Mo⁴⁺ state. When nitrate binds to the active site, it forms the reaction intermediate by coordinating the Mo atom with one of its O atoms, displacing a water molecule which was previously bound to Mo. The formation of the reaction intermediate causes Mo electrons to shift towards the created Mo–O bond. As a consequence, Mo is oxidized to Mo⁵⁺ and the bond between Mo and the coordinating O atom from nitrate becomes a double bond. This causes the bond between this O atom and nitrate to break, forming nitrite which is subsequently released. Finally, transfer of two electrons and two protons from NAD(P)H via a cytochrome b₅ domain cause regeneration of Mo⁴⁺ and reduction of the O atom bound by a double bond to a water ligand [125].

4.8. EC 1.8.1: oxidoreductases acting on a sulfur group

This group also consists of a diverse set of oxidoreductases whose only common characteristic is that they act on a sulfur-containing group of their substrate, which can be of very different types. These include both inorganic forms of sulfur (such as sulfite and sulfide), and a variety of sulfur-containing organic molecules (including cysteine, glutathione, coenzyme A and many others). As in the case of enzymes belonging to EC 1.7.1, the role of NAD(P)H is to provide electrons to regenerate the other cofactors directly involved in the catalysis.

NADPH-dependent sulfite reductase (EC 1.8.1.2), such as E. coli sulfite reductase, catalyzes the reduction of sulfite to sulfide by transferring electrons provided by NADPH, one of the key steps of the assimilation of inorganic sulfur into organic molecules. This enzyme shares structural and mechanistic features with nitrite reductase, including the presence of a sirohaem cofactor and a [4Fe-4S] cluster. Additionally, both of them catalyze the transfer of six electrons (which leads to the formation of ammonia in the case of nitrite reductase). In fact, purified sulfite reductase is also able to catalyze the reduction of nitrite to ammonia [126,127]. The reaction mechanism involves three successive transfers of two electrons, provided by NADPH, and protons to the oxygen atoms of sulfite, which binds as an axial ligand of the sirohaem Fe atom [29].

4.9. EC 1.10.1: dehydrogenases of diphenols

EC class 1.10 includes all oxidoreductases able to act on diphenols and related products. There are five subtypes this EC class, which vary in the redox partner they use to transfer the electrons from the diphenol substrate: NAD(P)⁺, a cytochrome, oxygen, a quinone, or a copper protein. Those using oxygen as the electron acceptor comprise by far the highest diversity of enzymatic activities, while there is only one known enzymatic activity of such type dependent on NAD(P)⁺: trans-acentaphene-1,2-diol dehydrogenase (EC 1.10.1.1), which oxidizes said substrate to acenaphthenequinone. This NAD(P)H-dependent enzyme was identified and purified in 1973 from rat (and other mammalian) liver extracts [32]. However, the structure of the enzyme has not been solved, and no information about the reaction mechanism is available.

4.10. EC 1.11.1: peroxidases

Peroxidases catalyze the transfer of electrons from an electron donor to H₂O₂. Both NADH-dependent and NADPH-dependent peroxidases have been found (EC 1.11.1.1 and EC 1.11.1.2 respectively). The reaction they catalyze is NAD(P)⁺ + H⁺ + H₂O₂ = NAD(P)⁺ + 2 H₂O. NADH peroxidase has been well characterized, and its structure and
mechanism long known. In addition to NADH, the catalytic centre of the enzyme includes FAD, a Cys residue and a His residue (Fig. 10). The Cys residue is adjacent to the FAD cofactor. As a first step, NADH binds to the enzyme with FAD in its oxidized state. The binding of NADH causes the electron density of the isoxaloxazine group of FAD to shift towards the Cys residue. Then, H$_2$O$_2$ binds to the enzyme forming hydroperoxy derivatives of fatty acids with the Cys and His residues, and other amino acids close to the active site. The Cys residue performs a nucleophilic attack on H$_2$O$_2$, cleaving the O=O bond to yield an OH$^-$ anion and a sulfenic acid derivative. The hydroxide anion abstracts a proton from the His residue, and is released as a water molecule. The sulfenic acid derivative is reduced by the transfer of two electrons and a proton from NADH through FAD, leading to the formation of a second water molecule which is released together with NAD$^+$. Finally, the His residue receives a proton from the solvent, to regenerate its initial protonated state [33].

Another class of NAD(P)H-dependent peroxidases catalyzes the reduction of hydroperoxy derivatives of fatty acids to the corresponding hydroxy acids (EC 1.11.1.22). The enzyme was originally found in Synechocystis PCC 6803 [128], and uses electrons from NADPH to reduce peroxidized lipids to hydroxy acids. These enzymes share sequence similarity to glutathione peroxidases, but no catalytic activity can be detected when reduced glutathione is provided as the electron donor. One of the major differences with glutathione peroxidases is the absence of a selenocysteine residue, which is replaced by a standard Cys residue. This type of NADPH-dependent peroxidases has been hypothesized to serve as a defense system against hydroperoxide derivatives of unsaturated fatty acids, which can lead to oxidative damage [128].

**4.11. EC 1.12.1: oxido-reductases of H$_2$ (hydrogenases)**

Hydrogenases catalyze the oxidation of molecular hydrogen or reduction of protons and are classified into three main phylogenetically distinct classes, based on the metals found at their active site: [NiFe]-hydrogenases, [FeFe]-hydrogenases, and the lesser-studied [Fe-only]-hydrogenases. In all cases, the catalytic mechanism of hydrogen oxidation or proton reduction requires electron transfer to and from the active-site metals, which are directly responsible for the oxidation of hydrogen or reduction of protons. The electrons can be transferred to or from a variety of donors/acceptors, which can include NAD(P)/H/NAD (P)$^+$. In all cases, the transfer of electrons occurs via an iron sulfur (Fe-S) cluster relay.

The active site of [NiFe]-hydrogenases is ligated by four Cys residues. Two of the Cys are bridging ligands of both metal ions, while the other two are bound to the Ni atom alone [129]. The Fe atom binds three diatomic ligands [130], two CN$^-$ ligands and one CO ligand [131] (Fig. 11). The bridging ligand between the metal ions is either a hydroxo or a hydro-peroxide depending on the redox state of the active site [132].

The catalytic core of [FeFe]-hydrogenases consists of four domains [133], which help with electron transfer to and from the active site and cofactors. The active site of [FeFe]-hydrogenases consists of a 4Fe-4S cluster ligated by four Cys, which is coordinated via a single conserved Cys thiolate sulfur atom to the two Fe core. Each of those two iron ions is attached to a CO and CN$^-$ ligand and a bridging CO ligand [134].

A small number of NAD(P)H-dependent hydrogenases have been described. All of the NAD(P)H-dependent hydrogenases are multimeric enzyme complexes [135], including a subunit with a flavin moiety which carries out the oxidation/reduction reaction of the nicotinamide cofactor. The best studied member is the bidirectional soluble [NiFe]-hydrogenase of Cupriavidus necator H16 [136], which oxidizes or produces H$_2$ in response to changes in cytoplasmic reduction states.

**4.12. EC 1.13 and 1.14: oxygenases and monooxygenases**

Monooxygenases catalyze the transfer of a single atom of oxygen to a substrate, requiring first that the oxygen molecule be activated, which takes place by transferring electrons to the oxygen molecule. Depending on the type of monooxygenase, electrons can be donated either from the substrate or from a cofactor, which determines the type of reactive oxygen intermediate. Here, we focus on NAD(P)H-dependent monoxygenases.

Cytochrome P450 are haem B monooxygenases (CYPs), which are able to catalyze a wide range of reactions (such as epoxidations, hydroxylations, heteroatom-dealkylations/oxidations, dehalogenations, dehydrations, reductions, dehalogenations and oxidative deaminations). The haem group activates molecular oxygen by transferring electrons to it, which in the case of NAD(P)H-dependent CYPs are ultimately provided by NAD(P)H. The mechanism by which the electrons are transferred to the haem group from the nicotinamide cofactor varies between different CYPs.

The general mechanism of NAD(P)H-dependent CYPs can be divided
into four main steps. Firstly, the substrate binds to the enzyme, and an electron is transferred to the haem group from NAD(P)H via a flavin-containing cytochrome P450 reductase, to reduce Fe$^{3+}$ to Fe$^{2+}$. Secondly, an oxygen molecule binds to the complex, forming a complex known as oxy-P450. Thirdly, the transfer of a second electron and two protons to the oxy-P450 complex causes the cleavage of the O–O bond, leading to the formation of a reactive intermediate and a water molecule. Then, the reactive intermediate inserts the oxygen atom into the substrate. Finally, product dissociation regenerates the initial state of the enzyme. In this state, a molecule of water coordinates the Fe atom of the haem-group as an axial ligand. The other ligands of the Fe atom are the nitrogen atoms of the haem group and a Cys residue present in the active site of all CYPs, which makes it more reactive [137]. The effect of the Cys ligand on reactivity is due to two factors, it lowers the reduction potential of the active site, and it increases the pKa of the oxygen-bound intermediate, which facilitates its protonation, resulting in the cleavage of the O–O bond [138].

In most CYP systems, the cytochrome P450 component and the reductase component, which transfers the electrons to the cytochrome P450 from the electron donor, are separate proteins. However, some simpler systems have been found in which both components are fused in a single polypeptide chain, making heterologous expression and protein engineering more facile. An example of this is the well-characterized BM3 enzyme from Bacillus megaterium, which is a soluble enzyme able to hydroxylate fatty acids of varying length between 12 and 18 carbon atoms, as well as the corresponding amides and alcohols. BM3 also displays a weak hydroxylation activity towards shorter chain alkanes, and has been modified, by means of random mutagenesis, to obtain several variants with enhanced activity towards octane, being up to five times more efficient than the wild type enzyme. One of the variants contained a single point mutation of a Glu residue in the surface of the protein by a His residue. The effect of this mutation was surprising, given the fact that this residue is located relatively far from the haem group [139].

Flavin-dependent monoxygenases catalyze a very wide range of oxidative reactions, and use either FMN or FAD as cofactors. In order to introduce an oxygen atom to the substrate, oxygen first binds to the flavin in its reduced form, to form peroxoflavin or hydroperoxoflavin. Then, the flavin derivative performs a nucleophilic or an electrophilic attack, depending on the protonation state of the flavin derivative, which leads to the oxygenation of the substrate, the production of one water molecule and the formation of the oxidized state of the flavin. The role of the NAD(P)H is to restore the reduced form of the flavin in the catalytic site [37,140].

There are also some NAD(P)H-dependent monoxygenases where the cofactor that activates O$_2$ is not a haem group or a flavin, but other redox cofactors. An example is methane monoxygenase (MMO), which catalyzes the incorporation of an oxygen atom into methane (it is also active with other hydrocarbons), yielding methanol, which is the critical first step in methanotrophic carbon assimilation. In MMO, activation of molecular oxygen is carried out by a metallic cofactor consisting of two Fe ions each coordinated by six ligands (a combination of His and Glu residues and water molecules) [141]. After one oxygen atom is transferred to the substrate, the reduced form of the metallic centre is regeneratted by the transfer of two electrons from NAD(P)H [142]. There are two types of MMO, soluble MMO (sMMO) and membrane-bound particulate MMO (pMMO). Soluble MMO is dependent on NAD(P)H, and contains three subunits: MmoA, MmoB and MmoC. MmoA carries out the oxygenation reaction, while MmoC binds NAD(P)H and provides to MmOA the electrons required for the reaction through MmOB [143]. MMO has been extensively studied thanks to its ability to oxidize a variety of hydrocarbons under mild conditions, and carry out a gas-to-liquid transformation for natural gas (methane to methanol). However, protein engineering studies have been hindered by the fact that no functional MMO has been expressed in E. coli [144].

4.13. EC 1.16.1: oxidoreductases of metal ions

This class of enzymes comprises NAD(P)H-dependent oxidoreductases able to transfer electrons to or from metallic ions. The metallic substrate can be in a free form (as in the case of mercury reductase), chelated by relatively small molecules (for example, ferrichelate reductase or aquacobalamin reductase) or forming part of another protein (such as methionine synthase reductase and transferrin reductase).

In general, when NAD(P)H-dependent oxidoreductases of metal ions act to reduce the metal, they first transfer the electrons from NAD(P)H to FAD, and then the reduced FAD donates the electrons to the metallic ion. This electron transfer path allows the enzyme to transfer the electrons provided by NAD(P)H to the metallic atoms one at a time, as in most cases they require the transfer of one single electron in order to be reduced or oxidized. Mercuric reductase and cyanocobalamin...
reductase are exceptions, as they perform two-electron reduction of Hg²⁺ to Hg⁰ and Co³⁺ to Co⁺, respectively, with FAD as a cofactor. Human methionine synthase reductase, which reduces the Co³⁺ atom of the cobalamin cofactor found in methionine synthase, follows a variation of this path where electrons are first transferred from NADPH to FAD, then to FMN and finally to the metal [145].

Mercuric reductase is an enzyme of particular interest for phytochemical studies, as it could potentially be used to generate plants capable of detoxifying mercurocific compounds. Many soil and enteric bacteria have a detoxification system for organomercuric compounds where mercuric reductase plays a key role. This system allows these organisms to generate volatile mercury (Hg⁰) from ionic mercury (Hg²⁺) by a two-electron reduction, thereby avoiding toxicity associated with the tendency of Hg²⁺ to cause sulphhydryl chelation [146]. The first step of the reaction is the binding of Hg²⁺ to the active site of the enzyme, where it is chelated by the thiolate groups of two deprotonated Cys residues. Then, two electrons are transferred from NADPH to FAD. Finally, the reduced FAD reduces the Cys-Hg²⁺-Cys complex, and the resulting Hg⁰ is released [147] (Fig. 12).

4.14. EC 1.17.1: oxidoreductases acting on CH or CH₂ groups

These oxidoreductases catalyze the oxidation or reduction of CH or CH₂ groups, which in most cases results in the addition or removal of a hydroxy group. The only exception is xanthine dehydrogenase, which catalyzes the addition of an oxo group.

Xanthine dehydrogenase (EC 1.7.1.4) and nicotinate dehydrogenase (EC 1.7.1.5), which catalyze respectively the oxidation of xanthine to urate with NAD⁺ as the electron acceptor and the oxidation of nicotinate to 6-hydroxynicotinate with NADP⁺, employ similar reaction mechanisms. Both contain a molybdenum cofactor (molybdopterin), two Fe-S clusters of the [2Fe-2S] type and FAD [148]. All of these redox centres are positioned linearly, and close enough to allow sequential transfer of electrons through them. After substrate binding, an OH ligand of the Mo is deprotonated by a Glu residue. The resulting Mo=O⁻ performs a nucleophilic attack on the carbon atom to be hydroxylated and a hydride group is transferred to the Mo cofactor, reducing Mo⁶⁺ to Mo⁴⁺. Then, the bond between the Mo cofactor and the substrate is hydrolysed, releasing the free hydroxylated product. Finally, the hydride groups are transferred first to the Fe-S centers, then to FAD and eventually to NAD(P)⁺ to yield NAD(P)H [148] (Fig. 13).

Leucoanthocyanidin reductase (EC 1.17.1.3), which catalyzes the reduction of 2R,3S,4S-flavan-3,4-diol to 2R,3S,4S-flavan-3-ols (for example, leucocyanidin to catechin) coupled to the oxidation of NADPH to NADP⁺, uses a different mechanism. Firstly, a step of dehydration of the substrate takes place. In order to do so, a Lys residue deprotonates the phenolic O7 atom of the substrate. This makes the hydroxyl group of C4 act as a leaving group which takes a proton from the adjacent O5. The O5 atom recovers the proton from a His residue. This generates a quinone methide intermediate, to which a hydride is transferred directly from NADPH to generate catechin and NADP⁺ [44].

4.15. EC 1.18.1 and 1.19.1: oxidoreductases of iron-sulfur proteins and of flavodoxin

This group of oxidoreductases are able to catalyze the reduction and oxidation of the Fe-S clusters of iron-sulfur proteins using NAD(P)H/NAD(P)⁺ as the electron donor/acceptor. The iron-sulfur proteins on which they can act include ferredoxin, adrenodoxin, putidaredoxin and rubredoxin, with the latter usually being classified as an iron-sulfur protein even though it does not contain a canonical Fe-S cluster but instead contains a lone iron ion coordinated by several Cys residues. Iron-sulfur proteins can have very different redox potentials, and can thus participate in a variety of redox reactions. Consequently, the physiological role they play is quite varied, including participating as electron carriers in photosynthesis [149], donating electrons to cytochrome P450 for steroid biosynthesis [150] or providing electrons to carry out hydroxylation reactions during ω-oxidation of fatty acids [151]. All NAD(P)H-dependent oxidoreductases of iron-sulfur proteins require an FAD cofactor, which acts as an intermediary in the transfer of electrons. This is due to the fact that iron-sulfur proteins are one-electron donors/acceptors, while NAD(P)H/NAD(P)⁺ only participate in two-electron transfers. Since FAD can participate both in one-electron transfers and two-electron transfers, it can first receive two electrons from NAD(P)H to be reduced to its hydroquinone fully reduced form, and then transfer them one at a time to the substrate, being oxidized first to a semiquinone form and finally to the fully oxidized quinone form. Structures of these enzymes show FAD and NAD(P)H/NAD(P)⁺ to be close to each other, suggesting direct electron transfer between both redox cofactors (Fig. 14). The enzymes differ on the route followed by electrons from the Fe-S cluster to FAD, with some of them involving several intermediate residue side chains [152]. Additionally,
the NADP⁺-dependent ferredoxin oxidoreductase of some organisms are also able to catalyze redox reactions with flavodoxin (EC 1.19.1.1), another small, FMN-containing protein which participates in several redox reactions and can replace ferredoxin in some of its functions under low iron availability conditions [153]. This is the case, for example, of *E. coli* and *Anabaena* ferredoxin-NAD⁺ reductase [49].

4.16. EC 1.20.1: oxidoreductases of phosphorus or arsenic compounds

Phosphonate dehydrogenase (EC 1.20.1.1), also called phosphite dehydrogenase (PTDH), catalyzes the oxidation of phosphonates or phosphite to phosphates. During the reaction, electrons are transferred to NAD⁺, generating NADH.

The exact mechanism through which PTDH catalyzes the reduction of phosphite remains unclear, but there are some proposed reaction mechanisms based on the high degree of sequence homology between PTDH and 2-D-hydroxyacid dehydrogenases. There are three residues essential for catalysis: a His, a Glu and an Arg (Fig. 15). The His residue has been proposed to act as a general base to abstract a proton from a water molecule, generating a hydroxide ion. The hydroxide ion then carries out a nucleophilic attack on phosphite, and at the same time a hydride leaving group is transferred to NAD⁺. The Glu residue probably helps to orient the His residue for catalysis and modulates its pKₐ to facilitate its role as a general base. Finally, the Arg residue is believed to
be essential for binding the substrate and correctly orienting it. Mutations in any of these residues have been found to compromise catalytic efficiency [154,155].

4.17. EC 1.21.1: reductive dehalogenases

There are two enzymes classified as NAD(P)H-dependent reductive dehalogenases: iodotyrosine deiodinase and 2,4-dichlorobenzoyl-CoA reductase.

Iodotyrosine deiodinase catalyzes the reductive dehalogenation of mono- and diiodotyrosine side-products generated during the biosynthesis of thyroid hormones (the prohormone of triiodothyronine, the most active thyroid hormone). The reaction involves the removal of the iodine substituents from the tyrosine derivative and their reduction to iodide ions, coupled to the oxidation of NADPH to NADP⁺. This process allows the scavenging of iodide, which can then be used for the synthesis of more thyroid hormones or other processes. Defects in this enzyme can lead to iodide deficiency, which can have several detrimental effects to health such as hypothyroidism. The mechanism employed by iodotyrosine deiodinase has not still been fully established, although recent structures of the enzyme bound to its substrate have provided some insight [51,156]. The enzyme contains an FMN cofactor which plays a key role in the catalytic process by carrying out a step-wise reduction of the substrate by means of sequential one-electron transfers and not through a single two-electron transfer as previously proposed [51,157,158].

2,4-Dichlorobenzoyl-CoA reductase was identified in Corynebacterium sepedonicum, an organism able to grow with 2,4-dichlorobenzoate as the sole source of energy and carbon, which could be of potential interest for the remediation of sites contaminated by polychlorinated biphenyl [159]. In order to catabolise 2,4-dichlorobenzoate, it is first converted to the corresponding CoA thioester, 2,4-dichlorobenzoyl-CoA. The thioester is then subject to a reductive dehalogenation catalyzed by 2,4-dichlorobenzoyl-CoA reductase, where NADPH provides the electrons. This reaction leads to the production of 4-chlorobenzoyl-CoA, which undergoes an additional dehalogenation (independent of NAD(P)H) before the thioester bond is hydrolyzed. Subsequent hydroxylations and oxygenations complete the catabolic pathway. The reaction mechanism of 2,4-dichlorobenzoyl-CoA reductase has been speculated to involve a nucleophilic attack of the C2 position of the substrate by a hydride group, with the Cl atom acting as a leaving group. However, this has not been confirmed. It is also unclear whether the hydride group would be transferred directly from NADPH or from an intermediate species [159].

4.18. EC 1.23.1: oxidoreductases reducing C–O–C group

Pinoresinol-lariciresinol reductase (EC 1.23.1.1–4) is a multifunctional enzyme found in certain plants (such as Forsythia and Thuja) able to catalyze the reduction of pinoresinol to lariciresinol and of lariciresinol to secoisolariciresinol, with NADPH providing the required electrons. This enzyme is unique in its ability to directly reduce an ether group to an alcohol. The crystal structure of the enzyme from Thuja plicata has been determined, and shows that the NADPH and substrate binding pockets are close enough for direct electron transfer to occur. The exact mechanism and reaction intermediates through which catalysis proceeds are still unknown due to the lack of published structures of enzymes with substrate bound [53].

5. Metabolic and protein engineering of NAD(P)H-dependent oxidoreductases

5.1. Metabolic engineering

There have been numerous metabolic engineering studies in which the metabolic network of an organism has been altered by the addition of NAD(P)H-dependent oxidoreductase(s), or pathways including them [100,160–164]. One of the most frequent goals of this type of approach is the efficient production of a given chemical of interest without the need for chemical synthesis or purifying enzymes, and ideally from renewable feedstocks and with a lower energy consumption due to the mild conditions at which reactions can be catalyzed by NAD(P)H-dependent oxidoreductases. However, metabolic engineering provides a useful tool not only for this purpose, but also others such as improved biodegradation [165,166]. This is usually achieved by genetically manipulating a candidate organism to add, modify (commonly by over-expression) and/or remove the genes coding for one or more enzymes.

An example of successful metabolic engineering leading to the development of a synthesis process suitable for commercial application was the construction of an E. coli strain able to produce 1,3-propanediol (a precursor for the generation of several polymers, including fibers) at high titer when growing aerobically and using only glucose as the
engineered an NADH-dependent methylglyoxal reductase from *Saccharomyces cerevisiae* (GRE2) to have enhanced aldehyde reductase activity towards furfural and 5-hydroxymethylfurfural [170]. From a library of variants generated by means of error-prone PCR, two variants with the enhanced desired activity were identified. Interestingly, one of these variants also acquired the ability to use NADPH as a cofactor in addition to NADH. In another study, Li et al. aimed to improve the 3-hydroxypropionaldehyde (3-HPA) reductase activity of a 1,3-propanediol oxidoreductase (YqhD) with the goal of obtaining an efficient enzyme for the biological production of 1,3-propanediol [167]. After screening a library of mutants generated through error-prone PCR, two variants with improved kinetic properties were isolated. They displayed both a lower $K_m$ and a higher $k_{cat}$ towards 3-HPA.

There have also been multiple attempts to modify the specificity of NAD(P)H-dependent oxidoreductases for the cofactor they use for the electron transfer reaction, as controlling the cofactor specificity of this class of enzymes can be used to optimally engineer the cellular metabolism by achieving a better balance of cofactor availability [171,172]. During the last two decades, there have been a considerable number of successful cases of relaxation or even reversal of NADH/NADPH cofactor specificity. Altering an NAD(P)H-dependent oxidoreductase to use NADH/NAD$^+$ instead is of great interest for non-cell-based industrial applications since the phosphorylated cofactors are more expensive and less stable than their nonphosphorylated counterparts, making the enzymes that are dependent on NADPH/ NAD$^+$ less attractive for industrial-scale applications [173–175]. Examples of this include an NADPH-specific xylene reductase from *Candida boidinii* which was engineered to be able to use also NADH by means of computational design through minimization of the binding energy of the cofactor in variants were the residues of the cofactor binding pocket were mutated. A set of 10 candidate variants were identified, which were experimentally tested, leading to seven variants able to reduce xylene with NADH [176]. In general, cofactor specificity reversal must be done on a case-by-case basis. However, there have been some attempts at developing general methodologies for specific subsets of NAD(P)H-dependent oxidoreductases. For example, Brinkmann-Chen et al. managed to develop a general approach to reverse the cofactor specificity of ketol-acid reductoisomerasers from NADPH to NAD$^+$ combining information derived from crystal structures and multiple sequence alignments. By applying this approach, they obtained variants for several enzymes of this class whose catalytic efficiency with NADH was comparable to that of the wild type enzymes with NADPH or even higher [177]. This is noteworthy, as other studies attempting to change the cofactor specificity of an NAD(P)H-dependent oxidoreductase typically report engineered variants which do not reach a catalytic efficiency with the new cofactor as high as that of the original enzyme with its native cofactor. The opposite cofactor specificity reversal from NADH/NAD$^+$ to NADPH/NAD$^+$ has also been reported. This type of reversal has been applied to generate enzymes suitable to be used in systems where regeneration of NADPH is necessary. With this purpose, Lerchner et al. engineered the highly NADH-specific alanine dehydrogenase from *Bacillus subtilis* to use NADPH with nearly the same catalytic efficiency that the native enzyme has with NADH. This allowed them to couple the amino acid dehydrogenase to an alcohol dehydrogenase and an aminotransferase to efficiently produce dicyclic diamines from the corresponding dicyclic dialcohols. In order to obtain this variant, homology models of both the *Bacillus subtilis* enzyme and alanine dehydrogenase of *Shewanella*, which was known to be able to use both NADH and NADPH as cofactors, were generated based on the crystal structure of *Mycobacterium tuberculosis* alanine dehydrogenase [178]. Then, mutations were introduced in the *Bacillus subtilis* enzyme to mimic the cofactor binding pocket of the *Shewanella* enzyme [179].

Another frequent goal when engineering oxidoreductases is the modification of substrate specificity. There are several examples, often concerning alcohol dehydrogenases, where variants with activity towards a new substrate or a different isomer of one of the natural...
substrates were generated. For example, Laadan et al. identified a mutant of alcohol dehydrogenase 1 (ADH1) of *Saccharomyces cerevisiae* able to catalyze the NADP-dependent reduction of 5-hydroxymethylfurfural to 2,5-bis-hydroxymethylfuran, which could enhance the production of bioethanol through fermentation of lignocellulosic substrates by *Saccharomyces cerevisiae*. In order to do so, *Saccharomyces cerevisiae* cells where the ADH1 gene had been deleted were transformed with the library of variants and selected by their ability to grow anaerobically, and then activity assays for the reduction of 5-HMF were performed [180]. In another study, Rellos et al. generated a library of variants of *Zymomonas mobilis* alcohol dehydrogenase 2, an ADH-dependent, iron-activated alcohol dehydrogenase able to oxidize only ethanol, 1-propanol and allyl alcohol. The library of variants was generated by a combination of error-prone PCR and site-directed mutagenesis, and transformants were screened by a colorimetric assay with butanol. Variants with higher activity towards butanol than to ethanol, as well as variants able to use NADPH/NADP⁺ while still retaining the ability to use NADH/NAD⁺, were identified. Additionally, another variant was able to use Zn instead of Fe as the metallic cofactor [181].

There have also been several cases where the enantiospecificity of an alcohol dehydrogenase has been altered. However, most frequently the reversal of enantiospecificity is not total, comes at the cost of decreased catalytic efficiency or produces unexpected side-effects. For example, Akita et al. generated a thermostable NADP+-dependent D-amino acid dehydrogenase (DAADH) from *Ureibacillus thermospharicus* meso-diaminopimelate dehydrogenase (DAPDH) by introducing five point mutations in the active site that had been previously described to be responsible for converting another DAPDH from a mesophilic organism (*Corynebacterium glutamicum*) into a DAADH [182]. Interestingly, while the resulting variant was able to oxidize several D-amino acids, it was no longer able to oxidize meso-diaminopimelate [183].

Finally, another quality usually sought during the screening of variant libraries is increased thermal stability. Increasing thermal stability is not only desirable for achieving a reduced loss of catalyst during enzyme-catalyzed reactions, but also because the catalyzed reaction takes place at a higher rate when temperature is increased if the enzyme can withstand the higher temperature. For example, a dramatic increase in the thermal stability of phosphate dehydrogenase was achieved by Johannes et al., who identified a mutant with a T50 (required temperature to reduce the initial enzyme activity by 50%) higher than the wild type by 20 °C and a 7000-fold greater half-life at 45 °C, which could be used as an efficient way to regenerate reduced NAD(P)H in industrial biocatalysis applications. In this case, the approach involved a combination of error-prone PCR and site-directed mutagenesis. Three rounds of error-prone PCR were carried out, starting with the wild type gene. After each round of error-prone PCR, variants with improved thermal stability were identified. The best variants were sequenced, and the mutations found in all variants were incorporated into the template. The new template incorporating all the mutations served as the template for the next round of error-prone PCR [184].

Many attempts at modifying the substrate and cofactor specificity of NAD(P)H-dependent oxidoreductases have been carried out and these attempts have been successful to a certain extent. However, in many cases the mutations do not achieve the desired effect, cause the loss of catalytic activity or lead to unexpected results [185,186]. This is partially due to the lack of understanding of the relationship between the amino acid sequence of a protein, its structure and its function, which still prevents us from developing widely applicable methodologies to alter the substrate or cofactor specificity of oxidoreductases.

6. Conclusion and future perspective

NAD(P)H-dependent oxidoreductases constitute an extremely diverse group of enzymes able to act on a similarly vast range of substrates to catalyze electron transfer reactions. In recent years there have been a growing number of studies where novel applications based on these enzymes are proposed, or new methods proposed where these enzymes or organisms containing them can replace other less advantageous methods for catalysis.

Various factors underpin ongoing progress in research towards applications based on NAD(P)H-dependent oxidoreductases. The explosive growth in available genomic information about all types of organisms has proven to be very useful for the identification of NAD(P) H-dependent oxidoreductases, increasing the number of members of known types and thereby improving understanding of conserved features, but also leading to identification of new types such as the ATP and NADP-dependent CAR activity described in section 3, EC 1.2.1. In parallel, the acceleration in the rate at which protein structures are determined and made public is providing more insight into the structural basis of aspects such as cofactor and substrate specificity, information which is important to guide protein engineering. Furthermore, the ability to exploit this increasing knowledge base has been facilitated by the development of protein engineering and directed evolution approaches which allow the properties of enzymes to be tailored in rational and semi-rational ways. There has been practical progress in the prediction of enzyme function from structure: The new CSR-SALAD computational tool uses recently-established rules to interpret structural and sequence information in order to predict specific mutations which might allow reversal of the cofactor specificity of a given NAD(P)H-dependent oxidoreductase [66]. The rapid growth in sequence and structural information together with technological developments will undoubtedly expand still further the potential and usefulness of an already naturally versatile group of enzymes.

Transparency document

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Transparency document

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References

[1] J.D. Stewart, Future directions in alcohol dehydrogenase-catalyzed reactions, Future Directions in Biocatalysis, Elsevier, 2007, pp. 293–304.
[2] R. Meijers, R.J. Morris, H.W. Adolph, A. Merli, V.S. Laminz, E.S. Cedergren-Zappeauer, On the enzymatic activation of NADH, J. Biol. Chem. 276 (2001) 9316–9321.
[3] A.K. Holm, L.M. Blank, M. Oldiges, A. Schmid, C. Solem, P.R. Jensen, G.N. Vermuri, Metabolic and transcriptional response to cofactor perturbations in *Escherichia coli*, J. Biol. Chem. 285 (2010) 17496–17506.
[4] U. Saufer, F. Canoano, S. Heri, A. Ferrenoud, E. Fischer, The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*, J. Biol. Chem. 279 (2004) 6613–6619.
[5] M.R. de Graaf, S. Alexeeva, J.L. Snoep, M.J. Teixeira de Mattos, The steady-state internal redox state (NADH/NAD⁺) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*, J. Bacteriol. 181 (1999) 2351–2357.
[6] C. Auriol, G. Bestel-Corre, J.-B. Claude, P. Soucaille, I. Meynial-Salles, Stress-induced evolution of *Escherichia coli* points to original concepts in respiratory cofactor selectivity, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 1278–1283.
[7] T. Fuhrer, U. Saufer, Different biochemical mechanisms ensure network-wide balancing of reducing equivalents in microbial metabolism, J. Bacteriol. 191 (2009) 2112–2121.
[8] L. Hue, The role of futile cycles in the regulation of carbohydrate metabolism in the liver, Adv. Enzymol. Relat. Areas Mol. Biol. 52 (1981) 247–331.
reduce dehydrogenase for iron uptake from soils, Nature 397 (1999) 694–697.

[43] T. Nishino, K. Okamoto, Mechanistic insights into xanthine dehydrogenase oxidase from development studies of candidate drugs to treat hyperuricemia and gout, J. Biol. Chem. 269 (1994) 25178–25180.

[44] C. Maugé, T. Gruazier, B.L. d’Estaintot, M. Gargouri, C. Manigand, J.-M. Schmitter, J. Chaudière, B. Gallois, Crystal structure and catalytic mechanism of leukoanthocyanidin reductase from Vitis vinifera, J. Mol. Biol. 397 (2010) 1079–1191.

[45] N. Smets-Lepage, E.A. Cecchinato, I.E. Smiley, R.W. Schevitz, A.J. Wonacott, Structure of NAD(P) + reductase catalytic mechanism, Eur. J. Biochem. 270 (2003) 1900–1915.

[46] K. Ma, M.W. Adams, A hyperactive NAD(P)+/Rubredoxin dehydrogenase from the hyperthermophilic archaeon Pyrococcus furiosus, J. Bacteriol. 181 (1999) 5530–5533.

[47] L.F. Sevrioukova, H. Li, T.I. Poulos, Crystal structure of putidaredoxin reductase from Pseudomonas putida, the final structural component of the cytochrome P450cam monooxygenase system, J. Mol. Biol. 242 (1994) 791–801.

[48] H. Sakamoto, M. Ohta, R. Miura, T. Sugiyama, T. Yamano, Y. Miyake, Studies on the reaction mechanism of NAD(P)-adrenodoxin reductase with NADPH, J. Biol. Chem. 268 (1993) 8441–8450.

[49] C.M. Jenkins, M.R. Waterman, NADPH-flavodoxin reductase and flavodoxin from Esherichia coli: characteristics as a soluble microbial P450 reductase, Arch. Biochem. Biophys. 293 (1992) 110–117.

[50] H.A. Reylea, W.A. van der Donk, Mechanism and applications of phosphite dehydrogenase, Bioorg. Chem. 33 (2005) 171–189.

[51] J. Hu, W. Chuneshor, S.E. Roka, A switch between one- and two-electron chemistry during the flavoenzyme deiodinase is controlled by substrate, J. Biol. Chem. 290 (2015) 590–600.

[52] K.A. Payne, C.P. Quezada, K. Fisher, M.S. Dunstan, P.A. Collins, H. Sjüts, C. Levy, S. Hay, S.E. Rigby, Dehydrogenase-catalysed redox switch for a mechanism in B12-dependent dehalogenation, Nature 517 (2015) 513–516.

[53] T. Min, H. Kahanara, D.L. Bedgar, B. Youn, P.K. Lawrence, D.R. Gang, S.C. Halls, H. Park, J.L. Hilsenbeck, L.B. Devin, N.G. Lewis, C. Kang, Crystal structures of propionyl-lactate-ferredoxin hydrogenase, J. Mol. Biol. 364 (2006) 902–915.

[54] B. Arora, J. Mukherjee, M.N. Gupta, Enzyme promiscuity: using the dark side of specificity in enzyme white technology, Curr. Sci. 83 (2002) 25–29.

[55] R.D. Gupta, Recent advances in enzyme promiscuity, Sustain. Chem. Process. 4 (2016) 1–14.

[56] J. Hoff, W. van der Donk, Chemo-promiscuity of alcohols dehydrogenase: reduction of phenylacetaldoloxide to the alcohol, Tetrahedron 66 (2010) 3410–3414.

[57] P.J. Olifer, D. Herschlag, Catalytic promiscuity and the evolution of new enzyme activities, Chem. Biol. 10 (2003) 891–900.

[58] C. Lee, H. Görish, H. Kleinkauf, R. Zecher, A highly specific 5-hydroxysteroid dehydrogenase from the enantiom purifier Fusarium-ambucium, J. Biol. Chem. 269 (1994) 11741–11747.

[59] H. Vöhringer, F. Hollmann, A.C. Kleeb, K. Buehler, A. Schmid, TADH, the thermophilic enzyme from Pseudomonas putida, the final structural component of the cytochrome P450cam monooxygenase system, J. Mol. Biol. 242 (1994) 791–801.

[60] S. Kinoshita, T. Kakizono, K. Kadota, K. Das, H. Taguchi, Puri cation of NADP-binding sites in oxidoreductases, Eur. J. Biochem. 180 (1989) 479–484.

[61] F. Kaufmann, D.R. Lovley, Isolation and characterization of a soluble NADPH-dependent dehalogenase, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 5582–5586.

[62] I.R. Schnell, H.E. Wright, Structure, dynamics, and catalytic function of dehydrogenase, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 119–140.

[63] S.C. Tu, Reduced flavin: donor and acceptor enzymes and mechanisms of channeling, Antioxid. Redox Signal 3 (2001) 881–907.

[64] A. Pedersen, G.B. Karlsson, J. Rydstrom, Proton-translocating transhydrogenase: an update of unsolved and controversial issues, J. Bioenerg. Biomembr. 40 (2008) 467–473.

[65] G. Vooroud, S.M. van der Vies, A.P. Themmen, Why are there two different types of pyridine nucleotide transhydrogenase found in living organisms? Eur. J. Biochem. 131 (1983).

[66] C. Spannco-Watkins, J.F. Stoltz, P. Basu, Nitrite and periplasmic nitrate reductases, Chem. Soc. Rev. 43 (2014) 676–685.

[67] N. Castiglione, S. Rinaldo, G. Viardolo, S. Cutruzzolà, Nitrite and nitrate reductases: from molecular mechanisms to significance in human health and disease, Antioxid. Redox Signal 17 (2012) 684–716.

[68] M.D. Boldan, F. Pérez-Reinoz, F. Castillo, C. Moreno-Vivien, Reduction of poly-nitroaromatic compounds: the bacterial nitroreductases, FEMS Microbiol. Rev. 32 (2008) 474–500.

[69] B.R. Crane, L.M. Siegel, E.D. Getzo, Structure of the flavin adenine dinucleotide-binding protein, Nature 250 (1974) 194–197.

[70] H.A. Reylea, W.A. van der Donk, Chemo-promiscuity of alcohols dehydrogenase: reduction of phenylacetaldoloxide to the alcohol, Tetrahedron 66 (2010) 3410–3414.

[71] B.R. Crane, L.M. Siegel, E.D. Getzo, Structure of the flavin adenine dinucleotide-binding protein, Nature 250 (1974) 194–197.
[95] C. Dellomonaco, J.M. Clomburg, E.N. Miller, R. Gonzalez, Engineered reversal of hydrogenase isoenzymes in *Clostridium beijerinckii* and alcohol dehydrogenase II of *Zymomonas mobilis*, J. Biol. Chem. 269 (1994) 6592–6597 (ASMBM). (http://www.jbc.org/content/269/9/6592)

[104] C.K. Winkler, G. Tasnády, D. Clay, M. Hall, K. Faber, Asymmetric bioreduction of activated alkenes to industrially relevant optically active compounds, *J. Bacteriol.* 162 (2000) 381–389.

[105] O. Warburg, W. Christian, Über das gelbe Ferment und seine Wirkungen, Biochem. Z. 266 (1933) 377–411.

[111] R.E. Williams, N.C. Bruce, “New uses for an old enzyme”—the old yellow enzyme family of flavoenzymes, J. Biol. Chem. 162 (2012) 381–389.

[116] S.F. Queener, V. Cody, J. Pace, P. Torkelson, A. Gangjee, Trimethoprim resistance in bacteria: from clinical resistance to proteins of *Pneumocystis jiroveci*, Animicrob. Agents Chemother. 57 (2013) 4990–4998.

[118] G. Voordouw, S.M. van der Vies, A.P. Themmen, Why are two different types of pyridine nucleotide transhydrogenase found in living organisms? *Eur. J. Biochem.* 131 (1982) 527–533.

[127] J.B. Hoek, J. Rydstrom, Physiological roles of nicotinamide nucleotide transhydrogenase, *Biochem. J.* 254 (1988) 1–10.

[133] H. Hohfeldmeyer, B.T. Wolfstätter, D.F. Savage, P.A. Silver, J.C. Way, Improved synthesis of chiral alcohols with *Escherichia coli* adhE, *Appl. Environ. Microbiol.* 56 (1990) 1298–1301.

[138] C. Fichtner, C. Laurich, E. Bothe, W. Lubitz, Spectroelectrochemical characterization of *Pyrococcus furiosus* hydrogenase/reductase gene and protein families, *Cell. Mol. Life Sci.* 65 (2008) 1092–1102.

[142] A. Weckbecker, W. Hummel, Improved synthesis of chiral alcohols with *Escherichia coli* cells co-expressing pyridine nucleotide transhydrogenase, *NADP*^+^-dependent alcohol dehydrogenase and *NAD*^+^-dependent formate dehydrogenase, *Biotecnol. Bioeng.* 36 (1997) 16109–16115.

[149] Q. Wan, B.C. Bennett, M.A. Wilson, A. Kovalevsky, E.L. Howell, S. Datsenko, Toward resolving the catalytic mechanism of dihydrofolate reductase hemoprotein subunit and porphyrin: the roles of Asp77 and Tyr170, *Proc. Natl. Acad. Sci. U. S. A.* 114 (2017) 18231–18236.

[157] S.F. Queener, V. Cody, J. Pace, P. Torkelson, A. Gangjee, Trimethoprim resistance in *Escherichia coli*: the role of pyridine nucleotide transhydrogenase e, *J. Biol. Chem.* 277 (2002) 43433–43439.

[168] G. Gottschalk, The genome of *Clostridium beijerinckii* NRRL B592, *Appl. Environ. Microbiol.* 56 (1990) 1140–1149.

[175] H. Theorell, Preparation in pure state of the *Escherichia coli* dihydrofolate reductase of enterobacteria. IV. The *flaB* avoprotein: purification and properties, *Biochem. J.* 29 (1942) 744–752.

[182] M. Murphy, C. Saigo, Y. Tanaka, M. Nakatsubo, Spectrophotometric study of dihydrofolate reductase from *Pseudomonas aeruginosa* on fractionation of *P. aeruginosa* whole cells, *Biochem. J.* 82 (1962) 407–412.

[183] R. Sato, N. Kadowaki, M. Tashiro, H. Ashikaga, Kinetic study of dihydrofolate reductase from *Escherichia coli*, *FEBS Lett.* 141 (1982) 285–288.

[184] K.L. Kavanagh, M. Klimacek, B. Nidetzky, D.K. Wilson, Crystal structure of pyridine nucleotide transhydrogenase subunit of *Pyrococcus furiosus*, *BBA - Proteins and Proteomics* 1866 (2018) 327–347.

[185] K.L. Kavanagh, M. Klimacek, B. Nidetzky, D.K. Wilson, Crystal structure of dihydrofolate reductase hemoprotein subunit of *E. coli*, *BBA - Bioenergetics* 1102 (1988) 350–357.

[186] K.L. Kavanagh, M. Klimacek, B. Nidetzky, D.K. Wilson, Crystal structure of dihydrofolate reductase hemoprotein subunit of *E. coli*, *BBA - Bioenergetics* 1102 (1988) 350–357.

[187] S. Fumi, A. Wani, M. Shanker, M. Shrestha, X. Huang, S. Hammes-Schiffer, S. Perutz, M. Shallis, B. Pistorius, P. Torkelson, A. Gangjee, Spectroelectrochemical characterization of *Pyrococcus furiosus* hydrogenase/reductase gene and protein families, *Cell. Mol. Life Sci.* 65 (2008) 1092–1102.
