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DOTTORATO DI RICERCA IN ANALISI, PROTEZIONE E GESTIONE DELLE BIODIVERSITÀ
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Enzyme promiscuity in amino acid oxidases: a tool for sustainable processes

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Introduction

1 Enzyme promiscuity

For decades, the paradigm of one enzyme catalyzing one specific reaction on one specific substrate, dominated (and still dominates) the textbooks. However, the idea that enzymes are able to catalyze other reactions on substrates different from the ones for which they have physiologically evolved, is undeniably not new: in 1976 Jensen first hypothesized that, unlike "modern" enzymes, ancestral ones possessed extensive cross-reactivity toward a broad range of substrates [1]; thus, relatively few primitive enzymes could act on multiple substrates allowing a wider metabolic potential [2]. Traces of this peculiarity can still be witnessed today, in the form of low secondary activities toward "unnatural" substrates [3].

Enzyme promiscuity is defined as the presence of auxiliary catalytic activities, in addition to the one for which an enzyme has evolved, that are not part of the canonical organism's metabolism. Thus, enzymes which "naturally" evolved to transform a wide variety of substrates, must not be considered promiscuous: they are multispecific or broad-specificity enzymes [2]. Promiscuous activities generally rely on the same active site features responsible for the native activity, and although differences may exist, such as the mode of substrate binding, the reaction mechanism is, in many cases, similar. From a thermodynamic point of view, specific enthalpy-driven interactions, such as hydrogen bonds, play a relevant role in the binding of the native substrate, while aspecific hydrophobic interactions are thought to prevail for the promiscuous substrates. The existence of promiscuous functions can be explained by the fact that enzymes exhibit a

"rugged" energy landscape with many local minima: these minima correspond to a set of different protein conformations with similar but discrete energy levels, that exchange one another in an equilibrium regulated by the binding of different substrates (Fig. 1) [4]. The conformation of the enzyme which binds the native ligand is the "native" state, while other (less represented) conformers bind promiscuous ligands and catalyze alternative reactions [2].

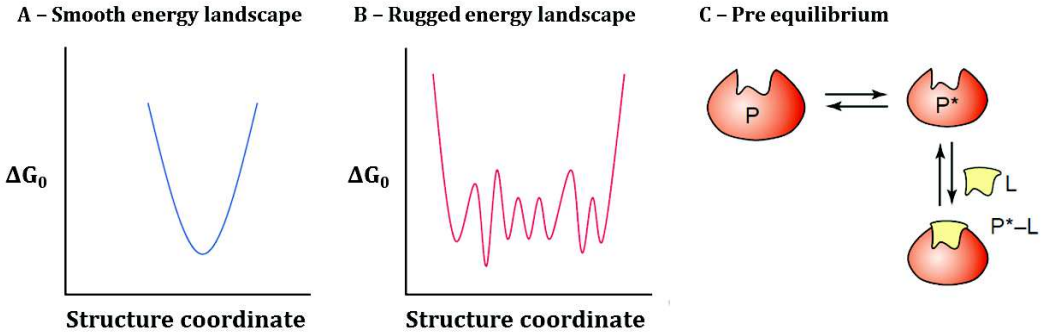

Figure 1 – Schematic representations of energy landscapes of enzymatic proteins. A) The classical protein landscape showing a unique global minimum (corresponding to a unique conformation). B) The 'modern view' assumes the existence of an ensemble of conformations possessing similar free energy. C) These conformations are in an equilibrium; the binding of different (e.g. promiscuous) substrates shifts this equilibrium towards different specific conformations [4].

The amplitude of such promiscuous activities can vary over several orders of magnitude in terms of catalytic efficiency (i.e. the ratio between turnover number and Michaelis constant), ranging from the same level of the native activity, as in the case of the promiscuous aryl esterase activity of serum paraoxonase PON1 [5] to an activity which is many orders of magnitude lower that the native one (e.g., the activity of dihydroorotase on phosphotriesters) [6].

The promiscuity of an enzyme can be quantified by a parameter called *degree of promiscuity*, which defines the level of "breach" of

the enzyme specificity, that is related to the extent promiscuous activities of a given enzyme differ from the native activity. The *degree of promiscuity* can be assessed by examining the differences in the catalytic mechanisms between the native and promiscuous reactions. Interestingly, an enzyme can possess more than one Enzyme Commission (EC) number; the comparison of the differences in these numbers could reveal promiscuous activities; differences from the native one in the first digit of the EC number indicate a different reaction mechanism and differences in the second or third digits indicate different classes of substrates [2].

Promiscuity has been also studied on non-enzymatic proteins, for example anbibodies or receptors. Antigen-antibody or receptor-ligand binding studies are much easier to perform in comparison to high throughput screenings for promiscuous enzymatic activities, due to the possibility to exploit a single detection protocol suitable for the whole range of studied interactions. These studies revealed that antibodies, thought to be strictly monospecific, are instead capable to bind, although with a lower affinity, a wide array of structurally unrelated antigens [7, 8], an observation also supported by theoretical or computational models [9]. It is reasonable to extend the results of these studies also to enzymatic proteins and, consequently, it is plausible that most enzymes exhibit a certain range of promiscuous functions, making promiscuity a general phenomenon which should be regarded as a rule, rather than an exception.

1.1 Classification of enzyme promiscuity

Three main kinds of promiscuity can be observed in enzymes: condition promiscuity, substrate promiscuity and catalytic promiscuity (Table 1) [3].

Table 1. Examples of different kinds of promiscuity. Catalytic promiscuity is split into accidental and induced promiscuity. Adapted from [3].

Example	Type of promiscuity				Reference
	Condition	Substrate	Catalytic		
			Accidental	Induced	
Maltase: reversal of reaction	X				[122]
Pig liver lipase: reactions in solvent	X				[123]
Subtilisin E: solvent tolerant mutants	X				[10]
Alanine dehydrogenase: cofactor usage		X			[124]
Lipase: resolution of chiral alcohols		X			[125]
Pyruvate decarboxylase: ynthesis of C-C bonds		X	X		[126]
Enolase superfamily: promiscuous ctivities as part of evolution			X		[127]
Lipase: C-C, C-N and C-S bond formation			X	X	[128]
Aminopeptidase as an oxidase				X	[129]
PLP-dependent racemase catalyzed aminotransferase				X	[130]

1.1.1 Condition promiscuity

Condition promiscuity is defined as the ability of an enzyme to catalyze reactions in an environment different from the one in which it was evolved, such as organic solvents, high temperatures, or extreme pHs. Indeed, taking to the extreme this assumption, every reaction performed in laboratory can be considered an example of "condition promiscuous" activity: outside of the cell enzymes are exposed to reaction environments different from the *in vivo* ones, at least because of the presence of a buffering system which is obviously different from the cytoplasm of the cell [3].

An early example of exploitation of the condition promiscuity of enzymes is represented by the studies on the stability and activity

of the serine protease subtilisin E in the polar organic solvent dimethylformamide (DMF). Under these conditions (i.e. in the absence of water) hydrolysis is suppressed and the enzyme is forced to catalyze the reverse reaction, that is the condensation reaction, leading to peptide synthesis. The performance of the enzyme in an organic solvent was improved by several rounds of directed evolution, which eventually produced an enzyme possessing an activity 256-fold higher in comparison to the wild type [10] (Table 1).

Also pH plays an important role in modulating the reactivity of an enzyme because the same active site residue can work in a different protonation state between the native and the promiscuous function, allowing the catalysis of different chemical reactions. As an example, proline 1 of of 4-oxalocrotonate tautomerase possesses a pKa of about 6.4 and mainly acts as a general base; however, at pH 7.8, a small fraction of proline 1 is present in the protonated state exhibiting a weak but detectable promiscuous hydratase activity by general acid catalysis [11].

1.1.2 Substrate promiscuity

In many cases, promiscuous activities share the same active-site configuration and features of the native activity. The differences between the higher catalytic efficiency on the native substrates versus lower activities on the promiscuous ones, originate from a different mode of interaction of the substrates with the active site residues. As an example, alkaline phosphatase efficiently catalyzes the hydrolysis of phosphate monoesters but it is also able to

promiscuously catalyze hydrolysis reactions of phosphodiesters, phosphoamides, sulfate esters and phosphite, using the same reaction mechanism which involves the nucleophilic attack of the substrate by Ser102 forming an intermediate which is stabilized by the interaction with a Zn^{2+} and Arg166. These interactions are much more favorable for the native substrates than for the promiscuous ones, explaining the observed different reaction rates [12].

Sometimes, changing the enzyme cofactor, especially in the case of metal ions, can alter the enzyme specificity; as an example, in carbonic anhydrase, the substitution of the native Zn^{2+} with Mn^{2+} allows the catalysis of styrene epoxidation, while the substitution of Zn^{2+} with Rh^{2+} converts this enzyme into a hydrogen-utilizing reductase [13, 14].

1.1.3 Catalytic promiscuity

The term catalytic promiscuity defines the ability of a single active site to catalyze more than one chemical reaction that can differ in the type of bond that is formed or cleaved during the reaction and/or in the catalytic mechanism; these differences result into the formation of different transition states [15].

An example is represented by aminopeptidase P, a metallopeptidase which natively catalyzes the hydrolysis of amide C–N bonds, but is also able to catalyze the hydrolysis of phosphate triesters (a P–O bond) which implies the stabilization of a transition state with different geometry [16].

Catalytic promiscuity can be further divided into two main categories: in most cases the wild-type enzyme is already able to catalyze more than one reaction; this property is defined as accidental catalytic promiscuity. On the other hand, often a new enzymatic activity is acquired or enhanced due to the accumulation of one or more point mutations that modify the reaction catalyzed by the wild-type enzyme. This event is defined as induced catalytic promiscuity [3].

1.2 Enzyme promiscuity and the evolution of molecular biodiversity.

It is now widely accepted that enzyme promiscuity might represent the starting point for the divergent evolution of new protein functions. Several evidences suggest that most, contemporary enzyme families and superfamilies have evolved from promiscuous multi-functional ancestral enzymes, from which specificity (first) and efficiency (later) stemmed through divergence and evolutionary optimization, respectively [17]. First, in many cases, the same promiscuous activity can be retrieved in more than one member of an enzyme family [18]: the native function of one family member represents often a promiscuous activity in other family members. This overlap is a consequence of the common catalytic strategy that defines these families as a consequence of the origin of the family members from the same gene (divergent evolution) [19]. Second, *in vitro* evolution of one promiscuous activity often yields a “generalist” intermediate that

follows a new specialization, indirectly leading to the appearance of other promiscuous activities [20] (Fig. 2).

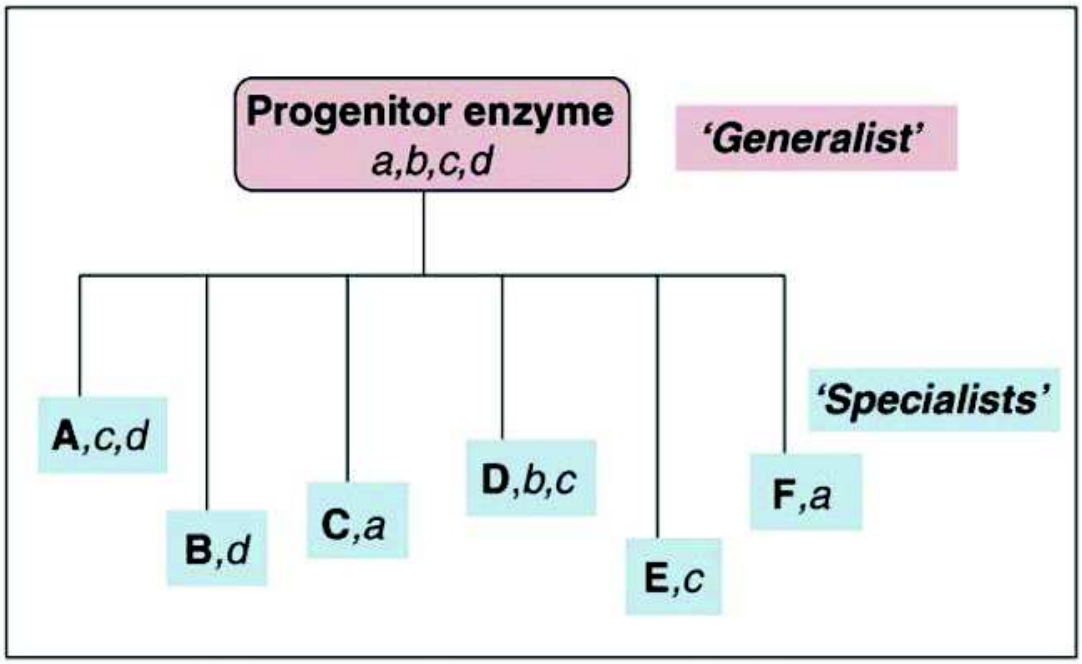

Figure 2. Ancestral enzymes are believed to display several low original activities (termed a, b, c, d); during evolution they have been subjected to selection pressures for those activities, thus duplicating and diverging into a family of highly specialized enzymes activities (denoted as uppercase letters A, B, C, D, E, F). 'Modern' enzymes may still retain some of the original activities of the ancestral ones (denoted in lower case), as promiscuous activities. These activities are shared among the members of the family, and in some case the native activity of one member is the promiscuous activity of another, and vice versa [25].

The above observations support the hypothesis that all existent proteins originated by divergent evolution from a small set of progenitors which conceivably formed the original protein pool of the ancestral microorganism defined as *last universal common ancestor* (LUCA) [1].

The existence of promiscuous activities is a prerequisite for the evolution of new functions. Thus molecular evolution is likely to happen through nature 'tinkering with' genes to generate new protein structures and functions by improving and by constantly

perfecting their promiscuous activities. The introduced mutations must confer a selective advantage to the organism from the very onset of the evolutionary process, otherwise they could not be positively selected and, in turn, transmitted during evolution [4]. Secondary activities eliminate the necessity to generate a new enzyme from scratch and their presence can potentially provide an immediate selective advantage to the organism. In fact, promiscuity plays a biological role in enabling organisms to survive changes in environment and contributes to the tolerance of some organisms to deletions of genes coding for proteins involved in metabolic pathways [21].

Once a promiscuous function acquires a physiological relevance, its efficiency can be enhanced by the accumulation of mutations in the corresponding gene. This process must happen, at least in the first stages, without any significant detrimental effect on the native function of the enzyme [2].

According to the most widely accepted model for divergent evolution of proteins, a gene duplication event results into the appearance of a redundant gene copy, that is relieved from selective pressure and, consequently, it is free to accumulate both beneficial and deleterious mutations [22]. It is estimated though, that more than 30 % of random mutations in a gene are deleterious for the function of the encoded protein [23], while beneficial mutations that enhance a novel function are much more rare, with an estimated frequency of $\sim 10^{-3}$. It follows that, if a gene could accumulate mutations in the absence of any selection, the probability to produce a non-functional protein would be some

orders of magnitude higher than that to obtain (or improve) a new function. Accordingly, enzymes, to be able to evolve, must display two crucial, yet apparently conflicting, features: plasticity, that is the ability to gain new functions through the accumulation of a relatively low number of mutations, and robustness, that is the ability to tolerate negative structural or functional changes induced by the effect of detrimental mutations [5].

Promiscuous enzymatic activities are highly plastic; they can be modified through mutations that dramatically increase or decrease them: following the introduction of only few mutations, these activities can be enhanced by 10–1000-fold, but reports of 10^4–10^6-fold increases, following a single mutation, have also been described [24]. The high plasticity of these activities is due to the fact that the mutated residues are often located on surface loops that are part of the substrate binding pocket and exhibit high conformational flexibility. Usually these residues are not part of the protein's scaffold or of the catalytic machinery of the enzyme. Their localization can also explain the different effects that mutations of these residues have on the native versus the promiscuous functions: in many cases, the enhancement of the promiscuous functions does not seem to correlate with an equal decreases in the original function's activity [5]: contrary to the large changes undergone by promiscuous activities, the effects of the same mutations on the native functions are limited. In most cases, the ratio between the increase in catalytic efficiency of a promiscuous function to the decrease in the native one is more than 10, and ratios greater than 100 observed in about 25% of

cases [25]. This behavior has been observed also in *in vitro* evolution experiments, despite the fact that, in these cases, only one selection criterion was applied for the screening of evolved variants.

The relative rates by which a new function is gained and the old one is lost have important consequences in molecular evolution; in principle, acquiring of a new enzymatic function by divergent evolution can proceed via a 'generalist' intermediate, that is an enzyme that exhibits a broad specificity, prior to generate a new specialist enzyme (Fig. 3) [25].

Since in a living cell, the "cost" of a generalist enzyme on fitness might be quite high, that is a broad specificity enzyme might be toxic for the organism [26]. Thus the driving force for specialization is likely to be quite strong [25]. The accumulation of favorable mutations can proceed only as long as the activity of the native function is retained at a level high enough to not severely weaken the fitness of the organism. However, at the end of the evolutionary process, the acquisition of an efficient new activity is reached at the expense of the native one (Fig. 3). Thus, during the evolution of a new enzymatic function, at some point a gene duplication event becomes necessary in order to offer a margin that allows more beneficial mutations to accumulate, enabling the complete re-specialization of the diverging function.

Thus it can be concluded that gene duplication is not a random event that happens in an early stage of the evolution of a novel enzymatic activity, but it is a positively selected event that happens in a later stage, to allow complete re-specialization of an

evolving protein. Furthermore, when an organism is forced to rely on a low promiscuous activity of an existing enzyme for its survival, the presence of a second gene copy that allows the production of an increased protein quantity, can grant an immediate selective advantage [2].

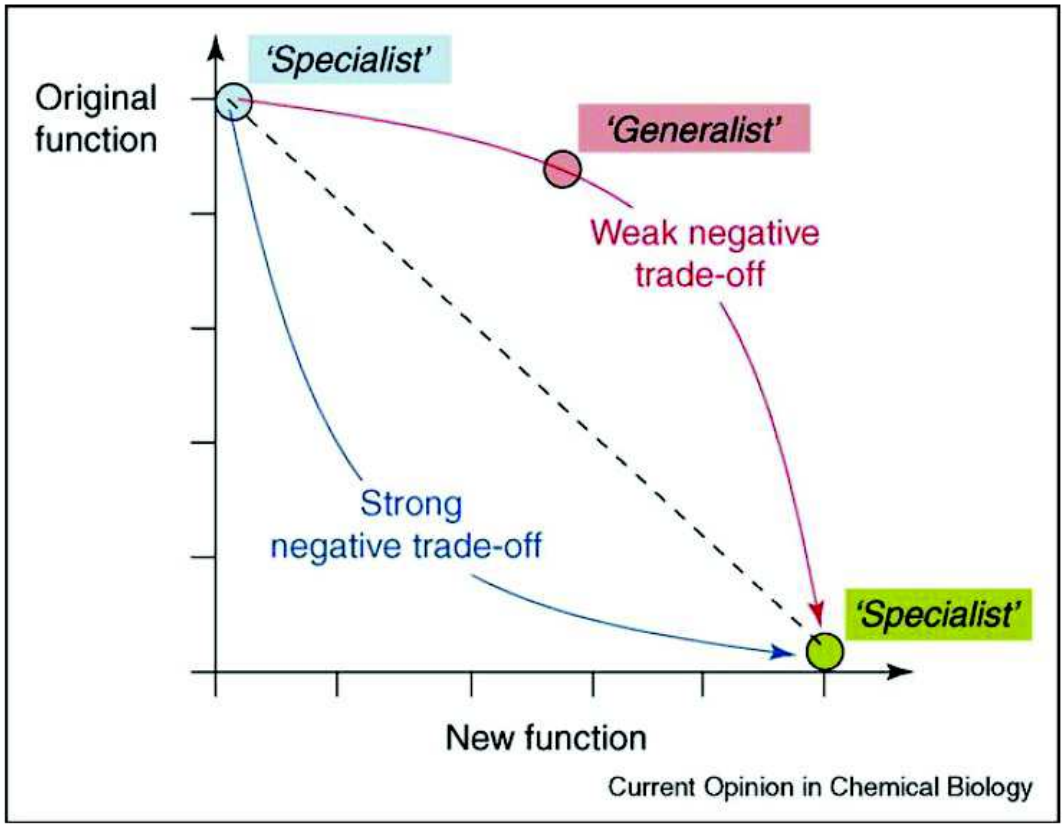

Figure 3. Possible routes to acquire a new enzymatic function. The conversion of one specialist enzyme into another may follow either the concave or convex routes. In the latter ('weak negative trade-offs') large increases in the promiscuous function ('new function') are accompanied by significantly smaller decreases in the native function. The intermediates of these route are generalist enzymes, and their evolution can proceed prior to gene duplication. In the concave route, gene duplication is a necessary prerequisite, because acquisition of even low levels of the 'new' function is accompanied by large losses of the original one. This route is observed in particular under a dual selection, for the gain of a new function and the loss of the old one [25].

Thus all existing proteins, no matter how different they can be in terms of sequence, structure or function, share a relatively low number of common ancestors and are related one to each other via

a small number of ancestral polypeptides. An example of such sequence motifs is the P-loop, which is present in a large number of proteins from unrelated superfamilies, suggesting that this motif was present in the early protein ancestors [27].

In summary, it can be concluded that, when the selective pressure of the environment requires the generation of new enzymatic functions, nature exploits existing enzymes that already promiscuously catalyze this new reaction, tinkering with their active sites, to render it more suitable for the new substrate and reaction. Subsequently, following gene duplication, new family members diverge from existing ones, thus yielding the large and functionally diverse enzyme families and superfamilies we see today [25].

2 Flavoproteins

Flavoproteins are proteins that use as cofactor a riboflavin derived nucleotide, such as flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN).

To date, several hundreds of flavin-containing enzymes has been discovered. Flavins are very versatile coenzymes, that catalyze with considerable efficiency a wide variety of enzymatic reactions involving either two-electron or one-electron transfers. These reactions range from typical redox catalysis such as the dehydrogenation of an amino acid, the activation of dioxygen, photochemistry, DNA damage repair or light emission [28].

2.1 The flavin cofactor and its chemical reactivity

From a chemical point of view, flavins can be considered amphipathic molecules: the isoalloxazine system is formed by a hydrophobic xylene moiety, prone to interact with hydrophobic regions of the protein, and a pyrimidine ring that is relatively electron-deficient and hydrophilic and usually involved in hydrogen bonds with the protein. These interactions can differ substantially between the three redox states in which the cofactor can exist: oxidized, semiquinoid, and fully reduced, each one possessing peculiar spectral features (Fig. 4) [28].

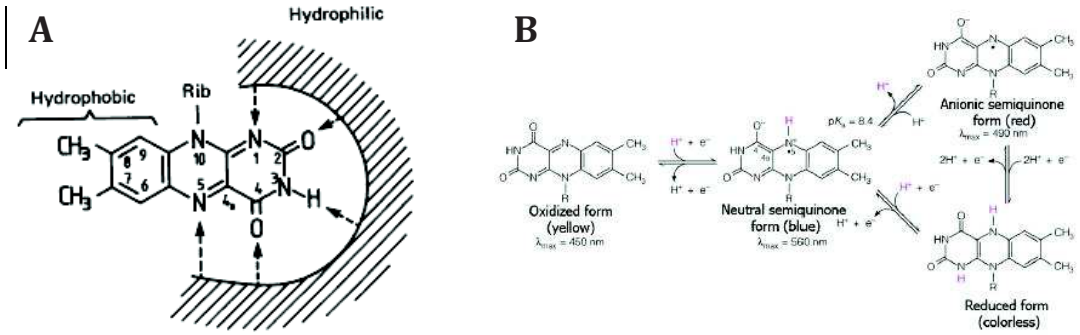

Figure 4. A) Representation of possible interactions between the isoalloxazine ring of the flavin and the protein. B) Different oxidation states of flavin cofactors [121].

The diversification of the reactions catalyzed by flavoproteins derives from the interaction of the isoalloxazine ring of the flavin cofactor with the protein scaffold. The redox potential of the two electron transfer at pH 7 is around -200 mV, but it can be lowered or increased, approximately in a 600 mV range, by the interaction with the protein: for example, a positive charge in the active site close to the pyrimidine ring contributes to raise the cofactor redox

potential, while a negative charge or a hydrophobic environment tends to lower it [28].

In free solution, the semiquinone form of the flavin is in very rapid equilibrium with the other two forms, with only a few percent of the total flavin being in this state [29, 30, 31], but when the flavin is bound to the protein, the semiquinone is generally stabilized and its neutral or the anionic forms can be observed; as an important consequence this allows the switch from two to one-electron transfer reactions [32].

In addition to the polarity of the environment, the apoprotein affects the conformation of the isoalloxazine ring, since it can increase or decrease the typical bending of the molecule in the free form. In particular a clear distinction can be made between "classical" oxidase flavoproteins, which bind the cofactor in a non-planar form and electron transferases which, in almost all cases, bind it in a planar state [32].

The variety of different reactions that can be catalyzed by flavoproteins is also reflected on the different reactivity toward molecular O_2. Enzymes belonging to class 2 (see section 2.2 for classification of flavoproteins), for example, directly catalyze a two-electron reduction of O_2 to yield H_2O_2 as product [33], while in class 3 monooxygenases the primary product of reaction is a flavin C4a-peroxide adduct [34, 35, 36]. Class 1 transhydrogenases and electron-transferases belonging to classes 4 and 5 (which carry out one-electron transfers) react with O_2 producing the superoxide anion (O_2^-) and the neutral semiquinone flavin radical [32, 33]. In

addition, another class of flavoproteins (i.e. amino acid deaminases) are not able to react directly with oxygen, but employ alternative electron acceptors [37].

According to recent data, O_2 appears to be able to directly interact with the flavin C4a atom, both in the monooxygenases and in the oxidases active sites, suggesting that, independently of the reaction mechanism, the C4a atom could be the locus directly involved in the transfer of electrons from the reduced cofactor to O_2 [38]. As a general rule, the different oxygen reactivity of the members of different flavoprotein classes resides in the charge distribution around the isoalloxazine ring of the flavin and in its accessibility [39].

Another important reaction that can be used for the functional characterization of flavoproteins is the formation of a covalent but reversible adduct between sulfite and the N5 of the flavin. This reaction is not thermodynamically favorable in free flavins and thus low affinity for sulphite is retained in the majority of protein-bound flavins [40]. There is, however, an exception: oxidases belonging to class 2 show a significant high reactivity with sulfite and in some cases the formation of the N5-sulfite adduct occurs with stoichiometric concentrations of this compound [41]. Interestingly, a correlation was observed between the reactivity with sulfite and the type of flavin radical which is stabilized by the enzyme: the stabilization of the anionic semiquinone flavin species coincides with high thermodynamic stabilization of the sulfite adduct, while the stabilization of the neutral semiquinone (or no

flavin radical stabilization) is concomitant with very low or no sulfite affinity [32].

2.2 Classification of flavoproteins

Since flavoproteins are mainly involved in dehydrogenation or oxidation reactions, the oxidized form of the cofactor must be regenerated at the expense of the reduction of an electron acceptor. The acceptor might be a disulphide containing molecule, an oxidized nicotinamide nucleotide or an unsaturated compound such as fumarate or crotonyl-CoA, but in the majority of cases, the acceptor is molecular O_2 or a redox protein such as an iron-sulphur containing protein or a cytochrome. In the latter case, the flavoprotein acts as a mediator between a two-electron and a one-electron transfer step; this peculiar feature is almost unique in the metabolism of the cell [32].

Flavoproteins can be classified according to their function, in five major classes:

Class 1 – Transhydrogenases. The enzymes catalyze a two-electron redox reaction by hydrogen transfer between two redox centers; class 1 flavoproteins can be further subdivided into 4 subclasses: carbon-carbon transhydrogenases, carbon-sulphur transhydrogenases, carbon-nitrogen transhydrogenases and nitrogen-nitrogen transhydrogenases.

Class 2 - Dehydrogenases/oxidases. Flavoproteins belonging to this class are considered the “canonical” oxidoreductases. They

combine the dehydrogenation of a substrate with the reduction of molecular O_2 to generate H_2O_2.

Class 3 - Dehydrogenases/oxygenases. These enzymes differ from the previous ones in the reactivity with molecular O_2. Class 3 flavoproteins do not produce hydrogen peroxide, but one atom of the oxygen molecule is reduced to a water molecule, while the other one is incorporated into the second substrate of the enzyme.

Class 4 - Dehydrogenases/electron-transferases. The enzymes accept two-electrons from a donor (e.g. a two electron-reacting nicotinamide nucleotide) and transfer them to a one-electron acceptor (e.g. a one-electron-reacting heme or iron-sulphur containing protein).

Class 5 - Pure electron-transferases. This class of flavoproteins is formed by enzymes such as flavodoxins, which are involved exclusively in one-electron transfers [32].

In addition to their function, flavoproteins can be classified also on the basis of the architecture of their three-dimensional structure [42].

2.3 D-amino acid oxidases

D-amino acid oxidase (DAAO EC 1.4.3.3), is a FAD-dependent peroxisomal enzyme, discovered more than 70 years ago by Krebs [43]. From a biochemical point of view, the enzyme has been extensively studied between 1950 and 1990 using the protein purified from pig kidney (pkDAAO) [44]. For this reason DAAO is considered a paradigmatic example of the class 2 of flavoproteins

[45]. DAAO catalyzes the strictly stereospecific oxidative deamination of D-amino acids to the corresponding imino acids which are non-enzymatically hydrolyzed to yield the corresponding α-keto acids and ammonium. The reduced FAD cofactor is then reoxidized by molecular oxygen, with production of hydrogen peroxide [46].

2.3.1 Sources and physiological role of D-amino acid oxidases

DAAO is mainly present in eukaryotic organisms. The enzymes from the yeast *Rhodotorula gracilis* (RgDAAO) and *Trigonopsis variabilis* (TvDAAO) have been purified and characterized in detail, also because of their biotechnological importance [47, 48].

D-amino acid oxidase activity has been identified also in almost all the higher eukaryotes, with the possible exception of plants. During the years, several mammalian DAAOs have been characterized (e.g., from pig, rat, human) and, the 3D structure of some of them is available [50, 51, 52].

In yeast this enzyme allows the use of D-amino acids as a carbon, nitrogen, and energy source [47]. In mammals the physiological role of DAAO has not yet been fully elucidated. It is plausible that even in the same organism it performs different tissue specific roles. For example, it was proposed that, in liver and kidney DAAO acts as a detoxifying agent by oxidizing endogenous or exogenous D-amino acids. In the human brain DAAO is involved in the regulation of the metabolism of D-serine, a modulator of the glutamatergic neurotransmission [53].

The highest level of mammalian D-amino acid oxidase overexpression in *E. coli* has been achieved for pig kidney DAAO (200-300 U/L of culture broth) [54]. On the other hand, better results have been obtained with RgDAAO which was expressed in *E. coli* with a volumetric yield of about 2300 U/L of culture [48].

2.3.2 Biochemical properties of D-amino acid oxidases

DAAO is specific of apolar D-amino acids whereas it shows little to no activity versus polar and charged D-amino acids. For example, the best substrates of RgDAAO are D-Met, D-Trp and D-Ala [55].

DAAO is able tostabilize the anionic semiquinone flavin species and shows a high reactivity with sulfite [32]. The protein is usually present in solution as a stable homodimer, with few exceptions (e.g. in the case of TvDAAO or pkDAAO) [44, 57]. DAAO monomer is formed by two domains, the FAD-binding domain, encompassing the Rossman fold, and the substrate binding domain. The FAD cofactor is non-covalently bound to the apoprotein dissociation constants that range from 0.02 μM for RgDAAO to 10 μM for hDAAO [46]. It has been shown that the dimerization in RgDAAO increases the protein stability and the affinity for the cofactor and also results into a 10-fold higher enzymatic activity [58].

2.4 L-amino acid oxidases

Similarly to DAAOs, L-amino acid oxidases (LAAOs, EC 1.4.3.2) are enzymes belonging to the class 2 of flavoproteins (dehydrogenases/oxidoreductases). According to the sequence and structure of the FAD-binding domain, LAAOs are classified as

members of the large glutathione reductase 2 (GR2) structural subfamily of flavoproteins [42]. The first LAAO has been described more than 70 years ago by Zeller and Maritz (1944) [59]. LAAOs catalyze the strictly stereospecific oxidative deamination of a wide range of L-amino acids to form the corresponding imino acids with the concomitant reduction of the flavin cofactor (reductive half reaction). The imino acid then undergoes a non-enzymatic hydrolysis, yielding an α-keto acid and ammonium. In most cases the reduced cofactor is reoxidized by molecular oxygen (oxidative half-reaction), generating hydrogen peroxide [60] (Fig. 5).

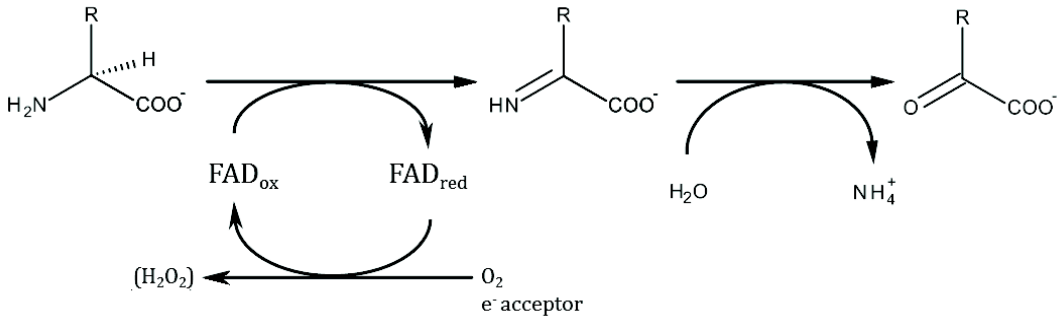

Figure 5. Reaction catalyzed by L-amino acid oxidases.

Some membrane-bound L-amino acid oxidases, such as the ones from the *Proteus* genus, are associated with the respiratory electron transport chain and their flavin cofactor is reoxidized by an electron acceptor different from O_2 [61, 62]. In turn this acceptor donates electrons to the membrane quinone pool through which they are transferred to a terminal cytochrome oxidase to reduce molecular oxygen to H_2O [63].

LAAOs are found in almost all taxonomic kingdoms, ranging from bacteria species to algae, fungi, insects, mollusks, fishes, as well as snakes and mammals (in which it is mainly expressed in liver,

kidney, nervous system, mammary gland and leukocytes) [64], [65]. From a biochemical point of view, LAAOs can be classified based on different properties: substrate specificity, molecular mass, regulation of expression, presence of post-translational modification and subcellular localization (i.e. intracellular, membrane bound, or extracellular) [66].

2.4.1 Snake venom L-amino acid oxidases

Snake venom LAAOs (svLAAOs) are the best-studied members of this enzyme family. These enzymes are generally classified as N-terminal FAD or FMN-bound reductases. They possess two highly conserved glutamine rich motifs involved in nucleotide-binding at the N-terminus of the protein [66]. The cofactor is usually non-covalently bound to the apoprotein moiety [67]. Their relative abundance in snake venom varies depending on the species of the reptile, but in general, they are present in high concentration especially in the venom of *Viperidae*, *Crotalidae* and *Elapidae* snakes, where they can account for up to 9% of total venomic proteins [68].

Most svLAAOs are active over a wide range of pH and temperature and show a preferential catalytic specificity toward long chain hydrophobic or aromatic amino acids such as L-Phe, L-Met, L-Leu and L-Ile [69, 70]. In general their affinity for polar and basic L-amino acids is low, especially for positively charged amino acids such as L-lysine and L-arginine, which form unfavorable electrostatic interactions with the residues of the catalytic site of the enzyme [71]. However, some LAAOs are active on charged

substrates: for example L-Lys is the best substrate for *Ophiophagus hannah* LAAO [72], and acidic amino acids L-Asp and L-Glu are the more efficient substrates for *Bungarus fasciatus* LAAO [73].

The isoelectric point of different svLAAOs ranges from 4.4 to 8.5; their isoelectric point also affects their pharmacological properties such as their anti-viral or bactericidal activity and their effect on the modulation of platelet aggregation. In the snake venom from the same species, acidic, neutral and basic forms of svLAAOs can coexist [74].

In general svLAAOs are homodimeric or, less frequently, monomeric proteins [68]; the molecular mass of each monomer ranges approximately from 50 to 70 kDa corresponding to a molecular mass of the dimer from 110 to 150 kDa [60].

SvLAAOs are extensively glycosylated proteins: they present a variable percentage of covalently linked sugars, which can widely vary according to the snake species: it can range from about 2% of the total protein mass in *Crotalus adamanteus* LAAO to up to 25% in the enzyme from *Bungarus caeruleus* [60]. This post-translational glycosylation is proposed to play a role in the enzymatic and biological activity of these enzymes: indeed, the activity of LAAO from *Agkistrodon halys pallas* was decreased by about 75% when treated with peptide-N-glycosidase F, an enzyme which removes carbohydrates covalently linked to the protein [75]. Nonetheless, the specific role of glycosylation and its effect on the modulation of svLAAO activity is still unclear.

The role of svLAAOs is to enhance the toxic effect of snake venom, by the extracellular oxidation of the free L-amino acids and

generation of hydrogen peroxide, a reactive oxygen species [64]. H_2O_2 then could act on cell membranes by altering the permeability of the attacked area, thus stimulating local necrotic or apoptotic processes, but the precise action mechanisms remain still unclear [76]. Consequences of svLAAO activity are edema and hemorrhage due to apoptosis of vascular endothelial cells and changing of the state of platelet aggregation [68].

2.4.1.1 Three-dimensional structure of eukaryotic L-amino acid oxidase.

In 2000 Pawelek solved the three-dimensional structure of LAAO from the Malayan pit viper *Calloselasma rhodostoma* (CrLAAO) in complex with two different inhibitors, citrate and anthranilate (2-amino benzoate) [67]. CrLAAO is a homodimer: each monomer possesses a molecular mass of about 55 kDa and non-covalently binds one molecule of FAD. CrLAAO is glycosylated at Asn172 and Asn 361 (Fig. 6A). Each monomer is composed of three domains: a FAD-binding domain which contains the typical nucleotide binding motif, a substrate binding domain and an additional α-helical domain which contributes to the formation of a funnel that constitutes the entrance to the active site. The isoalloxazine moiety of the FAD cofactor is located at the interface between the FAD and substrate binding domains and forms extensive interactions with the protein scaffold. In particular the xylene motif is surrounded by hydrophobic residues, (Ile374, Trp420 and Ile430), while the pyrimidine ring forms hydrogen bonds with Met89, Arg90 and several crystallographic water molecules. The structure of the

enzyme in complex with the inhibitor anthranilate gives information on the binding mode of the natural substrates and on the reaction mechanism: the C1 of the inhibitor molecule, which mimics the Cα of the substrate, is positioned in proximity of the N5 of the isoalloxazine ring of FAD, at an ideal distance for direct transfer of a hydride from the substrate to the cofactor [67].

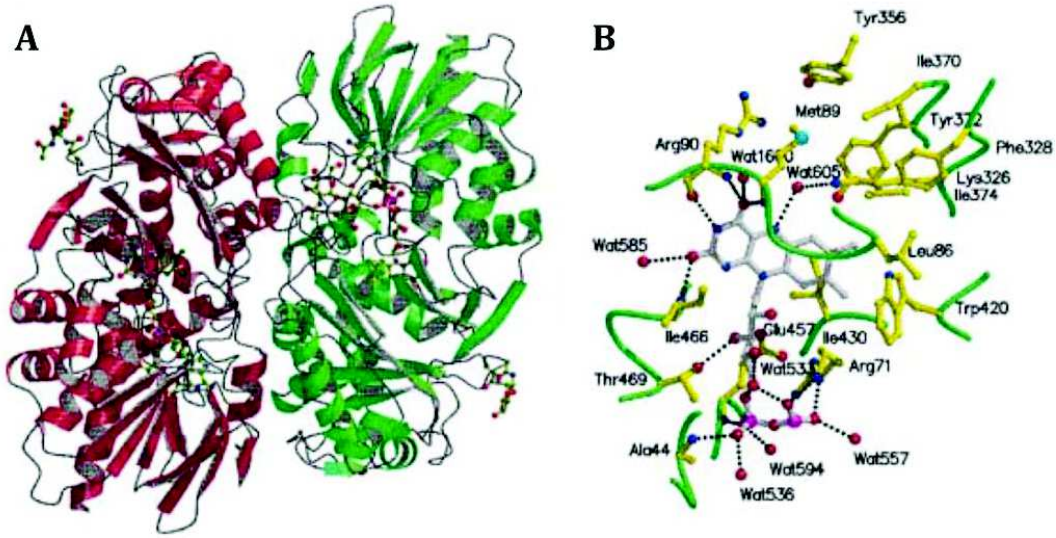

Figure 6. 3D structure of CrLAAO. A) Ribbon representation of CrLAAO dimer. Individual monomers are colored in red and green. FAD cofactor and glycosylation residues are shown in ball and stick representation. B) Detail of CrLAAO active site. The FAD cofactor is colored in gray and relevant interacting residues are labelled and colored in yellow. Water molecules are represented as red spheres, while dotted lines represent hydrogen bonds. Protein backbone is in green [67].

The ligand binding is stabilized by a salt bridge between Arg90 and the inhibitor carboxylate group, which is also hydrogen bonded with the hydroxyl group of the side chain of Tyr372. The aromatic ring of the ligand is sandwiched between the side chains of Ile430 and Ile374 and the amino group is positioned over the pyrimidine portion of the isoalloxazine ring, within hydrogen bonding distance with the backbone carbonyl oxygen atom of Gly464 (Fig. 6B) [67].

2.4.2 Microbial L-amino acid oxidases

The first microbial LAAO activity (from the bacterium *Proteus vulgaris*) was described by Stumpf and Green in 1944 [77]. Since then, an increasing number of LAAOs from different microorganisms have been reported, but only in 1994 the first LAAO activity from a microorganism (the Gram-positive bacterium *Bacillus carotarum*) could be purified to homogeneity and characterized [78].

2.4.2.1 General properties of microbial L-amino acid oxidases

Several microbial LAAOs exhibit a broad substrate specificity [79]. Typically they display a noticeable preference for hydrophobic amino acids such as L-Phe, L-Leu, L-Tyr and L-Trp [63]. A second group of LAAOs is formed by enzymes showing a very strict preference for a specific substrate (such L-aspartate oxidase). The stereospecificity of LAAOs is generally very strict; only few of them are active on some D-amino acid, but with negligible reaction rates in comparison to the ones of the corresponding L-amino acids [65]. Microbial LAAOs show an isoelectric point which varies from pH 4.0 to 9.4 and a molecular mass between approximately 50 and 300 kDa. They are usually present in solution as homodimers, formed by monomers that span from about 55 to 74.7 kDa and non-covalently bind one molecule of flavin cofactor in an extended conformation [63].

So far, four 3D structures of microbial LAAOs have been described: the structure of LAAO from *R. opacus* (RoLAAO), of L-glutamate oxidase (LGOX) from *Streptomyces* sp. X-119-6 and of L-aspartate

oxidases (LASPO) from thermophilic archaea *Sulfolobus tokodaii* and from *Escherichia coli* (the R386L variant). In all cases each LAAO subunit consists of three domains: a dinucleotide binding domain, containing the Rossman fold, a substrate binding domain and an additional helical domain. This topology is similar to the one described for snake venom LAAO [67].

All microbial LAAOs whose 3D structure is known are synthesized as precursors carrying a signal peptide and then converted into the mature proteins by limited proteolysis triggered by an endopeptidase [80]. This occurs after protein secretion and represents a strategy to prevent cellular apoptosis due to depletion of L-amino acids and H_2O_2 production in the cytoplasm.

2.4.2.2 Biological functions of microbial L-amino acid oxidases

Since most LAAOs from microorganisms are active versus a broad spectrum of substrates, it is plausible that they are involved in the utilization of such compounds as nitrogen and/or carbon source. In fact, microbial LAAOs (especially membrane bound forms), are functionally linked with metabolic pathways that allow an efficient catabolism of free amino acids [65]. As an example, in a number of unicellular algae, the presence of cell-surface LAAOs allow the growth of these organisms using free amino acids as the sole nitrogen source [81]. In addition, expression of LAAO in *Neurospora crassa* can be induced by the presence of L-Arg and L-Phe in the culture broth (in the absence of other readily available nitrogen compounds) [82].

The ability of LAAOs to produce H_2O_2 during the oxidation of L-amino acids is also by some microorganism to gain a selective advantage. For example, *Streptococcus oligofermentans* was proposed to use this enzyme to outcompete other microorganisms in the oral cavity [83].

2.4.2.3 L-amino acid oxidase from *Rhodococcus opacus* (RoLAAO)

R. opacus strain DSM 43250 is one of the best microbial LAAO producers. RoLAAO is purified from the native source by two ion exchange chromatography steps, separated by a hydrophobic interaction chromatography; this protocol allows to recover about 68% of the enzymatic units with, with a purification factor of 144. RoLAAO specific activity is about 4.6 U per mg of protein on L-Ala [79]. This flavoprotein is homodimeric in solution, with a molecular mass of 54 kDa for the monomer and has an isoelectric point of 4.8. Each protein monomer possesses a N-terminal signal peptide of 45 amino acids which is cleaved in the mature form of RoLAAO; in spite of the presence of this putative secretion sequence the mature protein was detected only in the cell cytoplasm [79]. The protein stability at 37 °C is 20-fold enhanced when the protein is incubated in a glycine/NaOH buffer, but in these conditions the specific activity on L-Ala was significantly decreased because of competitive inhibition by glycine. Interestingly, the storage of RoLAAO frozen at -20 °C completely inactivates the enzyme [79].

To date, RoLAAO possesses the broadest substrate specificity among known L-amino acid oxidases (Fig. 7).

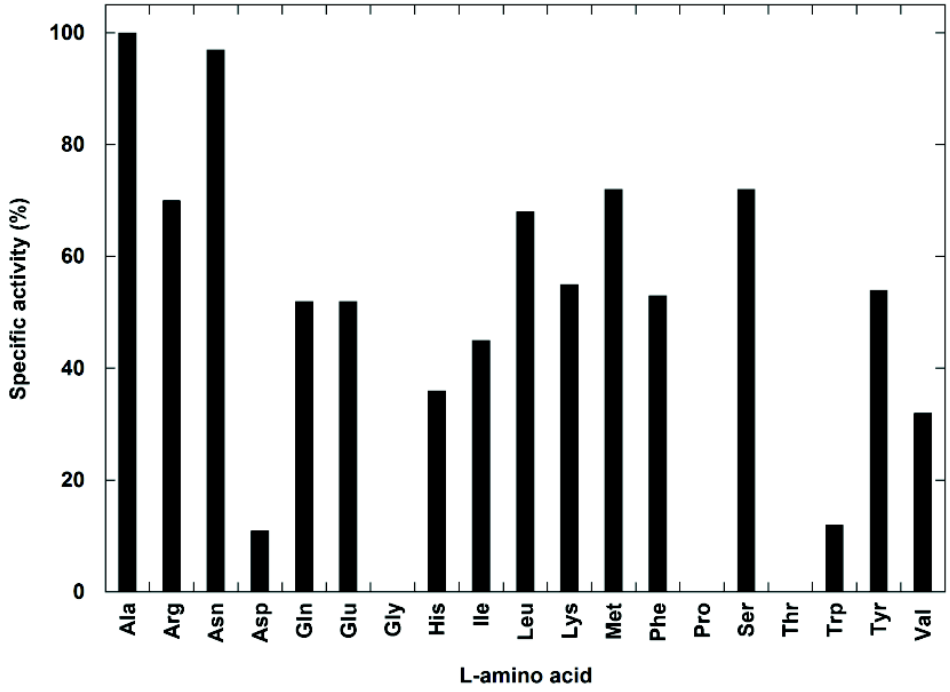

Figure 7. Comparison of RoLAAO specific activity versus the 20 proteinogenic L-amino acids.

This flavoenzyme is active on 39 different L-amino acids, including 17 of the 20 proteinogenic ones (the only exceptions being L-Thr, L-Pro and Gly). RoLAAO preferred substrates are aliphatic, aromatic and basic amino acids, namely L-alanine, L-1-naphtyl alanine and L-ornithine; for several of these substrate a K_m in the order of 10^{-6} M was calculated. RoLAAO is strictly stereoselective: in fact, in it is not able to oxidize D-amino acids. Given the very wide substrate specificity, RoLAAO turned out to be very interesting for biotechnological applications such as the resolution of racemic mixtures: preliminary trials were performed on D,L-Leu and D,L-Phe mixtures (in the presence of an excess of catalase to prevent inactivation of the enzyme by hydrogen peroxide). In both cases an excellent enantiomeric excess was obtained (>99.2% and >99.5%, respectively) [79].

The three-dimensional structure of RoLAAO has been determined at 1.4 Å resolution (Fig. 8).

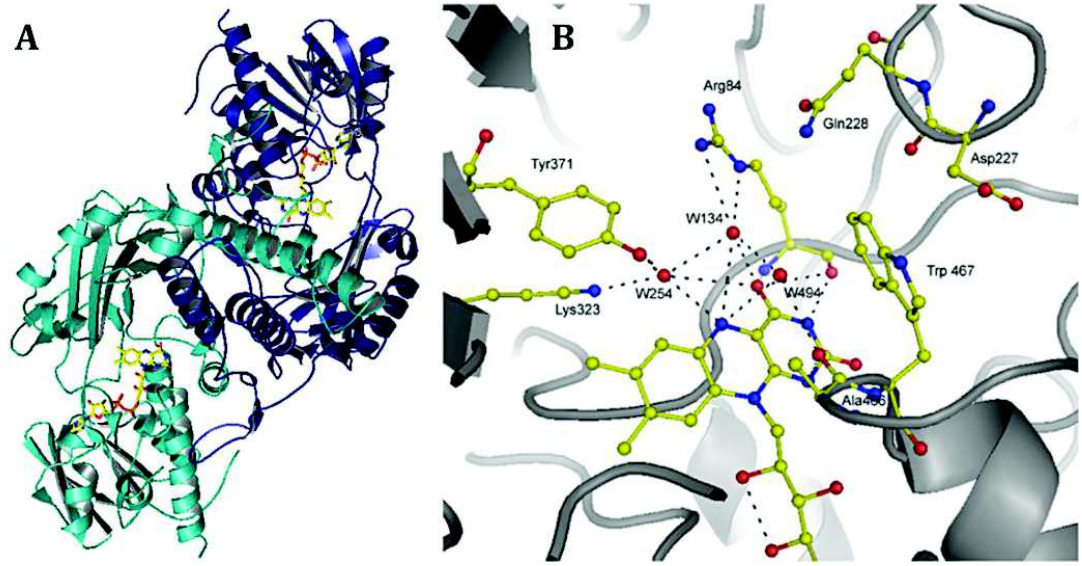

Figure 8. 3D structure of RoLAAO. A) Ribbon representation of the dimer of LAAO from *R. opacus*. The monomers are coloured in blue and cyan. The FAD molecules are shown as stick models. B) The active site of RoLAAO in binary complex with the FAD, shown as ball-and-stick models: carbon atoms are in yellow, nitrogen in dark blue and oxygen in red. Water molecules are shown as red spheres, H-bonds as dotted lines [84].

The general fold of RoLAAO is similar to the one of CrLAAO. It is a member of the gluthathione reductase (GR2) subfamily. Each monomer is composed of three domains: an FAD-binding domain which contains the Rossmann fold motif, a substrate binding domain and a helical domain, mainly responsible for protein dimerization.

One main difference with the LAAO from *C. rhodostoma* is the lack, in RoLAAO, of a narrow active site funnel; instead, the active site is exposed to the solvent and the substrate entry is limited only by diffusion, also explaining its broader substrate specificity [84].

Furthermore, the 3D structure of the complex between RoLAAO and the substrate L-Ala provided further evidence on the general

catalytic mechanism of L-amino acid oxidases: in fact, the proximity of the α carbon atom of the substrate to the FAD N(5) atom, and the absence of a functional group that could work in acid-base catalysis, clearly point towards a hydride transfer mechanism [84].

Attempts to overexpress RoLAAO in *E. coli* led to accumulation in inclusion bodies [85]. On the other hand, RoLAAO was successfully expressed in the actinomycete *Streptomyces lividans*, a microorganism evolutionarily close to the native strain *R. opacus*. The heterologous protein possesses a slightly better specific activity versus L-Ala than the native one (5.4 U per mg of protein), but the purification yield was lower: only 27 % of the enzymatic units could be recovered from the crude extract. Native and recombinant RoLAAO possess the same molecular mass and biochemical and spectral properties. Interestingly, RoLAAO is purified from *S. lividans* cultures in two different mature forms, with the deletion of 38 or 44 aminoacids at the N-terminus, respectively; this has been proposed to be due to the presence of different signal peptidases system of *S. lividans* [85].

2.4.2.4 Aminoacetone oxidase from *Streptococcus oligofermentans* (SoAAO)

The main strategy employed by *S. oligofermentans* to outcompete other *Streptococcus* species, such as *Streptococcus mutans*, is the production of H_2O_2 through oxidation of lactate catalyzed by lactate oxidase (lox). *S. oligofermentans* lox^- mutants, though, still retained the ability to inhibit *S. mutans* growth. The product of the

aao_{So} gene, an L-amino acid oxidase (SoAAO), was proposed to be responsible for this behaviour. In fact the Δaao_{So} mutant is not anymore able to outcompete *S. mutans* in interpecies antagonism experiments (Fig. 9) [83].

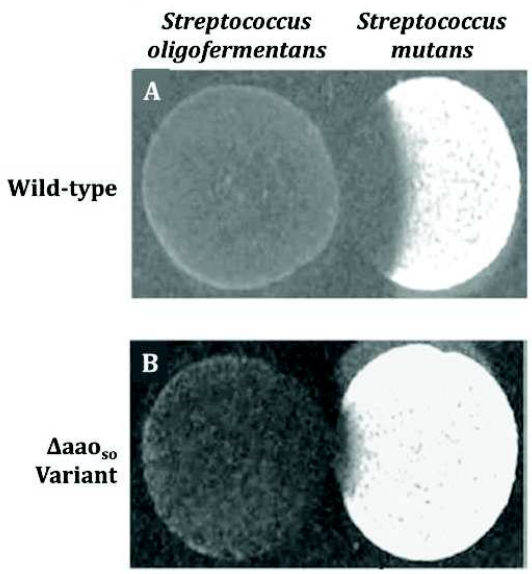

Figure 9. Growth inhibition of *Streptococcus mutans* by *Streptococcus oligofermentans*. 10 µl of overnight culture of *S. oligofermentans* and *S. mutans* at the same OD_{600} were spotted adjacently on peptone-present TPYG agar plates and incubated at 37°C for 24 hours. A) Inhibition of *S. mutans* by wild type *S. oligofermentans*. (B) Inhibition of *S. mutans* by Δaao_{so} *S. oligofermentans* variant. Adapted from [81].

SoAAO was overexpressed as a recombinant protein in *E. coli* BL21(DE3)pLysS strain with the addition His6-tag sequence at the N-terminus to allow its purification by immobilized metal affinity chromatography. The molecular mass of the protein is 44.9 kDa. Production of H_2O_2 by SoAAO was observe only in the presence of L-Asp, L-Trp, L-Lys, L-Ile, L-Arg, L-Asn and L-Gln, while no H_2O_2 was detected in the presence of other proteinogenic L-amino acids or the derivates *N*-acetyl-L-cysteine and *cis*-4-hydroxyl-L-proline [83].

In a more recent study, the aaoSo gene was shown to constitute an operon with MutT, a gene encoding for the 8-oxo-dGTPase, an enzyme involved in the elimination of harmful oxidized nucleotides. Deletion of both aaoSo and MutT genes causes a

significant reduction in S. oligofermentans growth rate, due to an increase in the concentration of reactive oxygen species in the cell, confirming that this operon might constitute a safeguard mechanism protecting cells from ROS damage in S. oligofermentans [86].

Since aminoacetone can be considered an analog of amino acids, the activity of SoAAO was tested on this compound: indeed, by using 25 mM of aminoacetone, a specific activity of about 48 mU per mg of protein could be detected. In parallel, SoAAO activity versus L-amino acids such as L-aspartate and L-lysine was found to be about 20-fold lower than the value reported in the previous work, while no activity on L-glutamine could be detected. For this reason SoAAO was newly classified as an aminoacetone oxidase (AAO) and its physiological role was revised [87]. In fact, this enzyme is possibly involved in aminoacetone detoxification, a role that in mammals is carried out by semicarbazide-sensitive amine oxidase (SSAO) [88]. Aminoacetone is a pro-oxidant by-product of threonine metabolism, and is able to react rapidly with oxygen through a superoxide-mediated mechanism [87] (Fig. 10).

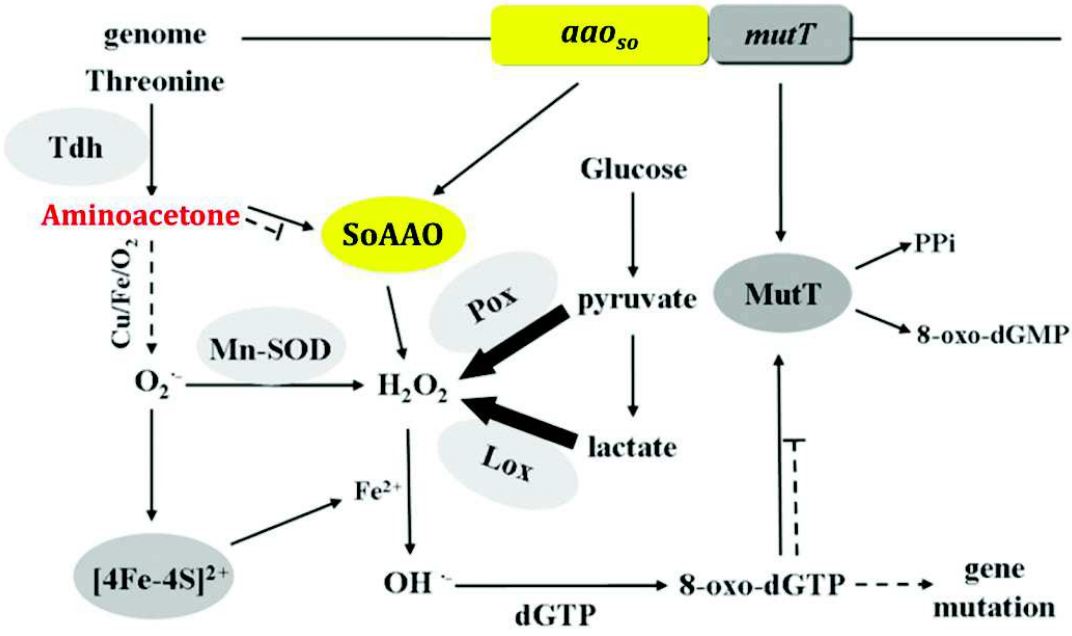

Figure 10. Metabolic pathway involving SoAAO and MutT in preventing oxidative damage to cells. The absence of SoAAO causes the accumulation of aminoacetone, which derives from threonine by threonine dehydrogenase(Tdh). It generates superoxide anions in the presence of oxygen and transition metals such as Fe^{2+} and the formation of hydroxyl radicals from H_2O_2. These hydroxyl radicals oxidize nucleic acids, like dGTP to 8-oxo-dGTP, which is hydrolized by MutT to the harmless 8-oxo-dGMP, and so prevents cell damage. Adapted from [86].

2.4.2.5 L-amino acid deaminase from *Proteus myxofaciens* (PmaLAAD)

L-amino acid deaminase is an enzyme that catalyze the oxidative deamination of L-amino acids without the production hydrogen peroxide.

Previous reports showed that the genome of microorganisms belonging to genus *Proteus* contains two separate L-amino acid deaminase genes [61, 62]. The enzymes coded by these two genes differ in their substrate specificity: one enzyme, termed PmaLAAD, prevalently deaminates a wide range of aliphatic and aromatic amino acids, whereas the second one, Pm1LAAD, shows activity on

a narrower range of amino acids, mainly basic ones such as L-arginine and L-histidine [89].

In 2001, the gene encoding for the LAAD from *Proteus myxofaciens* was cloned and overexpressed [90]. PmaLAAD is a 474 amino acids protein, with a molecular mass of about 51 kDa.

Like canonical amino acid oxidases, the enzyme requires oxygen for enzymatic activity and produces both NH_4^+ and phenylpyruvate (when L-Phe is used as the substrate) (Fig. 11). Differently from canonical LAAOs, the enzyme does not produce H_2O_2 [90].

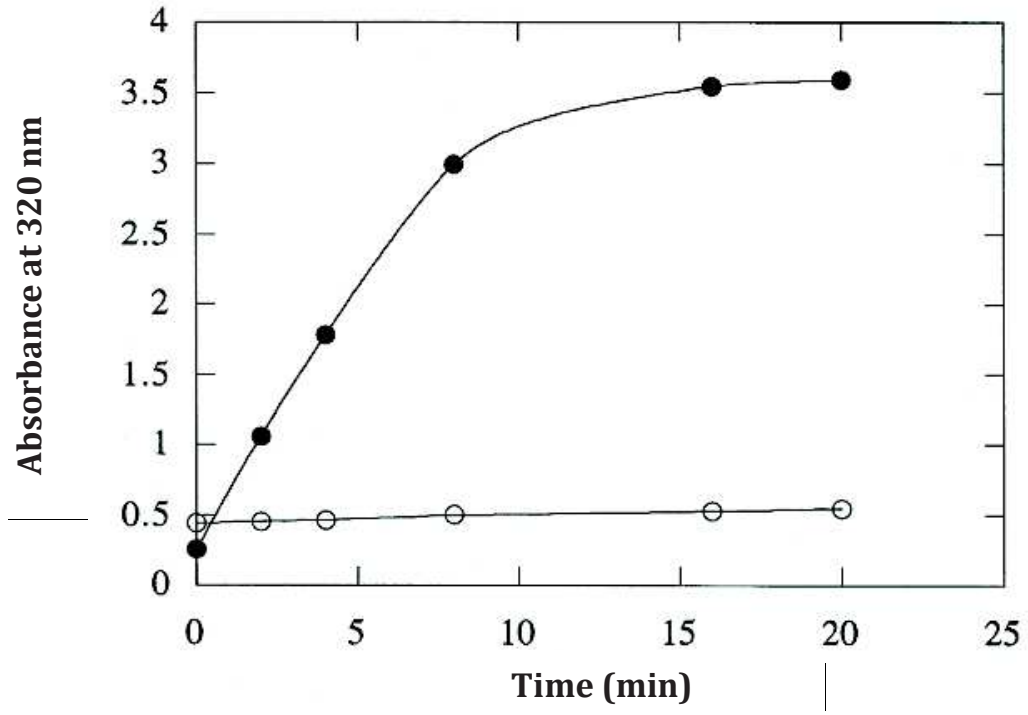

Figure 11. Dependence of the activity of PmaLAAD from O_2. The rate of phenylpyruvate formation from L-phenylalanine, monitored as the increase of absorbance at 320 nm; reactions were performed in the presence (filled circles) or in the absence (empty circles) of O_2 [90].

PmaLAAD is active on most of the 20 proteinogenic L-amino acids. Large apolar amino acids such as L-Phe, L-Met, L-Leu, L-Phe and L-Trp are the preferred substrates. In addition, marginal activity was measured also for the D-enantiomer of some of the substrates [90].

The enzymatic activity of PmaLAAD (on L-Phe as a substrate) is highest at pH 7.5 a pH optimum similar to the one determined for other deaminases from *Proteus* species. The K_m for L-Phe is 2.28 mM with a V_{max} of 0.26 U per mg of protein. This latter value is about 8-fold lower than the one reported for the LAAD from *Proteus rettgeri* (about 2 U/mg of protein) [61]. This discrepancy was related to a different grade of purity of the enzymes under investigations. In fact, LAAD is a membrane protein; computational analysis predicted that PmaLAAD is a type II membrane bound protein. Accordingly, following ultracentrifugation of the crude extract all PmaLAAD activity was retained in the membrane fraction (pellet). This centrifugation step resulted into a 3.4-fold purification of the protein [90].

In the last few years L-amino acid deaminases are gaining increasing interest for their application in biocatalytic processes, especially for the production of α-keto acids. In particular, the enzyme from *Proteus vulgaris* (active on apolar amino acids) has been recently employed in the production of two different compounds. In the first application, LAAD was employed for the production of α-keto-γ-methylthiobutyric acid (KMTB) from L-Met. This compound is gaining increasing interest in the pharmaceutical industry for the treatment of colon cancer [91]. In a laboratory scale process, 70 g/L of L-Met were incubated with 20 g/L of the whole-cell biocatalyst; the maximum yield (49.2 g/L of KMTB) was obtained after 20 hours of reaction at 40 °C and at pH 8.0 [92]. The same LAAD has also been employed for the production of α-ketoisocaproate (KIC) from L-Leu. This compound

could be used in the therapy for chronic kidney disease and hepatitis B virus infection, providing patients with their daily requirement of L-Leu [93]. A 97.8 % conversion of L-Leu to KIC was obtained by incubating 13.1 g/L of L-Leu with 0.8 g/L of whole-cell biocatalyst for 16 hours at 35 °C and at pH 7.5. The yield of KIC was 12.7 g/L [94].

The LAAD from *Proteus mirabilis* (Pm1LAAD), which is active on charged L-amino acids was also employed for the production of α-keto acids. The enzyme has been used in the whole-cell biocatalyst form for the production of α-ketoglutarate (KG), a compound that can be used as a building block for the synthesis of N-heterocyclic compounds possessing antitumor activity [95], as an antioxidant or to enhance wound healing [96]. A yield of 4.65 g/L of α-KG was obtained from 15 g/L of L-Glu, after 24 hours of reaction at 40 °C and at pH 8.0 [97]. Furthermore Pm1LAAD was recently employed for the production of phenylpyruvic acid (PPA) from L-Phe. This compound is widely used in the pharmaceutical, food, and chemical industry [98]. In this latter case, a comparison between whole-cell and enzymatic processes was performed. The performances of the two systems were similar: the process which employed the isolated enzyme allowed the production of 2.6 g/L of PPA (corresponding to a 87 % conversion) by incubation of 3 g/L of L-Phe with 0.2 g/L of Pm1LAAD for 5 hours at 35 °C and at pH 7.0, while whne the whole-cell biocatalyst was used, 3.3 g/L of PPA were obtained from 4 g/L of L-Phe (82.5 % conversion) after 6 hours of reaction at 40 °C and at pH 7.4employing 1.2 g/L of cells. In general, the isolated recombinant Pm1LAAD showed few

advantages in comparison to the whole-cell catalysts, because of the absence of side-reactions and because the substrate can interact more easily with the enzyme without the need to diffuse through the cell membrane. On the other hand, the whole-cell catalyst showed a better thermal stability (Hou et al., 2015).

3. Biocatalysis for the production of optically pure compounds

The biological activity of a compound often depends on the configuration of its chiral centers, because usually the active site of an enzyme (or a receptor) is strictly enantioselective [100]. It follows that the employment of enantiomerically pure chiral compounds could allow the employment of lower dosages, resulting, therefore, to fewer side effects and improved efficacy.

The market for chiral raw materials used as chemical building blocks or synthetic intermediates currently stands at $15 billion, with the pharmaceutical industry accounting for the major part of this value: about 80% of the active compounds that pharmaceutical companies have in the pipeline are chiral. Sales of optically active intermediates are increasing of 7–8% annually, making the production of such compounds a rapidly growing area in the fine chemical industry. This, in turn, caused the transformation of biocatalysis from a niche technology to a widely used manufacturing method, with the consequent increase of the demand for biocatalysts (especially for the industrial-scale manufacture of chiral compounds) [101, 102].

The methods for the preparation of enantiomerically enriched compounds are classified into two main categories:

asymmetrization of prochiral compounds and optical resolution of racemates. Kinetic resolution belongs to the latter category; it is a versatile process, already well-established, to prepare optically active compounds, but the inherent limitation of this approach of a maximum 50% yield represents an important obstacle for their use in manufacturing processes. In addition, also the separation of the product from the unreacted starting material is inevitable and could be laborious [100].

3.1 Dinamic kinetic resolution

Dynamic kinetic resolution (DKR) can overcome the limitations of standard kinetic resolution. This method involves the combination of an enantioselective transformation with an *in situ* racemization process at the equilibrium. As a consequence, as the reacting enantiomer is depleted by the enantioselective reaction, the racemization equilibrium of the two substrates is constantly shifted (Fig. 12). The racemization step be either enzymatic or non-enzymatic. Both enantiomers of the starting racemic mixture can be converted to the desired product with 100 % yield and very high enantiomeric excess giving an important advantage for industrial applications [103].

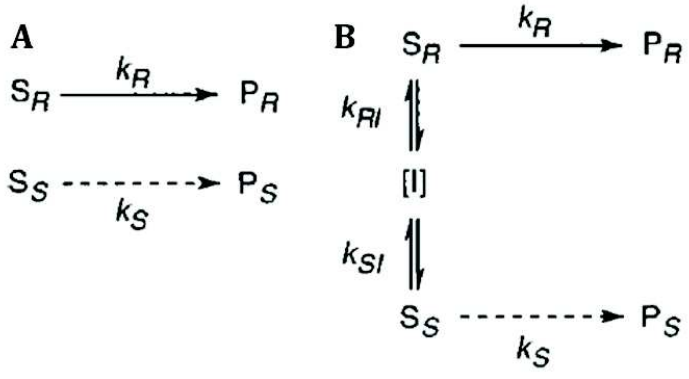

Figure 12. General scheme of A) kinetic resolution B) dynamic kinetic resolution. S_R, S_S = substrate enantiomers; P_R, P_S = product enantiomers; [I] = achiral transition state of the racemization k_R, k_S = rate constants; $k_R >> k_S$, preferably irreversible [104].

3.2 Deracemization

During deracemization a racemate is converted into a non-racemic product with 100% theoretical yield without intermediate separation of materials [104]. In this process one enantiomer of a racemate can be converted to the other via one or more reactions. In this way a racemic mixture can be transformed to an optically pure solution without any net change in the composition of the molecule [100]. An attractive feature of this process is the simplicity of the system, due to the fact that protecting groups are not required and because of the absence of product inhibition [103]. Deracemization reactions usually involve oxidation-reduction processes. In the first step, one enantiomer is enantioselectively oxidized to form an achiral intermediate, which subsequently is non-selectively reduced to give again the starting mixture in racemic form (Fig. 13).

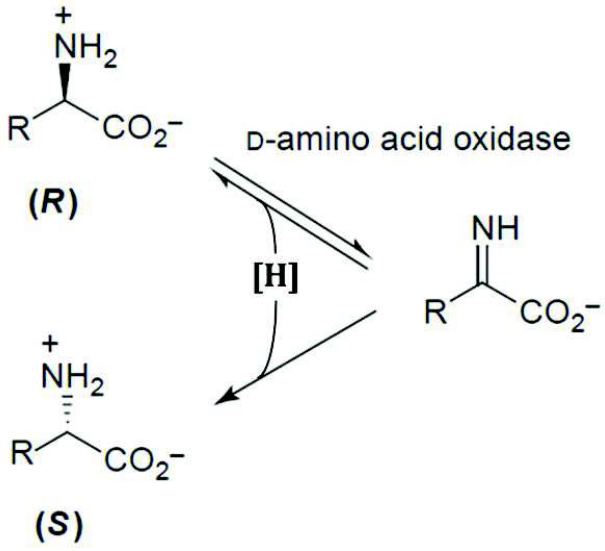

Figure 13. Scheme of deracemisation of racemic α-amino acid solution, by combining an enantioselective amino acid oxidase (e.g. DAAO) with a non-selective chemical reducing agent adapted from [101].

Cyclic repetition of this sequence of reactions leads to an overall chiral inversion of the reacting enantiomer to yield the mirror-image counterpart as the final product in a 100% theoretical chemical and optical yield. Clearly, since this system requires two reactions working simultaneously into opposite directions (an oxidation and a reduction) its feasibility relies mainly on their compatibility in a single reactor [104].

3.3 D-amino acids

D-amino acids (D-AAs) are rare in nature; they are present in peptide antibiotics synthesized by bacteria and fungi, including gramicidin D (D-Leu and D-Val). D-amino acids such as D-Ala, D-Asp, and D-Glu, are present in the milk of ruminants and in many fermented foods such as cheese, wine, yogurt, and fish products, where they can be derived by microorganisms during fermentation [105]. Furthermore in the human brain D-serine and D-aspartate have different roles in the development and functionality of the central nervous system (e.g. modulating the function of N-methyl-D-aspartate receptors) [106]. In 2011, the Global Industry Analyst, Inc., released a research report on the global market of D-AAs predicting that the global market for D-AAs will reach 3.7 billion dollars by 2017. D-amino acids are commonly employed in the pharmaceutical industry, food industry, and cosmetics. For example, they are used in the synthesis of semi-synthetic antibiotics, including traditional and new penicillin- and cephalosporin-derived antibiotics (i.e. D-Phe is used for the production of ampicillin and amoxicillin), giving them a longer

half-life. Additionally, D-AAs can enhance the activity of antibiotics against biofilms of clinical wound [105]. Interestingly several reports show that peptide inhibitors containing D-amino acids interfere with the aggregation of amyloid-β peptides, which are the major components of the plaque found in the brains of Alzheimer's disease patients. Also free D-amino acids are of great medicinal value: D-phenylalanine, in fact, could be used to treat pain, depression and Parkinson's disease [107], while D-proline derivatives could be used for the treatment of diseases such as Alzheimer's disease, diabetes mellitus, familial amyloid polyneuropathy, scrapie, and Kreuzfeld-Jacob disease [108]. In addition, unnatural D-amino acids now play an increasingly significant role in the development of synthesis protocols for pharmaceutical applications (for example as resolving agents) [109]. D-amino acids are also used as intermediates for the production of nutritional compounds (i.e. D-Ala is used for sweetener alitame) [110]. Given the high commercial importance of amino acids, recently a lot of efforts have been put in the development of processes for the production of these molecules [102].

3.3.1 Production of optically pure D-amino acids

The only efficient methods to produce enantiomerically pure D-amino acids are either chemical synthesis or biocatalysis.

The biocatalytic synthesis of D-amino acids can be performed by employing a number of different enzymes: oxidoreductases, aminotransferases, hydrolases, dehydrogenases. These enzymes

can act on different starting compounds, such as racemic mixtures of D,L-amino acid (also comprising N-acyl-D,L-amino acids), synthetic intermediates, such as D,L-hydantoin or D,L-amino acid amides, and prochiral substrates, such as α-keto acids [105].

i) One of the simplest strategies to synthesize D-amino acids from a racemic mixture is the employment of L-amino acid oxidase. The achiral imine produced by LAAO can be subsequently reduced by a non-enzymatic non-selective chemical reagent. Since only the L-enantiomer of the racemic mixture is utilized by the oxidase, at the end of the reaction the accumulation of the D-enantiomer is obtained. Theoretically, using a highly enantioselective LAAO, only 7 cycles are required to achieve an enantiomeric excess of > 99% [103, 111]. For example LAAO from *Rhodococcus* species AIU z-35-1 was employed for the oxidation of several L-amino acids (e.g. L-Glu, L-Phe, L-Lys, L-Leu, L-His L-Gln, L-Arg L-citrulline and L-homoserine). In a "model" reaction, 50 mM L-amino acid solutions were fully converted into optically pure D-amino acids in 24 h at 30 °C and pH 7.0, using 45 mU/ml of enzyme [112]. For the resolution of racemic mixtures of D,L-aspartic acid, a specific enzyme, L-aspartate oxidase from the thermophilic archaea *Sulfolobus tokodaii*, was employed; this enzyme possesses a high thermal and pH stability and shows no product inhibition under bioconversion conditions. In a typical reaction, a 50 mM D,L-Asp solution was quantitatively converted in 24 h at 37 °C and at pH 10, with an enantiomeric excess higher than 99.5 %, using a relatively low amount of enzyme (300 mU/mL) [113] (Fig. 14).

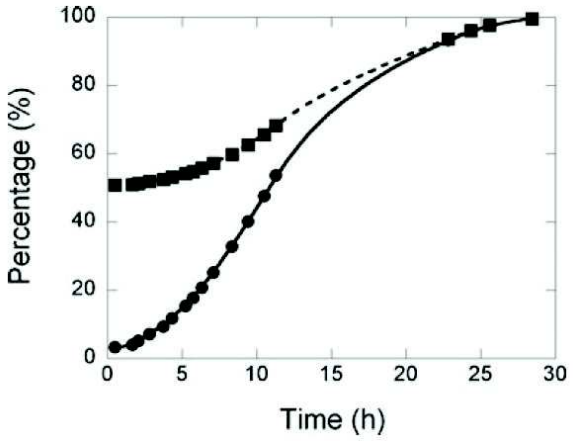

Figure 14. Time course of reaction of StLASPO with a D,L-aspartate mixture. Filled circle: conversion of L-aspartate. Filled square: enantiomeric excess. At different time points, 50 μL aliquots was taken from the reaction reservoir, derivatized with the OPA-NAC reagent, and analyzed by HPLC [113].

Further developments in the development of novel deracemization include the stereoinversion of beta and gamma substituted amino acids, in which the diastereoisomers of the substrate are interconverted [114].

ii) Enantioselective biotransformations are also possible with amino acid dehydrogenases. D-amino acid dehydrogenases are NADPH-dependent oxidoreductases, which can catalyze the asymmetric reductive amination of α-keto acids (using ammonium as a substrate) to form their corresponding D-amino acids [115].

iii) Also aminotransferases have been used for the production of enantiomerically pure D-amino acids. These enzymes produce α-amino acids from α-keto acids, using an amino donor such as L-Asp or L-Glu. Although they can achieve a theoretical 100% yield the actual outcome is typically lower, because the catalyzed reaction is at the equilibrium, unless the reaction product is removed from the reaction mixture. For example when L-Asp is employed as an amino donor, the generated oxaloacetate is decarboxylated to pyruvate, thereby shifting the equilibrium of the reaction in the desired direction [116].

iv) D-stereospecific amidohydrolases (e.g. N-acyl-D-amino acid amidohydrolase, D-amino acid amidase, D-aminopeptidase, and D-hydantoinases) can be used for resolution of racemic amino acid amides, which are inexpensive intermediates derived from chemical synthesis, to yield D-amino acids. These enzymes can be employed only in kinetic resolution processes, since they react only with the D-enantiomers of the substrates and thus the theoretical yield of the biocatalytic process is 50 % [117]. In addition D-hydantoinases can also be used for the production of non-natural amino acids. In this process D,L-5-substituted hydantoins as starting material, easily obtained by chemical synthesis, are first hydrolyzed into their carbamoyl derivatives by D-hydantoinase and then converted into corresponding D-amino acids through a decarbamoylation reaction catalyzed by D-carbamoylase [118].

3.4 Production and improvement of enzymes as biocatalysts

Enzymes can be employed in biocatalytic processes, either as components of metabolically inactive cells or as isolated proteins. Both the systems allow to exploit the strict enantioselectivity of the enzymes and usually allow to carry out processes under moderate temperature and pressure in aqueous media and are therefore ideal for the industrial manufacturing of chemicals. Purified enzymes can be used alone or as a part of reaction cascades; using this approach non-natural biocatalytic pathways can be easily assembled *in vitro* from purified enzymes and coenzymes [119]. The use of isolated enzymes allows an easier

monitoring and control of the reaction because of the direct substrate supply to the catalyst, without the necessity of substrate/product diffusion through the membrane when whole-cell systems are employed. In contrast, whole cells contain complex metabolic pathways that can interfere with the desired reaction (or series of reactions) and may suffer from reduced viability because of the potential toxicity of lab chemicals and/or reaction products [101, 120]. Currently a major inconvenience in the utilization of enzymes *in vitro* is their laborious and costly production, which often makes the biocatalytic processes unsustainable, from an economical point of view, beyond the laboratory scale. Nonetheless, the growing knowledge on the molecular biodiversity of enzymes (e.g. the exploitation of recombinant enzymes from thermophilic organisms) coupled with the development of modern techniques in molecular biology and in protein engineering will increase the possibility to produce effective, stable and affordable enzymes for biotechnological applications [120].

The "ideal" biocatalyst should possess the following properties: i) a broad substrate specificity ii) a high k_{cat} and a low K_m for its substrate (possibly in the millimolar range) iii) a good stability over a wide range of temperatures, pHs and organic solvents iv) a high enantioselectivity with a selectivity E value (i.e. the ratio between the specificity constants for the two enantiomers) of at least 50 [111]. However, enzymes identified in nature rarely possess all of these features, and frequently the *in vitro* improvement of a fine-tuned and optimized biocatalyst turns out

to be a necessary prerequisite for specific biotechnological utilization.

The strategy for the development of new catalysts is relatively simple and consists in coupling systematic alterations in the structure of the molecule (obtained by protein engineering), with effective methods for high-throughput screening of the produced variants for the desired properties (e.g., a higher turnover number or more strict enantioselectivity).

A typical enzyme consists of up to 1000 amino acids and thus the exhaustive variation of each of its amino acids is practically impossible since the number of possible variants that could, in principle, be produced exceeds even the potential of ultra-high-throughput screening methods. Moreover, usually it is not obvious which residue, or combinations of residues, is important for determining specific features of the enzyme. For this reason in the last few years a protein engineering approach called “directed evolution” has been established. This process mimics natural evolution *in vitro* in order to evolve enzymes possessing improved properties for biotechnological applications. In the first stage of directed evolution of an enzyme, mutations are randomly incorporated into the corresponding gene to generate a library of enzyme variants which are then screened for the improvement of specific functional or structural features (e.g., a higher activity or reduced K_m)[101, 111].

The advantage of this approach is that it does not require prior knowledge of the three-dimensional structure, or, in some cases, even of the sequence of the enzyme that has to be improved, since

the variants are selected simply by the possess of a specific (improved) feature (e.g. the ability to efficiently carry out a specific chemical reaction), without taking into account the structural determinants at the base of the observed changes. During the *in vitro* evolution processes, the high enantioselectivity of the native enzymes is usually retained, or even improved; in fact, variants of amino acid oxidases, screened primarily for an increase of their activity against a single enantiomer of the substrate, often retained also their high enantioselectivity [111].

Successful directed evolution projects rely on efficient and simple screening procedures. For this reason, this approach is especially suitable for enzymes that catalyze the production of colored products, easily detectable by means of colorimetric methods. For example, D- or L- amino acid oxidases produce hydrogen peroxide, which can be easily detected by a coupled spectrophotometric assay [102]. This makes the engineering of these enzymes (such as PmaLAAD) an ideal starting point for the generation of improved enzymatic variants by directed evolution approaches [103].

Aim of the work

In order to allow rapid adaptation to changing environmental conditions, such as the availability of different nutritional sources, organisms need to quickly develop new metabolic functions, which, in turn, require new enzymatic functions. Enzymatic promiscuity, that is the ability of enzymes to catalyze auxiliary reactions different from those for which they have evolved (and that are not part of the organism's physiology), plays a key role in the divergent evolution of novel enzymes from existing ones [2].

Enzymes belonging to the large family of flavoproteins share a remarkable degree of condition, substrate and catalytic promiscuity. This property allowed flavoproteins to rapidly evolve and specialize to catalyze a wide range of new and different reactions, which range from metabolic redox reactions, electron transport, gene expression regulation, signal transduction and light running [28]. Among flavoproteins, amino acid oxidases (AAOs) are a group of FAD containing enzymes that catalyze the oxidative deamination of amino acids. During evolution, AAOs evolved from a common ancestor to fulfill very different physiological roles (e.g. the catabolism of amino acids or the regulation of neuromodulators). This remarkable divergent evolution was obtained by the reshaping of the functional and structural features of these enzymes. Thus AAOs represent thus an ideal model to understand the mechanisms that originated molecular biodiversity in modern enzyme families. In addition, the strict enantioselectivity of these enzymes renders AAOs interesting biocatalysts for the development of processes for the production of enantiomerically pure amino acids [45, 46, 63].

D-amino acids are valuable compunds commonly used in semi-synthetic antibiotics synthesis, including traditional and new penicillin- and cephalosporin-derived antibiotics, and they can be exploited to enhance the activity of antibiotics against microbial biofilms in clinical wounds. Moreover several reports show that D-amino acid based peptide inhibitors interfere with the aggregation of amyloid-β peptides. In the food industry D-amino acids are used as intermediates in the production of nutritional compounds, such as D-alanine in the sweetener alitame [105].
Unfortunately, up to now the lack of overexpression of known LAAOs as recombinant proteins, prevented their use for the devolpment of processes for the preparation of pure D-amino acids.

The aim of this PhD project was the identification (through extensive literature and database research) and the characterization of novel microbial LAAOs possessing interesting characteristics, from a biochemical, structural and biotechnological point of view. The thorough investigation of these proteins will allow us to understand the structure/function relationships in this group of proteins, to clarify their physiological role and to obtain new insights on the underlying mechanisms of their molecular evolution. The information obtained from these studies will also allow to form a background for the structure-guided engineering of these proteins, in order to obtain a novel, improved biocatalyst.

Results

4. L-amino acid oxidase as biocatalyst: a dream too far?

Pollegioni, L., Motta, P., and Molla, G. Appl Microbiol Biotechnol, 2013. (21): p. 9323-41.

5. Aminoacetone oxidase from *Streptococcus oligofermentans* belongs to a new three-domain family of bacterial flavoproteins

Molla, G., Nardini, M., Motta, P., D'Arrigo, P., Panzeri, W., and Pollegioni, L. Biochem J, 2014. 464(3): p. 387-99

5.1 Supplementary material

6. Structure-Function Relationships in L-Amino Acid Deaminase, a Flavoprotein Belonging to a Novel Class of Biotechnologically Relevant Enzymes

Motta, P., Molla, G., Pollegioni, L., Nardini, M. Manuscript submitted

7. Expression of the L-amino acid oxidase from Rhodococcus opacus in Streptomyces, a novel heterologous expression system

Unpublished results