Structures of bacterial tyrosinases

Tyrosinases oxidise phenolic hydroxyl groups of small molecules or large polymeric substrates such as proteins. Tyrosinases catalyse first the *ortho* - hydroxylation of the phenolic substrate and second its subsequent oxidation to quinone (Figure 1) with the concomitant reduction of oxygen to water. The reaction is chromogenic as the quinones produced can undergo further non-enzymatic polymerisation to form black eu-melanins and, when reacting with thiol groups, brownish pheo-melanins [14]. This process can be inhibited by antioxidants such as ascorbic acid, for example to prevent the browning reaction in food preparations [15]. Tyrosinase activity is generally measured by either determining the consumption of oxygen during the reaction or spectrophotometrically by following the increase of absorbance at 475 nm due to dopachrome formation. The cross-linking activity of tyrosinase on proteins is usually analysed by SDS PAGE, size-exclusion chromatography, UV spectroscopy or mass spectrometry [16].

The active site of tyrosinases interacts with both the phenolic substrates and the co-substrate oxygen and it alternates among three different oxidation states. When in the *oxy* state, tyrosinase binds oxygen and is able to catalyse the hydroxylation of monophenols to diphenols, thus changing into the *met* form. The *met* form of tyrosinase is responsible for the oxidation of diphenols to quinones and the reaction turns the enzyme into the *deoxy* form that, upon binding molecular oxygen, returns to the *oxy* form. The *met* form is the resting state of the enzyme and it has been calculated that up to 85% of the enzyme is in this state when in solution [17, 18]. The inability of most of the enzymes in an enzyme population to act on monophenols

explains why a significant lag phase is detected in the activity when monophenols are the substrate of the reaction.

Bacterial tyrosinases have been divided in five types according to the organisation of domains and the possible requirement of a caddie protein for enzyme activity [12]. The necessity of a secondary helper protein (caddie protein) for secretion, correct folding, assembly of the copper atoms and activity of the enzyme is common to tyrosinases of type I, e. g. the enzyme from *S. castaneoglobisporus* and *S. antibioticus* [19, 20]. Type II tyrosinases are small, monomeric enzymes containing only the catalytic domain, which do not require additional helper proteins and are possibly secreted. An example is the tyrosinase from *B. megaterium* [6]. Type III tyrosinases are represented by the enzyme from *Verrucomicrobium spinosum* . Like the fungal tyrosinases it carries a C-terminal domain whose removal led to about 100-fold higher activity [21]. This supports the theory that the role of the C-terminal extension in plant and fungal tyrosinases is to keep the enzyme in an inactive form inside the cell [22-24]. Among the smallest bacterial tyrosinases reported (Type IV) are the ones produced by *Streptomyces nigrifaciens* (18 kDa) and *Bacillus thuringiensis* (14 kDa) [25, 26]. However, it is debated whether these proteins are true tyrosinases [12]. Type V tyrosinases include enzymes that do not carry the sequence features of tyrosinases but show features typical of laccase and have only marginal activity on tyrosine. For example, a membrane-bound tyrosinase active on the typical laccase substrate ABTS (NCBI ID: AAF75831. 2) has been isolated from *Marinomonas mediterranea* . A tyrosinase with a classical substrate

specificity that is activated by SDS (NCBI ID: AAV49996. 1) has also been reported from the same organism [27].

Similar to catechol oxidases and the oxygen carrying haemocyanins, tyrosinases are type-3 copper proteins, containing two copper atoms in the active site. The absorbance spectrum of *oxy* -tyrosinases has a characteristic maximum in the UV region (330-345 nm). As reported for the structurally similar catechol oxidases, a fluorescence intensity maximum at 330 nm upon excitation at 280 nm is also detected [28, 29]. Copper is essential for the catalytic activity of tyrosinases. The crystal structure of these enzymes has demonstrated the presence of two copper ions in the catalytic core (Table 1). In all tyrosinases of different origins and in the haemocyanins each of the copper ions is coordinated by three histidine residues that are found in a characteristic pattern in the primary structure (Figure 2). In the tyrosinase from *Streptomyces glaucescens* , for example, the key role of histidines at position 37, 53, 62, 189, 193 and 215 in the coordination of copper, and thus in catalytic activity, was confirmed by the decrease of activity upon their substitution with other amino acids [30, 31].

Various additional residues have been identified to have a function in fungal and bacterial tyrosinases, either being essential for or modulating tyrosinase activity. Sequence analysis and various mutagenesis studies have been performed in order to identify the residues necessary for the activity of the enzyme. In tyrosinase sequences from plants and fungi, the N-terminal signal peptide, when present, is followed by a conserved arginine residue that marks the beginning of the central catalytic domain and that forms a *pi* -cation interaction with a conserved C-terminal Y/FXY tyrosine motif, where X

is any amino acid [32]. These residues are conserved also in bacterial tyrosinases (Supplementary file 1). Substitution of the N-terminal conserved arginine (R40) has been reported to abolish the production of tyrosinase from *V. spinosum* [21]. Two single-amino acid substitutions have been reported to improve the catalytic activity of the tyrosinase from *Rhizobium etli* CFN42. The independent replacement of proline at position 334 and of aspartic acid at position 535 (Supplementary file 1) with a smaller residue such as serine (P334S) or glycine (D535G), respectively, led to a significant enhancement of the catalytic activity and melanin formation [33-35]. In the tyrosinase from *B. megaterium* , a single substitution of arginine by histidine within the copper B binding region (R209H) has been sufficient for a 1. 7-fold improvement of the activity towards tyrosine (monophenolase) and for a 1. 5-fold reduction of activity on L-DOPA (diphenolase), whereby the overall protein stability was not affected [36]. The crystal structure of the tyrosinase from *B. megaterium* showed that this arginine is positioned at the entrance of the active site in a flexible position and plays a role in the docking of the substrate [6]. However, the conservative substitution of the corresponding residue asparagine 190 to glutamine (N190Q) in *S. glaucescens* tyrosinase abolished the catalytic activity, indicating that this residue was possibly involved in hydrogen bonding at the active site [30]. Moreover, the conservative substitution of the residue aspartic acid 209 (D209E) has been reported to stabilise the oxy-form of the same enzyme [37]. To our knowledge, no study has investigated the role of the oxygen binding motif PYWDW [38] with regards to the affinity for oxygen in tyrosinase. The affinity for the co-substrate oxygen has been evaluated for the tyrosinase from *Streptomyces antibioticus* that carries the PYWDW motif. It was found that

this enzyme had a three-fold lower dissociation constant (k_D) for oxygen than the *A. bisporus* tyrosinase [39, 40] that carries a PFWDW motif, i. e. 16. 5 μM compared to 46. 6 μM. The analysis of the characterised bacterial tyrosinases evidenced the presence of functionally active variants of this motif (Supplementary file 1 and 2), e. g. PYWNY in the tyrosinase from *M. mediterranea* , PFWDW in tyrosinase from *R. etli,* PYWEW in the tyrosinase from *B. megaterium* , PYWRF and PYWNW in the tyrosinases from *Ralstonia solanacearum* . Mutational studies have also addressed the interaction of tyrosinases from streptomycetes and their caddie protein. In *S. antibioticus,* the two histidine residues at positions 102 and 117 of the caddie protein MelC1 have been found to be crucial for the biosynthesis of active tyrosinase [41].

The available crystal structures of bacterial tyrosinases and their mutant forms have been obtained from Gram-positive *S. castaneoglobisporus* and *B. megaterium* (Table 1). While the *B. megaterium* tyrosinase formed crystals containing only the enzyme, the *S. castaneoglobisporus* tyrosinase required the presence of a second protein, referred to as caddie protein, to stabilise its structure [4]. Moreover, the structure of the *Streptomyces* tyrosinase has been solved in different states of oxidation. Aiming at understanding the interaction between tyrosinase and caddie protein, tyrosinase has been crystallised in the presence of mutant forms of the caddie protein (Table 1). Likewise, the fungal tyrosinase from *A. bisporus* was crystallised as a tetramer in a complex with a second protein, a lectin-like protein [7].

Both intracellular and secreted bacterial tyrosinases have been isolated and characterised. For example, the tyrosinases from *Streptomyces nigrifaciens,*

Bacillus thuringiensis, M. mediterranea, R. solanacearum and Thermomicrobium roseum were isolated from cell biomass and the ones from *S. antibioticus, S. glaucescens, S. castaneoglobisporus, Streptomyces albus, B. megaterium, Sinorhizobium meliloti, Aeromonas media, R. etli* and *V. spinosum* were either isolated from the culture medium or predicted to be secreted [19, 21, 25, 26, 42-51]. The twin-arginine signal peptide is often found in cofactor-binding oxidoreductases that undergo complete folding in the cytoplasm prior to secretion to the periplasmic or extracellular space. Twin-arginine type signal peptides [52] could be identified in the N-terminal region of tyrosinases from *R. solanacearum* (34-amino acid long) and *V. spinosum* (33-amino acid long). A more detailed analysis of the sequence retrieved for the tyrosinase from *R. etli* and the alignment with the other sequences of tyrosinases (Supplementary File 1) suggests the possibility of incorrect open reading frame prediction. The true N-terminal methionine may be M112 (underlined in Supplementary file 1) as it aligns with the initial residue of the tyrosinase from *R. solanacearum* (number 15 in Supplementary file 1) and is followed by a predicted twin-arginine signal peptide of 31 amino acids [51]. Thus, we suggest that these proteins purified from the cell biomass but carrying a signal peptide for secretion are localised in the periplasm.

Tyrosinases, also from bacteria, and their caddie proteins generally lack conserved cysteine residues (for comments see [11, 12]). The paucity of cysteine residues, and thus disulphide bonds, allowed, however, the isolation of tyrosinases with significant thermal stability, e. g. the enzyme from *B. megaterium* had an optimum temperature of 50°C [48]. A single cysteine

residue is conserved in proximity of the second histidine residue of the copper A binding motif in the characterised tyrosinases from *M. mediterranea* , *R. solanacearum* , *S. meliloti* , *R. etli* and *V. spinosum* (Supplementary file 1). A cysteine residue at this position has been found to be covalently bound to a histidine residue two positions forward in, for example, the fungal tyrosinase from *Neurospora crassa* [53], the plant catechol oxidase from *I. batata* [8] and haemocyanins from the snail *Helix pomatia* [54] . The function of this unusual cysteine-histidine bond is not established, but it could confer structural rigidity to the copper-binding region and affect the redox potential [8]. Replacement of this cysteine residue (C84) with serine abolished the production of the tyrosinase from *V. spinosum* [21]. Type-3 copper proteins carrying six conserved cysteines (forming three *in silico* predicted disulphide bonds) and characterised by significant thermal stability have been reported in fungi [26]. No mutagenesis study has addressed a possible improvement of the thermal stability of bacterial tyrosinases by introducing disulphide bonds. However, *i n silico* analysis revealed the possible presence of one disulphide bond in the tyrosinases from *R. solanacearum* and *S. meliloti* and two in the enzymes from *M. mediterranea* and *R. etli* (Dianna software, http://clavius. bc. edu/~clotelab/DiANNA). The tyrosinase from *S. castaneoglobisporus* and the one from *B. megaterium* share approximately 30% sequence similarity with a catechol oxidase from *Aspergillus oryzae* that showed a melting temperature above 70°C and a half-life of 20 hours when incubated at 50°C [29].

It should be noted that the tyrosinase from *A. media* exhibits different sequence features when compared to the other enzymes. The sequence

alignment with bacterial tyrosinases shows that none of the typical signature motifs (copper A and B regions, oxygen binding motif and tyrosine motif) are present (see Supplementary file 1). Moreover, this enzyme has a predicted 23-amino acid long signal peptide [50] and shows strong sequence similarity to bacterial periplasmic proteins that are responsible for the uptake of peptides and involved in nutrition and sensing of the environment [55].