Crystal structure of Agaricus bisporus tyrosinase
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Summary

This thesis discusses the elucidation of the three-dimensional structure of tyrosinase from the mushroom *Agaricus bisporus* (champignon, button mushroom) by means of X-ray crystallography. Tyrosinase is a copper-containing enzyme that catalyzes the conversion of monophenols to *ortho*-diphenols and their subsequent conversion to *ortho*-quinone derivatives. The final product is the main precursor in the biosynthesis of melanin, a pigment that is widely distributed in nature and that is present in various organisms from all phyla. Therefore, the enzyme is commonly associated with pigmentation and pigment-related diseases or disorders in humans and animals, such as melanoma, albinism and vitiligo. In fruits and vegetables, tyrosinase is associated with the browning process that causes depreciation of the value of agricultural produce. A potent inhibitor of the enzyme’s activity is therefore highly desired to prevent this browning. Tyrosinase inhibition is also of interest for the cosmetics and pharmaceutical industry, i.e. for skin whitening and for treatment of post-inflammatory hyper-pigmentation due to burn or to other skin abrasion. The studies for these applications are mostly performed using the commercially available mushroom tyrosinase, which therefore has been studied extensively. However, the interpretation of the results is often hampered by the lack of knowledge of the enzyme’s three-dimensional structure. Therefore, the elucidation of its structure has become highly desirable, and several groups in the world have already attempted to determine its three-dimensional structure. Moreover, the availability of a three-dimensional structure will also allow rational, structure-based design of inhibitors.

However, the elucidation of the enzyme's 3D-structure has been hampered by difficulties in obtaining pure enzyme because of contamination from melanin and because mushroom tyrosinase appears to be heterogeneous. The sources for this heterogeneity conceivably originated from protein polymerization by quinones, purification procedures, posttranslational modifications, or enzyme sources. Later, it was discovered that at least four genes exist that encode the expression of mushroom tyrosinase, namely *ppo1, ppo2, ppo3,* and *ppo4*. This indicates that the heterogeneity of the enzyme may also originate from the presence of isoforms. These facts show that obtaining a pure tyrosinase for structural study is very challenging.
The molecular mass of mushroom tyrosinase (from commercial preparation or directly isolated from mushroom fruit bodies) upon analysis with various methods is consistently found to be ~120 kDa. However, the view of its quaternary structure has evolved from a homo-tetramer with ~30 kDa subunits to a hetero-tetramer consisting of two ~45 kDa (heavy, H) and two ~14 kDa (light, L) subunits. Furthermore, a peptide mass-spectrometry fingerprinting (PMF) experiment using commercial mushroom tyrosinase revealed that the heavy subunit of the commercial preparation would probably be PPO2. The identity of the light subunit, on the other hand, is unclear. It could be part of tyrosinase or a completely other, unrelated protein, which was proposed to be a lectin occurring in the mushroom. Thus, the identity of commercial mushroom tyrosinase, which is widely used in research, is not unambiguously established.

Because several ppo genes are now available, each mushroom tyrosinase isoform can be produced independently, and thus the problem with heterogeneity may be overcome. This was achieved by cloning the ppo2 gene into a bacterial system for overexpression of recombinant protein (chapter 2). Recombinant PPO2 with a histidine tag was produced in Escherichia coli for convenient purification using a nickel affinity chromatography column. However, the enzyme was found to be insoluble, forming protein aggregates called inclusion bodies. The inclusion bodies were solubilized by denaturation of the protein, and the PPO2 was then recovered via a refolding procedure whilst bound to the nickel affinity column. The refolded PPO2 was monomeric in solution with a molecular mass of 64 kDa, which is the size of ppo2 gene product. Spectroscopic analysis showed that the copper centre was present in the refolded enzyme. However, the refolded enzyme had a very low activity in comparison to both the commercial preparation and the enzyme isolated directly from mushroom. Moreover, no protein crystals were obtained using the refolded PPO2.

Following an alternative route, mushroom tyrosinase was successfully purified directly from the commercial preparation. Using enzyme material from this purification procedure, we could obtain mushroom tyrosinase crystals (chapter 3). Mushroom tyrosinase could only be crystallized at low ionic strength conditions. The crystals were obtained at room temperature with 8% polyethylene glycol in 10 mM sodium acetate buffer, pH 4.6 as crystallizing agent. The protein crystals grew in competition with aggregation and precipitation, and suffered from browning; harvesting the protein crystals was therefore also challenging. From a thermal stability analysis, we discovered that the stability of the enzyme was increased in the presence of calcium ions. Therefore, 10 mM calcium chloride was included in the purification buffer, but the calcium was removed prior to the crystallization experiments. Interestingly, the quality
of the crystals was improved when the enzyme was crystallized in the presence of holmium chloride. Although holmium is a calcium analogue, no crystal was obtained in the presence of calcium chloride. For this reason, 5 mM holmium chloride was added to the crystallization solution. The crystals were then subjected to X-ray diffraction experiments and diffraction data were collected. The enzyme had crystallized in two different spacegroups, \( P2_1 \) (monoclinic) and \( P2_12_12 \) (orthorhombic). The analysis of the content of the protein crystals showed that both the heavy and light subunits were present in both crystal forms. Moreover, when a crystal that had been exposed to X-ray radiation was dissolved, significant tyrosinase activity was observed. This indicates that we crystallized active enzyme.

The success with obtaining X-ray diffraction data from the crystals allowed us to take the next step in the structure elucidation of the enzyme (chapter 4). An initial structural model was generated employing the PPO2 amino acid sequence and the conserved parts of structures of PPO2 homologs, which are the mollusc hemocyanins from \( Octopus \) \( dofleini \) (giant octopus) and \( Rapana \) \( thaliana \) (sea snails), catechol oxidase from \( Ipomoea \) \( batatas \) (sweet potato), and the tyrosinase from \( Streptomyces \) \( castaneoglobisporus \) (bacterial tyrosinase). This initial model contained only part of the heavy subunit of the enzyme. The tyrosinase structure was built using both automatic model building programs and by hand. The data set from orthorhombic crystal was employed, because its resolution was higher. The model was regularly transferred to the monoclinic crystal data set. This practice beneficially reduces the presence of model bias because of the independency of the two data sets. Nevertheless, the model building and refinement did not progress because the PPO2 amino acid sequence did not fit to the electron density map at various places. When a higher resolution data set was obtained, the automatic model-building program ARP/wARP was employed to extend the structural model. We found that modifications had to be introduced to the PPO2 sequence. This modified sequence matched perfectly with the N-terminal region of the PPO3 sequence. The N-terminal amino acid sequence of PPO2, identified by PMF analysis, was very similar to that of PPO3 (figure 1), and since at the start of the modelling the PPO3 sequence was not yet known, the enzyme had been misidentified as PPO2. The structural model for the heavy subunit was then completed using the PPO3 sequence. The amino acid sequence derived from structural model of the light subunit did not fit to the amino acid sequence of PPO1, PPO2, PPO3, PPO4, the proposed mushroom lectin, or even any other protein in the public databases. This sequence was then submitted to the mushroom \( A. \) \( bisporus \) genome database, which is recently published. One clear hit was found and the model could be completed with the amino acid sequence of this protein, assigned as ORF239342. Thus, the elucidation of the
crystal structure of mushroom tyrosinase revealed the true identities of the mushroom tyrosinase H and L subunits, which had been unclear until then.

Figure 1. Alignment of the N–terminal regions of the PPO1 - PPO4 amino acid sequences. The box highlights the PPO2 fragment identified upon PFM analysis that is nearly identical to that of PPO3 and has the same mass. The numbering refers to the PPO3 sequence.

The overall structure of PPO3 is similar to the tyrosinase domain of mollusc hemocyanin, plant catechol oxidase, and bacterial tyrosinase. The tyrosinase domain is marked with an interaction between a strictly conserved arginine residue in the N–terminal region and a tyrosine or phenylalanine residue, part of the conserved Tyr/Phe-X-Tyr/Phe sequence motif, in the C–terminal domain. PPO3 structure, however, is 100-120 amino acid residues larger than the tyrosinase domain of its homologs. These additional residues reside mostly in the loops connecting the secondary structures. A binuclear copper centre resides in the heart of a bundle of four helices, which form the core of the enzyme. This copper centre is the catalytic centre of the enzyme; it is located in a spacious cavity on the surface of the protein, making it readily accessible from the solvent. The tyrosinase domain of PPO3 is followed by a 27 residues long extension that contains two α-helices, which are kept close to the core region via hydrogen bonds. This extension ends with a tyrosine–glycine sequence motif, which is highly conserved in fungal tyrosinases. Interestingly, in other fungal tyrosinases, the removal of the C–terminal part, via proteolytic cleavage upon maturation of the enzyme, has occurred at an amino acid residue immediately after this sequence motif. Unfortunately PPO3 structure provides no further information of amino acids beyond this sequence motif. Whether and where the cleavage upon maturation has occurred after this sequence motif can be explained when the structure of the full-length of PPO3 or its isoforms is elucidated.

One major question on the structure of tyrosinase has been the function of a thioether bond between Cys83 and His85 (PPO3 numbering) in the active site. His85 is one of the ligands that coordinate the first copper ion (Cu-A). This bond is conserved in hemocyanin and catechol oxidase (figure 2), with Cys-X-His and Cys-(X)_{17}-His sequence motif, respectively. Hemocyanin is an oxygen-carrier protein in molluscs and arthropods whilst catechol oxidase catalyzes the conversion of ortho-diphenols to ortho-quinones in plants. However, the thioether bond is absent in the structurally characterized bacterial tyrosinases from S.
**castaneoglobisporus** and **Bacillus megaterium**. Furthermore, the recently reported structures of these bacterial tyrosinases show that His85 is flexible. Therefore, in the current reaction mechanism based on their structures, the displacement of His85 is proposed in the ortho-hydroxylation step. However, the PPO3 structure shows that His85 displacement is unlikely because the thioether bond severely limits the rotational freedom of His85.

More insight into the reaction mechanism can be gained by studying the structure of tyrosinase in the presence of substrate or inhibitors. Unfortunately, the PPO3 as well as the PPO3–tropolone complex structures are in the deoxy-state, where the enzyme is not active. Furthermore, the tropolone inhibitor molecule is not bound specifically in the active site. Nevertheless, tropolone occupies a similar position as the phenylthiourea (PTU) inhibitor in catechol oxidase and the tyrosine side chain of the caddie protein in the **S. castaneoglobisporus** tyrosinase structure. The information on the steps in the reaction may be obtained by the elucidation of a structure of the oxy-state of PPO3, preferably in the presence of a substrate or substrate analogue.

The light subunit was identified as ORF 239342, which is a protein from mushroom with unknown function. Its architecture resembles proteins with agglutinating functionality but the carbohydrate binding is not conserved. Therefore, the function of this subunit as well as its
incorporation into the $H_2L_2$ tyrosinase tetramer remain questions because it is always found associated with the H subunit (tyrosinase). To find answers on these questions, new research can now be undertaken to studying the localization of this protein in mushroom and its regulation/expression in the cell, and to further characterize its biochemical characteristics.