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SEQUENCE AND STRUCTURAL FEATURES OF PLANT AND FUNGAL TYROSINASES

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Key Word Index—tyrosinase; polyphenol oxidase; enzyme activation; protein structure.

Abstract—Tyrosinases from various organisms are compared with respect to enzymatic structure, primary, secondary and tertiary structure, domain structure, Cu binding sites, maturation mechanism and activation mechanism. On the basis of these comparisons, and by using hemocyanin structure as a template, a structure model for the active site of tyrosinases is proposed. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Tyrosinase (EC 1.14.18.1), is a copper monooxygenase widely distributed in nature and is mainly involved in the formation of pigments such as melanins and other polyphenolic compounds [1]. The enzyme catalyzes the orthohydroxylation of monophenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity) (see Fig. 1). Physiologically, the function of tyrosinase in plants and in fungi is not yet understood, but it may be protective. In higher plants, the enzyme protects the plant against insects and microorganisms by catalyzing the formation of an impervious scab of melanin against further attack, as is well documented for the tyrosinase from the trichomes of Solanaceae species [2, 3]. Tyrosinase is upregulated in wounded tissue in apple [4], systemic wound induction of tyrosinase is found in potato [5], and system in activates wound inducible tomato leaf tyrosinase [6]. Although such findings suggest a defense-related role of tyrosinase in higher plants, the discussion is likely to continue as, for instance, no PPO-induction occurred upon infection of *Nicotiana* hybrids with various micoorganisms [7] or upon challenge of watermelon (*Citrullus lanatus*) with *Fusarium* [8].

In insects, tyrosinase is involved in sclerotization of the exoskeleton and in protection against other organisms by encapsulating them in melanin. In mammals, it is responsible for skin pigmentation. In most fruits and vegetables, tyrosinase is responsible for enzymatic browning, following bruising, cutting or other damage to the cell. For example, mushroom tyrosinase is responsible for the undesired browning of mushrooms that takes place during senescence or damage during post harvest handling. The principal endogenous substrates of mushroom tyrosinase are L-tyrosine, *p*-aminophenol and its condensation product with glutamate, γ-glutaminyl-4-hydroxybenzene (GHB), all originating from the shikimic acid pathway [9]. Besides this role in undesired browning, however, the activity of tyrosinase is needed in other cases (e.g. raisins, cocoa) where it produces distinct organoleptic properties.

To understand the details of the browning process and how to control it, studies on tyrosinases, their substrates and inhibitors are needed. Here an overview will be provided of our current knowledge concerning sequences and structural features of tyrosinases. The known sequences will be discussed, as well as the different protein domains that can be identified within these tyrosinase sequences. Furthermore, known structural aspects of the active site of tyrosinase will be described, together with structural features which we predict for this region. Special attention will be given to plant and fungal tyrosinases, since they are our main research interests.

By using all the sequence and structure information available, our research aim is to construct a structural model for the active site of (mushroom and plant) tyrosinase. Such a model will contribute to our knowl-

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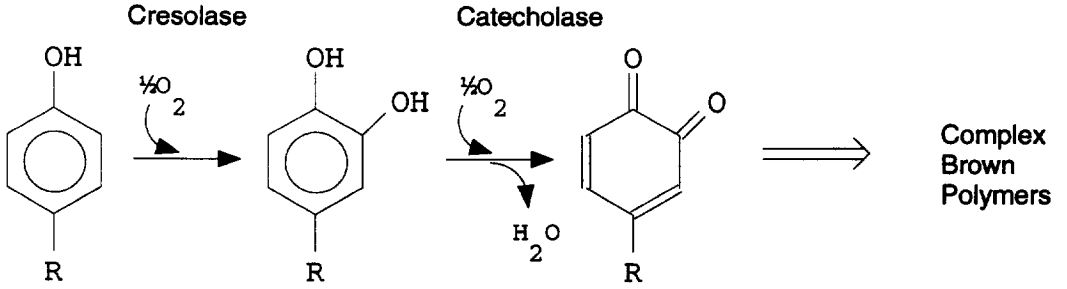

Fig. 1. Reaction scheme of tyrosinase; both the cresolase and catecholase activity are shown.

edge of tyrosinase and its reactions; it can also direct the design of mutagenesis experiments and may be useful in designing potent regulators of tyrosinase activity.

FUNGAL AND PLANT TYROSINASES IN A HISTORIC PERSPECTIVE

Early studies on the tyrosinase from the common mushroom *Agaricus bisporus* suggested that enzyme to possess a molecular weight of 110–130 000 [10–12] and occur as a tetrameric protein composed of identical subunits of *ca* 32 000. In the 1970s, the tetrameric structure was revised and suggested to consist of two subunits of 43 000 (H) and two subunits of 13 000 (L) [13].The quaternary structure was assumed to be H2L2 with a native molecular mass of approx. 110 000 and containing 4 Cu atoms [13]. Importantly, the isolated H and L subunits were found not to possess enzymatic activity [13]. On SDS–PAGE, a band with an apparent molecular weight (M_W) of 69 000 was observed (presumably L_2H) which possessed both cresolase and catecholase activity [13]. Electrophoretic data further suggested that the high M_W forms of the enzyme are aggregates in which interpolypeptide S–S linkages are not involved [13].

The tyrosinase from *Agaricus bisporus* has been found in active and latent forms. The ratio between latent and active enzyme may range from 3 to approximately 20, possibly depending on assay method and strain selection [14, 15]. Activation *in vitro* can be effected for instance by detergents such as SDS or by protease treatment [14, 16–18, see also below].

Recently, we isolated two monomeric tyrosinase isoforms from the fruiting bodies of *A. bisporus* U1, possessing not only catecholase activity but cresolase activity as well [19]. Both isoenzymes exhibited a molecular mass of 43 000 under denaturing conditions and 47 000 under native conditions, suggesting that both isoenzymes occur as monomeric single chain polypeptides. Furthermore, a putative tyrosinase cDNA was cloned that encoded a *ca* 64 000 protein [20, see further below].

In the case of tyrosinases from the fungus *Neurospora crassa* and from higher plants, a rather similar story can be told. Early studies indicated that the enzyme from *N. crassa* occurred as 33 000 subunits [21]. Subsequently, mixtures of aggregates up to tetramers were described, with fully active monomers of 47 000 [22, and references therein]. Currently, *N. crassa* tyrosinase is regarded as a 46 000 monomer with full enzymatic activity that is formed upon proteolytic processing of a 75 000 precursor. The complete coding sequence for this 75 000 precursor has been elucidated [23]. In the mould *Aspergillus oryzae*, tyrosinase is described as a tetrameric protein consisting of 67 000 subunits [24]. The enzyme can be activated by acid shock (pH 3.0) and the monomeric enzyme is active and shows a molecular weight of 67 000 by gel electrophoresis, both with and without β-mercapoethanol [24]. Acid activation of latent tyrosinases was described as early as in 1972 by Lerner *et al.* for grape catechol oxidase and suggested to result from conformational change [25].

In the 1950–1970s higher plant tyrosinases were thought to be tetramers of *ca* 30 000 monomers. In the 1980s, the first reports on the isolation of plant tyrosinases to apparent homogeneity appeared, describing *ca.* 45 000 monomers which might eventually associate in tetramers [26, 27]. In the late 1980s and early 1990s knowledge on preforms of plant tyrosinases increased, as *ca* 68–73 000 preproteins, which were later processed to 58–68 000 mature proteins, were isolated. Proteolysis of the mature proteins, *in vitro* and *in vivo*, can yield monomeric active forms of *ca* 40–45 000 [28–32].

Tyrosinase sequences

Circa 20 tyrosinase sequences are known at the moment, ranging from humans to prokaryotes. Table 1 summarizes tyrosinases for which the complete amino acid sequence is known. Recently, we established the sequence of a full-length tyrosinase clone for *Agaricus bisporus* [20, see also Fig. 2(C)]. A homology search with this sequence vs the protein sequence database resulted in the retrieval of many tyrosinases with highly significant similarity, strongly suggesting that the mushroom sequence represents tyrosinase. Furthermore, the protein was expressed in *E. coli* and was shown to crossreact with polyclonal antibodies raised against a 43 000 tyrosinase isolated from mushroom powder (data not shown).

Within different taxa the sequence homology of the tyrosinases is high and conserved domains can be identified for each group [see Fig. 2(A)]. This might

Domains in tyrosinase

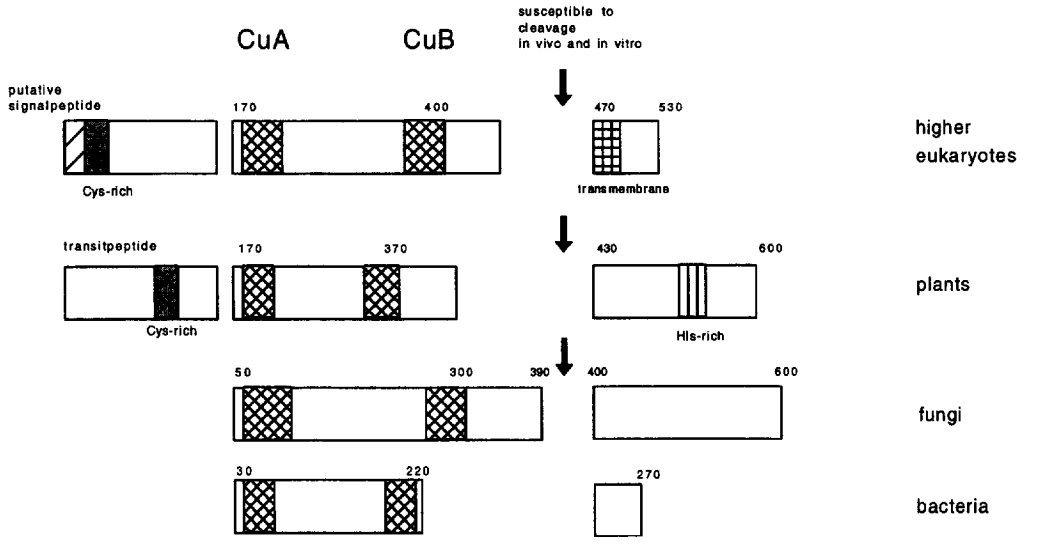

Fig. 2. (A) Domain structure of tyrosinases from different groups of species. The conserved Cu-binding sites CuA and CuB are indicated, as well as other conserved domains. An arrow indicates the known or predicted cleavage site for the generation of the protease-resistant fragment from the mature protein (see text). In plant tyrosinases, recently a third histidine-rich region was determined, which was postulated to be a putative Cu binding domain [43]. Also shown in the N-terminal region of animal and plant tyrosinases is a conserved cysteine-rich region (see text). (B) Sequence alignment of 11 plant tyrosinases. The conserved CuA and CuB regions are boxed as well as the third histidine-rich region in plants [43]. Indicated are furthermore the location of cleavage site of the transit peptide located in the N-terminal part of the protein. Cysteines are shown in bold. Asterisks below the amino acids indicate identity of amino acids between all 11 tyrosinase sequences, while dots indicate nine or more identical amino acids (out of 11 sequences) or conservative substitutions between the 11 sequences. (C) Sequence alignment of *N. crassa* and *A. bisporus* tyrosinase. The conserved Cu-binding sites CuA and CuB are boxed. The thioether bond in CuA is shown, as well as the known and predicted cleavage site for processing of *N. crassa*, and *A. bisporus* tyrosinases, respectively. Asterisks indicate terminal amino acids in the two sequences, while dots indicate similar amino acids.

indicate structural similarity with respect to subunit composition within a group. In agreement with this view is the observation that polyclonal and monoclonal antibodies against *Vicia faba* tyrosinase react with both active and inactive forms of tyrosinase from other plant sources [31–33]. Note that the recently established insect tyrosinase sequences (see Table 1) have not been included in Fig. 2(A), since they have some peculiar properties (see also below).

DOMAINS IN TYROSINASE

Roughly speaking the tyrosinases can be divided in three domains, of which the central domain contains the Cu-binding sites [Fig. 2(A)]. In this way their overall design is similar to that of arthropodean hemocyanins (see further below).

Several conserved regions can be identified, some of which are found in all tyrosinases, while others are found only in one group, e.g. the plant tyrosinases. Figure 2(B) gives an alignment of several known plant tyrosinases. The conserved CuA and CuB regions are indicated, as well as the location of the cleavage site of the transit peptide (see further below). When one of the potato tyrosinase sequences (potato-b [34]) is compared with one of the tomato sequences (tomato-e [35]), 92% identity (94% similarity) in amino acids is found. The homology between *A. bisporus* and *N. crassa* tyrosinase is 32% identity (54% similarity), and their alignment is shown in Fig. 2(C). However, the homology between *N. crassa* tyrosinase and one of the tomato tyrosinase sequences is only 11% identity (23% similarity). In fact, when all tyrosinase sequences are compared, the only conserved domain seems to be the central Cu-binding domain.

Central domain: Cu *binding sites*

The most prominent features observed in all tyrosinase sequences are the two Cu binding sites, called CuA and CuB, which are indicated in Fig. 2(A)–(C). For additional reading regarding the structure of the copper sites, recent reviews by Steffens *et al.* [36] and Lax and Cary [37] may yield further details. The active site of tyrosinase consists of a pair of copper ions, which are each bound by three conserved histidine

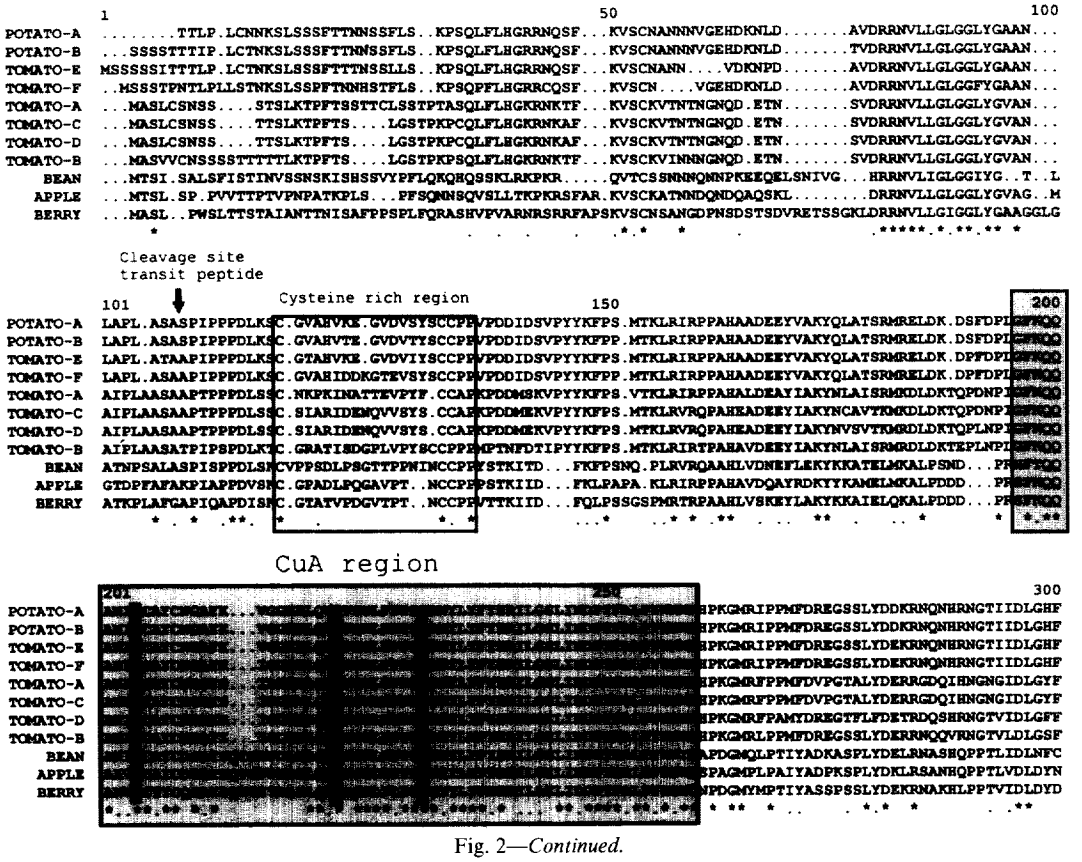

Fig. 2—*Continued.*

Table 1. Known tyrosinase amino acid sequences

Species	No. of aa	References
Homo sapiens (human)	529	[74]
Mus musculus (mouse)	533	[88]
Gallus sp. (chicken)	530	[89]
Rana nigromaculata (Japanese frog)	532	[90]
Caenorhabditis elegans (nematode)	485	[91]
Pacifastacus leniusculus (crayfish)	706	[69]
Oryzias latipes (medaka fish)	540	[92]
Manduca sexta (tobacco hornworm)	695	[93]
Drosophila melanogaster (fruit fly)	690	[42]
Bombyx mori (silkworm)	696	[70]
Lycopersicon esculentum (tomato)	585–630	[35]
Malus domestica (apple)	593	[4]
Vitis vinifera (grape berry)	607	[68]
Solanum tuberosum (potato)	583–588	[34, 94]
Vicia faba (broad bean)	606	[29]
Phytolacca americana (pokeweed)	586	[43]
Spinacia oleracea (spinach)	639	[51]
Agaricus bisporus	569	[20]
Neurospora crassa	620	[23]
Aspergillus oryzae	539	[24]
Rhizobium meliloti	494	[95]
Streptomyces antibioticus	272	[96]
Streptomyces glaucescens	273	[97]

residues (His) [38]. This copper pair is the site of interaction of tyrosinase with both molecular oxygen and its phenolic substrates. In Fig. 3(A) and (B), a sequence alignment is shown for the CuA and CuB sequences of several tyrosinases, respectively. For example, the CuA site of bacterial and human tyrosinase show 42% identical amino acids (53% similar amino acids), while their CuB site shows 42% identity (74% similarity). When looking at the complete central domain of these proteins these percentages decrease to 31% identity (51% similarity) on amino acid level. For *Neurospora* and *Agaricus* tyrosinases, the CuA site shows 44% (identity) and 58% (similarity) homology, for the CuB-site these values are, respectively, 49% (identity) and 62% (similarity).

The regions in tyrosinase around the copper-binding ligands also share sequence homology with hemocyanins (Hcs), which are copper-containing oxygen carriers from the hemolymph of many molluscs and arthropods [39]. For this reason, several Hc sequences are also included in Fig. 3(A) and (B). The CuB motif is found twice in arthropod hemocyanins, and once in molluscan hemocyanins and tyrosinases. The CuA site [Fig. 3(A)] is found in molluscan hemocyanin and in tyrosinases (see also below). Besides the three histidines in each Cu-binding site, several other amino acids are conserved in all the sequences, strongly indi-

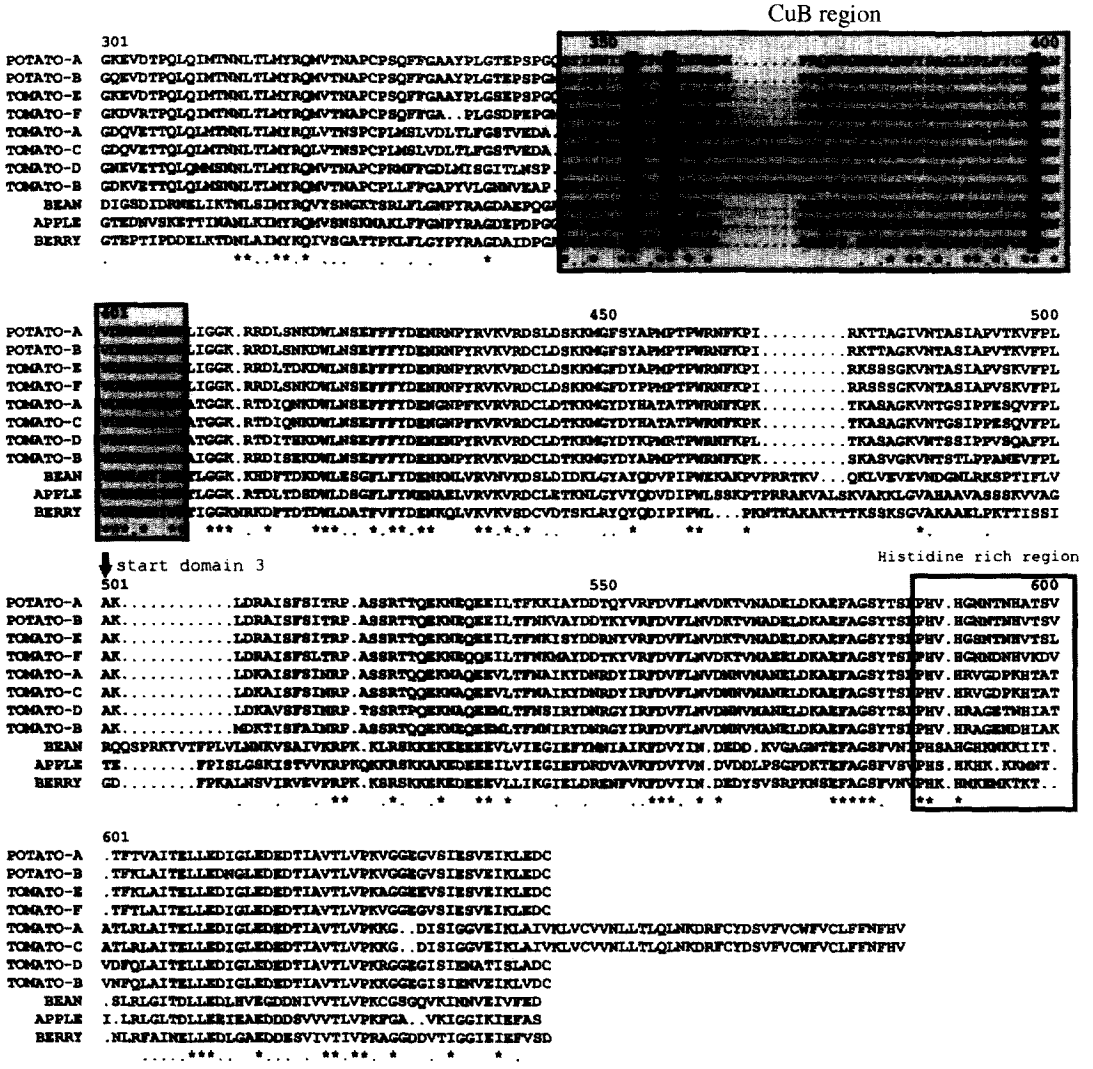

Fig. 2—*Continued.*

cating structural similarity between the hemocyanins and the tyrosinases. This relationship between tyrosinases and hemocyanins will be further discussed below.

In plant tyrosinases, a third histidine-rich domain was first identified by Steffens *et al.* [26]. It was postulated to also bind copper, and it is indicated in Fig. 2(B). However, the significance of this domain has yet to be determined; site-directed mutagenesis is being carried out to establish the role of this domain in enzymic activity and import and processing into the plastids of PPO pre-proteins [36].

An interesting feature in *N. crassa* tyrosinase is a thioether bridge between the second histidine residue in CuA and a cysteine residue located two amino acids towards the N-terminus [40]. This thioether linkage [see Figure 2(C)] was proposed to play a role in the regulation of tyrosinase activity [1]. When looking at the sequences of mushroom [20] and *Aspergillus* [24] tyrosinases, it can be seen that such a thioether bridge is also possible. This is not the case in the known prokaryotic, plant and mammalian tyrosinase sequences known sofar. Furthermore, a thioether bridge is also present in a molluscan hemocyanin (*Helix pomatia*) [41], strengthening the similarity between hemocyanins and tyrosinases. Interestingly, and in contrast to tyrosinases from prokaryotes, fungi and vertebrates, the recently sequenced insect tyrosinases revealed two CuB-like Cu-binding sites, which suggest a different evolution [42]. For this reason, the insect tyrosinases are excluded from Fig. 3(A) and (B).

N-terminal domain: signal peptides and transit peptides

As do all nuclearly encoded plastid enzymes, higher plant tyrosinases possess plastid transit peptides that post-translationally direct the protein to the chloroplast envelope for subsequent transport and processing to the correct molecular size for the mature protein [30]. The transitpeptide consists of 80–90

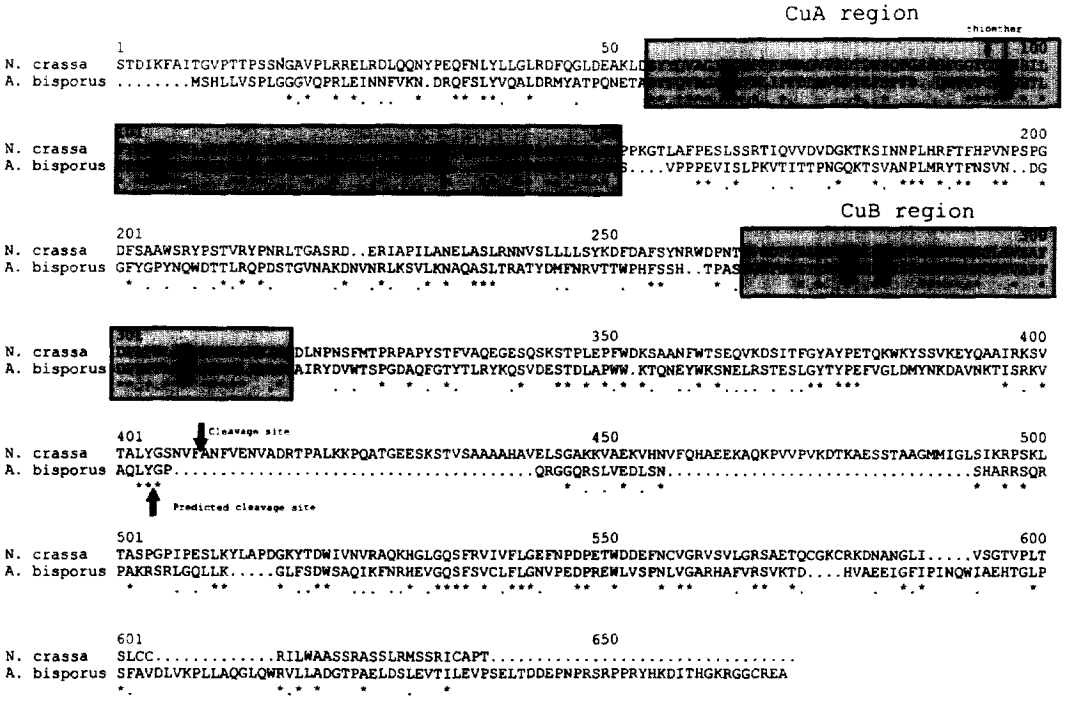

Fig. 2—*Continued.*

amino acids, and is located in the N-terminal part of the plant tyrosinase sequence [see Fig. 2(A) and (B)]. The peptide bond cleaved by the processing proteases was identified as alanine-serine or alanine-alanine in all plant protyrosinases examined sofar [2]. The transit peptide is characterized by the presence of three domains [35, 43]. The N-terminal 25 amino acids contain many hydroxyl containing amino acids. The middle part is more loosely conserved and called the 'n'-region, while the C-terminal 25 amino acids form a hydrophobic domain, which is the thylakoid transfer domain (lumen targeting). Tomato tyrosinase is routed to the thylakoid lumen in two steps. A 67 kDa protein is first imported into the stroma, then processed to 62 kDa by a stromal peptidase. Subsequently, translocation into the lumen, which is light dependent, involves processing to yield a 59 kDa soluble product [30].

In the three known fungal tyrosinases we found no prediction of an N-terminal signal peptide by the Von Heijne method [44]. This is in agreement with the fact that no function similar to that of the transit peptide of higher plant tyrosinases could be postulated, as the fungal tyrosinases are supposed to be cytoplasmic [20, 45].

In human and mouse tyrosinase the N-terminal 18 amino acids are putative signal peptides, and it was suggested that they were involved in the transfer of the enzyme into the melanosome [46]. Tyrosinases from either *Streptomyces glaucescens* or *Streptomyces antibioticus* do not possess a signal peptide; their intracellular and extracellular forms are identical in molecular weight and N-terminal amino acid sequence [47]. The insect tyrosinase sequences known so far do not contain a signal peptide [42].

Another interesting region in the N-terminal domain might be a small cysteine-rich region, which is conserved in both plant and animal tyrosinases [see Fig. 2(A) and (B)] and it is located directly after the transit or signal peptide.

Transmembrane domains in tyrosinases

Agaricus bisporus tyrosinase is a soluble cytosolic enzyme [20], as is *Neurospora crassa* tyrosinase [45]. In accordance with this observation, calculation of the transfer free energy according to Engleman [48] indicates no evidence for transmembrane regions, and neither does the transmembrane region predicting program TMAP [49].

In higher plants, the enzyme is mostly membrane bound in non-senescing tissues [45]. Evidence for membrane-bound tyrosinase in plants is based on the fact that detergent extraction of the tissue chloroplasts substantially increases tyrosinase activity and/or the release of tyrosinase from membranes [50]. However, more enzyme becomes soluble as the fruit ages, and soluble as well as membrane-bound plant tyrosinases have been described [45]. Only two of the at least seven tomato tyrosinase genes show two predicted α-helices in the C-terminal 60 amino acids, which could span the thykaloid, anchoring the protein in the membrane [35]. The bulk of the protein is in the lumen, while the C-terminal part is on the stroma side [30] (import of

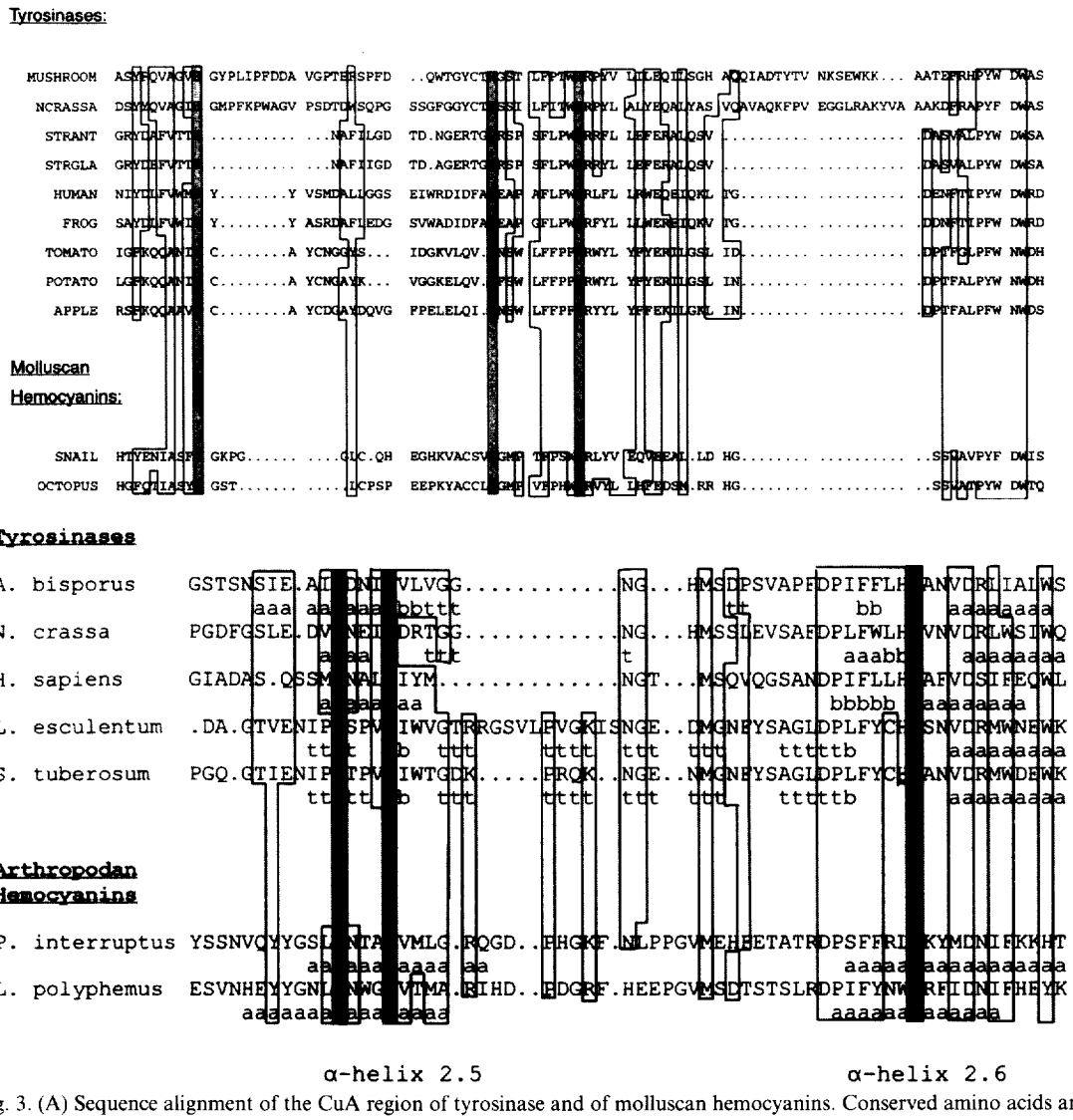

Fig. 3. (A) Sequence alignment of the CuA region of tyrosinase and of molluscan hemocyanins. Conserved amino acids are boxed and the three conserved histidines, which are implied in Cu binding are shown in grey. Strant and Strga indicate the two bacterial tyrosinases from *Streptomyces antibioticus* and *Streptomyces glaucescens*. (B) Sequence alignment of the CuB region of tyrosinases and arthropodan hemocyanins. *P. interruptus* = spiny lobster and *L. polyphemus* = crab. Also indicated are the locations of α2.5 and α2.6, the two helices which contain the conserved histidines in Hc. Secondary structure predictions are also indicate: a = α-helix,b = β-strand, t = turn. The predictions of the potato-a and tomato-c sequence are equal, because the PHD program uses a sequence alignment of all plant sequences as input and returns one prediction for all the sequences.

this protein into the plastids is treated above in the section 'N-terminal domain: signal peptides and transit peptides'). All known sequences for tomato and potato tyrosinases were used together as input to the TMAP program, which yielded a predicted transmembrane region which largely coincides with the thylakoid transfer (lumen targeting) domain of the transit peptide (data not shown), but no evidence for a C-terminal transmembrane domain was found. However, in two of the seven tomato tyrosinase sequences a transmembrane helix was suggested in the C-terminal domain, as well as in a few other plant tyrosinases [35, 51].

Mammalian tyrosinases are melanosomal membrane proteins with a carboxyl tail oriented to the cytoplasm and a single membrane-spanning helix located in the C-terminal part of the proteins [Fig. 2(A)]. The bulk of the protein is located inside the melanosome [52]. The enzyme is present in melanocytes in humans, and it is responsible for skin pigmentation, including freckles.

Latent tyrosinase and its activation

Many tyrosinases from plant, fungal and invertebrate origin exist as latent enzymes which have to be

activated. In case of mushroom tyrosinase, *ca* 99% of the total enzyme appears to be present in its latent form [53]. Most of the plant PPOs described seem to be latent in the mature form [29, 32, 33, 54]. In this inactive form the enzyme seems to be very stable [31], and upon activation it becomes more sensitive to temperature. SDS, which can activate tyrosinase, was shown to give rise to increased thermolability in *Vicia faba* tyrosinase [55].

One of the striking characteristics of tyrosinases is the broad spectrum of substances by which they can be activated in crude tissue preparations. Although *in vivo* regulatory mechanisms are as yet unknown, it is thought that endogeneous protease(s) might be involved, based on *in vitro* evidence [15, 56]. In plants, when protease inhibitors are added during extraction and fractionation of plant tyrosinases, the number of isoforms observed decreases [31, 57, 58]. An *in vivo* role of proteases in plants was suggested in grape [28]. In the report by Kuwabara [59], structural similarities between a protease and PPO from spinach chloroplasts is suggested. Since this report has not yet been confirmed by other authors, its implications are to be established. Perhaps part of the confusion may be caused by the apparent close association between PPOs and proteases that may occur, as described for instance for *Aspergillus oryzae* [60]. In case of the mould *Aspergillus oryzae*, two proteases have been isolated which appear to be involved in activation of tyrosinase [60]. Recently, a serine protease which plays a role in senescence has been isolated from mushrooms [61]. Since browning is one of the major hallmarks of senesense, it could well be that this protease is important in regulating tyrosinase (K. Burton, personal communication).

In vitro activation of tyrosinase can also be achieved by anionic detergents as SDS [45, 52, 55, 62]. Furthermore, activation by acidification [24, 25, 63] and by lipids [26, 52] has been described. Latent mushroom tyrosinase appears to be sensitive to activation preparations containing tolaasin [64]. Tolaasin is a bacterial lipodepsipeptide which is produced by the causal agent of brown blotch disease, *Pseudomonas tolaasii* [65]. The activation brought about by several seemingly unrelated substances is a phenomenon often attributed to conformational changes of the enzyme molecule, solubilization of the enzyme or the removal of an inhibitor. In the case of *Vicia faba* (broad bean) tyrosinase, it was shown that the active site is blocked in absence of SDS, but open in presence of SDS [55]. SDS was shown to bind tightly to *Xenopus* tyrosinase and in this process, which causes activation, SDS monomers were shown to be involved [66]. An interesting question in this respect is what might be the biologically relevant counterpart of the detergents. Lipids might fulfil this role. Remarkably, recently a human tyrosinase inhibitor was described which turned out to be a fatty-acid binding protein of 13–14 kDa [67].

In Table 2 an overview is given of the current knowl-

Table 2. Properties of plant and fungal tyrosinases

Species	No. genes identified	No. cDNAs identified	mRNA (kb)	Precursor predicted (kDa)	Precursor SDS-PAGE (kDa)	Mature predicted (kDa)	Mature SDS-PAGE (kDa)	Protease-resistant predicted (kDa)	Protease-resistant SDS-PAGE (kDa)	N-Terminus	References
Plants											
Apple	⩾4		2	66	60 67	57	63–65	43	42		[4]
Broad bean	⩾3	⩾3	2	68		58	60–68	43	42–45	free	[29, 54]
Grape	1	>2	2	67			60	41–42	40	blocked	[28, 54, 62]
Potato	⩾6	5	*ca* 2	67		57–60		42			[34, 54, 94]
Spinach	1	1	2.2	73		62–64	64		42		[51]
Tomato	⩾7	>2	2.2	66–71	67 (IVT)	57–62	59	43			[30, 35]
Carrot							59		43, 36	blocked	[31]
Fruits							63		43		[32]
Pokeweed	2	2.1–2.3	65			54					[43]
Fungi											
N. crassa	2		2	NA	NA		75	46	46	blocked	[23]
A. bisporus	⩾2	⩾2	<2	NA	NA	64	68	43	43 (47 GPC)	blocked	[20]
A. oryzae			1.6	NA	NA	67	67			blocked	[24]

edge about gene structure, mRNA size and (pro) protein size for tyrosinase in plants and fungi. In plants, most tyrosinases described are encoded by multigene families (potato, tomato, broad bean and apple), coding for 60–65 kDa peptides which can be converted to a 40–45 kDa form by proteolysis *in vitro* and *in vivo*. These proteolyzed forms are still enzymatically active [31, 32]. In broad bean (*Vicia faba*) tyrosinase, a 18 kDa protein is cleaved off from the C-terminal end of a 60 kDa precursor to generate a 42 kDa protein [54]. In grape tyrosinase a 16 kDa C-terminal domain is removed from 67 kDa precursor during processing [68]. In fruit tyrosinase a 63 kDa mature protein is cleaved to a 45 kDa product [32].

In the case of *N. crassa* tyrosinase, the mature form is 407 (46 kDa) amino acids, which is derived from cleavage of 213 amino acids (21 kDa) from the C-terminal end of the 620 amino acid long protyrosinase. This phenylalanine (Phe) 407 is a chymotrypsin cleavage site. In case of mushroom tyrosinase, the size of tyrosinase is 64 kDa, which is postulated to be cleaved to a 43 kDa protein [20]. No data on the enzymatic activity of the 64 kDa form is available yet. When looking at the sequence of mushroom tyrosinase in the sequence alignment of Fig. 2(C), a putative cleavage site for the conversion from the 64 kDa tyrosinase to the proteolyzed form can be postulated. Tyrosine 381 (a chymotrypsin cleavage site), yielding a cleavage product of 43.0 kDa, is located in the sequence alignment at approximately the same position as the cleavage site (Phe 407) found in *N. crassa* tyrosinase. Furthermore, in the partial cDNA sequence of a second mushroom cDNA clone a tyrosine is located at the exact same position (data not shown). In the *Aspergillus* sequences also a Phe residue is located at this position in the sequence [24]. Mammalian tyrosinase can be solubilized by mild trypsin digestion to yield a fully active soluble form. This treatment is thought to release the bulk of the molecule by cleaving the transmembrane fragment, thus removing the hydrophobic domain and the carboxylic cytosolic tail [52]. Also in this case the postulated site of cleavage is located at a similar position in the tyrosinase sequences as is seen in plant and fungal tyrosinases [Fig. 2(A)], i.e. on the border of the second and third domain.

In invertebrates, tyrosinase has been found to exist in the blood in an inactive form, which is activated in a stepwise process involving serine proteinases activated by microbial cell wall constituents [69]. Several endogeneous serine proteases that activate insect tyrosinase have been identified [69, 70]. However, in the case of crayfish protyrosinase, activation is established by cleavage of 177 amino acids from the N-terminus of the protein [69]. Also in other arthropod tyrosinases there is cleavage of a *ca* 50 amino acid peptide from the N-terminus of the protein. Thus, these tyrosinases not only differ in sequence but apparently also in domain structure as compared to vertebrate, fungal and prokaryotic tyrosinases.

STRUCTURAL ASPECTS OF TYROSINASE AND ITS RELATION WITH HEMOCYANIN

Spectroscopic data

Spectroscopic studies have revealed the surroundings of the two Cu ions in tyrosinase. EPR, UV/VIS and Raman spectroscopy data classify tyrosinase as a coupled binuclear Cu protein (type 3 Cu) (reviewed in [1, 71]). All these studies have led to a detailed picture of the electronic structure of the binuclear copper cluster. The Cu–Cu distance in several forms of tyrosinases and Hcs is known from these measurements [1]. The oxygenated form of tyrosinase (oxytyrosinase) consists of two tetragonal Cu(II) atoms, each coordinated by two strong equatorial and one weaker axial N_{His} ligand [71, see Fig. 4]. The exogenous oxygen molecule is bound as peroxide. For tyrosinase it is known that the protein pocket surrounding Cu-active site contributes to stabilizing substrate binding [72]. The excellent matching of the UV/VIS and Raman data for both arthropodan and molluscan Hcs and tyrosinases indicates a near identity of Cul ligation and oxygen binding in these two families of copper proteins [1].

Active site accessibility

Kinetic studies between several compounds such as CN-, phenol, azide or mimosine and the binuclear copper site of tyrosinase or Hc have shown that the tyrosinase copper site has a greater accessibility towards (large) ligands than that of Hc [72, 73]. Considering the large size and diversity of known substrates and inhibitors of tyrosinase this is an expected behaviour. Therefore, it was suggested that tyrosinases can be viewed as a hemocyanin with an exposed binuclear copper site. Furthermore, hemocyanin *in vitro* is able to oxidize DOPA, although the activity is 50- to 100-fold lower than in tyrosinase, and this activation can be enhanced 5-fold by perturbing the protein conformation [73].

Besides structural similarities of the Cu-site between Hc and tyrosinase, differences between tyrosinase and hemocyanin active sites are also apparent from binding studies with substrate analogues, relating to the different biological functions of the two copper proteins [1]. These differences in behaviour were, however, suggested to be caused by this less buried character rather than by intrinsic differences in active site organization [73].

Identification of important amino acids

Several mutagenesis and photoinactivation studies have been performed with tyrosinases, thus revealing amino acids which are important for enzymatic activity. The conserved histidines in CuA and CuB were mutated and shown to be important for Cu-binding and/or enzymatic activity in mouse [74]. In

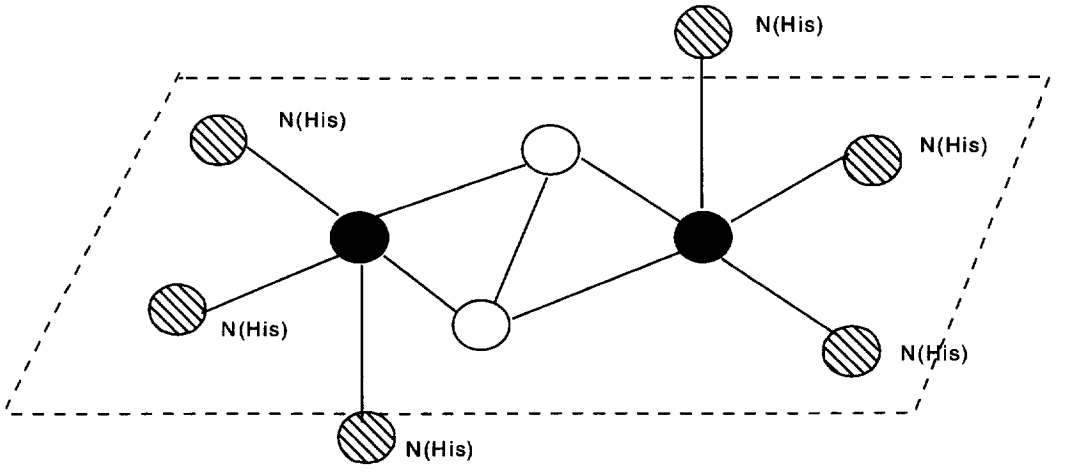

Fig. 4. Schematic representation of the binuclear copper center. The oxygen is bound as peroxide and each Cu ion is bound to three histidine nitrogen atoms. Black symbols—Cu-ions; white symbols—oxygen; symbols dashed vertically—His-N.

prokaryotic tyrosinases, mutation of the conserved histidines leads to albino mutants [38], and the same result was obtained with photo-inactivation studies [39, and references therein].

When the invariant asparagine 190 (adjacent to the CuB His189) in the CuB binding site of *Streptomyces glaucescens* tyrosinase was mutated to a glutamine, the enzymatic activity was 2000-fold lower and there was loss of copper binding [38]. However, the luminescence properties resembled that of native tyrosinase, indicating that no substantial active-site conformational changes had occurred. In the corresponding position in *Limulus polyphemus* (crab) hemocyanin and asparagine 325 is involved in an active-site hydrogen-bonding network that bridges the two Cu atoms [75].

Neurospora crassa tyrosinase shows two forms in wild type strains, one thermostable, with an aspartic acid (Asp) at position 201, and one thermolabile form, with an asparagine at this position [76]. Since the thermostability increases with ionic strength this Asp residue is probably involved in a salt bridge, and the strong pH dependency of thermostability suggested the involvement of a histidine side chain [76].

Studies on pH effects on the reaction of palmito tyrosinase indicated that oxidation of substrate depends on the ionization of two groups in the enzyme-substrate complex with apparent pK values of 3.1 and 7.3 [77]. The highest pK_a was suggested to be an imidazole of histidine while the acidic pK might refer to an acidic amino acid (aspartic or glutamic acid).

Cysteines (Cys), and their location are also interesting, because they may be involved in disulfide bridges, which can stabilize protein structures. In human and mouse tyrosinase there are 17 cysteines which perfectly align with each other and there are four S–S bridges postulated [74]. They are located in the N-terminal and central part of the proteins, while in the C-terminal part only 1 cysteine is found. In plant tyrosinases the same phenomenon is found, although the total number of cysteines is lower here (*ca* 11, two of which are found in the transitpeptide). In broad bean tyrosinase, the molecular weight of the protein estimated by gel electrophoresis is 45 000 under partially denaturing conditions, *ca* 55 000 in absence of reducing agents, and *ca* 63 000 under fully reduced denaturing conditions [58, 68]. This behaviour also suggests the presence of disulfide bridges [54]. Disulfide bonds were also suggested in apple tyrosinase [78].

Interestingly, the *Agaricus bisporus*, *Neurospora crassa* and prokaryotic tyrosinases contain zero or one Cys in the mature protein. In fact, the only Cys in the mature fungal tyrosinases is the one involved in the above mentioned thioether bridge with a histidine residue. In *N. crassa* tyrosinase the C-terminal extension shows six clustered cysteines and a relatively large number of aromatic amino acids. It was suggested that at least some of these cysteine residues may form disulfide bridges, resulting in a tightly packed structure [23]. However, in the mushroom tyrosinase sequence only two cysteine residues are found in the C-terminal domain.

Tertiary structure of hemocyanins

The X-ray structure is known for two arthropodan hemocyanins, *Panulirus interruptus* (spiny lobster) and *Limulus polyphemus* [75, 79, 80]. Arthropodan hemocyanins form hexameric or oligohexamers built from individual subunits of about 75 kDa. Each subunit consists of three domains, the first of which is an N-terminal variable domain of $\approx$180 amino acids (aa). The second domain is the central domain (*ca* 225 aa; 25 kDa), which is the most conserved domain and contains the binuclear copper site. The connection between domain 1 and 2 is easily accessible to proteolytic cleavage. The third domain (*ca* 265 aa) contains a 7 stranded β-barrel. Three disulfide bridges are present in *P. interruptus* Hc and two in *L. polyphenus*

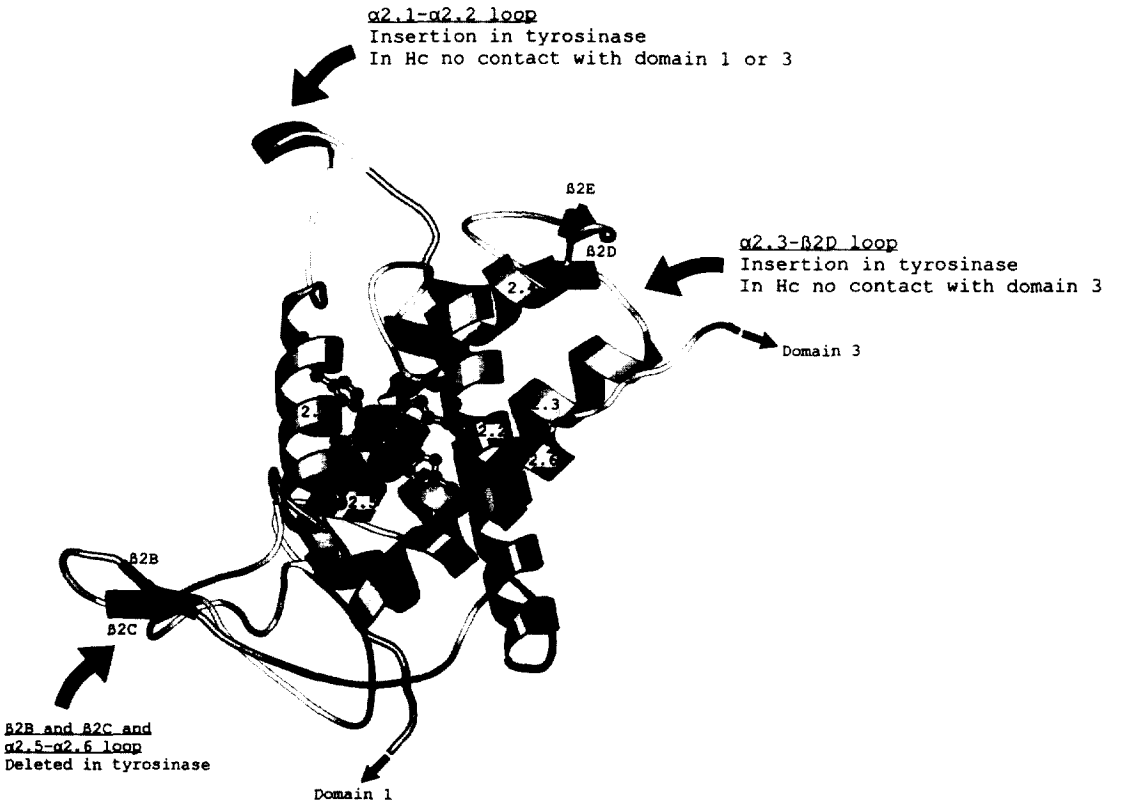

Fig. 5. Structure of the Cu-binding domain of crab hemocyanin [80]. The α-helices containing the conserved histidines are 2.1, 2.2, 2.5 and 2.6, in which the histidines are shown as ball-and-stick figures. β-strands indicated are β2B, β2C, β2D, β2E. The two copper ions are shown as dark-grey circles. Parts of tyrosinase which are predicted to be very similar to Hc are shown with stripes. Furthermore, predicted insertions and deletions in mushroom tyrosinase as compared to crab Hc, are indicated. Part of this figure was prepared with MOLSCRIPT [98].

Hc. Only one of these S–S bridges is conserved between the two arthropodan Hcs.

In domain 2, the two copperbinding sites CuA and CuB are very similar. Each Cu ion is strongly ligated to two equatorial histidine residues and coordinates a third axial histidine residue weakly (see also Fig. 5). Two of the histidine ligands are separated by three amino acids with the side chains projecting from the same side of an α-helix, and the third is furnished by a second α-helix running antiparallel to the first one [see Fig. 3(B)]. This leads to a 4-helix bundle topology, an arrangement of four antiparallel α-helices, which is a common structural motif in globular proteins.

The active site is located in a rather hydrophobic core and it is evident from the hemocyanin X-ray structure that domains 1 and 3 are shielding the copper active site from the solvent. This may also prevent the interaction of hemocyanin with organic molecules, which is typical for tyrosinase. Hence, domains 1 and 3 may be predominantly responsible for hemocyanin binding only molecular oxygen as opposed to the monooxygenase properties of tyrosinase [1].

The hemocyanins do not form a homogeneous class of proteins, since there are large differences between arthropodan and molluscan hemocyanins [80]. The molluscan Hc are *ca* 450 amino acids in length (*ca* 45–55 kDa) and form large cylindrical molecules of 10–20 subunits (total mass *ca* 350–400 kDa). Based on their length they probably contain one domain less than arthropodan Hc. The 50 kDa molluescan Hc was suggested to contain an oxygen binding domain similar to the one in arthropodan Hc and a second domain which might correspond to the first or third domain of arthropodan Hc; the two would be in quite intimate contact [75].

At amino acid sequence level the molluscan hemocyanins do not reveal high similarity with arthropodan hemocyanins except for the CuB region [39]. As mentioned earlier, in arthropodan hemocyanins CuA and CuB are very similar, being of the CuB-like type [39]. In contrast, both the CuA and CuB regions of molluscan Hc and tyrosinases resemble each other. Molluscan hemocyanin and our mushroom tyrosinase clone [20] share *ca* 25% sequence identity (50% similarity), and the matches are distributed over the complete sequence of both proteins (data not shown).

Despite the sequence differences in arthropodan and molluscan hemocyanins, their oxygen binding sites are quite similar spectroscopically [41, 81]. The molluscan and arthropod hemocyanins show a similar

pattern of Cu–N(His) vibrations, making it likely that the hemocyanins from the two phyla have the same set of terminal copper ligands [41]. In arthropodan hemocyanin, the His-ligands and the α-helices that donate them for ligating CuA and CuB show a pseudo two-fold symmetry [80]. This symmetry is also found for two conserved phenylalanines which play an important role in stabilizing the His-residues [80]. These Phe-residues are also present in CuA of molluscan hemocyanin and tyrosinase, suggesting that CuA site in these proteins also has its most C-terminal His donated by an α-helix [80].

As mentioned earlier, the fungal tyrosinases as well as molluscan hemocyanins contain a thioether linkage, in which one of the conserved histidines is covalently attached to a cysteine residue. It was suggested that this modified histidine would be more weakly coordinated to Cu and be in an axial position [41].

Secondary structure of tyrosinase

Secondary structure predictions were performed for the Cu-binding domain of for several tyrosinases by using several prediction programs (PHD [82], Chou-Fasman [83] and Garnier [84]), and Fig. 3(B) shows the results for the CuB region. This sequence alignment was guided by the known secondary structure of the arthropodan hemocyanins and the predicted secondary structure for the tyrosinases.

In the human tyrosinase there are clear α-helix predictions for the CuA and CuB regions (see also ref. [85]). However, in case of for instance *N. crassa* and mushroom tyrosinase the predictions are not that clear. For example, in the CuB region of the fungal tyrosinases, the first and second histidine are predicted to be located in or near an α-helical region, but the third histidine is predicted to be located between a β-strand and an α-helix in a region of less defined secondary structure [Fig. 3(B)]. For the plant sequences the secondary structure predictions are even less clear. The first and second histidine are predicted to be largely located in turn regions. This is probably due to the fact that a proline residue is present between the two histidines. Proline amino acids are known for their helix-breaking properties [86], and are not often present in the centre of a helix. Therefore, the two histidines are not expected to be located together in the same α-helix.

Considering all other similarities in experimental data mentioned before, however, it is expected that the CuB regions of tyrosinase show high structural similarity of that of Hc. It must also be noted here that the prediction algorithms also predicted β-strands for the copper-binding sites in Hc, which in the crystal structure were shown to be α-helical [87]. In Hc, α2.6 starts with a proline, and a proline residue is found exactly at the same position in the tyrosinase sequences. This further suggests the presence of an α-helix in tyrosinase CuB.

Several secondary structure predictions were made for the C-terminal region of tyrosinases. *Neurospora crassa* tyrosinase shows mainly β-strands [23], which is reminiscent of the secondary structure of domain 3 of *P. interruptus* Hc (7-stranded β-barrel). For mushroom tyrosinase, there are also several β-strands predicted in the C-terminal domain, several of which are consistent with the predicted β-strands in *N. crassa* tyrosinase (data not shown). However, it must be remembered that the sequence similarity between (fungal) tyrosinases and hemocyanins in this protein domain is low. In analogy to hemocyanin, the C-terminal extension of fungal tyrosinase could fulfill a similar function by interacting with the active site. Whether this interaction prevents binding of the active site copper ions or interferes with substrate binding is not yet known [23]. In the known plant tyrosinase sequences, there are also several β-strands predicted in the C-terminal domain [51, our unpublished data] which could also form a β-barrel analogous to Hc. In this case also an amphiphatic helix was predicted in the C-terminal domain [51]. In contrast to this, several α-helices are predicted in the C-terminal domain of mammalian tyrosinases, but no β-strands are predicted (data not shown).

Structure model for active site of tyrosinase

As indicated above, similarity in sequence, chemical and spectroscopic behaviour suggests that the tertiary structure of the binuclear copper site of tyrosinase is very similar to that found in the hemocyanins. Therefore, knowledge of the structure of arthropodan hemocyanin may be helpful in suggesting a structure model for the active site of tyrosinase.

We have investigated in more detail whether a structural model for the fungal and plant tyrosinases can be constructed by using the arthropodan Hc crystal structure as a template. For this we examined the sequence alignments of hemocyanin with tyrosinases, which are based on both sequence and secondary structure similarity. With the aid of the X-ray structure of the Cu-binding domain of *Limulus polyphemus* Hc [80], shown in Fig. 5, we have started the construction of a model for the active site of mushroom tyrosinase. Because of the similarity in sequence and secondary structure, the CuB region [α2.5 and α2.6; Fig. 3(B)] is presumed to be similar in tyrosinase and crab Hc. However, the loop between these helices is shorter in mushroom tyrosinase than in Hc [see Fig. 3(B)].

Interestingly, this deletion can be accommodated without disturbing the positions of the two α-helices (Fig. 5). Furthermore, the β2B and β2C strands, which in Hc interact with this loop between α2.5 and α2.6, are also not present in the mushroom tyrosinase sequence.

As mentioned above, modeling the CuA region presents some problems, because homology around CuA is only conserved between tyrosinases and molluscan Hcs. Although the histidines in CuA of tyrosinases have been shown to ligate Cu, their detailed environ-

ment in the structure is not known yet. However, as stated above, the presence of the third histidine in CuA of molluscan Hc and tyrosinase in a FXXXH motif [Fig. 3(B)], which in arthropodan Hc is important for Cu-binding, suggests that at least the most C-terminal histidine is present in an α-helix. Several prolines are present between the second and the third histidine in the tyrosinase sequences. As stated earlier, prolines are helix-breakers, and therefore the second and third histidine in CuA are not expected to be located together in the same α-helix, but each could be located in a different α-helix segment.

DISCUSSION

Our interest in the tyrosinase enzymes is two-fold. Since the physiological function of tyrosinase in plants and fungi is not yet fully understood, studies on the tyrosinase (iso)enzymes of various species, their expression and mechanism of activation are needed to further elucidate the role of this ubiquitous enzyme. Secondly, there is a need to diminish the severe economic losses due to tyrosinase-mediated browning of for instance mushrooms and potatoes, either in the fresh or the processed product.

Furthermore, in other plants, the activity of tyrosinase is desirable because it contributes to positive sensory properties. Taken together, this compels the need for insight into the structural aspects of this intriguing enzyme.

In this review, current knowledge about tyrosinases of various species, ranging from bacteria to man, is discussed, with emphasis on taxon-specific properties, particularly for plant and fungal tyrosinases. We have postulated a working hypothesis on the structure and (posttranscriptional and posttranslational) processing of tyrosinases, which is consistent with the current knowledge about the relationship between protyrosinase and the active enzyme. Furthermore, by using the known crystal structure of hemocyanins as a model for the copper-binding domain of tyrosinase, we are in the progress of constructing a tertiary model for the active site of mushroom and plant tyrosinase, which will integrate all available structural, spectroscopic and biochemical data. Our aim is to generate a structure model of the electronic and geometric structure of the Cu-binding domain of tyrosinase in the absence of X-ray crystallographic information. The derived model will shed more light on the mechanism of action of tyrosinases and can direct the design of mutagenesis experiments to test its validity. Furthermore, it will be useful in designing or improving modulators of enzymatic activity, e.g. inhibitors of tyrosinase that can stop or reduce the undesired browning.

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