Identification of Active Site Residues Involved in Metal Cofactor Binding and Stereospecific Substrate Recognition in Mammalian Tyrosinase. Implications to the Catalytic Cycle†

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ABSTRACT: Tyrosinase (Tyr) and tyrosinase-related proteins (Tyrps) 1 and 2 are the enzymes responsible for mammalian melanogenesis. They display high similarity but different substrate and reaction specificities. Loss-of-function mutations lead to several forms of albinism or other pigmentation disorders. They share two conserved metal binding sites (CuA and CuB) which, in Tyr, bind copper. To define some structural determinants for these differences, we mutated Tyr at selected residues on the basis of (i) conservation of the original residues in most tyrosinases, (ii) their nonconservative substitution in the Tyrps, and (iii) their possible involvement as an endogenous bridge between the copper pair. Two mutations at the CuA site, S192A and E193Q, did not affect Tyr activities, thus excluding S192 and E193 as endogenous ligands of the copper pair. Concerning CuB, the H390Q mutation completely abolished Tyr activity, whereas Q378H and H389L mutants showed 10−20% residual specific activities. Their kinetic behavior suggests that (i) H390 is the actual third ligand for CuB, (ii) H389 is critical for stereospecific recognition of *o*-diphenols but not monophenols, and (iii) the involvement in metal binding of the central extra H residue at the Tyrps CuB site is unlikely. However, replacement of Q (in Tyr) by H (in Tyrps) greatly diminished the affinity for L-dopa, consistent with the low/null tyrosinase activity of the Tyrps. These are the first data showing a physical difference in docking of mono- and *o*-diphenols to the Tyr active site, and they are used to propose a revised scheme of the catalytic cycle.

Melanin synthesis is a complex pathway involving enzymatic and chemical reactions, which, in mammals, is restricted to melanocytes. At least three enzymes, tyrosinase (Tyr)[1] and the tyrosinase-related proteins Tyrp1 and Tyrp2 (Dct) are involved. The melanogenic pathway starts with the Tyr-catalyzed conversion of L-tyrosine into L-dopa quinone (L-DQ), so that mutations in the *Tyr* gene are associated with type I human oculocutaneous albinism (*1*). The reaction involves two steps, the rate-limiting hydroxylation of L-tyrosine to L-dopa (monophenolase activity, so that Tyr is described as EC 1.14.18.1) and the oxidation of this intermediate *o*-diphenol to L-DQ (*o*-diphenol oxidase activity, so that Tyr is also described as EC 1.10.3.1). It has been shown that Tyr catalyzes the direct transformation of L-tyrosine into L-DQ without releasing L-dopa (*2*, *3*). L-DQ evolves to several intermediates which polymerize with the involvement of Dct and Tyrp1 to finally render melanins (*4*, *5*).

Sequence comparison of Tyr, Tyrp1, and Dct reveals that these proteins share many key structural features, due to their common origin from a single ancestral gene (*6*). They display a single membrane-spanning fragment near their C-terminus and two very similar metal ion binding sites. They also undergo posttranslational processing, including several glycosylation steps that appear to be crucial for acquisition of full enzymatic activity (*7*). Despite their extensive sequence similarity, Tyr, Tyrp1, and Dct show remarkable differences in their metal ion binding and enzymatic properties. As far as the mouse proteins are concerned, Tyrp1 displays a residual dopa oxidase activity but is able to oxidize DHICA (*8*, *9*), whereas Tyr is efficient in tyrosine hydroxylation but does not oxidize the carboxylated indole. Finally, Dct is not involved in redox reactions but catalyzes a tautomerization. Therefore, the Tyr family is a good model to study structure−function relationships as well as divergent functional evolution.

Tyr contains a pair of coupled copper ions at the active site (*10*, *11*). Some data based on the damage of H residues after photoinactivation of fungal Tyr, the sequence similarity among Tyr and hemocyanins (*11*, *12*), and the recently available crystallographic data of a plant catechol oxidase (*13*) indicate that two H-rich regions named CuA and CuB are involved in copper binding. The involvement of human TYR CuA and CuB sites in copper binding has also been demonstrated by site-directed mutagenesis studies (*14*). Both sites are similar in Tyr and Tyrps, but the conservation is higher in the CuB site (Figure 1), thus suggesting that this site is particularly important for full enzymatic activity.

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[1] Abbreviations: Dct, dopachrome tautomerase; dopa, 3,4-dihydroxyphenylalanine; DO, dopa oxidase; DQ, dopa quinone; MBTH, 3-methyl-2-benzothiazolinone hydrazone; TH, tyrosine hydroxylase; Tyr(s), tyrosinase(s); Tyrp(s), tyrosinase-related protein(s); wt, wild type.

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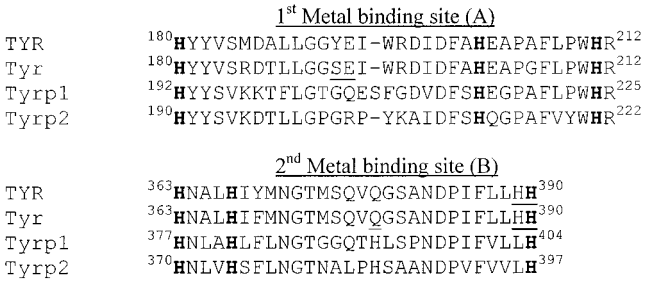

FIGURE 1: Alignment of the CuA and CuB binding sites of human TYR and mouse Tyr, Tyrp1, and Tyrp2 (Dct). The five amino acids mutated in this study are underlined, and the H copper ligands are shown in bold.

Both CuA and CuB sites contain three conserved H residues bound to copper. By virtue of the folding of the protein, both sites are adjacent and form the enzyme active center. The two sites differ in that CuA has a H-x(*n*)-**H**-x(8)-**H** motif whereas CuB has a **H**-x(3)-**H**-x(*n*)-HH motif. In both cases, the distances between two H ligands (shown in bold) are constant, whereas the position of the other H is variable. The situation is further complicated by the presence in the CuB site of Tyr, but not of the Tyrps, of a fourth H residue vicinal to the third one. According to the consensus sequence of the CuB site, the nature of the first pair of H residues is clear, but there are some doubts as to which one of the two vicinal H's is the authentic third copper ligand. Mutagenesis studies on human TYR (*14*) would suggest that the first one (H389 in the mouse and human enzymes) is more directly involved in copper binding. Recently, Nakamura et al. (*15*) proposed a new structure of the active site of *Aspergillus oryzae* Tyr where each copper would be in fact bound to four ligands. CuA would be bound to one Cys and three His residues, and CuB would be bound to four His including the two vicinal corresponding to H389 and H390 in mouse and human Tyr.

Tyrp1 and Dct display a **H**-x(3)-**H**-x(*n*/2)-H-x(*n*/2)-LH CuB motif. Therefore, they have the additional fourth H centered between the first H pair and the last H. In Tyr the central H is replaced by Q378 (Figure 1). This central H residue is, on theoretical grounds, another potential metal ion ligand in Tyrps. Should this be the case, the residues involved in metal binding might be different in Tyr and Tyrps, and this might account for the different metal specificity (*16*).

Despite the direct transformation of L-tyrosine into L-DQ by Tyr, it is clear that L-dopa is an alternative substrate and that the reactions underlying tyrosine hydroxylation (TH) and dopa oxidation (DO) must differ. It has long been debated whether the hydroxylase and oxidase activities of Tyr share a common catalytic site. Both activities have been usually inseparable, but early studies pointed out subtle differences between both reactions (*17*, *18*), suggesting different requirements at the reaction site. In this regard, TH activity shows a lag period before the reaction reaches maximal rate, whose length increases with the concentration of L-tyrosine (*19*, *20*). L-Dopa, the immediate product of L-tyrosine hydroxylation, is also a cofactor for TH, and low amounts of the *o*-diphenol abrogate the lag period (*21*). The affinity of Tyr is about 100-fold higher for L-dopa acting as cofactor for TH than as substrate for DO, suggesting that dopa might bind to two sites of the enzyme (*21*). The dual role of L-dopa as substrate for DO activity but cofactor for TH activity has greatly complicated the understanding of the Tyr mechanism of reaction. Current evidence suggests that although the enzyme has a single binding site for both substrates, monophenols would bind through different structural elements than *o*-diphenols (*22*). In summary, neither the structural determinants of the metal ion binding nor the residues involved in a possible differential interaction with monophenols and *o*-diphenols are adequately characterized. To gain further insight on these points, we performed a site-directed mutagenesis study of selected residues in mouse Tyr. The mutated amino acids were selected mainly on the basis of their conservation in a wide range of Tyrs including human, but being nonconservatively replaced in the Tyrps.

Table 1: Primers Used throughout This Study[a]

Name	Sequence
mTyrFw	$^{-16}$TGATGAATTC**GAGAAA**ATG**TTCTTGGCTGT**^{14}
mTyrRv	^{1616}GTTTTCTAGAATGT**TCA**CAGATGGCTCTGA^{1587}
378HFw	^{1129}GTA**CAC**GGATCGGCCAACGAT^{1149}
389LFw	^{1162}CTT**CTC**CATGCTTTTGTGGAC^{1182}
390QFw	^{1165}CAC**CAG**GCTTTTGTGGACAGT^{1185}
192ARv	^{580}TTTC**AGC**GCCCCCAAGCAGT^{561}
193QRv	^{583}ATAT**TTG**AGAGCCCCCAAG^{565}
SF5Fw	^{867}CGATGGAACACCTGAGG^{883}

[a] Names make reference to the mutated amino acid position, and Fw and Rv mean respectively forward and reverse orientations in PCR. The mutated codons are in bold, except for mTyrFw and mTyrRv, where bold characters indicate respectively the start and stop codons. Underlined sequences denote a restrictase site for cloning. SF5Fw was used as the paired primer to CuB mutagenic primers.

EXPERIMENTAL PROCEDURES

Reagents. The radioactive substrate L-[3,5-^{3}H$_2$]tyrosine, specific activity 50 Ci/mmol, was obtained from Amersham Pharmacia (Little Chalfont, England). The specific αPEP7 antiserum recognizing the C-terminal tail of mouse Tyr was a gift from Dr. V. J. Hearing (NIH, Bethesda, MD). Electrophoresis and Western blot reagents and materials were from Bio-Rad (Hercules, CA) or Amersham Pharmacia. Reagents and plasticware for cell culture were obtained from either Nunc (Roskilde, Denmark) or Gibco (Gaithersburg, MD). Proteinase K, restrictases, and other plasmidic DNA-handling enzymes were from Roche Molecular Biochemicals (Mannheim, Germany). Substrates, inhibitors, and other reagents were from Sigma (St. Louis, MO), Merck (Darmstadt, Germany), or Prolabo (Barcelona, Spain).

Site-Directed Mutagenesis. Expression constructs for wt-T*yr* (full-length tyrosinase), Δ3*Tyr* [inactive alternatively spliced *Tyr* lacking exon 3 (*23*)], and all mutant Tyr were directly prepared or subcloned in the pcDNA3 expression vector (Invitrogen, Amsterdam, The Netherlands). For wt*Tyr* and the Δ3*Tyr*, cDNA from B16 cells was obtained and amplified by PCR, using the proofreading *pfu* polymerase (Stratagene, La Jolla, CA) as previously described (*5*). Both were cloned into pBKSII (Stratagene, Mannheim, Germany) and then subcloned into pcDNA3. For mutant species, fragments with point mutations were obtained by PCR, using wtTyr-pBKSII as template. Each mutation was introduced with the appropriate primer mutated at the desired codon (Table 1). Amplicons were digested with *Sph*I and *Xba*I (for CuB mutations) or with *Eco*RI and *Sph*I (for CuA mutations)

and cloned into wtTyr-pBKSII after removing the corresponding homologous wild-type fragment with the same restriction enzyme pair. The mutated genes were subcloned into pcDNA3. Mutations and identity of the products were always checked by complete sequencing.

Cell Culture, Transfection, and Preparation of Crude Solubilized Extracts. B16 and COS7 cells were grown in DMEM supplemented with 1% streptomycin, 1% penicillin, and 5% fetal calf serum. HEK 293T cells were grown in RPMI-1640 containing the same antibiotics plus 1% fungizone and 10% fetal calf serum. Cells (10^4 cells/cm^2) were seeded in six-well plates, incubated at 37 °C in a water-saturated 5% CO_2 atmosphere, and allowed to grow to approximately 80% confluence. Transfection was performed with the SuperFect transfection reagent (Qiagen, Hilden, Germany). Cells were trypsin-harvested 24 h after transfection, washed twice with saline phosphate buffer, and solubilized in 10 mM sodium phosphate, pH 6.8, containing 1% Igepal CA-630 and 0.1 mM PMSF using a ratio of approximately 1 mL solubilization buffer/10^7 cells. The extracts were centrifuged at 20000*g* for 10 min, and the supernatants were used for enzyme activity determinations and Western blotting. Protein concentration was determined by the bicinchoninic acid assay.

Enzyme Activity Determinations. TH activity was determined by a radiometric method described elsewhere (*18*, *24*). One unit was defined as the amount of enzyme catalyzing the hydroxylation of 1 μmol of L-tyrosine/min, in the presence of 50 μM L-tyrosine and 10 μM L-dopa as cofactor. When appropriate, other conditions were used as detailed. DO activity was measured spectrophotometrically at 500 nm in the presence of MBTH (*25*) using final concentrations of L-dopa and MBTH of 2.0 and 3.8 mM, respectively.

Electrophoretic and Immunochemical Procedures. For activity stain of SDS−PAGE gels, electrophoresis was performed in 9% or 12% acrylamide gels under nonreducing conditions and at 4 °C to preserve enzymatic activity. Samples were mixed in a 2:1 ratio with sample buffer (0.18 M Tris-HCl, pH 6.8, 15% glycerol, 0.075% bromophenol blue, 9% SDS). A sensitive and specific DO stain was carried out by equilibrating the gels at pH 6.0 with 50 mM sodium phosphate buffer, followed by incubation at 37 °C in 1.5 mM L-dopa and 4 mM MBTH, in 10 mM phosphate buffer, pH 6.8, from 15 to 30 min (*26*). For immunochemical detection and quantification of Tyr, samples were mixed with the same sample buffer as above, but containing 2-mercaptoethanol, and heated at 95 °C for 5 min before electrophoresis. Gels were transferred to PVDF membranes, and the specific αPEP7 antiserum was used as previously described (*5*). Before blocking, the lower portion of the membrane was cut and stained for total protein with Amido Black to ascertain comparable loading and transfer. Staining of immunoreactive bands was done with a chemiluminescent substrate from Amersham Pharmacia and quantitation was performed in a Gel Doc system (Bio-Rad, Hercules, CA).

RESULTS AND DISCUSSION

High, Comparable, and Quantifiable Expression of wt and Mutant Tyr in Transfected Cells. The major aim of this study was to analyze the effect of selected mutations on the Tyr activities. Such mutations are likely to result in partial or total loss of function. Therefore, two essential prerequisites for such a study are (i) to set up an accurate method for quantitation and comparison of the expression levels of mutant forms and (ii) to ascertain high and reproducible expression of wt and mutant proteins.

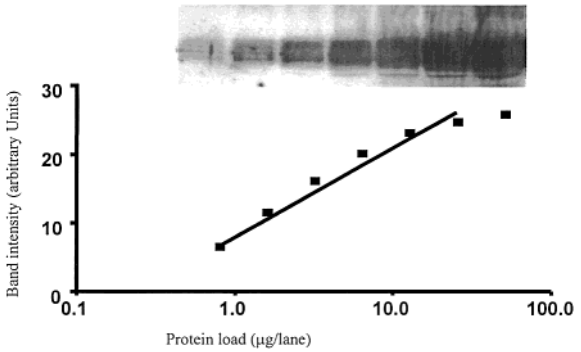

FIGURE 2: Quantitative detection of transiently expressed Tyr. Serial dilutions of COS7 cell extracts transfected with wtTyr were analyzed by Western blot, using the αPEP7 antiserum.

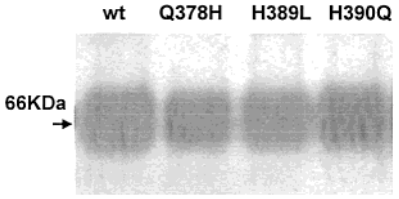

FIGURE 3: Comparison of the expression levels of wtTyr versus Q378H, H389L, and H390Q constructs. An equal protein amount (15 μg) was electrophoresed, transferred to PVDF membranes, and probed with αPEP7. Bands were visualized with a chemiluminiscence detection kit. Variations lower than 30% were found in five independent experiments.

The feasibility of an accurate quantification of the relative expression levels was checked by Western blot. Serial dilutions of extracts from COS7 cells transfected with the wt gene were analyzed using the Tyr-specific αPEP7 antiserum. The intensity of the resulting band was linear for protein loads (in a log scale) ranging approximately from 1 to 20 μg, but densitometric measurements suffered a saturation for higher loads, evident for the maximal amount tested, 52 μg (Figure 2). We performed all experiments with total protein amounts ranging from 10 to 20 μg to normalize enzymatic rates. Under our conditions, the lowest levels of Tyr protein that could be detected by Western blot corresponded to an enzymatic activity of approximately 0.36 microunits of TH. In addition, TH and DO activities were not detected in cells transfected with Δ3*Tyr* lacking the CuB region. Thus, this method allowed for a sensitive detection of Tyr expression and for an accurate comparison of the expression levels in different samples. As expected, neither DHICA oxidase nor Dct activities were found in transfected cells.

We next examined by the same technique whether expression of the different constructs was comparably effective and checked for possible major alterations in the structure or size of the mutated proteins. The expression efficiencies of the wt and all CuB mutant Tyrs were comparable, and their electrophoretic mobilities were indistinguishable (Figure 3). Similar results were obtained for mutants in the CuA site (data not shown). Therefore, any activity changes observed for the mutant Tyrs should be related to the catalytic role of

Table 2: Comparison of the Specific Activities (% Relative to wtTyr Expressed in COS7) of wtTyr and Mutant Tyr Species[a]

wtTyr	TH (microunits /mg)[b]	DO (milliunits/mg)[b]	TH × 10^3/ DO ratio
in B16	136 ± 16 (19)	8.1 ± 2.9 (27)	16.8
in COS7	711.3 ± 163.5 (100)	29.9 ± 6.5 (100)	23.2
mutant Tyr (in COS7)			
S192A	649.6 ± 97.6 (93)	27.5 ± 4.3 (92)	23.6
E193Q	687.4 ± 85.4 (98)	28.2 ± 4.9 (94)	24.4
Q378H	121.2 ± 26.8 (17)	2.7 ± 0.7 (9)	44.9
H389L	129.0 ± 25.5 (18)	5.7 ± 1.4 (19)	22.6
H390Q	0	0	

[a] Relative expression levels were normalized by Western blots. [b] Values in parentheses are in percent.

the mutated residues rather than to artifacts arising from alterations in protein folding and/or processing (*27*).

The enzymatic activities of extracts from COS7 cells transfected with wtTyr were much higher than those normally found in B16 mouse melanoma cells (711 ± 163 versus 136 ± 16 microunits/mg for TH activity and 30 ± 6.5 versus 8.1 ± 2.9 milliunits/mg for DO, $n \geq 4$; see Table 2). Further preliminary experiments showed that the efficiency remained constant by using a constant ratio of DNA amount to cell number. The Tyr expression levels using COS7 and HEK 293 cells as hosts were also compared. Similar results in terms of enzymatic activities and electrophoretic mobility of the expressed protein were obtained. Therefore, COS7 cells were routinely used in this work. The high and reproducible levels permitted the determination of enzymatic activities in mutant forms with diminished or residual catalytic capabilities, so that cotransfection of COS7 with calnexin was not necessary to improve Tyr processing (*27*, *28*).

The Q378H, H389L, and H390Q Mutations in the CuB Site Have a Higher Impact on Tyr Activity Than the S192A and E193Q Mutations in CuA. A comparison of the specific activities of the mutant Tyrs normalized by Western blot determination of the protein amount is shown in Table 2. The two mutations at the CuA site, S192A and E193Q, were isosteric substitutions to detect the possible involvement of the native residues as endogenous ligands bridging the two coppers in the binuclear active site. The existence of an oxygenated endogenous ligand bridging the two copper ions was postulated on the basis of spectroscopic studies on the hemocyanin and Tyr active site (*11*, *29*). This putative bridge would provide an antiferromagnetic coupling and thus would account for the lack of a detectable EPR signal (*30*). According to the hemocyanin model (*31*), the ligand should be the side chain of a residue located in the coil fragment between the two helical regions containing the H bound to CuA. Alignment of all Tyr sequences pointed out to a hydroxylated candidate, the S192 in the mouse enzyme. This residue is highly exposed since it follows a GG motif and it shows a polymorphism in human TYR, S192Y (*1*). Both amino acids contain a hydroxyl/phenolic group, enabling them to act as the postulated bridge. Furthermore, the next residue E193 changes to Q in Tyrp1 and to R in Dct (Figure 1). Mutation in Dct 193R greatly decreases its activity and is responsible for the *slaty* phenotype (*32*, *33*). Taking into account the fact that Tyr is a copper enzyme but Dct is a zinc enzyme lacking the endogenous bridge (*34*), it was also reasonable to explore this residue. However, neither the S192A nor the E193Q mutants showed significant differences in their catalytic activities as compared to wtTyr. Thus, mutations on the CuA binding site indicated that the existence of a residue bridging the two copper ions at the Tyr active site is very doubtful. The same conclusion was recently reported for Tyr from *Streptomyces antibioticus* (*35*). The lack of an endogenous bridge at the dicopper center seems to be a general feature of all Tyr.

On the other hand, the three mutations in the CuB site resulted in major enzymatic activity changes. The Q378H and H389L mutants retained low but significant levels of both TH and DO activities (10−20%), enough for analysis of some kinetic aspects. H390Q Tyr was totally devoid of any measurable enzymatic activity. None of the mutants displayed detectable Dct activity. Interestingly, the Q378H mutant showed a TH/DO ratio about twice as high as that of the wt and other mutant forms.

The residual activity of the Q378H and H389L mutants strongly supported the fact that they are still able to bind copper ions at their respective active site and pointed to H390 as the actual third copper ligand. Thus, the second H within the HH pair found at the end of the CuB motif is most likely the copper ligand in mammalian Tyr, as described for the microbial and fungal enzyme (*11*, *12*). Note that the two first mutations make the CuB site of Tyr more similar to Tyrps. The observation that H389L, but not H390Q, Tyr is catalytically active is consistent with the binding of a metal ion to Tyrps active site despite the LH sequence found at their CuB site.

However, this interpretation is in contrast with data on direct ^{67}Cu binding to wt and mutant human TYRs (*14*). In this study, the H389A and H390A mutant species were both enzymatically inactive, but the H390A was able to bind twice as much ^{67}Cu as wt. At least three possible explanations may account for this discrepancy. The first one relies in differences in the nature of transfected cells, HeLa (*14*) versus COS7 cells (this work). The chaperone repertoire in both systems is likely different, and HeLa cells have recently been described as an inefficient system for human Tyr processing and exit from the endoplasmic reticulum (*36*). Second, differences in the nature and stability of mouse Tyr and human TYR should also be considered. We have recently reported that the human, but not the mouse, enzyme shows DHICA oxidase activity (*5*), indicating subtle differences at the active site of both species. In addition, and in our hands, Tyr activities showed a remarkable unstability in transfected extracts, likely due to the absence of Tyrp1 in nonmelanocytic cells. This last protein has a marked stabilizing effect on Tyr (*37*, *38*), and its absence might lead to Tyr activity levels below the detection limit when the efficiency of the expression system is not as high as the one reported here. Third, direct copper binding assays should be interpreted with caution, because protein misfolding could yield enzymatically inactive species, which, nevertheless, could still be able to bind copper. This artifactual binding could even involve residues located outside the active site, thus accounting for higher metal loads in mutant proteins as compared to wtTyr. In keeping with this, Tsai et al. (*39*) also obtained controversial data on copper binding to mutant *Streptomyces* Tyr, where each of the six H residues of the copper sites were replaced by Q. The three mutations in CuA and one in CuB

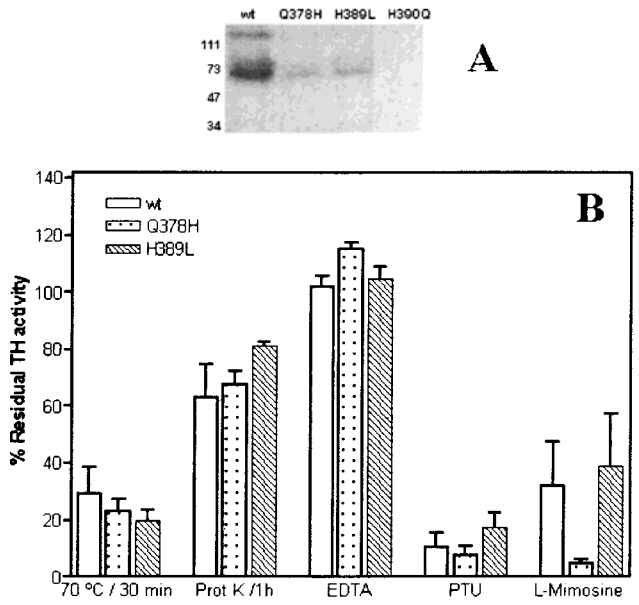

FIGURE 4: (A) Gel staining for DO activity of wtTyr and mutant Tyr species. Equal protein amounts were resolved by nondenaturing SDS−PAGE to correlate relative intensity of the bands to residual enzymatic activities of the mutant forms. The H390Q mutant was devoid of enzymatic activity, whereas the other forms displayed similar mobility. (B) Resistance of wtTyr (empty bars), Q378H (dotted bars), and H389L (dashed bars) mutant forms to thermal inactivation (70 °C, 30 min), proteinase K digestion (1 mg/mL, 1 h), EDTA (2 mM, 3 h), and the active site-directed inhibitors phenylthiorea (0.5 mM) and L-mimosine (0.5 mM).

blocked copper incorporation and abolished enzymatic activity, but, conversely, mutation of the two remaining H in CuB (including the one homologous to the human/mouse H390) was still compatible with copper binding to the enzyme.

Changes in Tyr Activity Caused by CuB Mutations Are Not Related to a Significant Alteration of the Protein Structure. We further examined whether the reduced specific activity of the Q378H and H389L mutants could be due to kinetically irrelevant effects such as decreased stability. These and other possibilities were explored by (i) probing the resistance to chaotropic agents by specific activity stain of SDS−PAGE gels run under nonreducing conditions, (ii) analysis of the thermal stability and susceptibility to proteolytic digestion, and (iii) inhibition by active site directed chelating compounds. The residual DO activity of the Q378H and H389L mutants, as determined by SDS−PAGE followed by a specific activity stain (*26*), was within the range of the one obtained by the usual spectrophotometric method (around 10−20% of the wt enzyme), thus showing that these mutant Tyrs are equally resistant to SDS than the wt form (Figure 4A). The H389L and Q378H species retained residual activity levels comparable to those of wtTyr after thermal shock or treatment with proteinase K, suggesting a compact protein folding. The comparable resistance of wt and H389L Tyrs to 2 mM EDTA (Figure 4B) indicates a similar affinity of the two proteins for copper, pointing again to H390 as the actual copper binding residue of the ^{389}HH^{390} pair. Finally, the residual activities of H389L and wtTyrs after treatment with phenylthiourea or L-mimosine also supported a similar structure and accessibility of the active site. Taken together, these data show that the conformations of the wt and mutant Tyrs are very similar. This agrees with recent results reporting that, generally, mutations affecting Tyr folding produce ER retention and degradation of the protein (*27*). Thus, the lower specific activities of H389L and Q378H Tyrs actually reflect an impairment in the catalytic potential of the mutants.

Kinetic Parameters and Stereospecific Properties of wt and Mutated Tyrs. To understand the structural basis for the low specific activity of Tyr mutants at CuB, the K_M, V_{max}, and catalytic efficiency (V_{max}/K_M ratio) parameters of wt and mutant Tyrs for several *o*-diphenolic substrates and L-tyrosine were analyzed (Table 3). Concerning *o*-diphenols, the affinity of wtTyr is decreased by decarboxylation (around 4-fold), supporting the role of the carboxyl group in substrate recognition. The maintenance of the carboxyl group on the substrate, but with different stereospatial conformation, decreases the affinity even more, as shown by comparison of the K_M for D-dopa and L-dopa (approximately 10 times higher for D-dopa). However, esterification of the carboxyl group has a poor effect, thus excluding an electrostatic enzyme−substrate interaction. Catalytic efficiencies for the different substrates correlate with affinities, D-dopa being the poorest substrate.

The differences in the kinetic parameters for the four *o*-diphenols were much smaller for the H389L mutant species. The replacement of the 389H by a hydrophobic L (resembling Tyrps) decreased the affinity of Tyr for L-dopa but increased it for D-dopa in comparison to wtTyr. This confirms that H389 is involved in a stereospecific interaction with the carboxyl group of the substrate, rather than in copper binding. On the other hand, the Q378H mutant showed a very low affinity for L-dopa (around 50 times lower), suggesting that the imidazole group at that position would create a strong steric hindrance that impairs L-dopa docking. The K_M for the other *o*-diphenols could not be accurately estimated due to the very slow reaction rates.

The effect of the H389L mutation on the stereospecificity for L-dopa prompted us to investigate its effect on TH activity. The first difference with DO was the similar K_M for L-tyrosine of the wtTyr and the mutant forms. The strong effect of the mutations on the K_M for L-dopa but not for L-tyrosine supports the fact that the residues located in the CuB site are more closely related to DO than to TH activity.

We next tested the kinetic pattern of the decarboxylated analogue tyramine and D-tyrosine. As compared to *o*-diphenols, the monophenolic substrates presented additional difficulties. As TH activity has a lower turnover number than DO (*19*, *20*), the reaction rates must be measured by radiometric assays using L-[3,5-$^3H_2$]tyrosine as substrate (*18*, *24*), but radioactively labeled tyramine and D-tyrosine are not commercially available. Thus, we assayed tyramine and D-tyrosine hydroxylation by an indirect method based on the rationale that, if these substrates are recognized by Tyr, then they should behave as competitive inhibitors of tritium release from L-[3,5-$^3H_2$]tyrosine. D-Tyrosine and tyramine were not good inhibitors of the TH activity of wtTyr. D-Tyrosine (0.2 mM) did not inhibit at all the hydroxylation reaction of L-tyrosine under standard conditions, and a 20-fold molar excess of D-tyrosine over L-tyrosine (1 mM versus 50 μM) was needed to detect a noticeable slight inhibition of the reaction rate (Figure 5A). Therefore, wtTyr is also stereospecific for L-tyrosine. The same behavior was found for mushroom Tyr (*40*). The inhibition by tyramine was more significant. The order of affinity for the monophenols tested

Table 3: K_M (mM), V_{max} (milliunits/mg), and V_{max}/K_M Ratio for wtTyr and the H389L and Q378H Mutant Tyr Species for Four *o*-Diphenols[a] and L-Tyrosine[b]

	wtTyr			H389L			H378Q		
	K_M	V_{max}	V_{max}/K_M	K_M	V_{max}	V_{max}/K_M	K_M	V_{max}	V_{max}/K_M
L-dopa	0.46	38.8	84.34	1.83	38.9	21.25	22.2	32.67	1.47
dopamine	1.64	23.4	14.27	2.01	36.43	18.12	nd[c]	nd	nd
D-dopa	4.57	41.3	9.09	2.41	31.50	13.06	nd	nd	nd
L-dopa methyl ester	0.58	44.6	76.90	2.26	41.62	18.42	nd	nd	nd
L-tyrosine	0.079	0.9	11.39	0.106	0.88	8.30	0.076	1.47	19.34

[a] DO activity; values are the mean of two determinations. [b] TH; values are the mean of three determinations. [c] nd = not determined.

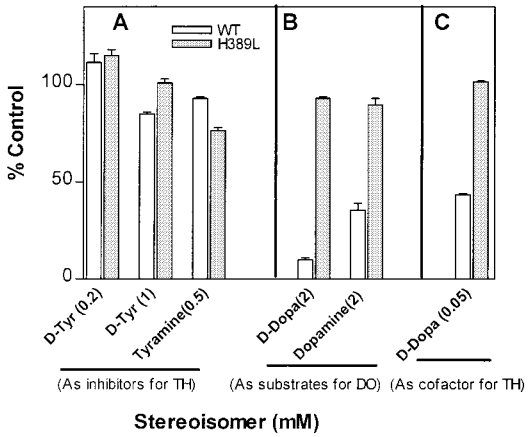

FIGURE 5: Stereospecificity of wtTyr and H389L Tyr species for different substrates. Data are expressed as percent of the control reaction rates. (A) Addition of D-tyrosine (0.2 and 1 mM) and tyramine (0.5 mM) referred to the TH standard assay using radioactive L-tyrosine. (B) Replacement of the substrate L-dopa (control) by D-dopa and dopamine (2 mM) in the DO standard assay. (C) Replacement of the cofactor L-dopa (control) by D-dopa in the TH assay using 50 M L-tyrosine and L- or D-dopa. H389L loses the stereospecificity for the DO activity and dopa binding as cofactor, but it does not for the TH reaction.

was L-substrate > decarboxylated substrate > D-substrate, the same as with *o*-diphenols.

Surprisingly, the H389L mutant showed the same behavior in the TH assay as wtTyr. Both forms displayed stereospecificity for monophenols (Figure 5B), pointing out two relevant aspects of the catalysis. First, the monophenol hydroxylase and diphenol oxidase activities have different structural requirements. In agreement with that, the H378Q mutant presented a significant increase in the catalytic efficiency for L-Tyr (Table 3), being more efficient for the TH activity than for the wtTyr. Second, H389 is involved in *o*-diphenol docking to the bicopper center but not in monophenol docking. The stereospecificity of Tyr for monophenols should reside in residue(s) different from H389, yet to be identified.

Finally, we also explored the efficiency of D-dopa as cofactor for the TH activity. It has been reported that D-dopa can substitute L-dopa as cofactor of the hydroxylase reaction without competing significantly as alternative substrate for DO (*25*). This suggested that the behavior of L-dopa acting as cofactor for TH or as substrate for DO would be different. We compared the results obtained using L- or D-dopa in a 1:1 ratio to L-tyrosine (50 μM both). D-Dopa was less efficient than L-dopa for wtTyr but not for the H389L mutant species (Figure 5C).

A Model for the Catalytic Cycles of Tyr Activities. Our results allow for the proposal of a reaction mechanism (Figure 6) that includes some modifications to former models (*11*, *30*). According to those models, the most abundant enzymatic form is oxy-Tyr, and the substrates, L-tyrosine and L-dopa, initiate the TH or DO cycles, respectively. Both phenols probably bind to the catalytic center in an axial orientation, but L-tyrosine binds preferentially to the CuA site, whereas L-dopa binds to CuB. The following data support this view: (i) H389Q mutation in mouse Tyr (at CuB) abolishes stereospecificity for L-dopa but does not for L-tyrosine (this work), (ii) the A206T mutation in human TYR (at CuA) increases TH activity 200% at the permissive temperature for correct Tyr folding but does not increase DO (*22*), (iii) the order of specificity of mushroom Tyr toward a series of monophenols is different from that of *o*-diphenols (*41*), and (iv) crystallographic data for the sweet potato catechol oxidase show that the active site of this enzyme has an aromatic residue (F261) blocking the CuA access (*13*). Accordingly, all plant catechol oxidases so far sequenced have an aromatic residue in the equivalent blocking position, and they do not display TH activity. Conversely, fungal or animal Tyrs have no blocking aromatic residue at the equivalent position, and they do show TH activity.

In the TH cycle, monophenol would labilize the oxygen bound in the catalytic center in a side-on manner (*42*). The resulting polarized molecule can ortho-hydroxylate the monophenol, and the substrate undergoes a Berry pseudorotation to an equatorial position (*43*). Oxidation of the *o*-diphenolic product would then occur at the equatorial position and the *o*-quinone would leave the reduced bicuprous site (*30*), allowing the entrance of a new oxygen molecule.

In the DO cycle, the *o*-diphenol bound to CuB would also labilize the oxygen, resulting in oxidation of the organic substrate and release of L-DQ. The enzyme is left in a bicupric state, met-Tyr. This species can next bind L-tyrosine or L-dopa with higher affinity than oxy-Tyr since the active center is not occupied by oxygen, and all substrates and aromatic inhibitors such as L-mimosine or phenylthiourea compete for the same binding site than oxygen (*13*). L-Dopa would dock to the two copper ions by both hydroxyl groups, with higher affinity than when the binding proceeds only through CuB. This higher affinity would account for the observation that the K_a measured for L-dopa as cofactor is in the micromolar range (*21*), approximately 100-fold higher than the K_M for diphenolic substrates, that is related to their affinity for oxy-Tyr. The different affinity ratio of the met and oxy enzymatic forms also accounts for the fact that D-dopa is a relatively efficient cofactor but a poor substrate (*25*, this work). The binding of both hydroxyl groups to the copper ions allows for an easy transfer of two electrons from

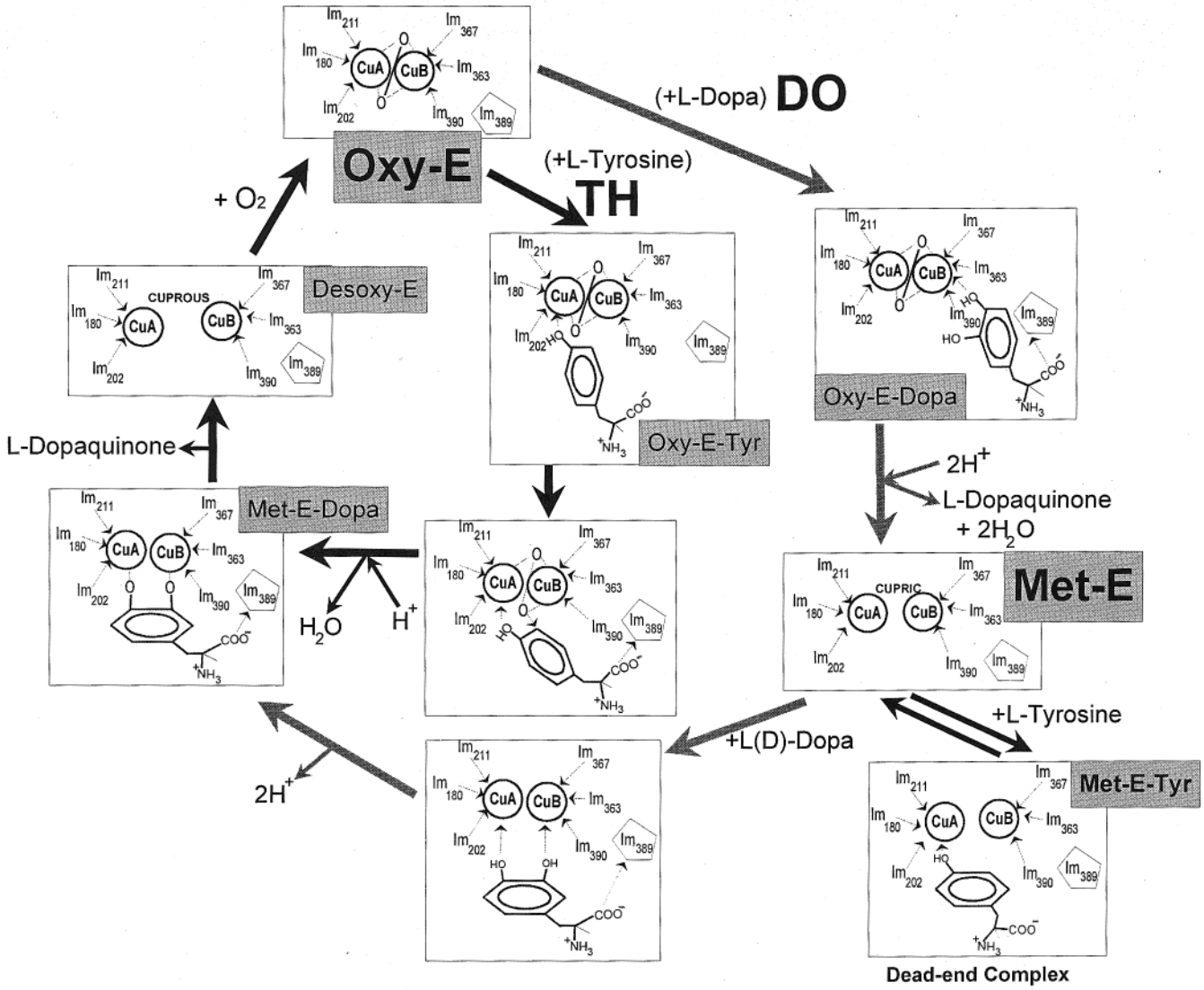

FIGURE 6: Proposed catalytic cycles for TH and DO to differentiate both activities. Tyr presents two forms (oxy- and met-Tyr) with different affinities and structural requirements for both substrates. Tyrosine acts as substrate for the oxy-Tyr and also as an dead-end inhibitor for met-Tyr. Dopa acts as cofactor when bound to the met form and competes with tyrosine for the inhibition of this species. H389 is essential for L-dopa recognition and orientation to CuB, but it is not involved in the docking of L-tyrosine to CuA.

the *o*-diphenol to the binuclear site, leading to the oxidized quinone and the reduced deoxy form, which is again oxidized upon oxygen binding.

Docking of L-tyrosine to met-Tyr would lead to a dead-end complex. As L-tyrosine binding to met-Tyr could theoretically occur through any copper ion, although with different affinities, the axial binding of two molecules of L-tyrosine to each copper at the catalytic center is also possible. This might also account for the inhibition by substrate excess that has been reported for Tyr (*24*, *44*), but such a possibility should be further explored by determining whether two aromatic rings could be located in a distance compatible with the binding of each molecule to each one of the copper ions in the met form.

The competition of L-tyrosine and L-dopa for met-Tyr also explains the characteristic lag period of the TH activity in the absence of cofactor (*3*, *19*, *20*). L-Dopa is generated in situ as a consequence of the reaction of L-DQ and L-cyclodopa during the melanogenic pathway (*4*, *5*). Therefore, the L-dopa/L-tyrosine ratio increases continuously as the reaction proceeds, and this leads to a parallel decrease in the fraction of enzymatic species reversibly captured in the dead-end complex. Thus, the proportion of Tyr molecules recruited to the productive catalytic cycle rises with the subsequent acceleration of the hydroxylase reaction rate.

In summary, the mechanism outlined at Figure 6 accounts for all kinetic features of Tyr so far described and reconciles some discrepancies in former mechanisms (*11*, *30*, *41*). It also contains evidence for the actual occurrence of physical differences in the catalytic requirements of TH and DO activities previously suggested (*18*, *22*). We propose that monophenols would dock to CuA but *o*-diphenols would dock to CuB at the Tyr active site. The inequivalence of the two copper ions was already reported by XANES (X-ray absorption near edge structure) spectra (*45*). Thus, the different spatial docking of mono- and *o*-diphenols to the Tyr active site would be an essential determinant for the course of the catalytic cycle. This means that the existence of two hydroxyl groups on the *o*-diphenol substrate orientates the binding of one of them to CuB. The interactions and residue(s) responsible for this orientation remain to be determined. On the other hand, the absence of a second

hydroxyl group in monophenolic substrates allows the interaction with CuA. In this case, the driving force for the high redox potential process would come from the presence of a negative group around CuA (*43*) and the concomitant uptake of the hydroxyl proton. The nature of this group, which is also a candidate to be directly involved in TH activity, remains to be determined too. Experiments are underway to determine those crucial residues.

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