Role of Conserved Asn-Tyr-Asp-Tyr Sequence in Bacterial Copper/2,4,5-Trihydroxyphenylalanyl Quinone-containing Histamine Oxidase*

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Copper amine oxidase contains a covalently bound quinonoid cofactor, 2,4,5-trihydroxyphenylalanyl quinone (TPQ), which is synthesized by post-translational modification of a specific tyrosyl residue occurring in the highly conserved sequence, Asn-Tyr-(Asp/Glu)-Tyr. To elucidate the role(s) of the conserved sequence in the biogenesis of TPQ, each of the corresponding residues at positions 401–404 in the recombinant histamine oxidase from Arthrobacter globiformis has been replaced with other amino acids by site-directed mutagenesis. When Asn-401 was changed to Asp or Gln, the rate of TPQ formation by copper-dependent self-processing was 103- to 104-fold slower than in the wild-type enzyme. When Tyr-404 was changed to Phe, TPQ was not formed at all, showing that Tyr-402 is essential as the precursor to TPQ. In contrast, Asp-403 could be replaced by Glu without changes in the rate of TPQ formation, whereas its replacement by Asn led to a marked decrease. Furthermore, when Tyr-404 was changed to Phe, TPQ was formed swiftly on incubation with copper ions, but the TPQ enzyme exhibited very low activity with altered substrate specificity. These results collectively indicate that a very rigorous structural motif is required for efficient formation of TPQ and for the catalytic activity in the active site of copper amine oxidases.

Copper-containing amine oxidases (EC 1.4.3.6) utilize a covalently bound quinonoid cofactor in catalyzing the oxidative deamination of various biogenic primary amines (McIntire and Hartmann, 1993; Klinman and Mu, 1994). Following the identification of 2,4,5-trihydroxyphenylalanyl quinone (TPQ)1 as the cofactor of the bovine serum enzyme (Janes et al., 1990), TPQ has been shown to be the common cofactor in all members of the copper amine oxidase family (Janes et al., 1992; Knowles and Dooley, 1994). TPQ has consistently been found to be encoded by a Tyr codon in the genes coding for these enzymes in animals, plants, and microorganisms (Tipping and McPherson, 1995), and references cited therein), indicating post-translational modification of the particular Tyr for the biosynthesis of TPQ (Mu et al., 1992). Furthermore, flanking the precursor Tyr is a conserved Asn on the N-terminal side and an Asp or Glu on the C-terminal side. Thus, Asn-Tyr-(Asp/Glu) is the consensus TPQ site in all copper amine oxidases, with an additional Tyr located toward the C terminus of Asp/Glu in most cases (Janes et al., 1992). It is evident that Tyr-382 in the conserved sequence of phenylethylamine oxidase is essential as the precursor to TPQ (Tanizawa et al., 1994; Matsuzaki et al., 1994). However, the significance of the conservation of the other residues remains unknown, except for the finding that Glu-406 in the conserved sequence of the yeast copper amine oxidase does not play a role in the TPQ biogenesis (Cai and Klinman, 1994a).

We reported recently that the inactive, copper-free form of the recombinant histamine oxidase from Arthrobacter globiformis was capable of forming TPQ when incubated aerobically with copper ion (Choi et al., 1995). This shows that the inactive enzyme is a precursor protein that can form TPQ through a self-processing mechanism, as first demonstrated with phenylethylamine oxidase from the same bacterial species (Matsuzaki et al., 1994). To elucidate the role(s) of the consensus sequence in the TPQ biogenesis, we have prepared a series of histamine oxidase proteins specifically mutated at residues 401–404 in the conserved Asn-Tyr-Asp-Tyr sequence. These mutant proteins were purified in their copper/TPQ-free precursor form, examined for their capacity for TPQ formation, and kinetically characterized in the active TPQ form. Asn-401 and Asp-403 were found to be important for the self-processed formation of TPQ. Furthermore, the essential role of Tyr-402 as the precursor to TPQ was confirmed. The roles of these conserved residues in the biogenesis of TPQ in copper amine oxidases are discussed on the basis of the recently determined x-ray crystallographic structure of the Escherichia coli amine oxidase (Parsons et al., 1995).

EXPERIMENTAL PROCEDURES

Construction of Improved Expression Plasmid—To achieve higher expression of histamine oxidase than that of pTrc99HAO constructed previously (Choi et al., 1995), a hybrid vector (named pUT) was constructed, consisting of an 1811-bp AuII-AflII fragment from pUC19 (containing a high copy number replicon and the ampicillin-resistance gene) (Yanisch-Perron et al., 1985) and a 652-bp SspI fragment from pDv95A (containing a strong trc promoter, a ribosome-binding sequence, a multiple cloning site, and an rnb transcription terminator) (Aman et al., 1988). The histamine oxidase gene (2.4 kilobase pairs) in pTrc99HAO was subcloned between NcoI and BamHI sites of pUT to give pUTH (about 4.9 kilobase pairs). The amount of the enzyme produced by the E. coli cells transformed with pUTH corresponded to about 30% of the total soluble protein in the crude extract.

PCR-based Mutagenesis—Mutant enzymes of histamine oxidase were prepared by two successive polymerase chain reactions (PCR) (Saiki et al., 1985) with the plasmid pUTH as a template. The first PCR was performed with each pair of one of the following synthetic primers

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1 The abbreviations used are: TPQ, 2,4,5-trihydroxyphenylalanyl quinone; bp, base pairs; PCR, polymerase chain reaction; Bicine, N,N-bis(2-hydroxyethyl)glycine.
containing mismatched bases (underlines) and one of the sequencing primers used in our previous studies (Choi et al., 1995) in the antisense strand, which is about 300-bp 3′-downstream from the mutagenic primer.

N401D: 5′-CACGCTGCGGCTAACCATGACGGG-3′
N401Q: 5′-CACGCTGCGGCTAACCATGACGGG-3′
Y402F: 5′-GTTGGCAACTTCCGACTACGGC-3′
D403E: 5′-GCAACTGACGACTACGGCTTC-3′
D403N: 5′-GCCAATAACGACTACGGCTTC-3′
Y404F: 5′-AATACGACGTCCTGCTTCAG-3′
Y404N: 5′-AATACGACGTCCTGCTTCAG-3′

SEQUENCE 1

The amplified product was separated on a 1% agarose gel and used as the antisense strand primer (“megaprimer”; Sarkar and Sommer, 1990) in the second PCR; the sense primer, which is about 200-bp 5′ upstream from the megaprimer, was taken from sequencing primers used in previous studies (Choi et al., 1995). A 385-bp MboI-MluI fragment excised from the amplified DNA (about 500 bp) was substituted for the corresponding region in the wild-type plasmid pUTH. The resulting constructs were used to transform E. coli JM109 cells for expression of mutant proteins.

Purification of Wild-type and Mutant Enzymes—E. coli JM109 cells carrying pUTH or one of the mutant plasmids were grown at 37 °C in a copper-depleted medium (Matsuzaki et al., 1994; Choi et al., 1995) supplemented with 50 μg/ml sodium ampicillin. Cells were cultivated until the cell density reached an A600 = 0.8 (about 3 h); then 50 μM isopropyl-1-thio-β-D-galactopyranoside was added and the bacteria were further cultivated at 30 °C for 6 h. The wild-type and mutant enzymes were purified to homogeneity in the copper-free form using the procedure reported previously (Choi et al., 1995). The copper-containing form of the wild-type and mutant enzymes were prepared by overnight incubation at 4 °C with an excess of 5–10 μM equivalents of CuSO4 over the enzyme subunit. They were dialyzed thoroughly against 50 mM HEPES, pH 6.8, containing 3 mM EDTA, followed by dialysis against buffer alone to remove the unbound copper. The enzyme and protein assays were performed as described previously (Choi et al., 1995).

Measurement of Oxygen Consumption—Oxygen consumption was measured polarographically at 25 °C with a standard Clark oxygen electrode (Toko Chemical Laboratories Co., Ltd., Tokyo). To the water-jacketed sample chamber was added the purified wild-type or mutant enzyme (5 mg/ml) in 50 mM HEPES buffer, pH 6.8 (total volume, 0.7 ml). The incubations were allowed to equilibrate for 10 min prior to the addition of CuSO4 (20–30 μl). The dissolved oxygen concentration was calculated as about 0.25 μM in an air-saturated solution at 25 °C.

Titration with Phenylhydrazine—The method of titration with phenylhydrazine was identical to that previously reported (Choi et al., 1995). For the determination of stoichiometry, the enzyme concentration was calculated using the subunit concentration of the dimeric enzyme with an approximate M, of 75,000 (Choi et al., 1995). The phenylhydrazine solution used was freshly prepared by dissolving reagent recrystallized from methanol.

Atomic Absorption Analysis—The copper contents of the enzyme proteins were analyzed with a Nippon Jarrell-Ash AA-880 mark II atomic absorption spectrophotometer (acetylene/air flame) at 324.8 nm.

RESULTS

Expression and Purification of Wild-type and Mutant Histamine Oxidases—An improved expression plasmid (pUTH) for the recombinant histamine oxidase from A. globiformis (McPherson, 1995) was constructed in the same manner). The purified enzyme (80 μM subunit) in 50 mM HEPES, pH 6.8, was incubated with 0.32 mM CuSO4 in a 1.0-cm path length cell placed in a thermostated cell holder at 30 °C, and the absorption spectrum was recorded at indicated times with a Hewlett-Packard 8452A diode array spectrophotometer.

because all plant enzymes sequenced so far contain Asn in place of Tyr at the corresponding position (Tipping and McPherson, 1995). The wild-type and N401D, Y404F, D403E, D403N, and Y404F mutant enzymes that were overproduced were all purified to homogeneity by SDS-polyacrylamide gel electrophoresis in the copper/TPQ-free, inactive form. CD spectra of these mutant enzymes overlapped almost completely with those of the wild-type enzyme in the wavelength region of 200-250 nm (data not shown), suggesting that the mutations caused few, if any, global conformational changes. In the case of the Y404N mutant, however, the E. coli cells harboring pUTH/Y404N grew very slowly at 37 °C. Because the expression efficiency of this mutant was also extremely low, it could not be analyzed further.

Copper-dependent Formation of TPQ—Using the copper/TPQ-free, inactive forms of phenylethylamine and histamine oxidases, we unequivocally demonstrated that the TPQ cofactor is produced through a copper-dependent self-processing mechanism (Matsuzaki et al., 1994; Choi et al., 1995). The time course of the overall process of TPQ formation in phenylethylamine oxidase was conveniently monitored by the increase in the TPQ-derived absorption peak around 475 nm (Matsuzaki et al., 1994). Analogously, the absorption peak at 498 nm due to TPQ (Choi et al., 1995) increased during incubation of the wild-type histamine oxidase with a 4-fold molar excess of CuSO4 (Fig. 1). Thus the capacity for TPQ formation by the wild-type histamine oxidase and the enzymes mutated in the conserved Asn-Tyr-Asp-Tyr sequence were compared by the rate of increase in absorbance at 498 nm (Fig. 2). As reported for phenylethylamine oxidase, the increase in the absorption at 498 nm could be best fitted to a biphasic curve composed of two parallel first-order reactions; the rate constant, k, of the faster phase, which was dependent on the copper concentration (data not shown), was estimated to be 0.18 min−1 for the wild-type enzyme. The rates of TPQ formation in the D403E and Y404F mutants at the same enzyme and copper concentrations were similar to or even higher than the rate of the wild type (Table 1). In contrast, the D403N mutant exhibited a marked decrease (nearly 50-fold) in the rate of TPQ formation. These results suggest that an acidic residue (Asp or Glu) is required at the position of residue 403 for the rapid formation of TPQ, consist-

FIG. 1. Spectral change of the copper/TPQ-free wild-type histamine oxidase during incubation with copper ions. The purified enzyme (80 μM subunit) in 50 mM HEPES, pH 6.8, was incubated with 0.32 mM CuSO4 in a 1.0-cm path length cell placed in a thermostated cell holder at 30 °C, and the absorption spectrum was recorded at indicated times with a Hewlett-Packard 8452A diode array spectrophotometer.
ent with the occurrence of Glu at the third position of the consensus sequence in some other copper amine oxidases.

The rapid formation of TPQ in the Y404F mutant seems to indicate that the phenolic hydroxy group of Tyr-404 has no role in TPQ formation. On the other hand, both mutants of Tyr-404 (N401D and N401Q) showed extremely reduced rates of TPQ formation with rate constants 10^3- to 10^4-fold smaller than that of the wild-type (Fig. 2, Table I). Hence, an Asn at this position is virtually essential for the efficient formation of TPQ. Furthermore, in the mutant of the second Tyr (Y402F), the TPQ cofactor was not formed at all, confirming the essential role of this Tyr as the precursor to TPQ (Matsuzaki et al., 1994).

**Oxygen Consumption**—The conversion into TPQ of a specific tyrosyl residue in the copper/TPQ-free, inactive enzyme is a copper-dependent autooxidation process (Matsuzaki et al., 1995; see also, Nakamura et al. (1996)). In the present study we measured oxygen consumption during the formation of TPQ with an oxygen electrode (Fig. 3). Although the time courses of oxygen consumption initiated by the addition of copper ions showed similar patterns to those of the 498-nm absorption increase observed for each of the wild-type and mutant enzymes (see Fig. 2), the oxygen consumption by the wild-type, D403E, and Y404F mutant enzymes appeared to cease within 10 min. Unlike the absorption increase, the oxygen consumption could be fitted to a monophasic curve, and the calculated first-order rate constants of oxygen consumption by the wild-type, D403E, and Y404F mutant enzymes were not largely different from the rate constants obtained from the increase in 498-nm absorption (Table I). Thus the dissolved oxygen is presumably consumed in the faster phase of TPQ formation.

Oxygen consumption was extremely slow in the N401Q and D403N mutants, consistent with their sluggish increase in the 498 nm absorption. No oxygen was consumed by the Y402F mutant, which was incapable of forming TPQ.

The total amounts of oxygen consumed by the wild-type, D403E, and Y404F mutant enzymes were estimated to be about 31, 26, and 35 nmol of the dissolved oxygen, corresponding to 0.67, 0.56, and 0.75 mol equivalents of the subunit (46.7 nmol), respectively. These values are roughly comparable to the amounts of TPQ titrated with phenylhydrazine (see below).

Although further studies are needed to confirm the exact stoichiometry of the oxidative modification leading to TPQ (Matsuzaki et al., 1995), the data suggest that 1 mol of molecular dioxygen is consumed during the formation of 1 mol of TPQ.

**Copper/TPQ Contents of Cu^{2+}-reconstituted Enzymes**—To examine whether the binding capacity for copper was affected by the mutation in the conserved Asn-Tyr-Asp-Tyr sequence, the copper content of the enzyme protein was determined by atomic absorption analysis. Before the analysis, the purified, copper/TPQ-free wild-type and mutant enzymes had been incubated overnight (for about 14 h) with excess copper ions and completely dialyzed (for about 24 h) to remove the unbound copper. As summarized in Table I, the wild-type and Y402F, D403E, and Y404F mutant enzymes contained nearly 1 mol atom of copper per subunit. Although the amounts of the bound copper in the N401Q, N401D, and D403N mutants were slightly less than the stoichiometric level, it may be concluded that the mutations in the conserved sequence did not greatly diminish the binding capacity for copper ions.

The ability of the mutant enzymes to form TPQ was also compared on the basis of the amount of TPQ produced per subunit of those Cu^{2+}-reconstituted enzymes. The amount of TPQ produced was determined by titration with the carbonyl reagent phenylhydrazine (Janes and Klinman, 1991). As shown in Fig. 4, the increase in absorbance at 438 nm was proportional to the amount of phenylhydrazine added and reached a plateau, indicating the formation of a phenylhydrazine adduct. At the intersection of the two lines, the phenylhydrazine/subunit molar ratio of the wild-type enzyme was 0.68, very close to the value (0.62) obtained previously (Choi et al., 1995). It is unknown at present whether less than one TPQ molecule has been produced by incubation with copper ions or one TPQ molecule has indeed been produced in each subunit, but TPQ in the dimeric enzyme has reduced reactivity to phenylhydrazine if one of the two is modified. A molar ratio of phenylhydrazine/subunit ranging from 0.15 to 0.61 was found in the mutant enzymes (Table I). The amounts of TPQ titrated with phenylhydrazine in the D403E and Y404F mutants are comparable to that of the wild-type enzyme. Thus the amounts

![Fig. 2. Time course of the increase in the 498-nm absorption of the wild-type and mutant histamine oxidases.](http://www.jbc.org/)

**Table I**

| Enzyme   | Rate of TPQ formation a | Rate of oxygen consumption b | Copper content | Specific activity | TPQ content | Specific activity/TPQ-enzyme |
|----------|-------------------------|-------------------------------|----------------|------------------|-------------|-----------------------------|
|          | min⁻¹                   | mol atom/subunit              | units/mg       | mol/mol subunit  | unit/mg     | unit/mg                     |
| Wild-type| 0.18                    | 0.28                          | 0.92           | 18.0             | 0.68        | 26.5                        |
| N401Q    | 0.000064 c              | 0.00003                      | 0.69           | 0.46             | 0.15        | 3.1                         |
| N401D    | 0.000132 d              | ND d                         | 0.76           | 3.0              | 0.31        | 9.7                         |
| Y402F    | 0                       | 0                             | 0.99           | 0                | 0           | 0                           |
| D403E    | 0.24                    | 0.19                          | 0.91           | 16.0             | 0.61        | 26.2                        |
| D403N    | 0.00355 f               | 0.0026                        | 0.74           | 0.58             | 0.31        | 1.9                         |
| Y404F    | 0.38                    | 0.35                          | 0.91           | 0.46             | 0.59        | 0.8                         |

| a Rate constant of the faster phase of the two parallel first-order kinetics calculated from the increase in absorbance at 498 nm, fitted to a biphasic curve (see Fig. 2).
| b Rate constant for the oxygen consumption fitted to a single first-order kinetics (Fig. 3).
| c On the basis of the amount of enzyme corrected for the TPQ content.
| d Fitted to a single first-order kinetics.
| e ND, not determined.
of TPQ formed are approximately proportional to the overall rates of the increase in absorbance at 498 nm.

UV-visible absorption spectra of the Cu^{2+}-reconstituted enzymes are shown in Fig. 5. The fully activated D403E mutant has an absorption maximum at the same wavelength (498 nm) as the wild-type enzyme. The Cu^{2+}-reconstituted Y404F mutant and the D403E mutant contained similar amounts of TPQ (Table I), but the absorption maximum of TPQ in the Y404F mutant is blue-shifted (around 480 nm) relative to those (498 nm) of the wild-type and D403E mutant enzymes. The 18-nm difference in the absorption maximum may reflect a slight difference in the active-site environment and/or in the copper-TPQ interaction and may correlate with the variations in the catalytic properties described below. The absorption maximum of TPQ is also known to differ among amine oxidases from various sources (McIntire and Hartmann, 1993).

**Catalytic Properties of Cu^{2+}-reconstituted Enzymes**—If a mutated residue in the conserved sequence plays a role in the formation of TPQ but not in the catalytic reaction of copper amine oxidase, the activity of the Cu^{2+}-reconstituted mutant enzyme should be proportional to the amount of TPQ formed.

**FIG. 3. Oxygen consumption during the TPQ formation.** Reactions were initiated by the addition of CuSO_4_ (final concentration, 0.27 mM) at the time point indicated with arrows into the reaction mixtures (0.7 ml) containing the copper/TPQ-free enzymes (67 μM subunit).

**FIG. 4. Titration of the Cu^{2+}-reconstituted enzymes with phenylhydrazine.** The freshly prepared solution of phenylhydrazine (0.1 mM) was added in 2- or 2.5-μl aliquots into each solution of the copper-reconstituted wild-type and mutant enzymes (5.9 μM) in 50 mM HEPES, pH 6.8 (0.5 ml). After incubation at 30 °C for 15 min, increases in the absorbance at 438 nm were measured. Absorbance values from the enzyme solution without phenylhydrazine were plotted against the molar ratio of phenylhydrazine added per enzyme subunit.

Therefore, it is particularly important to correct the specific activity of each mutant for its TPQ content and to compare it with that of the wild-type enzyme (Table I). The specific activity of the D403E mutant corrected for TPQ was essentially identical to that of the wild-type enzyme, whereas that of the D403N mutant was markedly lower, suggesting that an acidic residue at position 403 plays an important role in the catalytic reaction. The corrected activities of the other mutants (N401Q, N401D, and Y404F) were also considerably lower than that of the wild type. In particular, the Y404F mutant had markedly lower activity, even though it contained an appreciable amount of TPQ. Collectively, it is clear that the residues in the conserved sequence are important not only for the rapid formation of TPQ but also for efficient catalysis. This suggests that the process of TPQ formation is closely associated with the catalytic process.

To further investigate the role(s) of the conserved sequence in catalysis, we then performed steady-state kinetic analysis for all of the Cu^{2+}-reconstituted enzymes except the completely inactive Y402F mutant. Kinetic parameters were obtained with histamine and phenylethylamine as representative substrates; the wild-type enzyme catalyzes the oxidation, not only of histamine but also of other aromatic amines such as phenylethylamine (relative activity at 0.1 mM, 72% of histamine), tyramine (69%), tryptamine (13%), and benzylamine (0.5%) (Choi et al., 1995). With histamine as the substrate, k_cat values (corrected for TPQ content) for all of the mutant enzymes except D403E were markedly smaller than that of the wild-type enzyme, while K_m values for histamine varied insignificantly (Table II). These results suggest that the conserved residues are involved in the catalytic process and not in substrate binding. On the other hand, the wild-type enzyme had a very low K_m value for phenylethylamine but was also strongly inhibited by this substrate (K_i = 0.11 mM). Interestingly, the Y404F mutant showed much higher reactivity to phenylethylamine than it did to histamine; the ratio of the k_cat with phenylethylamine/k_cat with histamine emphasizes the effects of mutation on substrate specificity (Table II). In addition, the K_m value for Y404F for phenylethylamine was 25 times higher than that of the wild type, and showed no substrate inhibition even at 1 mM. Thus the conserved Tyr at position 404 is also most likely involved in the substrate recognition. The fact that copper amine oxidases from plant sources, which preferentially catalyze oxidation of the diamine substrates putrescine and cadaverine, contain Asn instead of Tyr at this position (Tipping...
TABLE II

Kinetic parameters of the wild-type and mutant enzymes

Steady-state kinetic analysis was performed at 25 °C in 50 mM Bicine, pH 8.0, with 0.01–1.0 mM histamine or 0.005–0.4 mM phenylethylamine as substrate for each wild-type and mutant enzyme. The $k_{cat}$ values determined as the maximum molecular activities (s$^{-1}$) were corrected for the TPQ content (see Table I).

| Enzyme | Histamine $k_{cat}$ s$^{-1}$ | Phenylethylamine $k_{cat}$ s$^{-1}$ | Ratio | Histamine $K_m$ mM | Phenylethylamine $K_m$ mM |
|--------|----------------------------|----------------------------------|-------|-------------------|---------------------------|
| Wild-type | 29.7 | 25.3 | 0.85 | 0.055 ± 0.012 | 0.004 ± 0.0005 |
| N401Q | 6.13 | ND | 0.018 | 0.139 ± 0.018 | ND |
| N401D | 16.8 | ND | 0.02 | 0.078 ± 0.01 | ND |
| D403E | 35.4 | ND | 0.02 | 0.064 ± 0.008 | ND |
| D403N | 1.90 | 4.06 | 2.14 | 0.022 ± 0.002 | 0.01 ± 0.001 |
| Y404F | 0.88 | 16.1 | 18.7 | 0.031 ± 0.003 | 0.102 ± 0.02 |

$^*$ Ratio of $k_{cat}$ with phenylethylamine/$k_{cat}$ with histamine.
$^*$ ND, not determined.

**FIG. 6. Stereoview of the active site region of copper amine oxidase.** Only TPQ and several nearby conserved residues were drawn by the viewer module of Insight II (Biosym Technologies, Inc.) using the E. coli copper amine oxidase coordinates (form II); the orientation of the TPQ ring (i.e. positions of O-2 and O-5) is unknown (Parsons et al., 1995) and so was fixed arbitrarily. Possible hydrogen bonds in the cofactor region, defined by a donor-acceptor distance shorter than 3.2 Å, are indicated by dotted lines. The amino acid residue numbers were applied to the histamine oxidase sequence (Choi et al., 1995). The His residue (H375B) of the other subunit is shaded.

and McPherson, 1995) led us to prepare the Y404N mutant enzyme of histamine oxidase and to examine its substrate specificity. Unfortunately, however, the mutant protein could not be produced in E. coli cells, as described before.

**DISCUSSION**

The crystal structure of copper amine oxidase from E. coli has recently been determined to 2.4-Å resolution (Parsons et al., 1995). The E. coli enzyme dimer is mushroom-shaped with the “stalk” composed of the 85 N-terminal amino acids of each polypeptide chain and the large “cap” composed of the remaining 640. The stalk domain (D1) is absent from some copper amine oxidases including histamine oxidase from A. globiforis studied here, but the cap consisting of two small peripheral domains (D2 and D3) and a large core with the active site is conserved throughout the copper amine oxidase family (Parsons et al., 1995). The main portion of the core domain is made up of a pair of extensive β sandwiches that form a disk. The active site lies between the two sheets, consisting of 8 and 10 antiparallel β strands (sheets S1 and S2) of each β sandwich. The TPQ residue and the C-terminal adjacent Asp with the following Tyr are located at the N terminus of the fourth strand of sheet S2 (S2.4), preceded by the invariant Asn on the β turn connecting the third strand (S2.3) and S2.4 (Parsons et al., 1995). Because the primary structures of copper amine oxidases from various sources are highly homologous in this core domain containing most of the invariant residues (Tipping and McPherson, 1995; Choi et al., 1995), it is reasonable to assert that the active site structure of histamine oxidase is essentially identical to that of the E. coli enzyme. The active site region comprised by TPQ and several conserved residues, including three His residues chelating the copper and an Asp residue putatively functioning as the catalytic base, is depicted in Fig. 6, where the residue numbers were applied to the histamine oxidase sequence.

In the structure of catalytically active crystals (form II) of E. coli amine oxidase grown from sodium citrate solutions, the TPQ side chain is positioned away from the copper and tilted toward the putative catalytic base, Asp-383 (Asp-318 in histamine oxidase; Fig. 6). The side chain of Asn-465 (Asn-401 in histamine oxidase), occurring at the base of the β turn to S2.4, is situated proximal to the TPQ ring (Parsons et al., 1995). We have shown here that the Asn-401 of histamine oxidase is extremely important for the rapid formation of TPQ and cannot be replaced, even by Asp or Gln. It is rather difficult to explain the decreased TPQ formation resulting from such small structural changes and it is not known whether the structure of the copper/TPQ-free precursor enzyme is similar to that of the active TPQ enzyme; but the evidence suggests that the amide group of Asn-401 may promote TPQ formation through an electronic interaction with the phenol ring of Tyr-402 that is oxidized to TPQ. Alternatively, Asn-401 may assist the rotation of the quinone ring about the β-carbon, which has been assumed to occur in the proposed mechanism for TPQ biosynthesis (Cai and Klinman, 1994b). In either case, the minuscule change of the position of the amide group caused by the insertion of only one methylene unit (i.e. replacement by Gln) is enough to cause the loss of the interaction with the quinone ring, which also appears to be important for the catalysis.

As first demonstrated with phenylethylamine oxidase (Matsuzaki et al., 1994), substitution of Phe for the precursor Tyr-402 in the conserved sequence of histamine oxidase leads to the loss of TPQ formation. Thus the phenolic hydroxyl group of the precursor Tyr is essential for the ring oxidation leading to TPQ; the first hydroxylation step is probably initiated by the attack of a hydroperoxide derived from O$_2$ at the ortho position of the hydroxyl group of the precursor Tyr (Nakamura et al., 1996). In the E. coli enzyme structure (Parsons et al., 1995), the O-4 oxygen of TPQ (corresponding to the phenolic oxygen of the
precursor Tyr) is placed within 2.5 Å of the Oγ of another Tyr residue at position 369 (Tyr-304 in histamine oxidase), which is also fully conserved in all known copper amine oxidase sequences. However, the distance between the two Tyr residues in the precursor enzyme and the relevance of such an interaction, if any, to TPQ biosynthesis remain to be elucidated.

The crystal structure of E. coli copper amine oxidase has also revealed that the acidic residue in the conserved sequence (Asp-467; Asp-403 in histamine oxidase) forms a hydrogen bond, via Oδ2, to the side chain Nε1 of a fully conserved His (His-440; His-375 in histamine oxidase, see Fig. 6) of the other subunit, which is involved in the intersubunit interaction (Parsons et al., 1995). The same Asp also hydrogen bonds via Oδ1 to the side chain hydroxyl (Oγ1) of another fully conserved Thr (Thr-462; Thr-398 in histamine oxidase) in the same subunit (Fig. 6). Cai and Klinman (1994a) reported that the replacement of Glu-406 at the corresponding position of the yeast Hansenula polymorpha methylamine oxidase by an uncharged Asn did not affect the capacity for TPQ biogenesis in vivo. However, we have shown here that Asp-403 of histamine oxidase can be replaced by Glu without loss of the ability to form TPQ, but replacement by Asn leads to a markedly lowered rate of TPQ formation; this indicates that the hydrogen bonding network provided by this conserved acidic residue is involved in maintaining the structural integrity of the active site, rather than directly participating in the formation of TPQ. In particular, the intersubunit hydrogen bonding (or electrostatic interaction) between these Asp and His residues appears to be essential for the structural links between the two active sites disclosed in the crystal structure (Parsons et al., 1995). Violation of the structural links could lead to small changes in the position of main chain atoms and in the side chain torsion angles of the TPQ residue (precursor Tyr) and its immediate neighbors in both active sites, thereby unfavorably affecting TPQ formation and catalysis.

Finally, it should be noted that the replacement of the highly conserved Tyr-404, adjacent to the acidic residue (Asp/Glu) in the consensus sequence, by Phe resulted in a significant alteration of the catalytic properties of the enzyme, but not in the capacity for TPQ biosynthesis. As shown in Fig. 6, Tyr-404 is located close to both TPQ and the putative catalytic base, Asp-318, which is thought to abstract a proton from substrate C1 of the TPQ-substrate Schiff base intermediate (Farnum et al., 1986). Further studies should be conducted before we discuss the mechanism of substrate recognition by the copper amine oxidases that act on a variety of mono- and diamines; but the proximity of Tyr-404 to TPQ, as manifested by the substan-

tial blue shift of the TPQ-derived absorption peak in the Y404F mutant enzyme, suggests that this Tyr somehow participates in substrate binding through its hydroxyl group. Considering the variations in stereospecificities of the proton abstraction at substrate C1 displayed by the enzymes from different sources (Coleman et al., 1991), it would be interesting to investigate the stereospecificity of proton abstraction by the Y404F mutant histamine oxidase with altered substrate specificity.

In conclusion, we have shown here that a very rigorous structural motif is required for the efficient formation of TPQ and for the catalytic activity in the active site of the copper amine oxidases. The significance of the conservation of the Asn-Tyr-(Asp/Glu)-Tyr sequence has now been made clear, with each residue playing an irreplaceable role.

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