Exploring a Channel to the Active Site of Copper/Topaquinone-Containing Phenylethylamine Oxidase by Chemical Modification and Site-Specific Mutagenesis†

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ABSTRACT: Copper amine oxidase contains an organic redox cofactor, 2,4,5-trihydroxyphenylalaninequinone (topaquinone, TPQ), derived by the post-translational modification of a specific tyrosyl residue. To identify amino acid residues participating in the biogenesis of TPQ in the recombinant phenylethylamine oxidase from *Arthrobacter globiformis*, we have modified the copper/TPQ-less apoenzyme and the copper/TPQ-containing holoenzyme with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F). In the apoenzyme modification, the Cu^{2+}-dependent, self-processing formation of the TPQ cofactor was retarded in accordance with the amount of NBD incorporated. The holoenzyme was also rapidly inactivated by incubation with NBD-F. The inactivation was prevented almost completely in the presence of an oxidation product from phenylethylamine, phenylacetaldehyde. Furthermore, the reaction of an inhibitor, phenylhydrazine, with TPQ was much slower in the NBD-labeled holoenzyme than in the native holoenzyme. Sequence analysis of the NBD-labeled holoenzyme has identified Lys184 and Lys354 as the labeled sites. The two Lys residues are located close to the entrance to a channel, which has been found by recent X-ray crystallographic studies to be suitable for the movement of substrates and products to and from the Cu^{2+}/TPQ-active site buried in the protein interior (Wilce, M. C. J., et al. (1997) *Biochemistry 36*, 16116−16133). However, site-specific mutant enzymes for Lys184, Lys354, and the neighboring invariant His355 had normal capacities for the TPQ formation in apoenzyme. These residues were also found to be dispensable for catalytic activity of holoenzyme. Thus, modification of Lys184 and Lys354 with NBD-F presumably causes structural perturbations of the substrate channel or steric hindrance for the access of small molecules to the active site through the channel.

Copper amine oxidases (EC 1.4.3.6) utilize a redox-active organic cofactor, 2,4,5-trihydroxyphenylalaninequinone (topaquinone, TPQ),[1] in catalyzing the oxidation of various biogenic primary amines (*1*−*3*). The TPQ cofactor is encoded by a Tyr codon in the genes coding for the enzymes from a wide variety of organisms (*4*−*6*) and is produced by the post-translational modification that proceeds in a Cu^{2+}-dependent, self-processing reaction (*7*−*11*). The X-ray crystallographic structures of the amine oxidases from *Escherichia coli* (*12*) and pea seedling (*13*) have shown that the Cu^{2+}/TPQ-containing active site of these enzymes resides deep inside the protein interior and is not directly accessible from solvent. However, inspection of the three-dimensional structure of phenylethylamine oxidase (PEAO) from *Arthrobacter globiformis* determined most recently (*14*) has revealed the presence of a channel, which appears suitable for the movement of reactants from the solvent-accessible surface to the Cu^{2+}/TPQ-active site. A similar channel has also been found to exist in the *E. coli* and plant enzymes (*14*), although the channel in the pea enzyme is narrower than in PEAO, and the channel in the *E. coli* enzyme is partly obstructed.[2]

In the PEAO structure, the entrance to the channel is surrounded by negative charges associated with the side chains of several acidic residues (*14*). Since amine substrates are recognized in the protonated, positively charged form (*16*), the electrostatic potential and surface topology at the entrance to the channel are thought to be important for substrate recognition. The internal surface of the channel is lined by residues which become more hydrophobic as they

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[1] Abbreviations: TPQ, 2,4,5-trihydroxyphenylalaninequinone (topaquinone); PEAO, phenylethylamine oxidase; NBD-F, 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole; ABTS, 2, 2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); DTNB, 5, 5′-dithiobis(2-nitrobenzoic acid); PCR, polymerase chain reaction; TNB, 5-thio-2-nitrobenzoic acid.

[2] An open channel that appears to function as a substrate pathway has also been found in the crystal structure of copper amine oxidase from a yeast *Hansenula polymorpha* determined very recently (*15*).

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approach the active site. At the end of the channel is the side chain of Tyr296 that likely acts as a "gate" to the active site (*14*). In the recently reported structure of the *E. coli* enzyme bound with an inhibitor 2-hydrazinopyridine (*17*), the TPQ-bound inhibitor extends from the active site into a region which corresponds to the channel in PEAO (*14*). However, there has been no evidence demonstrating that the presumed channel provides an actual route for small molecules such as substrates and inhibitors which are known to bind to TPQ (e.g., phenylhydrazine and semicarbazide) or copper (e.g., azide and cyanide) to enter the active site from solvent.

During the course of our studies on the mechanism of the TPQ biogenesis in PEAO, we have examined various chemical modification reagents to identify amino acid residues participating in the TPQ formation. In particular, a Lys residue, which has been hypothesized to exist in close vicinity to the precursor Tyr residue that is converted to TPQ and to interact electronically with an oxidation intermediate formed from the Tyr residue (*18*), has been targeted as a residue to be identified. Even though the crystal structure of PEAO unambiguously shows the absence of any Lys residues within the active site region (*14*), we have found that the enzyme is indeed inactivated efficiently by incubation with a Lys-directed reagent, 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F), which has been utilized for fluorescent labeling and identification of functional Lys residues in proteins (*19*). Sequence analysis of the inactivated enzyme has identified Lys184 and Lys354 as the NBD-labeled sites, both of which are located near the entrance to the channel. The results of chemical modification reported in this paper provide biochemical evidence for the existence of the channel, which likely functions as a pathway for substrates and inhibitors, leading to the Cu^{2+}/TPQ-active site located deep inside the protein.

EXPERIMENTAL PROCEDURES

Enzyme Purification and Assay. The recombinant PEAO was overproduced in *E. coli* BL21 (DE3) cells carrying plasmid pPEAO2 and purified to homogeneity in the inactive apo form (Cu^{2+}/TPQ-less, precursor form) by the procedure reported previously (*7*), which was modified recently not to include dithiothreitol in the purification buffer (*20*). Mutant enzymes for Lys184, Lys354, and His355 were also purified in the apo form by the same method as the wild-type enzyme. For enzyme assay, the apoenzyme was converted in advance into the active holo form by a 30-min aerobic incubation with 0.5 mM $CuSO_4$ (*7*). The enzyme activity was measured spectrophotometrically by monitoring H_2O_2 production coupled to the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (ϵ_{414} = 24.6 mM^{-1} cm^{-1}) by horseradish peroxidase (Wako Pure Chemicals, Osaka, Japan) as described previously (*21*). The Cu^{2+}/TPQ-containing holoenzyme was prepared by incubation of the apoenzyme with excess $CuSO_4$ (typically 0.5 mM) at 4 °C for about 12 h, followed by thorough dialysis (>24 h) against 50 mM Hepes (pH 8.0) to remove the unbound copper (*18*). Protein concentrations were determined using extinction coefficients at 280 nm of 12.3 and 13.2 for 1% (w/v) solutions of the apo- and holoenzymes, respectively (*7*). The method of titration of TPQ with phenylhydrazine was essentially identical to that reported previously (*22*); the phenylhydrazine solution used was freshly prepared by dissolving reagent recrystallized from methanol. For determination of the stoichiometry of enzyme inactivation by NBD-F, the enzyme concentrations were calculated using the subunit concentration of the dimeric enzyme with an approximate M_r of 70 600 (*23*). Absorption spectra were measured with a Hewlett-Packard 8452A diode-array spectrophotometer or a Pharmacia LKB Biochrom 4060 UV−vis spectrophotometer. The copper contents of the enzyme proteins were analyzed at 324.8 nm with a Shimadzu AA-6400G atomic absorption spectrophotometer attached with a GFA-4A graphite furnace atomizer, using an atomic absorption analysis grade $CuSO_4$ solution in 0.1 M HNO_3 as the internal and external standards.

Modification with NBD-F. To modify free Cys residues that could react with NBD-F (*24*), the enzyme (80 μM subunit) in 100 mM potassium phosphate buffer (pH 8.0) was first treated at 30 °C for 30 min with 1.6 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Nakarai Tesque, Kyoto, Japan) and dialyzed overnight against 0.1 mM sodium borate buffer (pH 8.5) to remove excess DTNB. The enzyme (8 μM) was then incubated at 30 °C for 30 min, unless otherwise stated, with various concentrations of NBD-F (Dojindo Lab., Co., Kumamoto, Japan); the stock solution of NBD-F (0.1 M) was prepared in acetonitrile and stored at −20 °C, protected from light. The reaction mixtures were filtered three times through Centricon-30 cartridges (Amicon, Inc., Beverly, MA) with repeated addition of 2 mL of 50 mM Hepes buffer (pH 8.0) to remove the remaining reagent. The NBD-labeled apoenzyme was incubated at 30 °C for 30 min with 0.5 mM $CuSO_4$ as above, prior to the measurements of the residual activity. The amounts of NBD incorporated into the enzyme protein were determined fluorometrically (excitation at 470 nm, emission at 530 nm) with a Hitachi F-4010 spectrofluorometer using *N*-ϵ-NBD-*N*-α-acetyllysine prepared from *N*-α-acetyllysine (Sigma) and NBD-F as a standard.

Isolation and Sequencing of NBD-Labeled Peptides. For identification of the NBD-labeled site(s), the apoenzyme was first treated with DTNB as above, second converted into the Cu^{2+}/TPQ-containing holoenzyme by incubation with excess Cu^{2+} ion, third incubated with 0.5 mM *p*-nitrophenylhydrazine at 30 °C for 1 h to protect the TPQ group, and finally labeled with NBD-F at a 100-times molar excess over the enzyme subunit at 30 °C for 30 min. The NBD-labeled holoenzyme (0.2 mM, 1 mL) was added with solid urea to 8 M, incubated at 40 °C for 10 min, and diluted with 100 mM Tris-HCl (pH 8.0) to a final urea concentration of 2 M. Lysyl endopeptidase was added to the enzyme solution in a protease/substrate ratio of 1:100 (w/w). After incubation at 37 °C for 2 h, the protease was further added in a ratio of 1:100, and the digestion was continued for 6 h. The peptides were separated on a Tosoh HPLC system equipped with a Capcell Pack C_{18} reverse-phase column (Shiseido Co., Tokyo, Japan) using a solvent system of 0.1% trifluoroacetic acid (A) and 0.1% trifluoroacetic acid containing 45% acetonitrile and 45% propanol (B). A 60-min linear gradient from 0% to 100% B was used to elute peptides at a flow rate of 1.0 mL/min. The absorbance at 215 nm and the fluorescence emitted at 530 nm by excitation at 470 nm were continuously monitored. The amino acid sequence was determined with an Applied Biosystems Model 477A protein

sequencer linked with an Applied Biosystems Model 120A PTH analyzer.

Site-Specific Mutagenesis. Site-specific mutagenesis for Lys184, Lys354, and His355 was performed by the two-step polymerase chain reaction (PCR) essentially according to the method of Ho et al. (*25*) using the following pairs of mutually complementary primers (+, sense strand; −, antisense strand) containing mismatching bases (underlined) for the codons to be mutated.

K184Q (+):
5′-GGACGTGGTCAGCCAGGAAGTCAC-3′

K184Q (−):
3′-GCACCAGTCGGTCCTTCAGTGGGCCC-5′

K354Q (+):
5′-GGGCATCCTGGCCCAGCACAGCGAC-3′

K354Q (−):
3′-GTAGGACCGGGTCGTGTCGCTGGAAAC-5′

H355A (+):
5′-CATCCTGGCCAAGGCCAGCGACCTTT-3′

H355A (−):
3′-GGACCGGTTCCGGTCGCTGGAAACCAGG-CCG-5′

Primer I (+): 5′-TTTTCATCCACGACGTCT-3′

Primer II (−): 3′-AGGTGGTCGTGTAGAAGT-5′

Primers I and II, which were used as the common forward (+) and reverse (−) primers in each PCR, corresponded to nucleotides 185−202 and 1288−1306, respectively, of the PEAO gene with the first A of the initiator ATG codon as nucleotide 1 (*23*). In the first PCR, two separate reactions were carried out using each pair of two primers (primer I plus one of the reverse (−) mutagenic primers; and primer II plus the other counterpart of the forward (+) mutagenic primers) in a 50-μL solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgSO_4$, 0.2 mM each of the deoxynucleotides (dATP, dGTP, dCTP, and dTTP), 1 μM each of two primers, about 2 ng of the template DNA (pPEAO2), and 0.5 units of *Taq* DNA polymerase (Takara Shuzo Co., Kyoto, Japan) with a program consisting of cycles 1−24 (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) and the last cycle (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 4 min) in a thermal cyclic reactor (Perkin-Elmer, GeneAmp PCR System 2400). The two PCR products were filtered through Suprec-02 cartridges (Takara Shuzo) to remove excess primers, annealed with each other (94 °C for 10 min, 40 °C for 10 min), and extended at 72 °C for 3 min by *Taq* DNA polymerase. The second PCR was done with the double-stranded fragment obtained above as a template and primers I and II for 15 cycles under conditions similar to those above. A 710-base pair *Bam*HI-*Kpn*I fragment excised from the amplified DNA (1121 base pairs) was substituted for the corresponding region in the wild-type plasmid pPEAO2. The sequence of the inserted region was confirmed with an Applied Biosystems 370A DNA sequencer. The resulting constructs were used to transform *E. coli* BL21 (DE3) cells for the expression of mutant proteins.

RESULTS

Prelabeling of Cys Residues with DTNB. Among various reagents generally used for chemical modification of protein lysyl residues, NBD-F was found to inactivate holo-PEAO most efficiently; others including pyridoxal 5′-phosphate (*26*), sodium 2,4,6-trinitrobenzenesulfonate (*27*), and methyl acetyl phosphate (*28*) were all ineffective. However, because NBD-F has a halogenobenzofurazan ring that could also react with sulfhydryl groups (*24*), the inactivation of PEAO might have resulted from modification of Cys residues; 4 Cys residues (Cys315, Cys317, Cys343, and Cys636) are present in each subunit of the dimeric PEAO (*23*). Therefore, we first studied the effect of modification of Cys residues with DTNB on the enzyme activity, prior to the modification with NBD-F. After incubation with 1.6 mM DTNB at 30 °C for 30 min, the Cu^{2+}/TPQ-less apoenzyme showed normal capacity for the Cu^{2+}-dependent TPQ formation and the resultant holoenzyme was fully active for phenylethylamine oxidation (data not shown), although about 2 mol of Cys residues was titrated with DTNB when measured at 412 nm by the amounts of 5-thio-2-nitrobenzoic acid (TNB) liberated (2.1 and 2.3 mol of TNB produced/mol of subunit in the absence and presence of 8 M urea, respectively). The crystal structure of apo-PEAO provided clear evidence for one intramolecular disulfide bridge, (Cys317)S−S(Cys343) (*14*). Thus, it is concluded that the two DTNB-titrable Cys residues are Cys315 and Cys636, both of which are nonessential for the TPQ-generating capacity of apoenzyme. In contrast, only less than 1 mol of Cys residue was titrated in the Cu^{2+}/TPQ-containing holoenzyme (0.55 and 0.46 mol of TNB produced/mol of subunit in the absence and presence of 8 M urea, respectively) and no catalytic activity was lost at all, even though the holo-PEAO structure indicated the presence of the same two free Cys residues (Cys315 and Cys636) and one disulfide bridge, (Cys317)S−S(Cys343), as the apoenzyme (*14*). The reason for the apparent absence of DTNB-titrable Cys residues in the holoenzyme is not obvious. Nevertheless, because the DTNB treatment affected neither the TPQ-generating capacity of apoenzyme nor the catalytic activity of holoenzyme, both of the apo- and holoenzymes were pretreated with DTNB before NBD-F modification to mask free Cys residues that could react with NBD-F. In fact, the DTNB treatment was required to achieve efficient inactivation of PEAO by NBD-F, as described below.

Modification of Apoenzyme with NBD-F. The DTNB-treated apoenzyme (8 μM) was incubated with 50 μM NBD-F at 30 °C, freed from the remaining reagent by repeated ultrafiltration, and then converted into the TPQ-containing holoenzyme by incubation with excess Cu^{2+} ion for measurement of the residual catalytic activity. As shown in Figure 1, panel A, apo-PEAO was inactivated by the progress of incubation time with NBD-F. Apoenzyme was also inactivated depending on the concentrations of NBD-F used (Figure 1, panel B). Nearly complete inactivation was attained by incubation with about a 30-times molar excess of NBD-F over the enzyme subunit. By quinone staining using the alkaline nitroblue tetrazolium/glycinate reagent developed for membrane-immobilized quinoproteins after SDS/polyacrylamide gel electrophoresis (*29*), the loss of

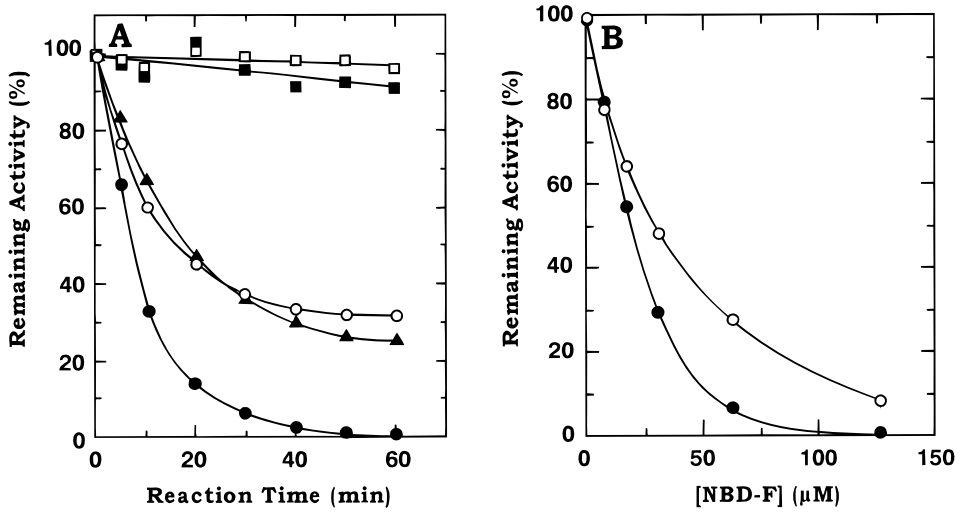

FIGURE 1: Inactivation of phenylethylamine oxidase by NBD-F. (A) Time course of inactivation. The reaction mixtures (1 mL) containing 25 mM Hepes (pH 6.8), 50 μM NBD-F, and 8 μM apoenzyme (○) or holoenzyme (●) were incubated at 30 °C. At the indicated times, aliquots were withdrawn and filtered three times through Centricon-30 cartridges or diluted with 25 mM Hepes (pH 6.8) containing 1 mM glycine. The remaining activities were assayed immediately for the holoenzyme or after further incubation with 0.5 mM $CuSO_4$ for 30 min for the apoenzyme (to convert into the holo form). The inactivation by NBD-F was also examined in the presence of 300 μM phenylacetaldehyde for both apo- (□) and holoenzymes (■) or 8 mM 2-phenylethyl alcohol for holoenzyme (▲). (B) Effect of concentration of NBD-F. The reaction mixtures (1 mL) containing 25 mM Hepes (pH 6.8), various concentrations (10−125 μM) of NBD-F, and 8 μM apoenzyme (○) or holoenzyme (●) were incubated at 30 °C for 30 min, and the remaining activities were measured as in A.

catalytic activity was found to correlate with the decrease in the amounts of TPQ produced by the subsequent incubation with Cu^{2+} ion (Figure 2, panel A); the lower the remaining activities, the lower the amounts of TPQ stained under the denaturing conditions. Spectrophotometric titration of TPQ with phenylhydrazine (*22*) also showed the decreases of titrable TPQ roughly corresponding to the remaining activities (Figure 2, panel A), although the reaction rate of phenylhydrazine with the NBD-F-modified enzyme was very slow under the nondenaturing conditions (vide infra). Therefore, the inactivation of apoenzyme by modification with NBD-F is ascribable at least partly to the loss of the capacity of apoenzyme for the Cu^{2+}-dependent, self-processing TPQ formation. The NBD-F-modified apoenzyme, on the other hand, retained the Cu^{2+}-binding capacity unless it had been inactivated to an extent of less than 1% of the remaining activity, as judged from the amounts of the enzyme-bound copper determined by atomic absorption (Figure 2, panel A). Thus, it is suggested that modification with NBD-F does not inhibit so deleteriously the binding of Cu^{2+} ion to the apoprotein, but retards the TPQ formation probably by restricting the structural rearrangement of active site residues including the precursor Tyr, occurring during the TPQ formation (*14*). In addition, the inactivation of apoenzyme by NBD-F is likely a combined consequence with the inactivation observed for the holoenzyme, as described below. The relationship between the remaining activities and the amounts of NBD incorporated into the enzyme protein determined fluorometrically (see Experimental Procedures) was not linear, but it appeared that incorporation of about 2 mol of NBD/mol of enzyme subunit was sufficient to lead the apoenzyme to the complete inactivation (data not shown).

Modification of Holoenzyme with NBD-F. The Cu^{2+}/TPQ-containing holoenzyme (8 μM; pretreated with DTNB as in the case of apoenzyme) was inactivated by incubation with NBD-F at concentrations lower than those in the apoenzyme modification (Figure 1, panel B). At 50 μM NBD-F, the inactivation proceeded in a time-dependent manner and was completed within 1 h (Figure 1, panel A). The TPQ cofactor in the holoenzyme remained intact after modification with NBD-F, as was evident from the constant stainability of the quinone group of TPQ (Figure 2, panel B). The amounts of TPQ titrated with phenylhydrazine and the copper contents were also essentially unchanged irrespective of the remaining activities (Figure 2, panel B).[3] However, it was noted that the reaction of phenylhydrazine with the TPQ cofactor in the NBD-labeled enzyme under the nondenaturing conditions was markedly slow as compared with the very rapid reaction in the unlabeled holoenzyme. The reaction rates continuously monitored by the increase in absorbance at 440 nm due to the phenylhydrazone peak decreased along with the decrease in remaining activities (Figure 3). This suggests that the incorporated NBD group inhibits the binding of phenylhydrazine to TPQ. Similarly, the mechanism of inactivation of holoenzyme by NBD-F is ascribable to the inhibition of substrate binding and/or product release, because the Cu^{2+}/TPQ contents in the holoenzyme were affected insignificantly by modification with NBD-F (Figure 2, panel B). The plot of the remaining activities versus the amounts of bound NBD determined fluorometrically gave a straight line (Figure 4). Extrapolation of the line to 0 activity yielded an approximate value of 2 mol of NBD incorporated/mol of enzyme subunit. These results indicate that labeling of the subunit protein with a total of 2 mol of NBD groups results in the complete inactivation of the holoenzyme. We then examined a protective effect of an oxidation product from phenylethylamine, phenylacetaldehyde, on inactivation by NBD-F; substrate amines could not be used because of their

[3] Titration of TPQ in various copper amine oxidases with phenylhydrazine usually gives a value less than 1.0 TPQ/subunit (*22*, *30*). It is unknown at present whether less than one TPQ molecule has been generated in holo-PEAO by incubation of the apoenzyme with copper 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.

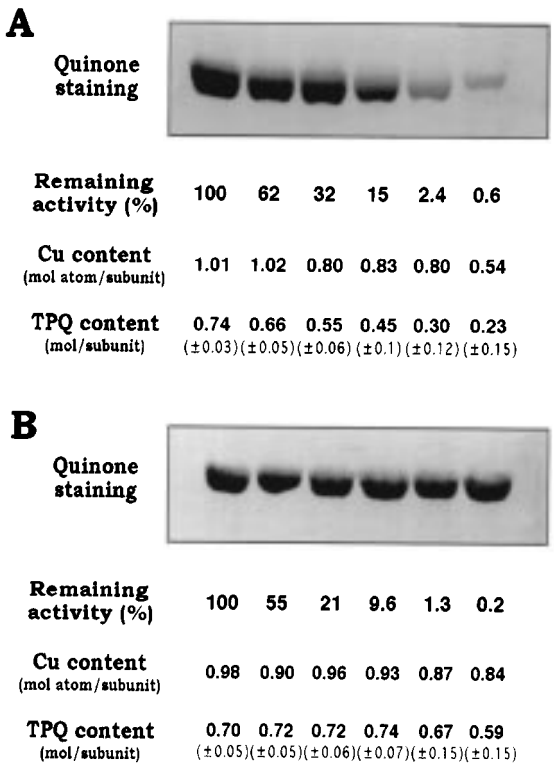

FIGURE 2: Quinone staining and copper/TPQ contents in the enzymes modified with NBD-F. The apoenzyme (A) and holoenzyme (B) (both at 8 μM subunit) were incubated with 0−512 μM NBD-F at 30 °C for 30 min. After the NBD-modified apoenzyme was reconstituted with excess Cu^{2+} as described in the legend to Figure 1, the remaining activity was measured. For quinone staining (*29*), 5-μg portions of the modified apo and holo proteins were subjected to SDS/polyacrylamide gel electrophoresis and were electrotransferred onto a nitrocellulose membrane. See Experimental Procedures for details of the measurements of copper/TPQ contents; SD in the measurements of copper content was 0.02. In the measurements of TPQ content, the absorption derived from the bound NBD group with λ_{max} at 480 nm was subtracted from the phenylhydrazone absorbance at 440 nm, and the titration reaction was carried out for at least 12 h because of the extremely slow reaction of phenylhydrazine with TPQ in the NBD-modified enzyme, as described in the text. Experimental errors are shown in parentheses.

reactivity toward NBD-F, but product aldehydes were confirmed to be unreactive with NBD-F. Holoenzyme as well as apoenzyme was protected almost completely from inactivation by NBD-F in the presence of 0.3 mM phenylacetaldehyde (Figure 1, panel A), suggesting the specific labeling by NBD of Lys residues located in the vicinity of the substrate- (and/or product-) binding region. Although the product aldehyde may covalently bind to the Lys residues through an aldimine linkage (reversibly), a nonoxidizable substrate analogue that does not bind to lysines, 2-phenylethyl alcohol (K_i = about 4 mM), also provided moderate protection (Figure 1, panel A). The marked protection of holoenzyme by phenylacetaldehyde is consistent with the strong product inhibition described later. The protection of apoenzyme shows that the apoenzyme can also bind the reaction product even without the Cu^{2+}/TPQ cofactors.

Identification of NBD-Labeled Lys Residues. It is unlikely that the inactivation by NBD-F is derived from denaturation of the enzyme protein, because little difference, if any, has been observed in the circular dichroism spectra of the NBD-

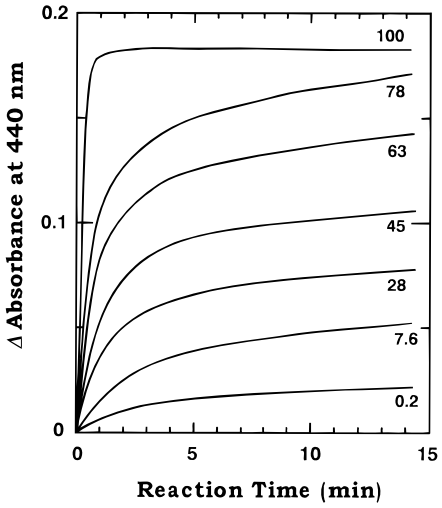

FIGURE 3: Time course of the reaction of the NBD-labeled enzyme with phenylhydrazine. The reaction mixtures (1 mL) containing 25 mM Hepes (pH 6.8), 5 μM phenylhydrazine, and 5 μM holoenzyme that had been modified with NBD-F to the indicated extents of the remaining activity (%) were incubated at 30 °C. Changes in the absorbance at 440 nm were continuously monitored.

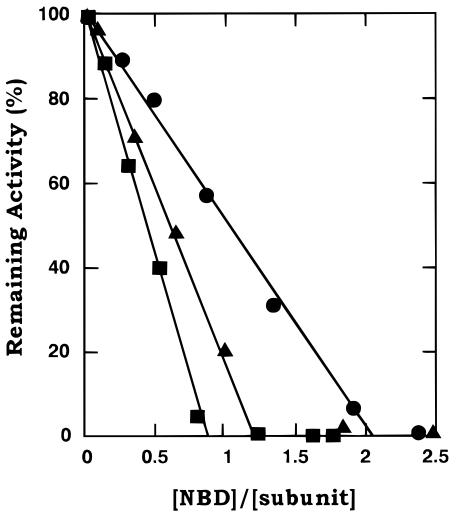

FIGURE 4: Stoichiometry of the enzyme inactivation by NBD-F and incorporation of the NBD group. The wild-type (●), K184Q (■), and K354Q (▲) mutant holoenzymes (all at 50 μM subunit) were incubated with various concentrations (0−300 μM) of NBD-F for 30 min. The remaining activities were assayed and plotted against the amounts of NBD incorporated into the enzyme protein, which was measured fluorometrically as described in Experimental Procedures.

labeled and unlabeled enzymes measured in the far-UV region (Figure 5, panel A). On the other hand, the NBD-labeled enzyme emitted strong fluorescence due to the NBD group with λ_{max} at 536 nm when excited at 470 nm (Figure 5, panel B); the reagent NBD-F itself emits almost no fluorescence. The maximum of fluorescence emission of the NBD-labeled enzyme is comparable with that of the synthetic compound, *N*-ϵ-NBD-*N*-α-acetyllysine, emitted at 539 nm in the same buffer. Since the fluorescence of protein-bound NBD is known to be very sensitive to its environment (*31*), the nearly identical maxima of fluorescence emission suggest a hydrophilic environment for the NBD moiety in the labeled enzyme. Thus, the NBD-labeled Lys residues are presumably located close to the enzyme surface.

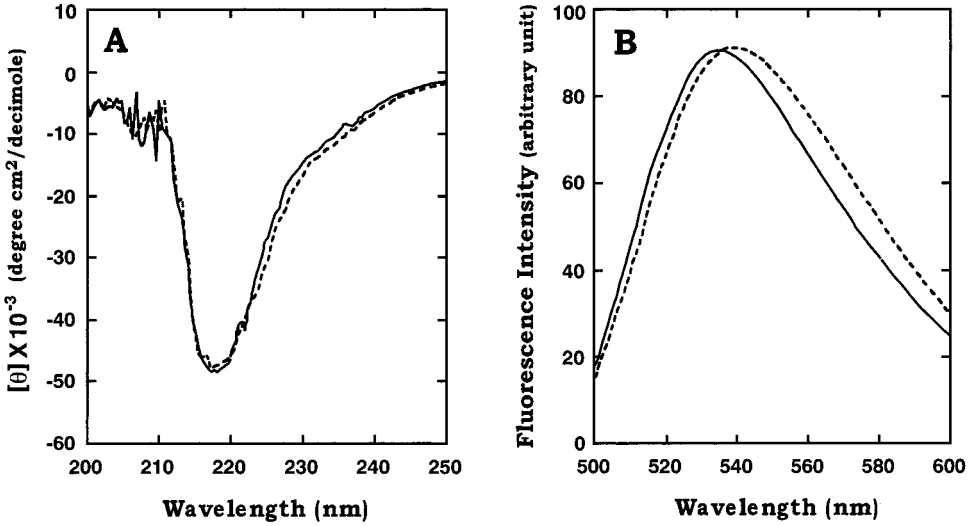

FIGURE 5: Circular dichroism (A) and fluorescence emission (B) spectra of NBD-labeled enzyme. (A) CD spectra of the native holoenzyme (solid line) and the holoenzyme treated with 100-fold molar excess of NBD-F at 30 °C for 30 min (broken line) were measured at a protein concentration of 0.1 mg/mL in 5 mM potassium phosphate buffer (pH 7.0) with a Jasco spectropolarimeter model J-600. (B) Fluorescence emission spectra of the NBD-labeled holoenzyme (10 μM subunit), containing about 2 mol of NBD/mol of subunit (solid line) and *N*-ϵ-NBD-*N*-α-acetyllysine (20 μM, broken line) were measured in 25 mM Hepes (pH 6.8) by excitation at 470 nm.

To identify the NBD-labeled Lys residues, the DTNB- and *p*-nitrophenylhydrazine-treated holoenzyme was extensively modified with NBD-F at 30 °C for 30 min (see Experimental Procedures) and several procedures were attempted for proteolytic digestion and peptide isolation. In the initial experiments, however, isolation of the NBD-labeled peptides by monitoring the NBD-derived fluorescence in the reverse-phase HPLC failed for all peptides obtained by digestion with trypsin, chymotrypsin, thermolysin, and lysyl endopeptidase. The failure was thought to be due to strong adsorption of the substantially long and hydrophobic, NBD-labeled peptides to a reverse-phase C_{18} column. Therefore, we then undertook an indirect way to identify the NBD-labeled sites by comparing HPLC elution profiles (monitored by 215-nm peptide absorption) of the NBD-labeled and unlabeled enzymes digested with lysyl endopeptidase (Figure 6). Because the PEAO polypeptide contains a total of 13 Lys residues (*23*), 14 peptides would theoretically be produced by the complete digestion with lysyl endopeptidase. In the elution profile of unlabeled enzyme, a total of 13 sharp peaks appeared (Figure 6, panel A), all of which could be assigned by amino acid sequence analysis to either one of the 14 expected lysyl endopeptidase peptides except for the N-terminal 37-residue peptide (data not shown). Four peptide peaks among them (designated L1−L4 in order of elution, Figure 6, panel A) were found to be decreased considerably in the elution profile of the NBD-labeled enzyme (indicated by arrows in Figure 6, panel B). It is evident that decreases in the peak height result from the inaccessibility of the protease at the NBD-labeled Lys residues. Because labeling of a single Lys leads to the disappearance of two peptides flanking the labeled Lys and conversion into a much longer, hydrophobic peptide firmly bound to the column, the above results indicate labeling of two Lys residues by NBD and are consistent with the stoichiometry between the remaining activities and the amounts of NBD incorporated into the enzyme protein described earlier. The results of amino acid sequence analysis for the four peptides (L1−L4) from the unlabeled enzyme are summarized in Table 1. The sequences of peptides L1 and L3 corresponded to those from

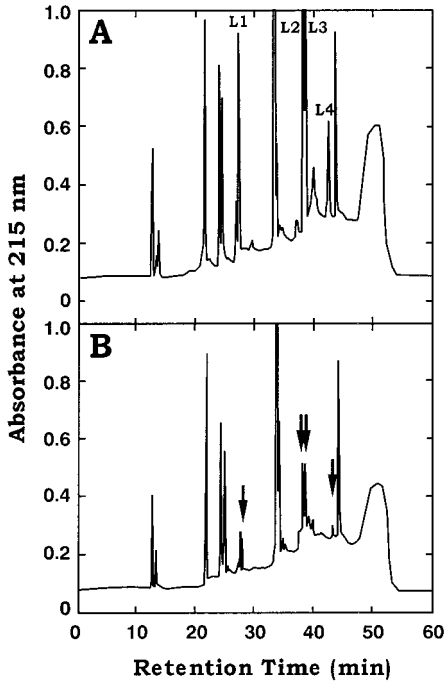

FIGURE 6: HPLC elution profiles of the NBD-labeled enzyme digested with lysyl endopeptidase. (A) Elution profile of the protease digest of unlabeled enzyme, and (B) elution profile of the protease digest of NBD-labeled enzyme. The chromatographic conditions are described under Experimental Procedures. The absorbances at 215 nm were continuously monitored. The peptide peaks found to be reduced in the elution profile of the NBD-labeled enzyme (indicated by arrows) are labeled L1−L4 in order of elution.

Val132 to Lys184 and from Glu185 to Lys218 in the PEAO sequence (*23*), respectively, showing that the two peptides in the labeled enzyme are connected at NBD-labeled Lys184 and not eluted from the column. Similarly, peptides L2 and L4 corresponding to those from Trp243 to Lys354 and from His355 to Lys401, respectively, are connected at NBD-labeled Lys354. In this way, we conclude that Lys184 and Lys354 are the NBD-labeled sites in the holoenzyme, whose modification leads to the enzyme inactivation. Proteolytic

Table 1: Amino Acid Sequences of NBD-Labeled Peptides[a]

peptide	amino acid sequence[b]	location in PEAO sequence
L1	EVTRVIDTGVFPVPAEHG	Glu185−Lys218
L2	WSLDVGFDVREGVV	Trp243−Lys354
L3	VRVAPLSAGVFEYAEERG	Val132−Lys184
L4	HSDLWSGINYTRR	His355−Lys401

[a] The NBD-labeled holoenzyme was digested with lysyl endopeptidase, and the resulting peptides were purified by HPLC as described in the text and Figure 6. The sequences of L1−L4 peptides from the unlabeled enzyme (Figure 6, panel A) were analyzed. [b] Only residues identified by amino acid sequence analysis are shown in one-letter codes.

digestion for identification of NBD-labeled Lys residues in the apoenzyme yielded similar but slightly complicated HPLC elution profiles, suggesting nonspecific labeling of a few additional Lys residues (data not shown).

Site-Specific Mutagenesis of Lys184, Lys354, and His355. To distinguish whether the inactivation of PEAO by NBD-F is caused by introduction of the bulky, hydrophobic NBD group into the enzyme protein or elimination of the free ϵ-amino groups of Lys184 and Lys354 with hitherto unknown functions, we have prepared mutant enzymes, in which Lys184 and Lys354 are replaced individually by Gln, using the two-step PCR method described under Experimental Procedures; Gln was chosen to examine the effect of removal of the amino group without significant changes in the side chain bulkiness. Also, His355, neighboring Lys354 and totally conserved in copper amine oxidase sequences reported to date (*6*, *32*), has been replaced by Ala. Lys354 and His355 occur in the β-ribbon arm (Arm I) that protrudes from one subunit to embrace the central domain of the adjacent subunit and link to several active site residues by a series of hydrogen bonds (*14*). The three mutant enzymes overproduced in *E. coli* cells were purified to homogeneity in the Cu^{2+}/TPQ-less, inactive apo form by the same procedure as that of the wild-type enzyme. Final yields of the purified proteins were about 30 mg/L of the culture medium, comparable to that of the wild-type PEAO.

The Cu^{2+}-dependent TPQ-generating capacities of the wild-type, K184Q, K354Q, and H355A mutant enzymes were compared by the kinetic analysis of the TPQ formation reaction (*7*, *20*). By using the apoenzyme that had been anaerobically preincubated with 0.5 mM $CuSO_4$ so that the initial rate of copper uptake and binding to the protein would not affect the subsequent kinetics (*20*), we initiated the reaction by addition of an appropriate volume of 50 mM Hepes buffer (pH 8.0) saturated with pure O_2 gas, and the increase in absorbance at 480 nm due to TPQ was monitored. The increase was fit to a first-order rate equation, yielding the rate constant k_{obs} ($= k[O_2]$) (*20*). As summarized in Table 2, similar values of k_{obs} were obtained for the wild-type, K184Q, K354Q, and H355A mutant enzymes, indicating that all of Lys184, Lys354, and His355 are dispensable for the Cu^{2+}-dependent TPQ formation. The TPQ and copper contents in these mutant proteins that had been converted into the holo form by overnight incubation with excess $CuSO_4$ followed by thorough dialysis were also determined by titration with phenylhydrazine and atomic absorption analysis, respectively, and the results are included in Table 2. Again, the Cu^{2+}/TPQ contents in the mutant proteins were similar to those in the wild-type protein. UV−vis absorption spectra of the wild-type and mutant holoenzymes were also almost identical with each other (not shown), although λ_{max} values of the TPQ chromophore were slightly different (Table 2), which may reflect a minuscule difference in the active site environment.

Table 2: Rate of TPQ Formation and Copper/TPQ Contents

enzyme	k_{obs} (min^{-1})	copper content[a] (mol atom/subunit)	TPQ content[b] (mol/subunit)	λ_{max} (nm)
wild type	1.4	1.00	0.76	481
K184Q	1.6	0.95	0.72	478
K354Q	1.3	1.01	0.64	481
H355A	1.2	0.94	0.76	482

[a] Determined by atomic absorption. [b] Determined by titration with phenylhydrazine. See text for details.

Table 3: Steady-State Kinetic Parameters[a]

enzyme	k_{cat} (max) (s^{-1})	K_m (μM)	$K_{i,sub}$[b] (μM)	$K_{i,pro}$[c] (μM)
wild type	131 ± 26	5.9 ± 2.0	31.1 ± 3.3	29.6 ± 1.2
K184Q	124 ± 23	9.3 ± 0.2	47.5 ± 2.8	24.8 ± 1.1
K354Q	108 ± 15	9.5 ± 0.06	182.0 ± 8.5	46.7 ± 4.8
H355A	42.1 ± 5.0	6.9 ± 1.3	ni[d]	28.3 ± 2.7

[a] Kinetic parameters were obtained in 50 mM potassium phosphate, pH 6.8. [b] Calculated from the data shown in Figure 7. [c] Calculated according to the noncompetitive inhibition. [d] No inhibition.

Steady-state kinetic parameters of the holoenzymes of the wild type and three mutants were determined at 30 °C with variable concentrations of phenylethylamine as substrate in 50 mM potassium phosphate buffer (pH 6.8) (*33*) at a fixed concentration of dissolved oxygen ($[O_2]$ in the air-saturated buffer = about 0.2 mM). Apparent k_{cat} (max) values measured in the range of 0.5−8.0 μM substrate were similar among the wild-type, K184Q, and K354Q enzymes (Table 3). Although the H355A mutant enzyme had a considerably decreased k_{cat} (max) value, these results clearly show that none of Lys184, Lys354, and His355 is directly involved in catalysis. Furthermore, K_m values for phenylethylamine of the mutant enzymes were only slightly larger than the value of the wild type (Table 3), showing that none of these residues is also involved in substrate binding. Above 10 μM phenylethylamine, the wild-type enzyme was strongly inhibited by the substrate with a K_i value of 31 μM, as calculated by a graphical method (*34*). This substrate inhibition was also observed with the K184Q mutant enzyme and, to a lesser extent, with the K354Q mutant (Figure 7; Table 3). However, the H355A mutant enzyme alone undergoes almost no substrate inhibition even at 100 μM (Figure 7), suggesting that His355 is implicated in substrate recognition in an undefined manner. On the other hand, a reaction product from phenylethylamine, phenylacetaldehyde, has also been found to cause strong inhibition in the steady-state reactions of phenylethylamine oxidation. Kinetic analysis of the wild-type enzyme reaction at different phenylacetaldehyde concentrations revealed a noncompetitive inhibition (data not shown). The K_i values of the wild-type and three mutant enzymes for phenylacetaldehyde, calculated according to the noncompetitive inhibition, were similar with each other and comparable to the values for substrate inhibition (Table 3). These results suggest that excess substrates and products inhibit the catalytic reaction by

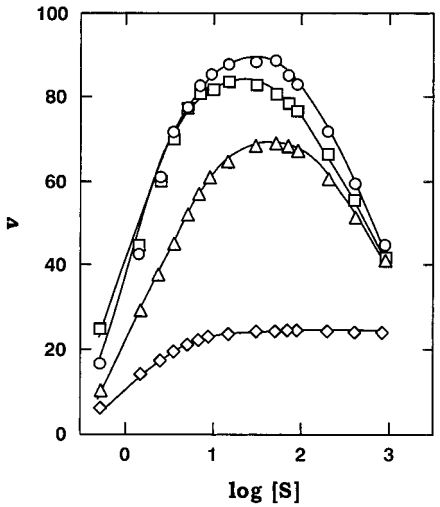

FIGURE 7: Effect of substrate concentrations on the rate of oxidation of phenylethylamine. Initial velocities (v, μmol/min/mg) of the oxidation reactions by the wild-type enzyme (○) and K184Q (△), K354Q (□), and H355A (◇) mutant enzymes were plotted against the logarithm of phenylethylamine concentrations (μM) used.

binding to the vicinity of the active site, noncompetitively with the reacting substrate.

The holo forms of K184Q and K354Q mutants were also inactivated by NBD-F as efficiently as the wild-type holoenzyme (data not shown). However, the amounts of NBD incorporated into the fully inactivated enzyme were reduced to about 1.0 ([NBD]/[subunit] = 0.9 for K184Q and 1.2 for K354Q; Figure 4), indicating that the remaining one of the two Lys residues (Lys354 in K184Q and Lys184 in K354Q) was modified by NBD-F concomitantly with the enzyme inactivation. In conclusion, the results of site-specific mutagenesis unequivocally show that the inactivation of PEAO by NBD-F is caused by introduction of the bulky, hydrophobic NBD group into the enzyme protein (specifically either Lys184 or Lys354) but not by elimination of the ϵ-amino groups of Lys184 and Lys354.

DISCUSSION

Recently, the X-ray crystallographic structure of PEAO has been determined to 2.2 Å resolution for both the apoenzymes and the holoenzymes (*14*). Significant differences between the two forms of PEAO are limited to the active site, strongly suggesting that the overall conformations of the polypeptide fold do not change extensively during the Cu^{2+}-dependent, self-processing formation of the TPQ cofactor. The crystal structure of PEAO is also closely similar to those of the copper amine oxidases from *E. coli* (*12*) and pea seedling (*13*), the shape resembling a "mushroom cap" (*12*), although their sequence identities are only 30−40% (*6*, *31*). One of the unique structural features common to these enzymes is the presence of two conspicuous antiparallel β-ribbon arms (Arms I and II) that protrude from one subunit of the dimeric enzymes to embrace the central 18-stranded β-sandwich domain (labeled D4) of the adjacent subunit, in which all active site residues including TPQ (residue 382 in PEAO), the three Cu^{2+}-ligating His residues (His431, His433, and His592 in PEAO), and the putative catalytic base (Asp298 in PEAO) are contained (*12*−*15*). In the orientation facing the mushroom cap, Arm I is in a groove along the bottom of domain D4 and is mostly invisible from the molecular surface, while Arm II is primarily along the surface at the top of domain D4 (*14*). The functional importance of the two arms is still uncertain, but it has been suggested that they (particularly Arm I) not only contribute to the stability of the dimeric structure but also affect the access of substrates to the active site (*14*), as also discussed below.

Another structural feature reported for the first time in the PEAO structure is the existence of a channel beginning from the molecular surface and reaching the active site that is buried deep inside the protein, over a distance of about 17 Å (*14*). As described previously in full detail (*14*), the channel likely provides a pathway for positively charged amine substrates (*16*) by electrostatically guiding them with predominantly negative surface charges, surrounding its entrance, and being associated with the side chains of several acidic residues including Glu102, Glu103, Glu106, Glu109, and Asp357. The internal surface of the channel becomes increasingly hydrophobic as it approaches the active site, which also appears advantageous for facilitating the catalytic reaction by directing the deprotonated neutral amines to the reactive C═O5 carbonyl of TPQ, although it is not obvious at which stage in binding the substrate becomes deprotonated and which base accepts the proton. A part of the entrance to the channel is formed by some residues at the end of the above-mentioned Arm I from the neighboring subunit. In addition, Arm I contains an invariant His residue (His355), which forms a hydrogen bond to the carboxyl oxygen of an active site residue, Asp383, adjacent to TPQ and occurring in the consensus sequence, Asn-TPQ-(Asp/Glu) (*35*), as depicted in Figure 8, panel A. The other carboxyl oxygen of Asp383 is in turn hydrogen-bonding to the hydroxyl group of Thr378, which is also totally conserved in copper amine oxidase sequences (*6*, *31*). This hydrogen-bonding network, linking Arm I with the active site of the neighboring subunit, runs along the channel at 5−9 Å behind its hydrophobic internal wall (Figure 8, panel A). The imidazole nitrogen of His355 also forms a hydrogen bond to the carboxyl oxygen of an Arm I residue, Asp357, which is one of the negatively charged residues constituting the channel entrance. Flanking His355 in Arm I is a conservatively substituted residue, Lys354, which forms a salt linkage or hydrogen bond to the carboxylate group of another conserved residue, Asp316, creating an additional link from Arm I to a different part of the central domain-D4 polypeptide. It has been reported recently that disruption of these hydrogen bonds by site-specific mutagenesis, particularly those linking Arm I and the active site, does not affect so deleteriously the capacity for the TPQ formation of copper amine oxidases but causes the yeast *Hansenula polymorpha* enzyme to be inactivated by its substrate methylamine (*36*) or the *A. globiformis* histamine oxidase to have very low catalytic efficiency (*37*), thus suggesting the importance of the hydrogen-bonding network in the catalytic process of amine oxidation.

We have shown here the efficient inactivation of PEAO by incubation with low concentrations of NBD-F. Although the X-ray structures of the Cu^{2+}/TPQ-less apoenzyme and the Cu^{2+}/TPQ-containing holoenzyme were found to be virtually identical (*14*), the former was slightly more resistant to the NBD-F modification than the latter (Figure 1). The reason for the different reactivities of the two forms to

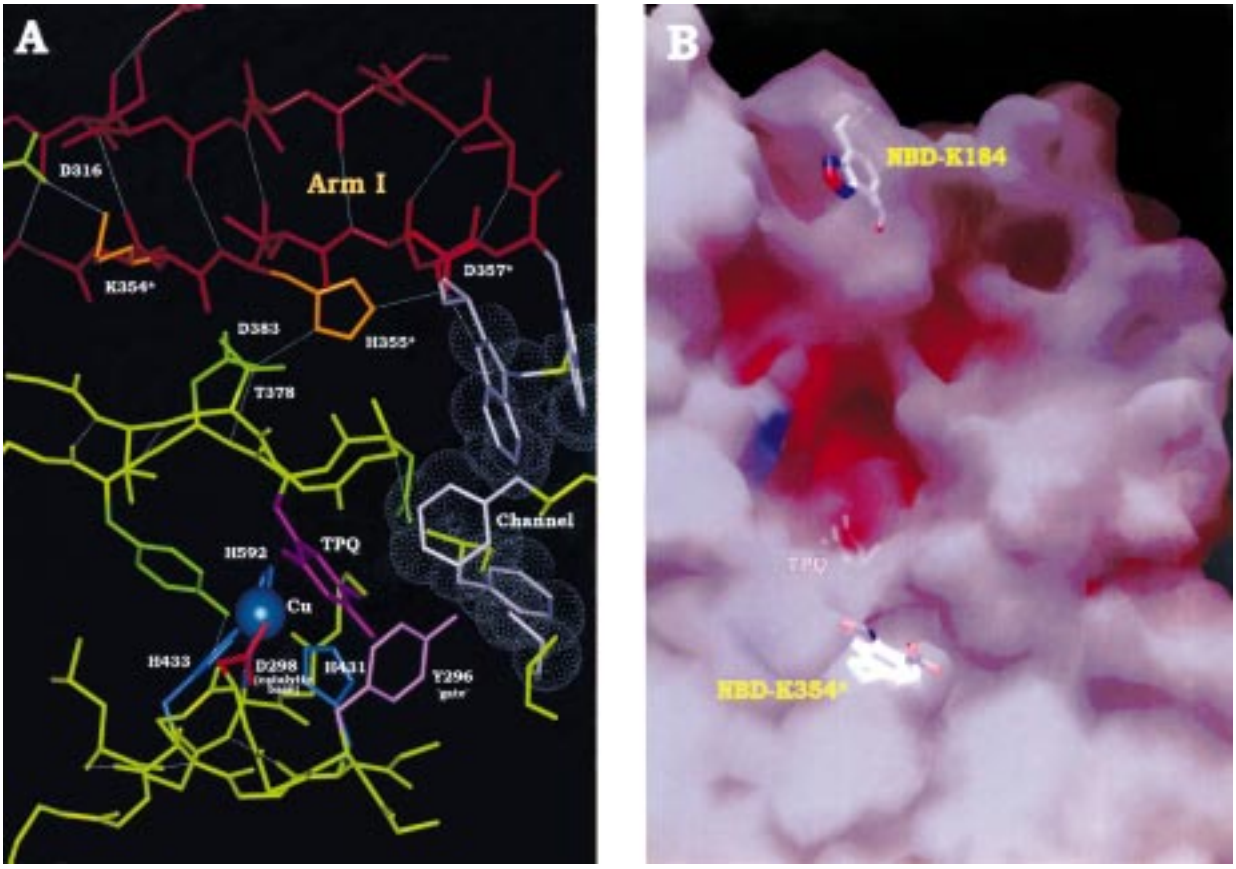

FIGURE 8: (A) Active site structure of holo-PEAO. Only the main chain and residues relevant to this paper were drawn in a stick model, colored light green for those of the central domain D4 of one subunit and dark brown for those of Arm I of the neighboring subunit, using INSIGHT II (Molecular Simulations Inc., San Diego) and the coordinates of holo-PEAO (*14*). The residues referred to in the text are also colored differently for clarity and annotated with residue numbers (with asterisks for those of Arm I of the neighboring subunit). The residues constituting a part of the internal hydrophobic wall of the channel are colored gray with their atomic surfaces shown by dots. Hydrogen bonds are shown by dotted lines. (B) A close view of the molecular surface around the substrate channel drawn in a half transparency mode by using GRASP (*39*), showing the relative charge distribution colored red (negative) and blue (positive). The computer-modeled structure of the NBD groups bound to Lys184 and Lys354 is shown in a stick model. The NBD groups were generated using a Builder module of INSIGHT II and connected to the ϵ-amino groups of Lys184 and Lys354. The TPQ cofactor is located behind the side wall at the bottom of the channel.

NBD-F is not obvious, but may be correlated with the fact that the apoenzyme crystal has a larger value of the average disorder parameter (temperature factor) [B] than the holoenzyme crystal (*14*). It is possible that the larger [B] indicates a less rigid structure, which would render the protein to suffer additional, nonspecific modification by NBD-F. Nonetheless, the inactivation by NBD-F does not result from denaturation of the enzyme protein. Rather, it is derived from modification of two specific Lys residues, Lys184 and Lys354.

The inactivation of apoenzyme was partly associated with the reduction of the TPQ-forming capacity (Figure 2, panel A). Because the NBD-F-modified apoenzyme retained nearly normal Cu^{2+}-binding capacities, the decreased TPQ formation in apoenzyme is likely due to small structural changes limited to a local region of the enzyme protein. The introduced NBD groups may restrain the structural rearrangement of active site residues including the precursor Tyr, required in the Cu^{2+}-dependent, self-processing formation of the TPQ cofactor (*14*), by perturbing the hydrogen-bonding network linking Arm I and the Cu^{2+}/TPQ-active site. The inactivation of holoenzyme by NBD-F, on the other hand, was found not to be caused by the loss of the redox-active TPQ and bound copper, their contents remaining practically unchanged (Figure 2, panel B). Furthermore, the reaction of an inhibitor, phenylhydrazine, with TPQ was markedly inhibited in the NBD-labeled holoenzyme (Figure 3). Therefore, it is conceivable that the inactivation of holoenzyme is due to the prevention of binding of small molecules such as substrates and inhibitors to the active site or the inhibition of release of reaction products by the incorporated NBD groups. The marked protection of enzyme by phenylacetaldehyde from the NBD-F inactivation (Figure 1, panel A) is also consistent with the interpretation that the reagent NBD-F and the reaction product phenylacetaldehyde compete in binding to the same region.

The NBD-labeled sites have been identified, indirectly though, as Lys184 and Lys354. In the crystal structure of PEAO, Lys184 is almost solvent-exposed and Lys354 is close to the molecular surface. They are located in the opposing sides, facing each other with an approximate distance of 30 Å, of the entrance to the above-described channel. It is puzzling that only Lys184 and Lys354 among a total of 10 solvent-exposed Lys residues existing in each monomer (*14*) are specifically modified with NBD-F. Moreover, the reasons for the failure of modification with other Lys-directed reagents such as pyridoxal 5′-phosphate, 2,4,6-trinitrobenzenesulfonate, and methyl acetyl phosphate

are unclear. Presumably, nearby negative charges around the two Lys residues (Asp316, hydrogen-bonding to Lys354, as described above; Glu109, Glu106, Glu102, and Glu102, constituting a major part of the entrance to the channel and being close to Lys184) (*14*) hinder those negatively charged reagents from approaching to the Lys residues to be labeled. As already described, Lys354 in Arm I is conservatively substituted in copper amine oxidases (Lys in most of the microbial enzymes and Arg in the enzymes from higher eukaryotes), whereas Lys184 is only weakly conserved (*6*, *31*, *38*).

The results of site-specific mutagenesis of Lys184, Lys354, and His355 clearly indicate that none of these residues is indispensable for both of the TPQ-forming capacity of apoenzyme and the catalytic activity of holoenzyme. The inactivation of PEAO by NBD-F is thus not due to the modification of the ϵ-amino groups of the two Lys residues but to the introduction of the bulky, hydrophobic NBD group into the channel entrance. However, the Ala mutant for His355 in Arm I has a considerably decreased activity in the holo form without undergoing the strong substrate inhibition that the wild-type holoenzyme encounters (Figure 7). The mechanism of substrate inhibition is yet unknown, but is suggested to be the competed binding of excess substrates to the channel in a nonproductive mode, which may also inhibit the product release. Disruption of hydrogen bonds from Asp357 in Arm I (located at the channel entrance; Figure 8, panel A) to the active site Asp383 via His355, by substitution of Ala for His355, may cause subtle structural perturbations of the channel so that it becomes unsusceptible to the substrate inhibition.

Finally, the NBD molecules bound to Lys184 and Lys354 have been generated and modeled in the PEAO structure on a graphic workstation, as shown in Figure 8, panel B. Their locations are compatible with the hypothesis that modification of the Lys residues at the channel entrance with the NBD group causes steric hindrance for the access of small molecules, such as substrates and inhibitors, to the active site through the channel, the existence of which has been revealed from the crystal structure of PEAO (*14*). Simultaneously or alternatively, such modification may also cause perturbations of the channel structure, altering the internal hydrophobicity, may affect the net negative charges surrounding the channel entrance, or may bring slight changes of the active site structure through disruption of the hydrogen-bonding network involving His355 in Arm I by NBD-labeling of the flanking Lys354. Our ongoing X-ray crystallographic studies on the NBD-labeled enzyme are expected to clarify these possibilities.

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