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Chemical Modification of L-Phenylalanine Oxidase from *Pseudomonas* sp. P-501 by Phenylglyoxal. Identification of One Essential Arginyl Residue

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L-Phenylalanine oxidase from *Pseudomonas* sp. P-501 was irreversibly inactivated by the arginine-specific reagents, phenylglyoxal (PGO) and *p*-hydroxyphenylglyoxal (HPG). The inactivation by PGO and HPG follows pseudo-first-order kinetics with second-order rate constants of 10.6 and 15.1 $M^{-1} \cdot min^{-1}$, respectively, and a single arginyl residue was modified specifically. The effective protection by substrate L-phenylalanine against the inactivation by these reagents strongly suggests that the arginyl residue is located in the substrate binding site. SDS/PAGE analysis of the enzyme modified with [^{14}C]PGO revealed that the arginyl residue was in the β subunit of the enzyme. The fragment containing the ^{14}C-labeled arginyl residue was purified from the enzymatic digests of the labeled β subunit by HPLC and sequenced. The modification of Arg-35 in the β subunit was identified. The sequence around Arg-35 shows homology to the corresponding regions of tryptophan-2-monooxygenases.

Key words: active site, chemical modification, flavoenzyme, peptide sequencing, phenylglyoxal.

L-Phenylalanine oxidase (PAO) from *Pseudomonas* sp. P-501, found by Koyama (*1*), has unique characteristics, that is, the enzyme catalyzes both oxidative deamination and oxygenative decarboxylation of L-phenylalanine simultaneously (*2, 3*). The enzyme has 2 mol of non-covalent FAD and consists of 2 mol each of α and β subunits (*4*). The amino acid sequence of the α subunit has been determined (*4*), and it was shown that the sequence contains a nucleotide binding domain for FAD (*4, 5*), but as a whole it shows no homology to any known protein sequence. These results suggest that this enzyme may have a novel type of sequence and structure with unique characteristics.

Among the enzymes with oxygenative decarboxylation activity, tryptophan 2-monooxygenases [EC 1.13.12.3] have been well studied (*6-9*). Though the involvement of a cysteine residue in the active site of this enzyme has been demonstrated (*7*), detailed characterization has not yet been achieved. On the other hand, there are many analogous enzymes with oxidative deamination activity, and among them D-amino acid oxidase (DAAO; EC 1.4.3.3) and the closely related enzyme D-aspartate oxidase (DASPO; EC 1.4.3.1) have been well characterized (*10-14*). That is, both DAAO and DASPO have at least one arginyl residue which has a crucial role in the catalytic activity (*11, 12*). Furthermore the crystal structure of DAAO (*13, 14*) demonstrated the presence of one arginyl residue at the substrate binding site (*13*). Although PAO from *Pseudomonas* sp. P-501 catalyzed both oxidative deamination and oxygenative decarboxylation of L-phenylalanine, the oxygenative decarboxylation is the major (~80%) reaction catalyzed (*3*). Suzuki *et al.* (*15*) proposed from a resonance Raman study of a reaction intermediate of PAO that the intermediate is similar to that of DAAO. Based on this background, we performed the chemical modification of PAO in the present study, to identify the residue(s) at the active site.

Phenylglyoxal (PGO) and *p*-hydroxyphenylglyoxal (HPG) are specific reagents for arginyl residues (*16, 17*). We studied the effect of these reagents on the enzymatic activity, and found that PAO was irreversibly inactivated and the inactivation was prevented by L-phenylalanine. We examined the kinetics of the inactivation of the enzyme with PGO and HPG. We also labeled PAO with [^{14}C]PGO, digested the labeled enzyme and analyzed the sequence of the ^{14}C-labeled fragment. The data suggest that one arginyl residue of the β subunit is located at the binding site of the substrate.

MATERIALS AND METHODS

Materials—PAO was purified to homogeneity from *Pseudomonas* sp. P-501 as described previously (*1*). Although PAO contains two FAD molecules as the prosthetic group, it is assumed that the oxidation of L-phenylalanine can be catalyzed by an enzyme unit containing one

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Abbreviations: PAO, L-phenylalanine oxidase from *Pseudomonas* sp. P-501; DAAO, D-amino acid oxidase; DASPO, D-aspartic acid oxidase; BD, 2,3-butanedione; CHD, 1,2-cyclohexanedione; HPG, *p*-hydroxyphenylglyoxal; PGO, phenylglyoxal; TFA, trifluoroacetic acid.

molecule of FAD (*18*). So the concentration of the enzyme was expressed in terms of enzyme-bound FAD, which was determined by measuring the absorbance at 465 nm, using the absorbance coefficient of 11.3 $mM^{-1} \cdot cm^{-1}$. PGO monohydrate and 1,2-cyclohexanedione (CHD) was purchased from Aldrich. Lysyl endopeptidase (3.3 AU/mg), *p*-hydroxyphenylglyoxal (HPG), and 2,3-butanedione (BD), were from Wako Chemicals. Ultra pure urea was from Schwarz/Mann. [7-^{14}C]PGO (27.0 mCi/mmol), ACS II and Amplify were from Amersham.

Enzyme Assay—The activities of the native and modified enzymes were assayed by measuring the oxygen uptake using a Clark-type oxygen electrode YSI model 5331 in the presence of 488 μM L-phenylalanine at 30°C. The buffer used was 50 mM potassium phosphate (pH 7.0).

Kinetics of Inactivation of PAO with PGO and HPG—PAO (2.37×10^{-5} M) was incubated with various concentrations of PGO or HPG at 30°C in 0.2 M sodium borate buffer (pH 9.0) for different times (0–20 min) in the dark. At the indicated times of incubation, 5 μl aliquots of the mixture were removed and diluted 100-fold with 50 mM potassium phosphate buffer (pH 7.0), then the enzymatic activity was assayed.

Labeling of PAO with [^{14}C]PGO and Measurement of Radioactivity—PAO (2.37×10^{-5} M) was incubated at 30°C in 0.2 M sodium borate buffer (pH 9.0) with 10 mM [^{14}C]-PGO (28.8 dpm/pmol) in the absence or presence of 20 mM L-phenylalanine for different times in the dark. At the indicated times of incubation, 5 μl aliquots of incubation mixture were mixed with 2 μl of 1 M PGO and the whole was boiled for 30 s in the sample buffer (10% glycerol, 0.0625 M Tris-HCl, pH 6.8, 2% SDS, and 5% β-mercaptoethanol). SDS/PAGE was carried out with SPU-15S gel (Atto) according to the manufacturer's protocols. The molecular weight markers used were SDS-PAGE standard (Broad) from Bio-Rad. Protein bands were stained with CBB R-250, and the gel was treated with Amplify. The dried gel was analyzed by fluorography.

To determine the ^{14}C-incorporation into PAO, 5 μl aliquots of incubation mixture were precipitated with 7% trichloroacetic acid and the precipitates were collected on GF/C filters (Whatman), and washed 3 times with 7% trichloroacetic acid and then with ethanol. The filters were dried and the radioactivity was measured.

Isolation of [^{14}C]PGO-Labeled Peptide—PAO (3.2 nmol, 6.32×10^{-5} M) was incubated with 10 mM [^{14}C]PGO for 20 min as described above. PGO was added to the mixture at the final concentration of 295 mM, then the whole was immediately subjected to reverse-phase HPLC to separate the α and β subunits as described previously (*4*) with minor modifications. The labeled β subunit was collected and suspended in 50 mM *N*-ethylmorpholine buffer (pH 8.7) containing 8 M urea and incubated at 37°C for 1 h. The subunit was digested with lysyl endopeptidase at the substrate/enzyme molar ratio of 30:1 in 50 mM *N*-ethylmorpholine buffer (pH 8.7) containing 4 M urea at 37°C for 7 h. The proteolytic fragments were separated by reverse-phase HPLC on an Inertsil ODS column (4.6×250 mm, GL Sciences) by eluting at 1 ml/min with a linear gradient of acetonitrile in 0.1% TFA (0–70% for 70 min).

Cyanogen Bromide Degradation—The sample was suspended in 70% formic acid and CNBr in the same solvent was added to the concentration of 1%. The mixture was placed in a hood at room temperature for 23 h in the dark.

Amino Acid Sequencing—The amino acid sequences of peptide fragments were analyzed with a gas-phase sequencer (Shimadzu, PPSQ-10). A sequencer (Applied Biosystems, 470A) was used for sequencing ^{14}C-labeled peptides.

CD Measurement of the Native and Modified PAO in the Far-Ultraviolet Region—All spectra were measured with a Jasco J-720 spectropolarimeter with a cell of 1 mm path length at 10°C. Each spectrum is the result of 8 accumulated scans. The enzyme was incubated with 10 mM PGO in the absence or presence of L-Phe for 20 min at 30°C, then a fivefold molar excess of arginine was added to remove the remaining PGO. The mixture was extensively dialyzed against 50 mM potassium phosphate, pH 7.0. The dialyzed enzyme solution was diluted with the same buffer and filtered through a 0.2 μm pore size filter, then the CD spectrum was measured and the residual activity was assayed. The concentration of each sample was determined by measuring the absorbance at 465 nm. The contents of α-helix and β-strand were determined by the use of the application "Secondary structure estimation for protein" supplied with the apparatus (*19*).

RESULTS

Inactivation of PAO by PGO and HPG—PAO catalyzes the oxidation of L-phenylalanine most effectively and that of tyrosine to a lesser extent (*1*). These substrates have phenyl and *p*-hydroxyphenyl groups, respectively, so we examined whether PGO and HPG, containing these groups, inactivate the enzyme. As Table I shows, PGO and HPG strongly inactivated PAO, though CHD and BD, which are also arginine-specific reagents, inactivated PAO only slightly. The results suggest that PGO and HPG interacted with the enzyme as substrate analogs. This is supported by the observation that the substrate L-phenylalanine protected PAO against the inactivation by PGO and HPG (Table I).

Treatment of PAO with PGO and HPG caused a time-dependent loss of activity. The time courses of inactivation by PGO and HPG are shown in Figs. 1A and 2A, respectively. The linear plots of the logarithm of residual enzymatic activity *versus* the reaction time indicate that the inactivation with these reagents follows pseudo-first-order kinetics. When the pseudo-first-order rate constant (k_{obs}) of inactivation was plotted against the reagent concentration, the plot was linear (Fig. 1B), indicating that the modification is simply bimolecular, and therefore the inactivation velocity v is represented by the equation $v = k_{obs}[PAO]$. The second-order rate constant of inactivation was determined to be 10.6 $M^{-1} \cdot min^{-1}$ for PGO from the plot. To confirm

TABLE I. **The effects of various arginyl specific reagents on the activity of PAO.**

Reagents	Concentration (mM)	Preincubation conditions	Residual activity (%)	
			L-Phe(−)	L-Phe(+)[a]
Control			100	100
Phenylglyoxal	10	20 min, 30°C	11.37	95.51
p-Hydroxyphenylglyoxal	10	20 min, 30°C	5.42	75.47
1,2-Cyclohexanedione	50	30 min, 37°C	87.32	83.94
2,3-Butanedione	50	30 min, 25°C	81.09	102

[a]The concentration of L-Phe in the preincubation mixture was 20 mM for PGO, 5 mM for HPG, and 30 mM for CHD and BD.

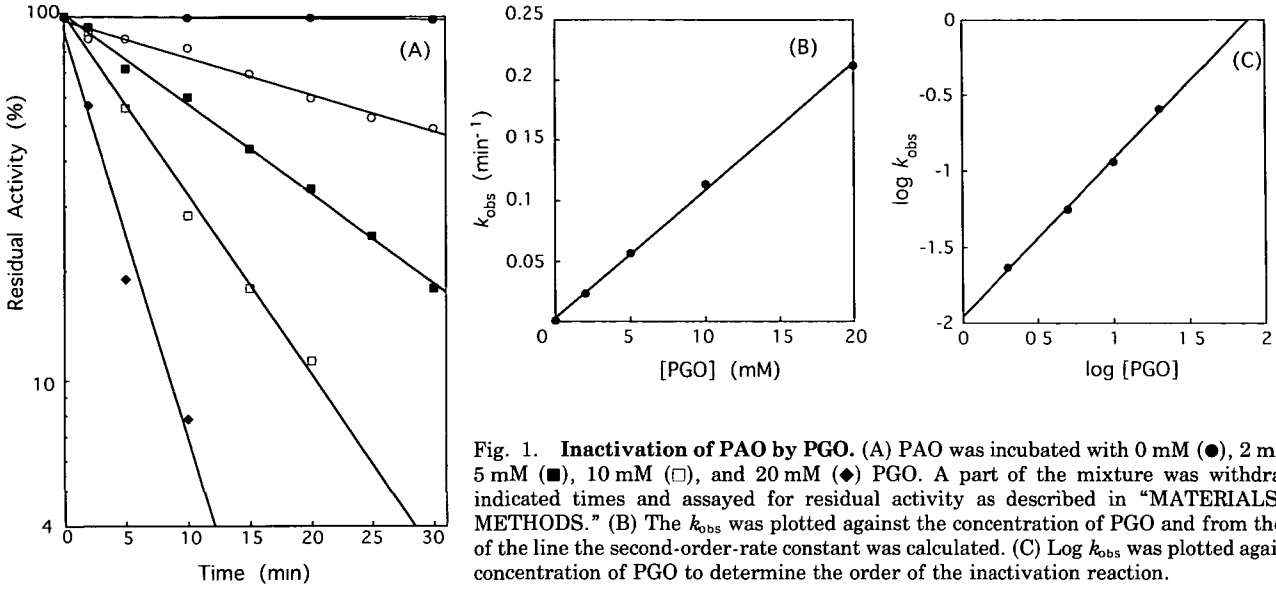

Fig. 1. **Inactivation of PAO by PGO.** (A) PAO was incubated with 0 mM (●), 2 mM (○), 5 mM (■), 10 mM (□), and 20 mM (◆) PGO. A part of the mixture was withdrawn at indicated times and assayed for residual activity as described in "MATERIALS AND METHODS." (B) The k_{obs} was plotted against the concentration of PGO and from the slope of the line the second-order-rate constant was calculated. (C) Log k_{obs} was plotted against log concentration of PGO to determine the order of the inactivation reaction.

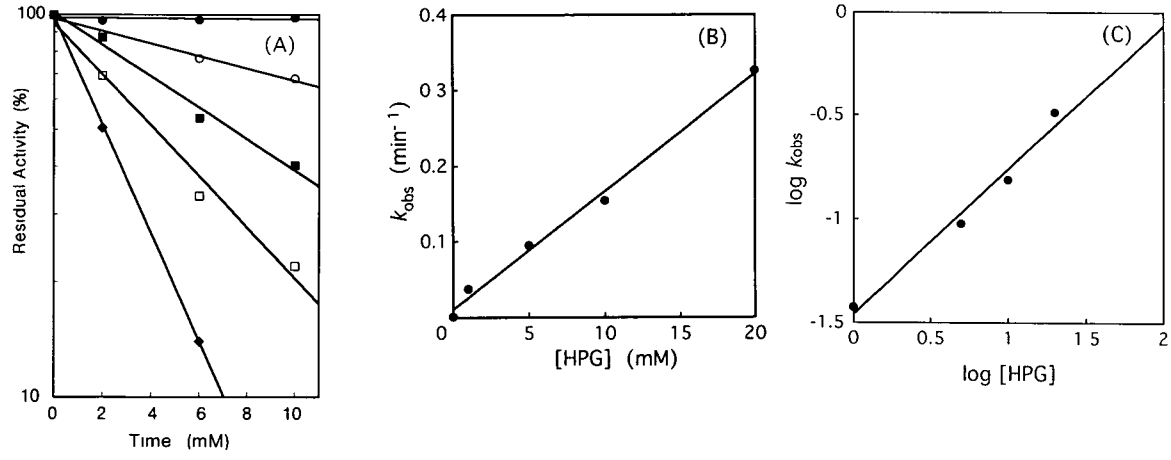

Fig. 2. **Inactivation of PAO by HPG.** (A) PAO was incubated with 0 mM (●), 1 mM (○), 5 mM (■), 10 mM (□), and 20 mM (◆) HPG. The activity of the enzyme was assayed as described in the legend to Fig. 1. (B) and (C) were plotted as in Fig. 1.

that the inactivation is bimolecular, log k_{obs} was plotted against log[PGO]. The plot gave the straight line with a slope of 1.03 (Fig. 1C), which suggested that one molecule of phenylglyoxal was needed to inactivate one molecule of the enzyme unit (*20*). Similar results were obtained with HPG, and the second-order rate constant for inactivation and the reaction order were determined to be 15.6 $M^{-1} \cdot min^{-1}$ (Fig. 2B) and 0.70 (Fig. 2C), respectively. As the inactivation by HPG was similar to that by PGO, the following experiments were performed using PGO.

Substrate Protection—The protection by the substrate was examined in more detail by incubating PAO with 10 mM PGO in the presence of various concentrations of L-phenylalanine (Fig. 3). The rate (k_{obs}) of inactivation decreased with increasing concentration of the substrate, then reached the limiting value. L-Phenylalanine also protected PAO against the inactivation by HPG (Table I). Combining the data with those in Figs. 1 and 2, it is suggested that the enzyme was inactivated by modification of a single arginyl residue per enzyme unit at the binding site of the substrate.

Modification of PAO with [^{14}C]PGO—PAO consists of 2 mol of each α and β subunits (*4*), so we labeled the enzyme with [^{14}C]PGO to identify which subunit is modified. PAO treated with [^{14}C]PGO in the absence and presence of L-phenylalanine was analyzed by means of SDS/PAGE (Fig. 4, A and B) as described in "MATERIALS AND METHODS." As Fig. 4B shows, the β subunit was labeled in the absence of L-phenylalanine (lanes 1-5), but the labeling was almost completely inhibited in the presence of L-phenylalanine (lanes 6-10).

Stoichiometry of Incorporation of [^{14}C]PGO into PAO—The amount of [^{14}C]PGO incorporated into PAO in the absence of L-phenylalanine was determined at given times as described in "MATERIALS AND METHODS" and represented as mol of [^{14}C]PGO incorporated per mol of enzyme unit (Fig. 5A), and the residual enzymatic activity was measured under the same conditions except that non-labeled PGO was used in place of [^{14}C]PGO (Fig. 5A). The loss of enzyme activity was plotted against the incorpora-

tion of PGO (Fig. 5B). Extrapolation of the plots to 100% loss of activity showed that the activity was completely abolished when 1.02 mol of PGO was incorporated into 1 unit of PAO. These results and those in Fig. 3 suggest that the binding site of the substrate is located in the β subunit and a single arginyl residue is present at the site.

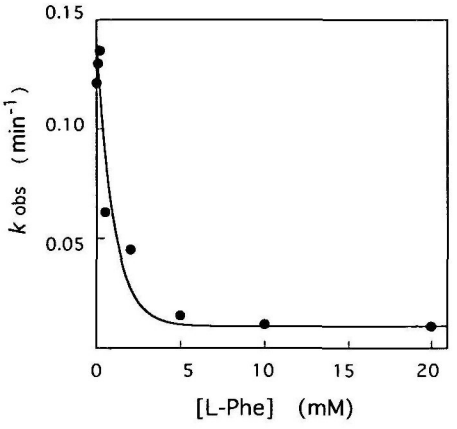

Fig. 3. **Protection against PGO inactivation by L-phenylalanine.** PAO was incubated with 10 mM PGO in the presence of various concentration of L-phenylalanine and a part of the mixture was withdrawn at given times, then assayed for residual activity. The rate constants (k_{obs}) of the inactivation were plotted against the concentration of L-phenylalanine.

Isolation of the Peptide Fragment Containing the [^{14}C]-PGO-Labeled Arginyl Residue and Sequencing of the Peptide—To locate the ^{14}C-labeled arginyl residue in the β subunit, PAO was incubated with 10 mM [^{14}C]PGO in the absence and presence of L-phenylalanine, and the reaction mixture was directly subjected to reverse-phase HPLC to isolate the ^{14}C-labeled β subunit. The isolated β subunit was digested with lysyl endopeptidase as described in "MATERIALS AND METHODS," then the proteolytic digests were fractionated by reverse-phase HPLC (Fig. 6A). One major ^{14}C-radioactive peak (a retention time of 51.5 min, indicated by an arrow in Fig. 6B) was observed for the digests of PAO incubated without L-phenylalanine, but the radioactivity of the corresponding peak was reduced greatly for the digests of PAO incubated with L-phenylalanine (indicated by an arrow in Fg. 6C). So it is reasonable to conclude that the peptide in this ^{14}C-radioactive peak contains the substrate binding site of the enzyme. To identify the sequence around the labeled arginyl residue, the ^{14}C-radioactive peak fraction was collected. Before sequencing the ^{14}C-labeled peptide, we analyzed the N-terminal sequence of the corresponding peak fraction of the

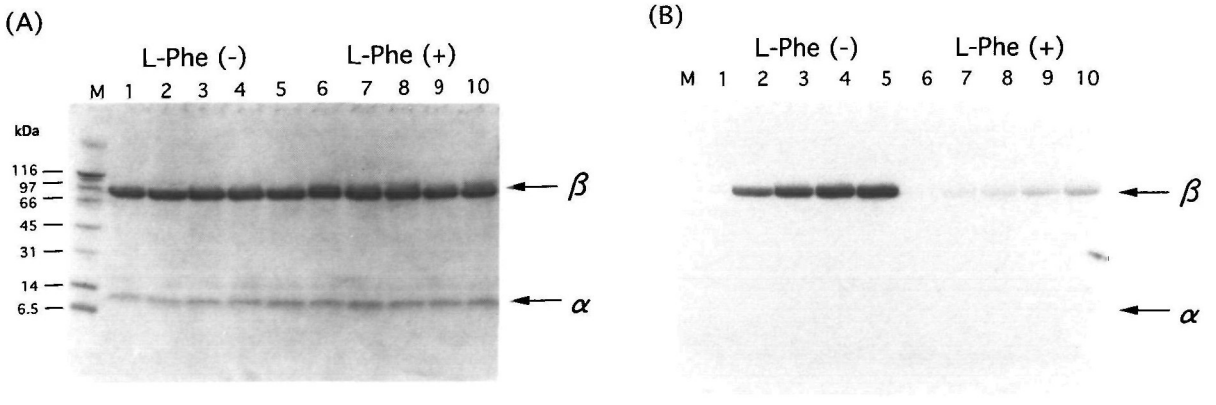

Fig. 4. **Incorporation of [^{14}C]PGO into PAO.** The enzyme was incubated with 10 mM [^{14}C]PGO in the absence and presence of L-phenylalanine, and a part of the mixture was withdrawn at 0 min (lanes 1, 6), 5 min (lanes 2, 7), 10 min (lanes 3, 8), 15 min (lanes 4, 9), and 20 min (lanes 5, 10), then analyzed by SDS/PAGE as described under "MATERIALS AND METHODS." (A) Stained gel. Lane M, molecular size marker. The positions of the α and β subunits are shown. (B) Fluorogram of the dried gel.

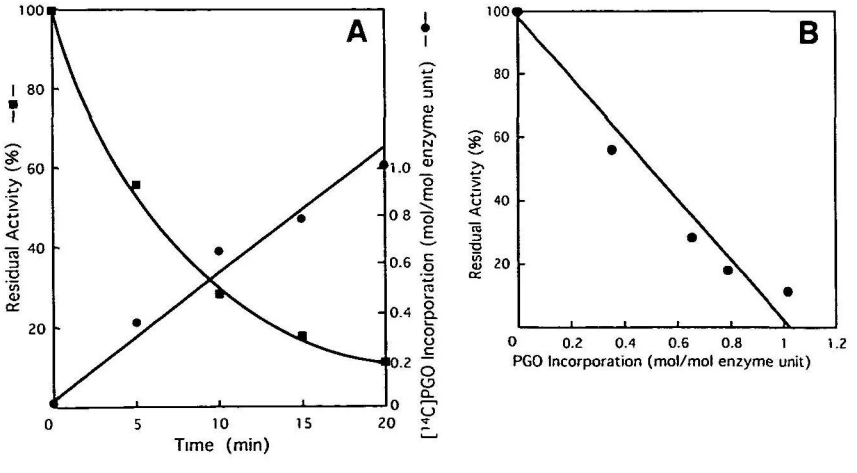

Fig. 5. **Correlation between the inactivation of PAO and the amount of PGO incorporated.** (A) ^{14}C-Incorporation into PAO after incubation with 10 mM [^{14}C]PGO for the indicated time was plotted (●), after subtraction of the background (950 dpm). The residual activity with 10 mM PGO was also plotted (■). (B) The residual activity was replotted against PGO incorporation.

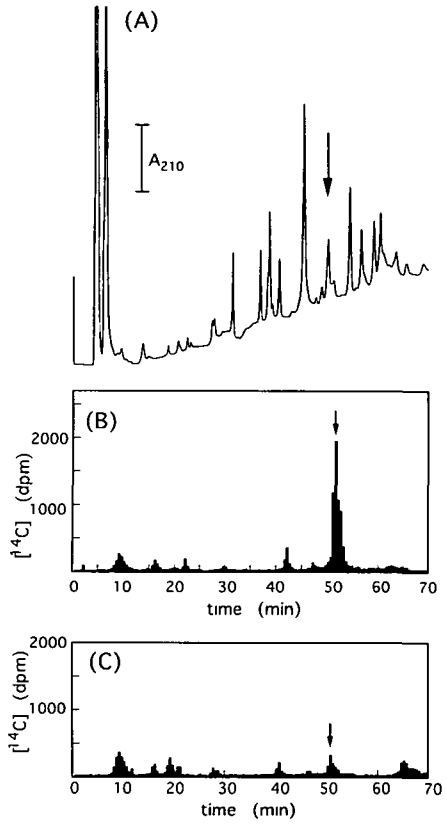

Fig. 6. **HPLC profiles of the proteolytic fragments of ^{14}C-labeled β subunit.** The β subunit labeled with [^{14}C]PGO in the absence (B) and presence (C) of L-phenylalanine was digested with lysyl endopeptidase as described in "MATERIALS AND METHODS." A part of the enzymatic digests was analyzed by reverse-phase HPLC. The amounts of samples analyzed were 1×10^4 dpm for (B) and 5×10^3 dpm for (C). The absorbance of the eluates was monitored at 210 nm (A), and the eluates were collected in 0.5 ml fractions. The ^{14}C-radioactivity of each fraction was measured after the addition of 1.5 ml of ACS II. The retention time of the major labeled peak is represented by arrows in (A), (B), and (C).

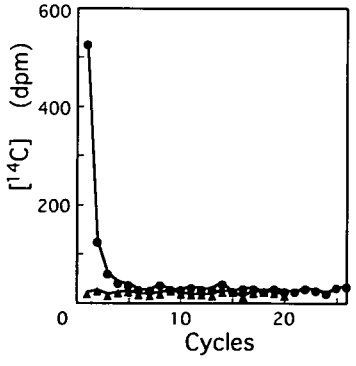

Fig. 7. **Edman degradation of CNBr fragments of the labeled peak fraction.** The ^{14}C-labeled fragments obtained as in Fig. 6 were collected, and then degraded by CNBr as described in "MATERIALS AND METHODS." The resultant mixture was sequenced and the radioactivity released at each Edman cycle was measured. The samples analyzed were derived from the modification in the absence (●) and the presence (▲) of L-phenylalanine.

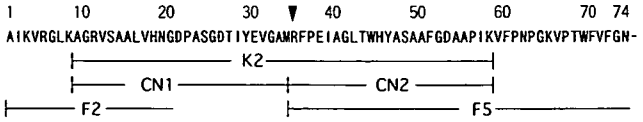

Fig. 8. **N-terminal sequence of the β subunit and the site of the modified arginyl residue.** The arrowhead represents the modified arginyl residue. K2 represents the peptide fragment derived from the digests of PAO with lysyl endopeptidase. CN1 and CN2 are the sequences of the CNBr fragments of K2. F2 and F5 are both previously determined N-terminal sequences of the CNBr fragments of the β subunit.

enzyme modified with nonradioactive PGO. From the sequence obtained and our preliminary sequence analyses of the β subunit (Fig. 8), it is suggested that the ^{14}C-labeled peptide fragment is the peptide K2 in Fig. 8. If this is the case, the sequence contains two arginyl residues at the 3rd and 27th positions from the N-terminus of the peptide. It is rather difficult to clarify by direct sequencing of the fragment which arginyl residue was modified, since the recovery of each Edman cycle is usually 90-95%. So we degraded the ^{14}C-labeled fragment with CNBr, since the peptide K2 contains one methionyl residue at position 26. The CNBr fragments were applied to the sequencer, and the ^{14}C-radioactivity of the fractions obtained after each Edman cycle was measured. The radioactivity was observed at the first Edman cycle (Fig. 7), but the corresponding fragments of PAO incubated in the presence of L-phenylalanine showed no radioactivity (Fig. 7). Moreover, in the sequence analysis of the CNBr fragments, nearly equimolar amounts of two kinds of PTH-amino acids were detected at each Edman cycle, and the PTH-amino acids agreed well with those derived from the CN1 and CN2 peptides (Fig. 8), except that the PTH-Arg was not detected in the first cycle (data not shown). These results confirm that the ^{14}C-labeled peptide fragment is the peptide K2 and the labeled arginyl residue is Arg-35 of the β subunit (see Fig. 8).

Far-Ultraviolet CD Spectroscopy—To see if the loss of the enzymatic activity with PGO was due to a large structural change, we analyzed the far-ultraviolet CD spectrum of the enzyme modified with PGO as described in "MATERIALS AND METHODS." The residual activity was assayed after dialysis of the modified enzyme, and the activity was 93% for the control sample, 5% for PAO modified in the absence of L-phenylalanine, and 66% for PAO modified in the presence of L-phenylalanine. From the spectrum shown in Fig. 9A, the native enzyme was estimated to contain 38% α helix and 41% β strand. The enzyme modified with PGO in the absence (Fig. 9B) and presence (Fig. 9C) of L-phenylalanine showed almost the same spectra as that of the native enzyme, implying that the modification of PAO did not induce a large change of the secondary structure of the enzyme.

DISCUSSION

PAO catalyzes both oxidative deamination and oxygenative decarboxylation of L-phenylalanine. In order to identify the active site residues, we modified PAO with arginyl-specific reagents, focusing on PGO and HPG (Table I and Figs. 1 and 2). These reagents inactivated PAO irreversibly, and

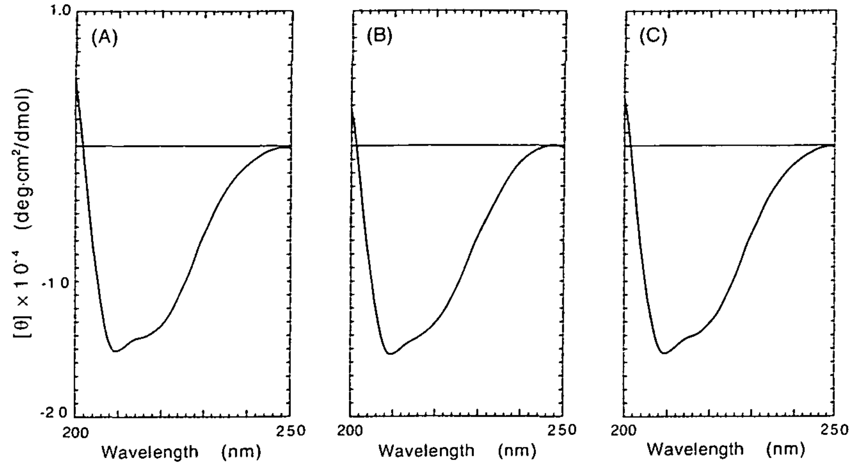

Fig. 9. **Far-ultraviolet CD spectra of native and modified PAO.** The spectra of the native PAO (A), the modified enzymes in the absence (B), and the presence (C) of L-phenylalanine were measured as described in "MATERIALS AND METHODS." The concentrations of PAO used were 3.45 μM for (A) and 3.63 μM for (B) and (C).

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                         1        10        20        30   ▼    40        50        60        70
     PAO-β             1:AIKVRGLKAGRVSAALVHNGDPASGDTIYEVGAMRFPEIAGLTWHYASAAFGDAAPIKVFPNPGKVPTWFVF-GN
(A)  TR2M AGRRA      247:DVT.YEASDRLGGKLWS.GF-KSAPNV.A.M......--RSESCLFFYLKKHGLDSVGL.....S.D.ALFYR.R
     TR2M AGRT3      262:DVTIYEAGDRVGGKLWS.AF-KDAPGVVA.M......--PAASCLFFFLERYGLSSMRP.....T.D.DL.YE.C
     TR2M AGRT4      262:DVTIYEASDRVGGKLWS.AF-RDAPSVVA.M......--PAAFCLFFFLERYGLSSMRP.....T.D.YL.YQ.V
     TR2M AGRVI      233:DVTIFEA.NVVGGR.HT.FF-KGEPSVCA.L......--RSQACLFYLLEYLGINAMTK.....T.D.GLYYR.R
     TR2M PSESS       64:DVVLYESRDRIGGRVWSQVF.QTRPRY.A.M......--PSA.GLFHYLKKFGISTSTT..D..V.D.ELHYR.K
                                                         * ******                     ***** * *     *
(B)  DAAO FUSSO      271:IANRIMQRIVEVRPEIANGKGVKGLSVIRHAVGM.PWRKDGVRIEEEKLDDETWIVHNYGHSGWGYQGSYGCAEN
     DAAO PIG        249:EINNIQDHNTIWEGCCRLEPTLKDAKIVGEYTGF.PVRPQVRLEREQLRFGSSNTEVIHNYGHGGYGLTIHWGCA
     DAAO HUMAN      249:ELNNIQDHNTIWEGCCRLEPTLKNARIIGEATGF.PVRPQIRLEREQLRTGPSNTEVIHNYGHGGYGLTIHWGCA
     DAAO RABIT      249:EGNSTDDHNTIWKGCCSLEPTLKDARIVGEWTGF.PVRPQIRLGREQLSAGPSKTEVIHNYGHGGYGLTIHWGCA
     DAAO MOUSE      248:GLNSVRDHNTIWKSCCKLEPTLKNARIVGELTGF.PVRPQVRLEREWLRFGSSSAEVIHNYGHGGYGLTIHWGCA
     DASPO BOVIN     244:LSPDAEISKEILSRCCALEPSLRGAYDLRERVGL.PTRPGVRLEKELLAQDSRRLPVVHHYGHSGGIAMHWGTA
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Fig. 10. **Sequence comparison of the conserved regions around the Arg-35 of PAO-β with corresponding regions of several tryptophan 2-monooxygenases (TR2M).** (A) TR2M from five different sources. Abbreviations: AGRRA (*23*), from *Agrobacterium rhizogenes*; AGRT3 (*24*) and AGRT4 (*25, 26*), from *Agrobacterium tumefaciens*; AGRVI (*27*), from *Agrobacterium vitis*; PSESS (*28*), from *Pseudomonas syringae*. (B) DAAO from six different sources is shown. Abbreviations: FUSSO (*29*), from *Fusarium solani*; PIG (*30*), from pig kidney; HUMAN (*31*), from human kidney; RABIT (*32*), from rabbit kidney; MOUSE (*33*), from mouse kidney. DASPO from bovine kidney was denoted as BOVIN (*34*). The arrowhead represents the modified arginyl residue in PAO. Positions of deletions are indicated by -. The residues indicated by dots are homologous with those of PAO-β.

kinetic studies of the inactivation indicated that a single arginyl residue per enzyme unit was involved in the catalytic activity (Figs. 1C, 2C, and 5B). The substrate L-phenylalanine protected PAO from modification by PGO and HPG (Fig. 3), so this arginyl residue is thought to be located in the binding site of the substrate. By labeling PAO with [^{14}C]PGO, we showed that this arginyl residue is located in the β subunit (Fig. 4), and identified it as the 35th residue from the N-terminus of the β subunit (Figs. 6-8).

The sequence shown in Fig. 8 was searched for the homology with sequences in the SWISS-PROT protein sequence database (*21*) using the BLAST program (*22*). Interestingly, the sequence around Arg-35 appears to have two homologous regions with that of tryptophan 2-monooxygenase (Fig. 10A, Glu-Xaa-Gly-Ala-Met-Arg35-Phe-Pro and Phe-Pro-Asn-Pro-Gly-Xaa-Val-Xaa-Thr). The amino acid sequences of tryptophan 2-monooxygenase from several organisms have been reported (*23-28*) and these homologous regions are conserved except for Asp-125 of tryptophan 2-monooxygenase from *Pseudomonas syringae* (TR2M PSESS) (*28*). As for tryptophan 2-monooxygenase, the presence of a reactive thiol in the active site (*7*) and the involvement of a histidyl residue as a carbanion (*9*) have been demonstrated, but the two conserved sequences do not include a cysteinyl or histidyl residue. As PAO is thought to be included in the same subgroup as tryptophan monooxygenase (*7*), it is suggested that the region containing the conserved sequences may have some role in recognition and/or binding of the substrate.

PAO should have similar characteristics to the enzymes that catalyze the oxidative deamination of amino acids. Among these enzymes, DAAO and DASPO are reported each to have one arginyl residue located at the active site (*11, 12*). Though DAAO has the opposite stereospecificity to PAO, resonance Raman spectra of purple intermediates of PAO suggested that a reaction intermediate of PAO resembles that of DAAO (*15*), so there may be some common structural feature at the active site. Therefore the N-terminal sequence of PAO, which contains Arg-35, was aligned with the sequences of these enzymes with respect to their essential arginyl residues (*29-34*), but the sequences around this residue are different from that of PAO (Fig. 10B). Moreover, PAO catalyzes the oxidative deamination of L-phenylalanine to a lesser extent (20%) (*3*), so it is conceivable that Arg-35 in PAO has a role different from that of the essential arginyl residue in DAAO. Further structural and mechanistic studies of this enzyme are required to characterize the active site of PAO.

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REFERENCES

1. Koyama, H. (1982) Purification and characterization of a novel L-phenylalanine oxidase (deaminating and decarboxylating) from *Pseudomonas* sp. P-501. *J. Biochem.* **92**, 1235-1240
2. Koyama, H. (1983) Further characterization of a novel L-phenylalanine oxidase (deaminating and decarboxylating) from *Pseudomonas* sp. P-501. *J. Biochem.* **93**, 1313-1319
3. Koyama, H. (1984) Oxidation and oxygenation of L-amino acids catalyzed by a L-phenylalanine oxidase (deaminating and decarboxylating) from *Pseudomonas* sp. P-501. *J. Biochem.* **96**, 421-427
4. Mukouyama, E.B., Suzuki, H., and Koyama, H. (1994) New subunit in L-phenylalanine oxidase from *Pseudomonas* sp. P-501 and the primary structure. *Arch. Biochem. Biophys.* **308**, 400-406
5. Wierenga, R.K., De Maeyer, M.C.H., and Hol, W.G.J. (1985) Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. *Biochemistry* **24**, 1346-1357
6. Hutcheson, S.W. and Kosuge, T. (1985) Regulation of 3-indoleacetic acid production in *Pseudomonas syringae pv. savastanoi.* Purification and properties of tryptophan 2-monooxygenase. *J. Biol. Chem.* **260**, 6281-6287
7. Emanuele, J.J., Heasley, C.J., and Fitzpatrick, P.F. (1995) Purification and characterization of the flavoprotein tryptophan 2-monooxygenase expressed at high levels in *Escherichia coli. Arch. Biochem. Biophys.* **316**, 241-248
8. Emanuele, J.J. and Fitzpatrick, P.F. (1995) Mechanistic studies of the flavoprotein tryptophan 2-monooxygenase. 1. Kinetic mechanism. *Biochemistry* **34**, 3710-3715
9. Emanuele, J.J. and Fitzpatrick, P.F. (1995) Mechanistic studies of the flavoprotein tryptophan 2-monooxygenase. 2. pH and kinetic isotope effects. *Biochemistry* **34**, 3716-3723
10. Curti, B., Ronchi, S., and Simonetta, M.P. (1991) D- and L-amino acid oxidases in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed.) Vol. III, pp. 69-94, CRC Press, Boca Raton, Ann Arbor, London
11. Gadda, G., Negri, A., and Pilone, M.S. (1994) Reaction of phenylglyoxal with arginine groups in D-amino-acid oxidase from *Rhodotorula gracilis. J. Biol. Chem.* **269**, 17809-17814
12. Tedeschi, G., Negri, A., Ceciliani, F., Biondi, P.A., Secchi, C., and Ronchi, S. (1992) Chemical modification of functional arginyl residues in beef kidney D-aspartate oxidase. *Eur. J. Biochem.* **205**, 127-132
13. Mattevi, A., Vanoni, M.A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M., and Curti, B. (1996) Crystal structure of D-amino acid oxidase: A case of active site mirror-image convergent evolution with flavocytochrome b_2. *Proc. Natl. Acad. Sci. USA* **93**, 7496-7501
14. Mizutani, H., Miyahara, I., Hirotsu, K., Nishina, Y., Shiga, K., Setoyama, C., and Miura, R. (1996) Three-dimensional structure of porcine kidney D-amino acid oxidase at 3.0 Å resolution. *J. Biochem.* **120**, 14-17
15. Suzuki, H., Koyama, H., Nishina, Y., Sato, K., and Shiga, K. (1991) A resonance Raman study on a reaction intermediate of *Pseudomonas* L-phenylalanine oxidase (deaminating and decarboxylating). *J. Biochem.* **110**, 169-172
16. Takahashi, K. (1968) The reaction of phenylglyoxal with arginine residues in proteins. *J. Biol. Chem.* **243**, 6171-6179
17. Yamasaki, R.B., Vega, A., and Feeney, R.E. (1980) Modification of available arginine residues in proteins by *p*-hydroxyphenylglyoxal. *Anal. Biochem.* **109**, 32-40
18. Koyama, H. and Suzuki, H. (1986) Spectral and kinetic studies on *Pseudomonas* L-phenylalanine oxidase (deaminating and decarboxylating). *J. Biochem.* **100**, 859-866
19. Yang, J.T., Wu, C.-S.C., and Martinez, H.M. (1986) Calculation of protein conformation from circular dichroism in *Methods in Enzymology* (Hirs, C.H.W., ed.) Vol. 130, pp. 208-269, Academic Press, New York
20. Levy, H.M., Leber, P.D., and Ryan, E.M. (1963) Inactivation of myosin by 2,4-dinitrophenol and protection by adenosine triphosphate and other phosphate compounds. *J. Biol. Chem.* **238**, 3654-3659
21. Bairoch, A. and Apweiler, R. (1996) The SWISS-PROT protein sequence data bank and its new supplement TREMBL. *Nucleic Acids Res.* **24**, 21-25
22. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410
23. Camilleri, C. and Jouanin, L. (1991) The TR-DNA region carrying the auxin synthesis genes of the *Agrobacterium rhizogenes* agropine-type plasmid pRiA4: nucleotide sequence analysis and introduction into tobacco plants. *Mol. Plant Microbe. Interact.* **4**, 155-162
24. Bonnard, G., Vincent, F., and Otten, L. (1991) Sequence of *Agrobacterium tumefaciens* biotype III auxin genes. *Plant. Mol. Biol.* **16**, 733-738
25. Gielen, J., De Beuckeller, M., Seurinck, J., Deboeck, F., De Greve, H., Lemmers, M., Van Montagu, M., and Schell, J. (1984) The complete nucleotide sequence of the TL-DNA of the *Agrobacterium tumefaciens* plasmid pTiAch5. *EMBO J.* **3**, 835-846
26. Klee, H., Montoya, A., Horodyski, F., Lichtenstein, C., Garfinkel, D., Fuller, S., Flores, C., Peschon, J., Nester, E., and Gordon, M. (1984) Nucleotide sequence of the tms genes of the pTiA6NC octopine Ti plasmid: two gene products involved in plant tumorigenesis. *Proc. Natl. Acad. Sci. USA* **81**, 1728-1732
27. Canaday, J., Gerard, J.-C., Crouzet, P., and Otten, L. (1992) Organization and functional analysis of three T-DNAs from the vitopine Ti plasmid pTiS4. *Mol. Gen. Genet.* **235**, 292-303
28. Yamada, T., Palm, C.J., Brooks, B., and Kosuge, T. (1985) Nucleotide sequences of the *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. *Proc. Natl. Acad. Sci. USA* **82**, 6522-6526
29. Isogai, T., Ono, H., Ishitani, Y., Kojo, H., Ueda, Y., and Kohsaka, M. (1990) Structure and expression of cDNA for D-amino acid oxidase active against cephalosporin C from *Fusarium solani. J. Biochem.* **108**, 1063-1069
30. Ronchi, S., Minchiotti, L., Galliano, M., Curti, B., Swenson, R.P., Williams, C.H. Jr., and Massey, V. (1982) The primary structure of D-amino acid oxidase from pig kidney. II. Isolation and sequence of overlap peptides and the complete sequence. *J. Biol. Chem.* **257**, 8824-8834
31. Momoi, K., Fukui, K., Watanabe, F., and Miyake, Y. (1988) Molecular cloning and sequence analysis of cDNA encoding human kidney D-amino acid oxidase. *FEBS Lett.* **238**, 180-184
32. Momoi, K., Fukui, K., Tada, M., and Miyake, Y. (1990) Gene expression of D-amino acid oxidase in rabbit kidney. *J. Biochem.* **108**, 406-413
33. Tada, M., Fukui, K., Momoi, K., and Miyake, Y. (1990) Cloning and expression of a cDNA encoding mouse kidney D-amino acid oxidase. *Gene* **90**, 293-297
34. Negri, A., Ceciliani, F., Tedeschi, G., Simonic, T., and Ronchi, S. (1992) The primary structure of the flavoprotein D-aspartate oxidase from beef kidney. *J. Biol. Chem.* **267**, 11865-11871