Structural Basis of Proteolytic Activation of L-Phenylalanine Oxidase from Pseudomonas sp. P-501*

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The mature form of L-phenylalanine oxidase (PAOpt) from Pseudomonas sp. P-501 was generated and activated by the proteolytic cleavage of a noncatalytic proenzyme (proPAO). The crystal structures of proPAO, PAOpt, and the PAOpt-o-amino benzoate (AB) complex were determined at 1.7, 1.25, and 1.35 Å resolutions, respectively. The structure of proPAO suggests that the prosedent peptide of proPAO occupies the funnel (pathway) of the substrate amino acid from the outside of the protein to the interior flavin ring, whereas the funnel is closed with the hydrophobic residues at its vestibule in both PAOpt and the PAOpt-AB complex. All three structures have an oxygen channel that is open to the surface of the protein from the flavin ring. These results suggest that structural changes were induced by proteolysis; that is, the proteolysis of proPAO removes the prosedent and closes the funnel to keep the active site hydrophobic but keeps the oxygen channel open. The possibility that an interaction of the hydrophobic side chain of substrate with the residues of the vestibule region may open the funnel as a putative amino acid channel is discussed.

L-Phenylalanine oxidase (EC 1.13.12.9: PAO) from Pseudomonas sp. P-501 catalyzes both the oxidative deamination and oxygenative decarboxylation of L-Phe, L-Tyr, L-Trp, and L-Met as a substrate (1–3). The enzyme was screened from bacteria in soil for the clinical determination of L-Phe (4, 5). The enzyme does not show any catalytic activity. Protease (Pronase-trypsin mixture) treatment of proPAO initially removes only the Ile107-Lys108 dipeptide from proPAO, but the removal of this peptide does not lead to the expression of enzyme activity. Further treatment with the protease removes the prosedent from proPAO, and the resultant protein shows activity. The Pronase-trypsin-treated proPAO (PAOpt)3 showed spectral and kinetic properties comparable with the native enzyme.

Several secreted enzymes are expressed as a proform. The prosedent usually acts as an intramolecular chaperone and/or an inhibitor of catalytic activity. The best studied examples are proteases, and these have been reviewed (10–12). Proteolytic enzymes are synthesized as inactive precursors or zymogens to prevent unwanted protein degradation. Zymogen conversion to the active enzyme typically involves limited proteolysis and removal of the prosedent. X-ray crystallographic studies of zymogens and comparisons with their counterparts have identified the structural changes that accompany this conversion. As for flavoproteins, several enzymes are expressed as proforms, such as succinate dehydrogenase from Saccharomyces cerevisiae (13), pea ferredoxin-NADP reductase (14), glucose-fructose oxidoreductase from Zymomonas mobilis (15), and cellulose dehydrogenase from Phanerochaete chrysosporium (16). The size of the prosedent of these enzymes is 18 residues long to 5-kDa molecular mass. Of these enzymes, the crystal structure of the prosedent of glucose-fructose oxidoreductase is known (15). Pre-glucose-fructose oxidoreductase consists of a tetramer, and the monomer has a prosedent...

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3 The abbreviations used are: PAO, L-phenylalanine oxidase; PAOpt, PAO activated by Pronase and trypsin; AB, o-aminobenzoate; proPAO, noncatalytic proenzyme of PAO; LAO, L-amino acide oxidase.
with 52 residues (17). N-terminal residues 1–83 extend from the monomer, although only residues 53–83, which represent the start of the mature enzyme, form an arm that wraps around a neighboring subunit of the tetramer (15). proPAO does not show enzyme activity (9), but pre-glucose-fructose oxidoreductase is fully active (18). On the other hand, pre-ferredoxin-NADPH reductase has a 5-kDa presequence at the N terminus and shows 1–5% activity of the mature form (14). Pre-ferredoxin-NADPH reductase gains its full activity by proteolysis, but the change in the three-dimensional structure by proteolysis is not known.

This work reports the crystal structures of proPAO, PAOpt, and the PAOpt-o-aminobenzoate (AB) complex at 1.7, 1.25, and 1.35 Å resolutions, respectively. Our results show that the 14-residue-long presequence occupies a possible channel of the substrate amino acid to the active site flavin and that the pre-sequence is removed from the channel by proteolysis. We discuss the unique mechanism of the proteolytic activation of proPAO and the possible channels of amino acid and molecular oxygen.

**EXPERIMENTAL PROCEDURES**

**Purification**—proPAO was expressed in *E. coli* as described previously (9). The cells were disrupted by sonication at 277 K. The supernatant was applied to HisTrap HP (GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0) containing 500 mM NaCl. The protein was eluted with a linear gradient of imidazole from 0 to 0.5 M. The eluted fractions were collected, and ammonium sulfate was added to the supernatant until 50% saturation. After keeping the solution for 16 h at 277 K, the precipitates were collected by centrifugation at 30,000 × g for 30 min and dissolved in 20 mM Tris-HCl (pH 8.0) containing 300 mM ammonium sulfate. The enzyme proteins in the solution were purified by several chromatographic procedures using HiTrap Phenyl FF (GE Healthcare), Resource Q (GE Healthcare), and finally HiLoad 16/60 Superdex 200 prep grade (GE Healthcare). The purity of the enzyme was confirmed by SDS-PAGE. PAOpt was obtained by the Pronase (Sigma-Aldrich) and trypsin treatment of proPAO. Immediately after treatment of proPAO, the activated enzyme was purified by HiTrap Q HP (GE Healthcare) and then by gel filtration as described for the purification of proPAO. Mass analysis of PAOpt was performed by the Peptide Institute using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and two peaks with the masses of 9243.3 and 65533.9 were observed. These data and our previous work (9) suggest that the amino acid sequences of α and β subunits are residues 16–106 and 109–711, respectively, of the proPAO polypeptide. In this work, the residue number of PAO is expressed as that of the proPAO polypeptide.

**Site-directed Mutagenesis**—Site-directed mutagenesis of desired residues (Met$^{142}$ and Lys$^{478}$) in proPAO was carried out by using a QuikChange XL site-directed mutagenesis kit, following the manufacturer’s recommendations (Stratagene). The desired mutation was introduced on the wild-type proPAO gene in the plasmid, pPAO$_{+15}$ (9). The mutagenic change was confirmed by sequencing the mutant plasmid gene. Sequencing was performed by the Genomic Research Department of Shizamizu-Biotech. The proPAO mutants were expressed in *E. coli* BL21 (DE3) and purified. The PAOpt mutants M142A and K478A were activated from the respective proPAO mutants and purified as described for the wild-type PAOpt.

**Assay of Enzyme Activity**—The rate of the overall reaction was estimated by measuring the consumption of oxygen dissolved in the reaction mixture with a Clark-type oxygen electrode (Strathkelvin Instruments). The reaction mixture contained various concentrations of substrate in 20 mM Tris-HCl (pH 8.0) at 25 °C. The rate ($v$) was expressed as the moles of oxygen consumed per second at a given concentration of enzyme. The enzyme concentration was determined by measuring the amount of enzyme-bound flavins, assuming that the extinction coefficient of flavin is 11.3 mM$^{-1}$ cm$^{-1}$ at 450 nm. The apparent kinetic parameters $k_{cat}$, $K_m$, and $K_i$ were calculated from the rates determined at various concentrations of L-Phe. The PAOpt used in this work showed an apparent $k_{cat}$ and $K_m$ for L-Phe to be 115.3 ± 2.5 s$^{-1}$ and 15.9 ± 0.7 μM, respectively. These kinetic constants are comparable with those of the native enzyme (1–3, 9).

**Crystallization**—The purified protein in 10 mM Tris-HCl (pH 8.0) was concentrated to 10 mg ml$^{-1}$ in an Amicon Ultra (Millipore) at 277 K. Crystallization was performed by a vapor diffusion method. The protein solutions were mixed with an equal volume of a reservoir solution consisting of 0.1 M HEPES (pH 7.5) and 1.7 M ammonium sulfate for proPAO and 0.1 M HEPES (pH 7.5) and 1.0 M ammonium sulfate for PAOpt at 293 K. The shapes of crystals are like gold ingots. The size of PAOpt crystal used was 1 mm$^3$. The crystal had a different space group from the proPAO crystal (proPAO: P2$_1$2$_1$2$_1$), PAOpt: P2$_1$2$_1$2$_1$), and the diffraction spots extended beyond 1 Å. The crystals were soaked in crystallization buffer containing 30% glycerol as a cryoprotectant prior to the x-ray experiment, and the PAOpt-AB complex was prepared by a soaking method with the cryoprotectant. The concentration of AB and soaking time are 1 mM and 1 min, respectively.

**Data Collection, Structure Solution, and Refinement**—Diffraction data sets were collected on NW12 in PF-AR and BL45XU at SPring-8. The quality of the diffraction data statistics are given in Table 1. The crystal structure of proPAO was solved using a selenomethionine-derivative crystal and the single-wavelength anomalous dispersion method. The structure could not be solved by the multiple-wavelength anomalous dispersion method, because the crystal was severely damaged by the radiation and could not maintain its isomorphism between the data sets. The experimental phase was calculated to a 2.1 Å resolution with SOLVE (19), and density modification was carried out with Resolve (20). Initial model building was carried out automatically by the program ARP/wARP (21), and additional model building was manually performed with O (22). A refinement of the model was carried out using CNS (23). The model at this stage was then used to estimate the phases of the native data set by the molecular replacement method using CNS, followed by phase extension to 1.7 Å resolution. Water molecules were assigned automatically by the program ARP/wARP. The initial phase of PAOpt was determined by the molecular replacement method using the atomic coordinates of the crystal structure of proPAO with MOLREP (24). The refine-
Proteolytic Activation of \( \text{L-Phenylalanine Oxidase} \)

**TABLE 1**
<table>
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<th>Data collections and refinement statistics</th>
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<tr>
<td>Data collection</td>
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<tr>
<td>( R_{merge} ) (%)</td>
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<td>( 1/\sigma )</td>
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**Reefinement**

\( R_{work} \) (%) | 16.6 | 10.1 | 10.2 |
\( R_{merge} \) (%) | 19.3 | 12.9 | 13.8 |
Root mean square deviations
Bond lengths (Å) | 0.017 | 0.028 | 0.031 |
Bond angles (°) | 1.600 | 2.335 | 2.470 |

* The values in parentheses correspond to the reflections observed in the highest resolution shell.
** \( R_{merge} \) = \( \sum_{i} \sum_{\text{hkl}} I_i - \langle I_{\text{hkl}} \rangle \) / \( \sum_{i} \sum_{\text{hkl}} \langle I_{\text{hkl}} \rangle \), where \( I_{\text{hkl}} \) is the observed intensity, and \( \langle I_{\text{hkl}} \rangle \) is the averaged intensity for multiple measurements.

**RESULTS AND DISCUSSION**

**Overall Structure of proPAO**—The structure of proPAO, which was determined at 1.7 Å resolution, demonstrated that proPAO exists as a homodimer (supplemental Fig. S1). The amino acid sequence of proPAO consists of an N-terminal 14 residues of prosequence, an \( \alpha \) subunit, a dipeptide, and a \( \beta \) subunit (Fig. 1). Each monomer is related by a noncrystallographic 2-fold axis. The overall dimension of the dimer is \( \sim 110 \times 80 \times 65 \) Å. Approximately 15% of the accessible surface area of the monomer is buried in the dimer interface consisting of 46 residues/monomer. Each monomer contains 18 \( \alpha \)-helices and 19 \( \beta \)-strands, and they are folded into one region and two domains, namely, the prosequence (residues 1–14), the FAD-binding domain (residues 29–120, 148–162, 346–472, and 621–C-terminal), and the substrate-binding domain (residues 15–28, 121–147, 163–345, and 473–620), respectively (Fig. 2A). Following a structural similarity search on the three-dimensional structure of PAOpt at 1.25 Å resolution by molecular replacement (Fig. 2B). The missing residues in the structure are 105, 522–525, and the 708 C-terminal of each subunit. Both PAOpt and proPAO exist as a homodimer (supplemental Fig. S2). The overall structures of proPAO and PAOpt by SSM (32) as described above. Polyamine oxidase showed the highest score (28; Z score, 4.2: proPAO, and 3.4: PAOpt) and had a “substrate tunnel” similar to that of proPAO and of PAOpt. The substrate spermic acid occupies the tunnel of polyamine oxidase just like the protein in the PAOpt structure. The following regions of proPAO could not be modeled because of poor electron density: residues 105–108, 520–526, 547–561, 612–614, and the 704 C-terminal of chain A, and residues 129–132, 519–526, 548–563, 612–614, and the 705 C-terminal of chain B. These missing regions are located around the dimer interface and are likely in a random conformation. The structures of the missing area differ slightly between monomers. These differences are possibly caused by crystal packing.

**Structural Changes of proPAO to PAOpt**—To understand the proteolytic activation of proPAO, we also determined the three-dimensional structure of PAOpt at 1.25 Å resolution by molecular replacement (Fig. 2B). The missing residues in the structure are 105, 522–525, and the 708 C-terminal of each chain. By proteolysis of proPAO, prosequence (Gly15–Glu109), Lys15, linker dipeptide (Ile107 and Lys108), and several C-terminal amino acid residues are removed (Fig. 1 and see “Experimental Procedures”).

The superposition of the Ca atoms of PAOpt and proPAO results in a root mean square deviation of 0.84 Å by using Chimera (33) (supplemental Fig. S2). The overall structures of proPAO and PAOpt are very similar, although there are two differences: one in the prosequence region in proPAO, and the other at Ala109 of the N terminus of the \( \beta \) subunit. Moreover, two loops, residues 547–561 and 612–614 in the substrate-binding domain, showed an electron density in PAOpt, but not in proPAO, suggesting that the conformation of the protein in...
these regions changes greatly by the proteolysis of proPAO to PAOpt.

In the proPAO structure, residues Gly1 to Ile6 of the prosequence peptide occupy a funnel from the outside of the protein to the interior flavin ring, making hydrophobic interactions with the residues Gly172, Lys173, Val214, Phe311, Gly312, Thr313, Leu319, Leu330, Trp538, and Trp660 (Fig. 3A). The long side chain of Arg7 might be an anchor of the prosequence. The aperture of the funnel is narrower than the length of the side chain of Arg7. Several water molecules are found around the prosequence. Thr3 is located near the internal portion of the cavity (Fig. 3B), which was identified with the program VOIDOO (34). The internal portion contains Thr3, Val4, and 10 water molecules in proPAO, whereas the cavity is lost and filled by Tyr335 in PAOpt (Fig. 3, A, C, and D). The N-terminal Gly residue of proPAO interacts with N-5 and C-4a atoms of the re-face of the flavin ring (Fig. 4A). The distances from the Gly1 amino nitrogen to the N-5 and C-4a atoms are 3.49 and 3.48 Å, respectively.

The flavin ring has a planar conformation in proPAO, whereas it has a slightly bent and twisted (sloped down to the C8M atom) conformation (−15°) in PAOpt (Fig. 4). This conformational change also induces Trp608 to cover the flavin ring and prevents the region from contact with solvent water (Fig. 4B). Two interactions induce the conformational change. One is the interaction between Asp602 upstream of Trp608 and Ala109 of the N terminus of the β subunit (supplemental Fig. S2). The other is the interaction between the Ne1 atom of Trp608 and the O atom of Val139 at the si-face of the flavin ring (Fig. 4B). At the re-face of the flavin ring, Phe417 and Trp660 form an “aromatic cage” (Fig. 4) similar to that observed in monoamine oxidase (Tyr398 and Tyr435) (35) and are separated by 7.7 Å (in monoamine oxidase 7.8 Å). This suggests that by proteolysis, the flavin ring

FIGURE 2. Overall structure of PAO. A view of proPAO (A) and PAOpt monomers (B). The prosequence, FAD-binding domain, and substrate-binding domain are colored magenta, blue, and orange (proPAO) or cyan (PAOpt), respectively. A and B are shown in the same orientation.

FIGURE 3. Cross-section views of funnels in proPAO (A and B) and PAOpt (C and D). The carbon atom in the prosequence is colored magenta. The molecular surface view of funnel spaces in proPAO (A) and PAOpt (C). The cavities are shown in green mesh (B and D). A and B are shown in the same orientation as C and D, respectively. SO4 and GOL represent sulfate ion and glycerol, respectively, and are shown by a stick model.
The bent flavin ring allows its N-5 atom to receive a hydrogen atom from the substrate easily, and the C8M of the ring bends in the direction of Trp608.

Structure of PAOpt-AB Complex and Amino Acid Channel—
The overall structures of proPAO and PAOpt are similar to each other (supplemental Fig. S2), although there is a difference at the prosequence region in proPAO. The flavin ring is buried deep inside the protein, and the funnel occupied by the prosequence of proPAO is probably a channel for the amino acid substrate, because the N-terminal Gly residue in proPAO interacted with the \( \text{re} \)-face of the flavin ring and is packed by the aromatic cage (Fig. 4A). We expected that the funnel in PAOpt interacts with the \( \text{re} \)-face of the flavin ring in a similar way that Gly\(^1\) of the prosequence interacts with the flavin ring (Figs. 4A and 5A). These observations allowed us to propose that the funnel is the channel for substrate amino acids. This proposal is supported by the observation that PAOpt contained a glycerol molecule and a sulfate ion in the funnel space (Fig. 4B and supplemental Fig. S3), because the sulfate ion and glycerol must enter from the outside of the protein molecule during the crystallization and soaking in cryoprotectant solution. The chemical modification study also supports the proposal (36). That is, phenylglyoxal specifically modifies Arg\(^{143}\), and the modification is almost completely prevented by L-Phe. Moreover, our proposal is supported by a previous report on the structure of the AB complex with L-amino acid oxidase (LAO) (37). In the LAO-AB complex, three AB molecules are positioned in the funnel, although the PAOpt-AB complex contains only one molecule of AB in the funnel. In both the PAOpt-AB and LAO-AB complexes, AB is positioned at the \( \text{re} \)-face of the flavin ring (Fig. 5B); that is, the ring of AB is in the vertical plane of the flavin ring and the orientation of the AB ring is supported by the aromatic cage. Moreover, the superimposition of two PAO models (proPAO and PAOpt) shows that the prosequence occupies the funnel (Fig. 3), and the superimposition of proPAO and the LAO-AB complex shows that the prosequence overlaps with the active site funnel of LAO (supplemental Fig. S4).

The above discussion allows us to conclude that the substrate amino acid entry channel is the funnel that the prosequence occupies in proPAO. However, an unknown still remains: how
is the substrate amino acid able to reach the active site flavin from the outside of the protein, because the channel is closed with the hydrophobic residues in the structure of PAOpt and its AB complex? Even though we do not have any conclusive evidence, we propose the following mechanism to explain the opening of the channel.

The vestibule of the funnel is mainly composed of hydrophobic residues (supplemental Fig. S3). Then the hydrophobic side chain of the substrate amino acid interacts with these hydrophobic residues and changes them from a closed to an open conformation. This opening mechanism explains the highly specific nature of the enzyme to hydrophobic amino acids (1).

In the case of L-Phe as the substrate, the benzene ring of L-Phe interacts with the hydrophobic region of the vestibule. Then L-Phe is led into the funnel placing the benzene ring of L-Phe at the front, allowing it to accommodate L-Phe and to be subsequently dehydrogenated from the Cα atom to the flavin ring. L-Phe, which enters into the funnel, moves the benzene ring into the pocket formed by Leu319, Ile322, and Leu619 and turns back to the active site similarly to a “switch back” and arrives at a proper position to be dehydrogenated. It is conceivable that Arg143, Tyr536, and the aromatic cage determine the position of the L-Phe that interacts with the flavin ring (supplemental Movie S1).

Oxygen Channel—Previous studies on PAO suggest that a purple intermediate (reduced FAD-imino acid complex) (7, 8) is formed during the reduction of PAO with the substrate amino acid. The complex reacts with an oxygen molecule to regenerate the oxidized form of the enzyme. PAO mainly catalyzes the oxygenation of L-Phe (1–3), and a peroxy flavin is formed during the reduction of PAO with the substrate amino acid. The complex reacts with an oxygen molecule to regenerate the oxidized form of the enzyme. As for PAOpt, the channel is closed in the PAOpt-AB complex. Even if the oxygen molecule is able to pass through the closed channel, it is not able to reach the flavin ring, because the re-face of the flavin ring is occupied by the imino acid intermediate as shown for the PAOpt-AB complex (Fig. 5A). Therefore, we searched for another channel for the oxygen molecule.

Using the program CAVER (42), many channels were found in the structures of proPAO and PAOpt, the widest of which is shown in Fig. 6). The channel is in a similar region in both proPAO and PAOpt structures. As for PAOpt, the channel is from the flavin ring through the gate residues, namely Met142 and Lys478 (supplemental Fig. S5), to the outside of the protein molecule. From these results, we propose this channel to be an oxygen channel (Fig. 6).

The channel gate of PAOpt was wringed more than that of proPAO; their radii were 0.70 and 1.09 Å, respectively. Our proposal of the oxygen channel is supported by the structure of the PAOpt-AB complex (Fig. 5A). This structure, the electron density of oxygen atoms in Fig. 5A may suggest the presence of one oxygen molecule, but the presence of one oxygen molecule is also possible. We propose that the electron density is derived from one oxygen molecule. If this is the case, the oxygen molecule stays at the end of the oxygen channel proposed above (supplemental Fig. S5). To see whether the residues Met142 and Lys478 are present at the oxygen-binding site, we performed site-directed mutagenesis experiments of these residues as described under “Experimental Procedures.” The PAOpt mutants, M142A and K478A, were prepared, and the activity was assayed. As for the M142 mutant, an apparent $k_{cat}$ was found to be $1/10$ of the wild-type enzyme, and an apparent $K_{m}^{O2}$ value was elevated (3.4 mM for M142A mutant compared with 1.8 mM for the wild-type enzyme in the presence of 0.5 mM L-Phe), suggesting that the mutation of Met142 to Ala decreases the affinity of oxygen with the enzyme. As for the K478A mutant, the yield of the purified enzyme was very low, and the catalytic activity was $1/200$ of the wild-type enzyme. These findings support the idea that oxygen binds to these residues. LAO has been reported to have an oxygen entry channel (43) that exists at the re-face of the flavin ring and is used as the amino acid channel near the flavin ring. This report is also consistent with our proposal of the oxygen channel. We further wish to comment on the candidate of the oxygen molecule. As an oxygen molecule, the bond length between two oxygen atoms is rather long (2.12 Å) (Fig. 5A). Although we do not have any evidence, this structure may suggest an intermediate state of an oxygen molecule. It is also possible. We propose that the electron density is derived from one oxygen molecule. If this is the case, the oxygen molecule stays at the end of the oxygen channel proposed above (supplemental Fig. S5).

**FIGURE 6. Possible channel for molecular oxygen.** The channels are displayed as a blue mesh calculated by CAVER with PyMol in proPAO (A) and PAOpt (B). The red dot displays the prosequence. The side chains around the active site are shown by a stick model.
positively charged Arg$^{337}$ side chain is proposed to stabilize C-4a-peroxyflavin. Furthermore, in p-hydroxyphenylacetate hydroxylase (41), the hydrophobic cage of histidine is also proposed to stabilize C-4a-peroxyflavin. However, structural information of the reaction intermediate with the substrate amino acids is clearly required before these links can be discussed further.

**Mechanism of Proteolytic Activation of PAO**—There are two notable points in the proteolytic activation of PAO. One is the environmental changes of the active site, that is, the reshaping of the oxygen channel and the conversion of the hydrophobic environment around the flavin ring, which is caused by cleaving the linkage between the $\alpha$ and $\beta$ subunits. In the overall structure, when the FAD-binding domain is fixed, the substrate-binding domain moves by proteolysis (Fig. 6 and supplemental Movie S2). The other is the formation of an amino acid channel, which is caused by the removal of the prosequence. The schematic representation of the activation of proPAO is shown in Fig. 7. That is, Pronase-trypsin treatment of proPAO initially cleaved the linkage caused by the removal of the prosequence. The schematic representation of the reaction intermediate with the substrate amino acids is clearly required before these links can be discussed further.

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