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Crystal Structure of *Pseudomonas aeruginosa* Lipase in the Open Conformation

THE PROTOTYPE FOR FAMILY I.1 OF BACTERIAL LIPASES*

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The x-ray structure of the lipase from *Pseudomonas aeruginosa* PAO1 has been determined at 2.54 Å resolution. It is the first structure of a member of homology family I.1 of bacterial lipases. The structure shows a variant of the α/β hydrolase fold, with Ser82, Asp229, and His251 as the catalytic triad residues. Compared with the "canonical" α/β hydrolase fold, the first two β-strands and one α-helix (αE) are not present. The absence of helix αE allows the formation of a stabilizing intramolecular disulfide bridge. The loop containing His251 is stabilized by an octahedrally coordinated calcium ion. On top of the active site a lid subdomain is in an open conformation, making the catalytic cleft accessible from the solvent region. A triacylglycerol analogue is covalently bound to Ser82 in the active site, demonstrating the solvent region. A triacylglycerol analogue is covalently bound to Ser82 in the active site, demonstrating the presence of the oxyanion hole and of the three pockets that accommodate the sn-1, sn-2, and sn-3 fatty acid chains. The inhibited enzyme can be thought to mimic the structure of the tetrahedral intermediate that occurs during the acylation step of the reaction. Analysis of the binding mode of the inhibitor suggests that the size of the acyl pocket and the size and interactions of the sn-2 binding pocket are the predominant determinants of the regio- and enantio-preference of the enzyme.

Lipases are triacylglycerol ester hydrolases (EC 3.1.1.3) that catalyze the hydrolysis of long chain acylglycerols. Since the beginning of the 1990s, the three-dimensional structures of an impressive number of lipases from mammalian, yeast, and microbial origin have been determined (1–3). In particular, structures of fungal and bacterial lipases have attracted interest because these enzymes are applied on a large scale in detergents and for stereoselective biotransformations. Their widespread biotechnological application is related to their cofactor independence, their broad substrate specificity, their high enantio-selectivity, and their stability in organic solvents (4).

Bacterial lipases have recently been classified into eight different families, with family I being the largest and consisting of six subfamilies (5). Families I.1 and I.2 contain lipases from the genus *Pseudomonas*. These lipases usually show pronounced differences in regio- and enantio-selectivity despite a high degree of amino acid sequence homology (more than 40%) (6, 7). They are widely used in industry, especially for the production of chiral chemicals that serve as basic building blocks in the synthesis of pharmaceuticals, pesticides, and insecticides (8).

The prototype enzyme of family I.1 is the 29-kDa extracellular lipase from *Pseudomonas aeruginosa*, which was the first *Pseudomonas* lipase to be purified to electrophoretic homogeneity (9). This enzyme has since been studied in great detail mainly because of the two following reasons. (i) It plays an important role as a virulence factor of *P. aeruginosa*, which is an opportunistic pathogen, causing a variety of infectious diseases in immuno-compromised patients. This lipase can act synergistically with other lipolytic enzymes, leading to damage of host cellular membrane lipids including lung surfactants (10). (ii) *P. aeruginosa* lipase is widely used for biotechnological applications because it is a versatile enzyme that can catalyze the hydrolysis and synthesis of a wide variety of industrially important substrates with broad specificity (4, 11–19).

Among *Pseudomonas* lipases only those belonging to family I.2 are known in atomic detail (5). These include the lipases from *Chromobacterium viscosum*, *Pseudomonas glumae*, and *Pseudomonas cepacia* (the latter two renamed *Burkholderia glumae* and *Burkholderia cepacia*) (14–17). The *C. viscosum* and *B. glumae* lipases are 100% identical in sequence, and their x-ray structures show the enzymes in a closed conformation, with an α-helical lid covering the active site and protecting it from the solvent phase (14, 15). In contrast, the *B. cepacia* lipase was crystallized in an open conformation, both in the absence and in the presence of a bound substrate-like inhibitor (16–19).

Here we report the x-ray structure of *P. aeruginosa* PAO1 lipase (PAL)1 at 2.54 Å resolution, in the open conformation. The enzyme has been co-crystallized with the substrate ana-

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1 The abbreviations used are: PAL, *P. aeruginosa* lipase; BGL, *B. glumae* lipase; CVL, *C. viscosum* lipase; BCL, *B. cepacia* lipase; R<sub>0</sub>-trioctyl, R<sub>0</sub>-(R<sub>0</sub>,S<sub>0</sub>)-1,2-dioctylethylammonium-glycero-3-O-p-nitrophenyl octylphosphonate.
A 1,2-diocetylcarbamoyl-glycerol-3-O-p-nitrophenyl octylphosphonate, which binds covalently to the enzyme. The structure provides a structural description of the enzyme in its activated state and allows a detailed explanation of the region- and stereoselectivity of the enzyme toward triglyceride substrates. These results can be generalized to other members of the bacterial lipase family I.1.

Experimental Procedures

Crystallization and Data Collection—The lipase from *P. aeruginosa* was cloned, overexpressed, and purified as described previously (20). Crystallization of the inhibited enzyme was achieved by the sitting drop vapor diffusion method at room temperature. The protein solution, containing 5 mg/ml lipase, 10 mM Tris/HCl (pH 8.0), and 1% (v/v) methanol, was filtered through a 0.2-μm filter and equilibrated against a 1L reservoir containing 25% methanol, 2.4 M ammonium sulfate, 0.1 M sodium acetate buffer (pH 5.6), and 20 mM CaCl2 in 100 mM citrate buffer (pH 5.6). Rod-shaped crystals, suitable for X-ray analysis, grew within months. They were highly x-ray-sensitive, and therefore the data collection was performed at cryo temperature (100 K), using the mother liquor as the cryoprotectant.

The crystals diffraction up to 2.5 Å resolution using synchrotron radiation. They belong to space group P212121, with unit cell parameters of a = 45.47 Å, b = 50.96 Å, c = 110.02 Å. This unit cell gives a V M = 24 366 Å3, cell volume which is very close to the structure calculated by the program O (25), and refinement, using the program CNS (26) (rigid body and simulated annealing at the beginning, conventional positional refinement and restrained individual B-factor refinement in the final stages), were necessary to improve the electron density map and to make the loops visible that in the initial maps were not visible at all. The best refinement results were obtained using a flat bulk solvent correction. Water molecules and a calcium ion were placed according to strict density and distance criteria.

**Model Quality and Accuracy**—The final model consists of 285 amino acid residues, one calcium ion, 112 water molecules, and one molecule of 

**RESULTS AND DISCUSSION**

**Overall Structure**—*P. aeruginosa* lipase has a nearly globular shape with approximate dimensions of 35 × 40 × 50 Å3. Its structure consists of a “core” domain (residues 1–108 and 164–285), showing the typical features of the α/β hydrolase fold topology (31–33), and a “cap” domain (residues 109–163), with four α-helices (α4, α5, α6, and α8) that shape the active site cleft (Fig. 1). Helix α5 and its neighboring loops can be considered as a “lid” in the open conformation, making the active site cavity accessible to solvent and substrate (Fig. 1A). Compared with the canonical α/β hydrolase fold (Fig. 1B), the first two β-strands are absent, and therefore, to be consistent with the numbering of the consensus α/β hydrolase fold, the first strand in the PAL structure is named β3. The helix corresponding to the canonical helix α6 is absent too. Furthermore, an additional small antiparallel β-sheet (β-strands b1 and b2) is inserted between strand β3 and helix α1, and an additional β-sheet (β′) lines up with the sixth strand (β6) of the central β-sheet, but in the opposite direction (Fig. 1B).

*P. aeruginosa* lipase is the first member of family I.1 of bacterial lipases of which a crystal structure has been elucidated. Its structure is similar to the family I.2 lipase structures from *B. glumae* (BGL) (15), *B. cepacia* (BCL) (16–19), and *C. viscosum* (CVL) (14), which show 42% amino acid sequence identity to PAL. The structural similarity is mainly localized in the core domain, where the secondary structure elements correspond well (Figs. 2 and 3). Nevertheless, major differences occur in the region following strand β7, where in PAL a deletion of more than 20 residues results in the absence of the antiparallel β-sheet formed by strands b3 and b4 (Fig. 3). As a consequence, helix α8 of PAL superimposes on helix α9 of homology family I.2 lipases. Most importantly, helix α10 in
The catalytic triad residues (Ser82, Asp229, and His251) and the position of the disulfide bridge are indicated, and a comparison with the canonical α/β hydrolase fold is given. α-Helices and β-strands are represented by rectangles and arrows, respectively. G1 and G2 are $3_10$ helices and are represented by squares. Locations where insertions in the canonical fold may occur are indicated by dashed lines.

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the three-dimensional structure has been elucidated (33), the nucloephile adopts unfavorable torsion angles (\(\phi = 57^\circ, \psi = -111^\circ\)) and imposes steric restrictions on the residues located in its proximity. An intricate hydrogen-bonding pattern similar to that found in family I.2 lipases stabilizes the active site residues. The main chain nitrogen atom of Ser\(^{252}\) is hydrogen-bonded to the carboxyl oxygen atom of Val\(^{258}\). The Ser\(^{252}\) side chain O\(_2\) atom, covalently bound to the trioyctyl inhibitor, is 3.0 Å from the catalytic histidine Ne2 atom, but the geometry is not suitable for a hydrogen bond between these two atoms. Instead, the His\(^{251}\) Ne2 makes a 2.8-Å hydrogen bond to the O1 of the phosphonate inhibitor (Fig. 4B). The His\(^{251}\) carboxyl oxygen and amide nitrogen atoms are hydrogen-bonded to the His\(^{251}\) Ne2 atom and Asp\(^{250}\) carboxyl oxygen atom, respectively. Most importantly, the His\(^{251}\) N\(_6_1\) is 3.1 Å from the O62 of the Asp\(^{259}\) side chain that is responsible for stabilizing the positively charged His\(^{251}\) side chain during catalysis. The Asp\(^{259}\) O\(_6_2\) atom is not only at hydrogen-bonding distance from the catalytic histidine but is also very close (2.7 Å) to the Glu\(^{254}\) O\(_6_2\) atom. The Glu\(^{254}\) O\(_6_1\) atom is 3.6 Å away from the His\(^{251}\) N\(_6_1\) atom and, therefore, does not interact directly with the triad histidine. However, it has been suggested for B. glumae lipase (15) that an acid residue at that position may assist the catalytic histidine during hydrolysis, either directly or via a water molecule, if the triad acid (Asp/Glu) is mutated. This could also be the case for the P. aeruginosa lipase.

Ca\(^{2+}\)-Binding Site—Approximately 15 Å from the nucleophile Ser\(^{252}\), at the same side of the enzyme where the catalytic His\(^{251}\) is located, a calcium binding pocket is present. The calcium ion is octahedrally coordinated, and its ligands are the two carboxylate groups of Asp\(^{259}\) and Asp\(^{250}\) (both absolutely conserved in all family I.1 and I.2 lipases), the two carboxyl oxygen atoms of Glu\(^{254}\) and Leu\(^{281}\), and two water molecules (Fig. 5). All the distances to calcium are within ~2.2–2.4 Å, and the low B\(_{factor}\) values of the ion and its ligands (17–30 Å\(^2\)) reflect a relatively rigid conformation of the calcium binding region. Despite the medium resolution (2.54 Å) of the PAL crystal structure, Val\(^{258}\) is unambiguously in a cis-peptide conformation. The cis-peptide bond between Glu\(^{257}\) and Val\(^{258}\) is stabilized by a conserved hydrogen-bonding network that extends to the catalytic His\(^{251}\). The Ca\(^{2+}\) ion bridges helix \(\alpha_8\), which forms part of the wall of the active site cleft, to the loop containing the catalytic histidine and, therefore, contributes to keep His\(^{251}\) at the correct position in the active site. The binding mode of the Ca\(^{2+}\) ion is conserved in BCL, BGL, and CVL, including the presence of a cis-peptide bond corresponding to Val\(^{258}\) in PAL. Furthermore, a structure-based sequence alignment of the bacterial lipases from families I.1 and I.2 shows the conservation of an Asn/Asp residue at the position preceding the catalytic histidine (Fig. 3). This Asn/Asp residue hydrogen bonds one of the two water molecules that coordinate the calcium ion. The other residues in PAL that interact with these water molecules (Thr\(^{205}\), Ser\(^{211}\), and Asp\(^{212}\)) are also highly conserved, with some variation that is not likely to influence the calcium binding. This can be deduced, for instance, from the fact that in the BCL, BGL, and CVL structures an alanine replaces PAL Thr\(^{205}\), with the calcium-coordinating water molecule hydrogen bonded to another solvent molecule located at a position equivalent to the Thr\(^{205}\) O\(_{1}\) atom in PAL.

Active Site Cleft and Inhibitor Binding—The PAL structure provides a rare example of a lipase complexed with a triglyceride-like inhibitor. The only other example is that of the family I.2 lipase from B. cepacia. Structural analysis of the open conformation of PAL and BCL in complex with the same R\(_1\)-trioctyl inhibitor (Fig. 4A) (21) offers the opportunity of a direct comparison of the binding mode of the substrate analogue in lipases from families I.1 and I.2.

In both PAL- and BCL-inhibited structures the R\(_1\)-trioctyl molecule is covalently linked at its phosphorus atom to the nucleophilic O\(_3\) atom of the catalytic serine residue and perfectly fits into similar hydrophobic active site clefts. In PAL this cleft is 15 Å deep and has an ovoid shape with approximate dimensions of 10 × 25 Å. It is bordered by helices 4, 5, 8 and by the loops of residues 16 to 29 and 255 to 258. Its walls are lined mostly with hydrophobic side chains, making it perfectly suited for lipid binding. At the base of the cleft the Met\(^{16}\) side chain divides it in two parts, thus giving a characteristic boomerang-like shape to the active site, with four binding pockets, an oxyanion hole and three pockets for the different branches of the triglycerylcerol substrate (Fig. 4B).

In PAL the Met\(^{16}\) and His\(^{88}\) main chain amide groups are 2.8 Å and 2.9 Å from one of the phosphorous oxygen atoms (O4) (Fig. 4B). A similar interaction is present in BCL involving Leu\(^{17}\) and Glu\(^{89}\) residues. Since the R\(_1\)-trioctyl-complexed structure represents the putative tetrahedral intermediate conformation during the acylation step of the reaction, the Met\(^{16}\) and His\(^{88}\) backbone nitrogen atoms localize the oxyanion binding pocket in a position very well conserved within the \(\alpha/\beta\) hydrolase-fold enzymes (31, 33, 34). Met\(^{16}\) is the second residue of the tetrapeptide motif Gly-Hyd-X-Gly (Hyd = Met, Leu, Val) located between strand \(\beta_3\) and helix \(\alpha_4\) and highly conserved in families I.1 and I.2 of bacterial lipases (Fig. 3). In PAL this
The tetrapeptide motif is stabilized by a hydrogen bond between the His¹⁴ carbonyl oxygen atom and the Arg⁵⁶ NH₂ atom. Arg⁵⁶ is a totally buried residue that lies at the beginning of helix a₂, close to the nucleophile elbow. It connects the oxyanion hole tetrapeptide motif to the loop between strand b₃ and helix a₂ via the hydrogen bond Arg⁵⁶ NH₁-Ser⁴⁸ O. Interestingly, Arg⁵⁶ is totally conserved in homology family I.1 as well as in homology family I.2 lipases (Fig. 3). In the open conformation of BCL the interaction pattern of this Arg (Arg⁶¹) strongly resembles that of PAL Arg⁵⁶, with the Arg⁶¹ side chain hydrogen-bonded to the carbonyl oxygen atom of His¹⁵ and Ser⁵⁰. In contrast, in the closed conformations of CVL and BGL, the Arg⁶¹ side chain is hydrogen-bonded to the carbonyl oxygen atoms of Leu¹⁷ and Gly⁵¹, and of Gly¹⁶ and Phe⁶², respectively. Since the movement of the loop between strand β₄ and helix a₂ (PAL residues 45–52), of the oxyanion loop (PAL residues 14–20), and of the lid region are correlated (17), the variation of the hydrogen-bonding pattern of this conserved Arg residue might play an important stabilizing role during the opening of the lid of these lipases.

In PAL the sn-3 octyl chain of the inhibitor (atoms C₄-C₁₁) is accommodated in a large groove (about 8.5 Å × 9.0 Å) formed by the side chains of Met¹⁶, Pro¹⁰⁸, Ser¹¹², Thr¹¹⁴, Ala¹¹⁵, Leu¹¹⁸, Leu¹³¹, Val¹³⁵, Leu¹⁵⁹, Leu¹⁶², Leu¹³¹, and Val¹³² (acyl chain pocket HA). The octyl group of the inhibitor fits snugly in this cleft and is bound via van der Waals interactions. Because of its size, the acyl binding pocket may easily accommodate up to about 12 fatty acid carbon atoms. The binding of the sn-3

**FIG. 3.** Structure-based sequence alignment of bacterial lipases from homology family I.1 and I.2. The comparison of the amino acid sequences of bacterial lipases from homology family I.1 (P. aeruginosa, V. cholerae, P. fragi, A. calcoaceticus, P. wisconsinensis, P. fluorescens, and P. vulgaris) and from homology family I.2 (B. cepacia, B. glumae, C. viscosum, and P. luteola) has been performed using the program CLUSTALW (36) and manually inspected and corrected on the basis of the three-dimensional structure comparison of PAL, BCL, BGL, and CVL. Shaded regions indicate the catalytic triad residues (blue) and identical (black) or similar (yellow) amino acids in all the aligned sequences. The two Asp residues that coordinate the calcium ion and the two Cys residues that make a disulfide bridge are highlighted by black and green triangles, respectively. α-Helices and β-strands are represented by rectangles and arrows, respectively. G1 and G2 are 3₁₀ helices. The color code of secondary structure elements is in accordance to Fig. 1. The figure has been prepared with ALSCRIPT (37).
moiety in the acyl-binding pocket (HA) is almost identical in PAL and BCL.

The alcohol binding pocket is separated from the acyl pocket by the side chain of Met\textsuperscript{16} and contains the bound lipid analogue in a bent “tuning fork” conformation (Fig. 4B). The sn-2 moiety (atoms N2-C30) fits in a hydrophobic pocket lined by Leu\textsuperscript{17}, Phe\textsuperscript{19}, Ile\textsuperscript{22}, Val\textsuperscript{25}, Tyr\textsuperscript{27}, Leu\textsuperscript{252}, Val\textsuperscript{255}, Gln\textsuperscript{257}, Val\textsuperscript{258}, and Phe\textsuperscript{259} (HH pocket). The latter residues are part of the calcium binding loop (residues 252 to 261). The HH pocket is slightly larger in PAL compared with that of BCL, and consequently, the enzyme-inhibitor interactions are less tight than in BCL. There is space for about 8–10 fatty acid carbon atoms to be accommodated in this pocket. Longer chains would partly stick into the solvent or micelle. In the HH pocket all the enzyme-inhibitor interactions are hydrophobic, and the NH group of the carbamoyl function of this chain does not have any specific hydrogen-bonding interactions with the protein. In particular, the hydrogen bond which in BCL connects the Thr\textsuperscript{18} O\text{y} atom to the carbonyl oxygen of the carbamoyl function of the sn-2 chain is absent in PAL due to the presence of a hydrophobic residue, Leu\textsuperscript{17}, instead of the polar Thr\textsuperscript{18} in BCL.

The sn-1 inhibitor moiety (atoms N1-C20) interacts less tightly with the enzyme than the sn-2 chain. It is located in a pocket more exposed to the solvent (HB pocket). The only specific interactions with the protein are van der Waals contacts with the side chains of Leu\textsuperscript{17}, Leu\textsuperscript{138}, and Phe\textsuperscript{214}. The location of the sn-1 chain is mainly determined by a hydrophobic clamp formed by Phe\textsuperscript{214} and Leu\textsuperscript{138}. The sn-1 chain diverges considerably with respect to the position found in the BCL complex since in the latter lipase the bulky side chain of Phe\textsuperscript{214} is substituted for an Ala\textsuperscript{247} (Fig. 3). As for the sn-2 moiety, the sn-1 NH group of the carbamoyl function does not specifically interact with the protein. Pro\textsuperscript{210} and Ser\textsuperscript{211} are located between the two sn-1 and sn-2 moieties but too far away to make any direct interaction. Since the HH pocket hosting the sn-2 chain provides a more intimate interaction with the substrate analogue than the HB pocket occupied by the sn-1 chain, the HH pocket is most likely the one that determines the regio-preference of the enzyme toward the primary position of triglyceride substrates together with the size of the acyl chain pocket HA.

In view of the differences in the inhibitor binding modes in the alcohol binding pocket, it is not surprising that PAL and BCL differ somewhat in their stereospecificity. BCL prefers the R\textsubscript{c}-trioctyl inhibitor over the S\textsubscript{c}-trioctyl compound by a factor of 7. It has been suggested that Leu\textsuperscript{287} and Ile\textsuperscript{290} play an important role in conferring this preference by providing unfavorable interactions with the carbonyl oxygen (O7) of the S\textsubscript{c}-trioctyl compound (18). Furthermore, the hydrophilic interaction of the sn-2 carbonyl oxygen (O6) with the O\text{y} atom of Thr\textsuperscript{18} favors this preference (18). In contrast, in PAL the residue corresponding to BCL Ile\textsuperscript{290} is Val\textsuperscript{255}, a less bulky residue, and BCL Thr\textsuperscript{18} corresponds to Leu\textsuperscript{17} in PAL, a non-polar residue (Fig. 3). These differences explain why PAL prefers the R\textsubscript{c}-trioctyl inhibitor only by a factor of 1.5 over the S\textsubscript{c}-trioctyl compound.\textsuperscript{3}

Conclusions—The x-ray structure of *P. aeruginosa* lipase provides the first three-dimensional structure of a member of homology family 1.1 of bacterial lipases. The molecule is in the open conformation and the active site contains a triglycerol analogue covalently bound to the catalytic nucleophile, Ser\textsuperscript{252}. The other catalytic residues are Asp\textsuperscript{229} and His\textsuperscript{251}. An octahedrally coordinated calcium ion stabilizes the loop containing the catalytic histidine. Sequence alignment with other lipases of homology family 1.1 shows that the residues important for catalysis and fold stabilization are conserved.

The bound inhibitor unambiguously identifies the position of the oxoanion hole and the three pockets that accommodate the sn-1, sn-2, and sn-3 fatty acid chains, thus providing a picture of the tetrahedral intermediate that occurs during the acylation step of the triglycerol hydrolysis reaction. At the base of the active site cleft the Met\textsuperscript{16} side chain divides the cleft into two branches, thus creating the acyl pocket for the binding of the sn-3 moiety of the inhibitor on one side and the alcohol pocket for the binding

\[\text{Fig. 4. Binding of the } R_c\text{-trioctyl inhibitor to } P. \text{ aeruginosa lipase.} \]

\[\text{A, schematic representation of the phosphonate inhibitor in comparison with a triglycerol molecule. B, stereo view of the binding mode of } R_c\text{-trioctyl in PAL active site. The inhibitor (black), the catalytic triad (gray), the oxoanion hole residues (gray), and the residues that line the three acyl chain binding pockets (HA, HB, HH) (white) are in ball and stick representation. Hydrogen bonds are shown by dashed lines. The figure was produced using MOLSCRIPT (35).}\]

\[\text{Fig. 5. Ca}^{2+}\text{-binding site. Stereo view of PAL calcium binding site. The catalytic triad (yellow) and the residues and water molecules that coordinate the calcium ion (black) are in ball and stick representation. The octahedral coordination of the Ca}^{2+}\text{ ion is shown by dashed lines. The figure was drawn using MOLSCRIPT (35).}\]

\[\text{K. Liebeton and K.-E. Jaeger, manuscript in preparation.}\]
of the sn-1 and sn-2 chains on the other side. The interactions between inhibitor and enzyme are mostly of a hydrophobic nature. Since the pocket hosting the sn-2 chain provides a more intimate interaction with the substrate analogue than the one occupied by the sn-1 chain, the sn-2 binding pocket and the acyl chain pocket are the ones that predominantly determine the regio-preference of the enzyme. Furthermore, comparison of PAL and \( B. cepacia \) lipase, both in complex with \( R_{\text{o}} \)-trioctyl, suggests Val\(^{255} \) and Leu\(^{17} \) to play a role in the differences in enantio-specificity of the enzymes toward the triacylglycerol analogue. These results pave the way for site-directed mutagenesis experiments to improve the enzyme for application in bioconversion reactions.

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