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Articles

Unusual Catalytic Triad of *Escherichia coli* Outer Membrane Phospholipase A†

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ABSTRACT: *Escherichia coli* outer membrane phospholipase A (OMPLA) is an integral membrane enzyme. OMPLA is active as a homodimer and requires calcium as a cofactor. The crystal structures of the monomeric and the inhibited dimeric enzymes were recently determined [Snijder, H. J., et al. (1999) *Nature 401*, 717−721] and revealed that OMPLA monomers are folded into a 12-stranded antiparallel β-barrel. The active site consists of previously identified essential residues Ser144 and His142 in an arrangement resembling the corresponding residues of a serine hydrolase catalytic triad. However, instead of an Asp or Glu that normally is present in the triad of serine hydrolases, a neutral asparagine (Asn156) was found in OMPLA. In this paper, the importance of the catalytic Asn156 is addressed by site-directed mutagenesis studies. All variants were purified at a 30 mg scale, and were shown to be properly folded using SDS−PAGE and circular dichroism spectroscopy. Using chemical cross-linking, it was shown that all variants were not affected in their calcium-dependent dimerization properties. The Asn156Asp variant exhibited a 2-fold lower activity than wild-type OMPLA at neutral pH. Interestingly, the activity of the variant is 1 order of magnitude higher than that of the wild type at pH >10. Modest residual activities (5 and 2.5%, respectively) were obtained for the Asn156Ala and Asn156Gln mutants, showing that the active site of OMPLA is more tolerant toward replacements of this third residue of the catalytic triad than other serine hydrolases, and that the serine and histidine residues are minimally required for catalysis. In the X-ray structure of dimeric OMPLA, the cofactor calcium is coordinating the putative oxyanion via two water molecules. We propose that this may lessen the importance for the asparagine in the catalytic triad of OMPLA.

Outer membrane phospholipase A (OMPLA),[1] encoded by the *pldA* gene, is a 31 kDa integral membrane protein present in Gram-negative bacteria. The enzyme catalyzes the hydrolysis of acylester bonds in phospholipids using calcium as a cofactor (*1*). OMPLA is known to be involved in secre-

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[1] Abbreviations: OMPLA, outer membrane phospholipase A; *pldA*, structural gene for OMPLA; 12-SB, *n*-dodecyl *N*,*N*-dimethyl-1-ammonio-3-propanesulfonate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid; C_{16}PC, hexadecylthioglycolphosphocholine; DODTPE, dioleoyldithiophosphatidylethanolamine; DODTPG, dioleoyldithiophosphatidylglycerol; DODTPC, dioleoyldithiophosphatidylcholine.

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tion of colicins (*2*, *3*) and to play a role in the pathogenicity of *Helicobacter pylori* (*4*) and *Campylobacter coli* (*5*).

In normally growing cells, all of the ingredients for catalysis, i.e., the enzyme, its substrate, and its cofactor are present, yet no activity can be detected (*6*). The need for a regulatory mechanism is obvious since uncontrolled breakdown of the cell envelope phospholipids would be lethal to the cell. It has been shown that in vitro OMPLA activity can be modulated by reversible dimerization (*7*). In vivo, activity can be induced by processes that disrupt the integrity of the membrane such as phage-induced lysis (*8*), temperature shock (*9*), and colicin secretion (*2*, *3*). The in vivo activation has also been shown to coincide with a dimerization process (*7*). Thus, OMPLA is present in the outer membrane in a dormant, monomeric state and becomes active only upon dimerization.

Recently, the X-ray structure of OMPLA has been determined (*10*). The structure consists of a 12-stranded antiparallel β-barrel with the exposed active center located in the outer leaflet of the membrane near the hydrophobic to hydrophilic membrane boundary. The active site contains Ser144 and His142, as had been deduced from chemical modification (*11*) and site-directed mutagenesis (*12*, *13*) experiments. On the basis of these results, OMPLA has been classified as a serine hydrolase. Serine hydrolases have a catalytic triad of a Ser, a His, and an Asp/Glu (*14*). Surprisingly, in the catalytic triad of OMPLA, a neutral asparagine is present at the location where usually an acidic residue is found in serine hydrolase triads. The existence of an asparagine in the catalytic triad is unique among all known serine hydrolases.

In the study presented here, we have investigated the importance of this Asn156 in the catalytic mechanism of OMPLA using site-directed mutagenesis.

MATERIALS AND METHODS

Bacterial Strains, Media, and Plasmids. Escherichia coli DH5α was the host strain for the propagation of plasmids; *E. coli* CE1433 is a *pldA*$^-$ derivative of BL21(DE3) and was used as the host strain for expression. Cells were grown in Luria-Bertani medium supplemented with ampicillin (0.1 mg/mL) at 37 °C. The plasmid used for the expression of wild-type OMPLA and the construction of variant OMPLAs was pND1, a vector that carries the *pldA* gene under the control of the T7 promoter. In this plasmid, the *Afl*III site present in ppL7.5 (*15*) was deleted by a single digest of the vector followed by filling in of the overhangs and subsequent religation of the newly created blunt ends.

pldA Mutations. With the QuikChange site-directed mutagenesis method (Stratagene) using the primer RK1 (5′-GGAACCGCCTTTATAC**G**CG**T**CTGATGGCAGAAAACG-3′) and the complementary primer RK2 (5′-CGTTTT-CTGCCATCAG**A**CG**C**GTATAAAGGCGGTTCC-3′), a silent mutation (indicated in bold) was introduced in pND1, creating a unique *Mlu*I restriction site (underlined) in the *pldA* gene. From this vector, the 107 bp *Pvu*II−*Bst*XI fragment was sequenced and subcloned into the *Pvu*II and *Bst*XI sites of the pND1 expression vector, resulting in pRK2. By cassette mutagenesis cloning of synthetic duplexes of oligonucleotides RK3 (5′-CTGG**GCC**CGCCTTTATA-3′) and RK4 (5′-CGCGTATAAAGGCG**GGC**CCAG-3′), RK5 (5′-CTGG**GAC**CGGTGTATA-3′) and RK6 (5′-CGCG-TATACA**ACCG**GTCCCAG-3′), or RK7 (5′-CTGG**CAG**-CGCTTGTATA-3′) and RK8 (5′-CGCGTATACA**AGCG**CT-**G**CCAG-3′) into *Pvu*II−*Mlu*I-digested pRK2, we created constructs pRK3, pRK4, and pRK5, respectively. These constructs encode OMPLA variants in which the codon for Asn156 is substituted with a codon for Ala, Asp, and Gln, respectively (depicted in boldface). Screening for insertion of the corresponding synthetic duplexes was facilitated by introduction of unique restriction sites (underlined). The nucleotide sequence was verified by DNA sequencing with the T7 DNA polymerase sequencing kit (Pharmacia).

Expression and Purification of OMPLA Variants. OMPLA variants were overproduced in strain CE1433 transformed with the appropriate expression plasmid by induction with isopropyl β-D-thiogalactopyranoside. Subsequent isolation of inclusion bodies, in vitro folding, and purification were carried out essentially as described for wild-type OMPLA (*15*) with some modifications. Approximately 200 mg of inclusion bodies was obtained from 1 L of bacterial culture. The inclusion bodies were dissolved in 10 mL of 8 M urea and 50 mM glycine (pH 8.3). Refolding was carried out by rapid dilution of this solution in 1 L of buffer [1.4 M urea, 2% (w/v) *n*-octylpolyoxyethylene glycol, and 5 mM glycine (pH 8.3)]. After refolding had been carried out for 16 h, the solution was loaded on a 80 mL DEAE-cellulose column which was equilibrated with buffer A [10 mM 12-SB and 20 mM Tris-HCl (pH 8.3)]. The column was washed with 5 column volumes of buffer A. OMPLA was eluted by applying a linear gradient of 0 to 0.6 M KCl in buffer A (2 × 10 column volumes). Fractions with a total amount of protein exceeding 20% of the top fraction were pooled and dialyzed twice against 10 volumes of buffer B [2.5 mM 12-SB and 20 mM imidazole (pH 6.5)] at 4 °C. Subsequently, the protein was loaded on a 120 mL DEAE-cellulose column which was equilibrated with buffer C [20 mM imidazole (pH 6.5) and 10 mM 12-SB]. The column was washed with 5 column volumes of buffer C and eluted by applying a linear gradient of 0 to 0.5 M KCl in buffer C (2 × 10 column volumes). Fractions were analyzed by SDS−PAGE. Fractions with a purity of >95% were pooled and dialyzed twice against buffer D [2.5 mM 12-SB and 20 mM Tris-HCl (pH 8.3)]. The protein was loaded on a Q-Sepharose column (loading capacity of 5 mg/mL), washed with 5 column volumes of buffer D [2.5 mM 12-SB and 20 mM Tris-HCl (pH 8.3)] and eluted with 10 column volumes of 1 M KCl in buffer D. Subsequently, the protein was dialyzed twice against buffer D.

OMPLA Activity Assays. Outer membrane phospholipase activities were determined in the standard assay [using hexadecylthioglycolphosphocholine (C_{16}PC) as a substrate] as described by de Geus et al. (*16*). Furthermore, specific activities were determined for the phospholipid analogues dioleoyldithiophosphatidylethanolamine (DODTPE), -phosphatidylglycerol (DODTPG), and -phosphatidylcholine (DODTPC) at saturating substrate conditions [10 mol % substrate, 900 μM TX-100, 50 mM Tris-HCl, 5 mM $CaCl_2$, and 100 μM dithiobis(2-nitrobenzoic acid) (DTNB)]. One unit corresponds to the hydrolysis of 1 μmol of substrate per minute.

pH Profile. A pH−activity profile was obtained by measurement of the enzymatic activities in the kinetic assay

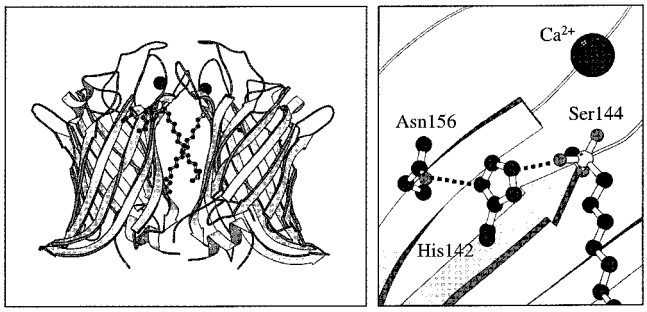

FIGURE 1: (Left) Crystal structure of a dimeric OMPLA−inhibitor complex (PDB entry 1qd6). The active site residues and the inhibitor (hexadecylsulfonyl covalently attached to Ser144) are depicted in a ball-and-stick representation. (Right) Closeup of the active site. The dashed lines represent hydrogen bonds. An interesting detail is the presence of a calcium ion 4.3 Å from the putative oxyanion.

in a mixed buffer (10 mM sodium acetate, 10 mM MES, 10 mM Hepes, 10 mM Tris, and 10 mM glycine) adjusted to pH values of interest with either HCl or NaOH. Activities at pH values below 8.0 were corrected for the lower molar extinction coefficients of DTNB at these values.

Chemical Cross-Linking. OMPLA was incubated at 0.2 mg/mL in buffer [50 mM Hepes (pH 8.3), 100 mM KCl, 3 mM 12-SB, and either 20 mM $CaCl_2$ or 20 mM EDTA] in a total volume of 100 μL. After 1 h, 10 μL of 1% glutaraldehyde in 2.5 mM 12-SB was added. The reaction was allowed to continue for 15 min at room temperature. Subsequently, 100 μL of gel loading buffer [0.1 M Tris-HCl (pH 6.8), 3% SDS, 15.4% glycerol, 7.7% β-mercaptoethanol, and 0.008% bromphenol blue] was added, and 20 μL of this solution (corresponding to 2 μg of OMPLA) was analyzed by SDS−PAGE. Visualization of the bands was achieved by staining with Coomassie Brilliant Blue.

Ca^{2+} Binding. The level of Ca^{2+} binding was determined indirectly by measurement of the enzymatic activities at different $CaCl_2$ concentrations. The apparent binding constant was determined by unweighted nonlinear regression fitting.

Circular Dichroism Spectroscopy. CD spectra were recorded as described by Dekker et al. (*15*).

RESULTS

Construction, Expression, and Purification of OMPLA Variants. In Figure 1, the active site geometry of OMPLA is shown, highlighting catalytically important residues Ser144, His142, and Asn156. These residues match the active site composition of typical serine hydrolases, but with the Asn156 side chain within hydrogen-bonding distance of the His142 Nδ atom instead of an Asp (*10*). Typically, the active site residues are among the most highly conserved residues in enzymes as is the case for Ser144 and His142 in OMPLA. Interestingly, Asn156 is *not* conserved. In *Enterobacter agglomerans* OMPLA (amino acid sequence 75% identical to that of *E. coli* OMPLA), an Asp is observed at the corresponding position, and it has been shown to be an active enzyme (*18*). In the more distantly related *H. pylori* OMPLA (27% identity), a Gln is found in strain 26695 (*19*) and an Asn in strain J99 (*20*).

To investigate the importance of Asn156 for catalysis by *E. coli* OMPLA, this residue was substituted with Asp, Gln, and Ala using cassette mutagenesis. All variants were expressed under control of a T7 promoter in a BL21(DE3)-

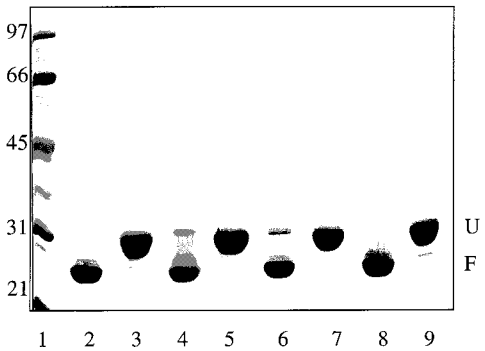

FIGURE 2: SDS−PAGE gel showing the purity and the heat modifiability of wild-type and variant OMPLAs: lane 1, molecular mass markers; lanes 2 and 3, WT OMPLA; lanes 4 and 5, N156D OMPLA; lanes 6 and 7, N156A OMPLA; and lanes 8 and 9, N156Q OMPLA. The samples in the odd lanes were boiled before electrophoresis, whereas those in even lanes were untreated.

Table 1: Specific Activities and Apparent Ca^{2+} Binding Constants for WT and Variant OMPLAs

	specific activity[a] (units/mg)				
protein	C_{16}PC	DODTPE	DODTPC	DODTPG	K_{Ca}[b] (μM)
WT	83	25	14	19	18
N156D	35	12	4.5	9.5	2.5
N156A	4	1.6	1.7	0.6	19
N156Q	2	0.7	0.6	0.3	30

[a] The error of the measurements was within 5%. [b] The error of the measurements was within 20%.

Δ*pldA* host. Overexpression of the mature protein without the signal sequence led to the accumulation of the protein in inclusion bodies. Expression levels were similar (30−40% of total cell protein) for wild-type (WT) and variant OMPLAs. The proteins were folded in vitro and subsequently purified by anion-exchange chromatography to a yield of approximately 30 mg per liter of cell culture. Next, they were analyzed by SDS−PAGE and appeared to be virtually pure (>95%, Figure 2, odd lanes). OMPLA has a stable β-barrel structure that does not unfold in SDS without subsequent heat treatment. The folded form of OMPLA is more compact than the heat-treated unfolded form and migrates slightly faster on SDS−PAGE as also found for other outer membrane proteins (*21*). Hence, the mobility of boiled and nonboiled samples on SDS−PAGE is an indicator of proper folding. Of each OMPLA variant, more than 95% was properly folded and migrated at 27 kDa (Figure 2, even lanes), whereas heat-treated, unfolded OMPLA migrated at 31 kDa (Figure 2, odd lanes). Furthermore, CD spectroscopy was used to confirm proper folding of the variants. The CD spectra for the wild type and variants were very similar (data not shown), indicating that mutation of Asn156 did not affect global folding of OMPLA.

Activities and Calcium Binding of OMPLA Variants. The specific activities of the purified enzymes are summarized in Table 1. Where wild-type OMPLA has a specific activity of 83 units/mg on the standard assay substrate C_{16}PC, the mutation of Asn156 to Asp results in a decrease in the specific activity to 35 units/mg, a value 2.4-fold lower than that of the wild-type enzyme under identical conditions. Replacement of Asn156 with Ala resulted in a 20-fold lower specific activity, whereas a 40-fold lower specific activity was found for the Gln156 variant. Various phospholipids

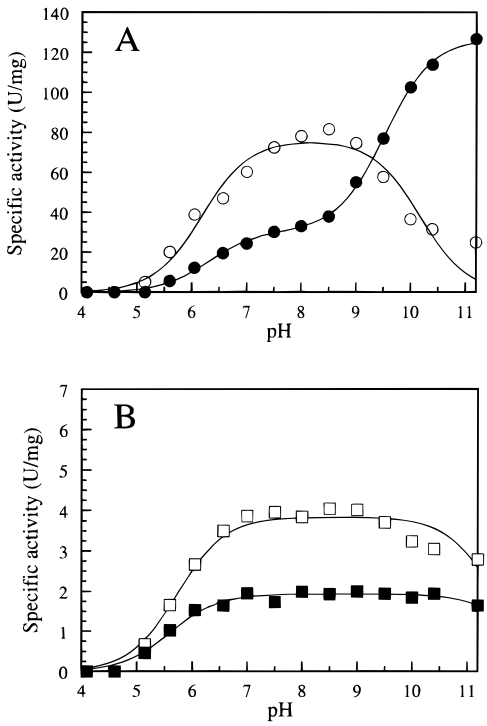

FIGURE 3: Specific activities of WT and variant OMPLAs as a function of pH. (A) Wild-type (○) and N156D OMPLA (●). (B) N156A OMPLA (□) and N156Q OMPLA (■). Note the 20-fold difference between the activities in panels A and B. The experimental data were fitted according to the model of Fersht and Renard (*38*).

were tested to check whether the mutations at position 156 also resulted in similar changes with natural substrates. All the variants exhibited similar behavior on these compounds compared to that of C_{16}PC (Table 1).

Previously, it has been shown that the active form of OMPLA is a dimer, and that dimer formation depends on the presence of the cofactor calcium (*7*). We used glutaraldehyde cross-linking to study the effect of Asn156 mutations on dimerization. All OMPLA variants could be cross-linked as well as the wild type in a calcium-dependent manner, showing that dimerization properties were unaffected (data not shown).

The importance of the cofactor calcium for OMPLA activity has been described previously (*1*). More detailed studies demonstrated that in the active, dimeric OMPLA complex two calcium ions are bound per OMPLA monomer (*17*). One of the calcium ions [Ca^{2+}(I)] binds to OMPLA with high affinity and is suggested to be the one essential for activity. The role of the other calcium ion [Ca^{2+}(II)] is unclear. The impact of our Asn156 mutations on the high-affinity site Ca^{2+}(I) was investigated by kinetic experiments that are summarized in Table 1. Replacement of Asn156 with Ala or Gln hardly influences this high-affinity calcium binding site (Table 1). Interestingly, the mutation of Asn156 to Asp leads to a 7-fold increase in affinity, suggesting that this calcium ion is bound near residue 156 and senses charge changes (Figure 1). This result is in accordance with the crystal structure of an OMPLA−inhibitor complex where a calcium ion is bound 11.4 Å from the Cγ of Asn156, allowing long-range electrostatic interactions with a charge present at position 156 (*10*).

pH−Activity Profile. Figure 3 shows the pH dependence of substrate hydrolysis by the wild type and the OMPLA variants. The pH profile of WT OMPLA takes the classical "bell-shaped" form with an optimum around pH 8.5 (Figure 3A). It has been suggested that the pK_a of 6.3 corresponds to deprotonation of the active site His142 (*1*). The drop in activity at alkaline pH values is not yet understood. Lower activity might result from deprotonation of one or more residues with a pK_a of around 9.5, e.g., tyrosines or lysines. Substitution of Asn156 with Ala or Gln yielded enzymes that resemble WT OMPLA in their pH profiles (Figure 3B). In contrast, the pH dependence of the variant N156D was biphasic, following the profile of WT OMPLA up to pH 8.0, after which a second ionization step with an apparent pK_a of 9.5 induced a strong increase in activity. In fact, the N156D variant is more active at basic pH values than the WT enzyme under its optimal conditions.

DISCUSSION

OMPLA has been classified as a serine hydrolase on the basis of site-directed mutagenesis and chemical modification studies (*11*, *12*). The active sites of serine hydrolases generally consist of a Ser-His-Asp/Glu triad (*14*), with the active site residues coming together from separate regions within the protein. OMPLA is unique among this class of enzymes with the active site Ser144 and His142 close together in the primary structure. Whereas the third member of the catalytic triad is invariantly an aspartate in serine proteases, a glutamate is sometimes found at the corresponding position in esterases. Some serine hydrolases have been reported with a main chain carbonyl (*22*) or a His (*23*) instead of the aspartic acid. In the X-ray structure of OMPLA, an uncharged asparagine residue (Asn156) was found at the position where normally an acidic residue is located, providing yet another way to stabilize the imidazole of the active site His in serine hydrolases. In the work presented here, we have investigated the contribution of Asn156 to the catalytic machinery of OMPLA.

Mutations of the active site Ser or His in serine proteases generally lead to rate reductions of 4−6 orders of magnitude (*24*, *25*). In OMPLA, similar results were obtained for Ser144 and His142 (*12*, *13*). The acidic residue usually contributes to a lesser extent to catalysis. Two roles in catalysis have been proposed for this residue: (1) neutralization of the positive charge on the histidine that is transiently present during the reaction and (2) fixation of the correct tautomeric state of the histidine. It has been predicted that in the case of charge neutralization, replacement of the charged Asp with the neutral Asn would reduce the rate of catalysis by a factor of 10 (*26*), provided that such a replacement is without structural perturbation. If a structural perturbation occurs, as for example was found for the D102N mutation in rat trypsin (*27*), then the decrease in activity is much larger (4 orders of magnitude) (*28*). In pancreatic phospholipase A2 (PLA2), which has an active site geometry comparable to those of serine hydrolases with a water molecule as a nucleophile rather than a Ser, mutation of Asp to Asn resulted in an only 20-fold lower activity (*29*). X-ray crystallography of this PLA2 D99N variant indicated that the Oδ of Asn99 points toward the active site His and that the tautomeric form of the His was the same as in the wild type (*29*). Therefore, in this case we are dealing exclusively with charge neutralization of the catalytic residue. On the basis of these results,

an increase in activity of 1 order of magnitude was anticipated for the Asn156Asp mutation in OMPLA. At pH 8.3, the optimum pH for WT OMPLA, a small reduction in activity was observed for the variant. However, at pH 10 the activity was 150% of the WT activity at its optimum pH, and 6-fold higher than the activity of WT OMPLA at pH 10. In the triad of classical serine hydrolases, the active site aspartate is hydrogen bonded not only to the active site His but also via the other oxygen in the carboxylate involved in an extensive hydrogen bonding network that maintains the structure of the catalytic pocket. In these hydrolases, introduction of a carboxyamide moiety with a hydrogen bond donor and acceptor group instead of the carboxyl group which consists of two hydrogen bond acceptor groups can result in a reorganization of the hydrogen bonding network and thus the catalytic pocket. In this respect, it is noteworthy that Asn156 in OMPLA is solely hydrogen bonded to the active site His142, making structural perturbations in the catalytic pocket less likely in the Asn156Asp variant. Moreover, the absence of an extended hydrogen bonding network suggests that the precise location of the side chain of Asn156 is less important than in other serine hydrolases.

Removal of the carboxylate function by mutation of the Asp to Ala invariably resulted in the complete loss of activity in both serine hydrolases (*30*, *31*) and pancreatic PLA2 (*32*). An Asn naturally occurs in the active site of the cysteine protease papain, where the geometry of the active site Asn-His-Cys is similar to that of the serine hydrolase triad (*33*). Mutation of this Asn to Ala resulted in a 150-fold decrease in activity (*34*). The relatively small effect for this enzyme compared to those of serine hydrolases was interpreted in terms of the intrinsically more reactive nature of the nucleophilic Cys, which is supposed to exist in its ionized state at the optimum pH. In view of these results, complete loss of activity would be expected for the Asn156Ala variant. However, the activity decreased only 20-fold. Considering the small effect of the Asn156Ala mutation on the activity of OMPLA, we conclude that Asn156 is not essential for activity and that other factors must be involved that aid catalysis by OMPLA.

The question of why OMPLA displays such tolerance in the catalytic triad remains. Inspection of the enzyme structure does not reveal any main chain carbonyls or side chains of other residues that could substitute for Asn in any of the variants. Although no experimental data are available on the rates of individual steps taking place during catalysis, we hypothesize that efficient hydrolysis of substrate is determined to a large extent by the presence of the cofactor calcium that has been observed in the vicinity of the active site in the X-ray structure (Figure 1). The positive charge on the calcium ion is not compensated by its ligands and may stabilize the negatively charged intermediate that develops during catalysis (*10*). A model for this oxyanion hole-aided transition state stabilization is shown in Figure 4. The distance of 4.3 Å between the proposed oxyanion and the calcium ion is too large to allow direct interactions. In the crystal structure, two water molecules have been observed that could mediate such stabilization. Oxyanion stabilization is of major importance in catalysis as has been shown experimentally for, e.g., cutinase (*35*) and subtilisin (*36*).

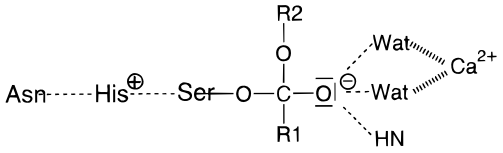

FIGURE 4: Model for the stabilization of the transition state by calcium during catalysis by OMPLA. R1 is the fatty acid alkyl chain, and R2 is the acylglycerolphosphoethanolamine part of the phospholipid substrate. WAT stands for water molecules observed in the X-ray structure of OMPLA (*10*). The oxyanion ligands are the backbone amide of residue Gly146 and two water-mediated interactions with the calcium ion. In the crystal structure of the OMPLA−inhibitor complex (*10*), the distance between the sulfur oxygen (proposedly corresponding to the oxyanion) and the calcium ion is 4.3 Å.

Surprisingly, the pH profile of the Asn156Asp variant was found to be biphasic rather than bell-shaped as is the case for the pH profile of WT OMPLA. For both the wild type and the Asn156Asp variant, the pK_a values of all titrating groups were calculated with the WHAT IF pK_a calculation package (J. E. Nielsen and G. Vriend, manuscript submitted for publication). This pK_a calculation package uses the hydrogen-bond optimization procedure of Hooft et al. (*37*) to model the organization of the hydrogen-bond network. These calculations predict a strong upward shift in the pK_a value of His142 when Asn156 is changed into Asp (J. E. Nielsen, personal communication). The contribution of solvent is not taken into account in the calculations. Therefore, the absolute magnitude of the pK_a shift cannot be determined with great accuracy. However, since pK_as were calculated for both the wild-type and variant enzyme, the relative change in the pK_a value of His142 is useful for a qualitative analysis. This calculated shift in pK_a is coincident with the increase in activity for this variant at alkaline pH. This outcome is even more surprising, considering the fact that in classical serine hydrolase triads, in which an Asp stabilizes the active site His, the pK_a of His is generally around 7. In OMPLA, the shift in the pK_a for His142 upon introduction of a negative charge may be explained by the isolated nature of residue 156 in the active site. In OMPLA, the Asp or Asn at position 156 is solely hydrogen bonded to the active site His142, whereas in other serine hydrolases, the active site Asp is part of an elaborate hydrogen bonding network, presumably leading to a delocalization of the negative charge. The hydrophobic nature of the exterior of the β-barrel and the location of the active center in the hydrophobic core of the membrane further contribute to the isolation of the triad in OMPLA. OMPLA represents one of the first well-documented examples for studying the effects of the biological membrane environment on structural and functional constraints in membrane enzymes.

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