Escherichia coli Outer Membrane Phospholipase A: Role of Two Serines in Enzymatic Activity†

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ABSTRACT: In the outer membrane phospholipase A (OMPLA) of *Escherichia coli*, Ser144 has previously been identified by chemical modification as the active site serine residue. In a specific OMPLA-negative mutant strain, the *pldA* gene coding for OMPLA was shown to differ from the wild-type gene by a single point mutation, resulting in the substitution of Ser152 by phenylalanine. The role in catalysis of these two serine residues in OMPLA was investigated by site-directed mutagenesis. Ser144 and Ser152 were replaced one at the time by either alanine, valine, phenylalanine, threonine, or cysteine. Ser152 was furthermore replaced by asparagine. Replacement of Ser144 by cysteine resulted in 1% residual activity, whereas the other substitutions at this position yielded virtually inactive enzymes. Substitution of Ser152 by threonine or asparagine resulted in 40% and 2% residual activity respectively, whereas all other substitutions at this position resulted in the loss of enzymatic activity. We propose that Ser144 is the nucleophile in catalysis, and that Ser152 is involved in hydrogen bonding either to the catalytic triad or in the oxyanion hole.

Most bacterial outer membrane proteins are involved in the transport of nutrients across this membrane by forming pores or receptors. Besides these transport proteins, the outer membrane also contains a few enzymes, one of which is the outer membrane phospholipase A (OMPLA or PldA protein).[1] This 30 kDa protein, encoded by the *pldA* gene, was demonstrated to be present in most members of the family of *Enterobacteriaceae* (Brok *et al.*, 1994). The *pldA* genes of *Escherichia coli* (Homma *et al.*, 1984), *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Proteus vulgaris* (Brok *et al.*, 1994) have been cloned and sequenced. These genes appeared to be highly conserved, indicating an important function, but the physiological role for the enzyme is still unknown. Although *E. coli* OMPLA has been shown to be required for efficient secretion of bacteriocins (Pugsley & Schwartz, 1984; Luirink *et al.*, 1986), it is unlikely that this is the primary function of the enzyme. The enzyme appears to be dormant in normally growing cells (Audet *et al.*, 1974), but high OMPLA activity can be induced by damaging the membrane, *e.g.*, by phage-induced lysis (Cronan & Wulff, 1969) or by temperature shock (de Geus *et al.*, 1983).

OMPLA harbors several enzymatic activities, *i.e.*, those of phospholipases A_1 and A_2 as well as of 1-acyl- and 2-acyllysophospholipase and diacylglyceride lipase (Horrevoets *et al.*, 1989). Phospholipases are widely spread in nature. The best studied enzymes are the phospholipases A_2 originating from mammalian pancreas or from snake venom. These enzymes have low molecular weights and contain seven disulfide bridges. They hydrolyze specifically the acyl ester bond at the *sn*-2 position of phospholipids in a calcium-dependent way. Next to the low molecular weight phospholipases, a 85 kDa phospholipase is present in the cytosol of mammalian cells. This cPLA2 is reported to be arachidonic acid selective and has a serine in the active site (Sharp *et al.*, 1994). Although OMPLA has phospholipase A_2 activity, it has no sequence homology with water-soluble phospholipases and it does not contain cysteines at all. There is no structural homology either: OMPLA probably has the β-barrel structure that is characteristic for porins (Brok *et al.*, 1994; Dekker *et al.*, 1995). Because of this unique structure, it is of interest to study the mechanism of action of this membrane-bound enzyme.

The water-insoluble inhibitor *n*-hexadecylsulfonyl fluoride, incorporated in micelles, inactivates OMPLA in a stoichiometric reaction (Horrevoets *et al.*, 1991). In this way, Ser144 was identified as the catalytic center serine residue. Because the inactivation of OMPLA by *n*-hexadecylsulfonyl fluoride was controlled by a group with a p*K* of 6.75, a histidine seems to be part of the active center, and OMPLA was suggested to belong to the class of serine hydrolases having a "classical" Asp-His-Ser catalytic triad (Horrevoets *et al.*, 1991). In all known OMPLA sequences, four histidine residues are conserved, and recently His142 has been

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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; Ap, ampicillin; Km, kanamycin; Ap^r, Ap resistance; Km^r, Km resistance; PCR, polymerase chain reaction; IPTG , isopropyl β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, *N*,*N*,*N*,*N*-ethylenediaminetetraacetic acid.

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identified as the only histidine residue essential for enzymatic activity (Brok *et al.*, 1995).

Previously, we have characterized the *pldA* allele of the most commonly used *pldA* mutant, strain S17 (Brok *et al.*, 1994). This strain expresses a correctly assembled, but inactive OMPLA. This mutant form deviates from the wild-type OMPLA by the replacement of the Ser152 residue by a phenylalanine residue. Therefore, the possibility was raised that the identification of Ser144 as the catalytic center residue (Horrevoets *et al.*, 1991) was at error. Alternatively, both Ser144 and Ser152 may be important for catalytic activity. In this respect, it should be noted that Ser144 and Ser152 are conserved in the four established OMPLA sequences (Brok *et al.*, 1994). In the present study, we have investigated the role of these two serine residues in the catalytic mechanism of OMPLA. Several substitutions were made at both positions, and the effect of these mutations on OMPLA activity was determined.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strains were grown at 37 °C in L-broth (Tommassen *et al.*, 1983), supplemented with 100 μg/mL ampicillin (Ap) when required for plasmid maintenance. In *pldA* mutant strain CE1348 (Brok *et al.*, 1994), the chromosomal *pldA* gene is replaced by a Km^r cassette. The *fabB* strain CE1302 (de Geus *et al.*, 1986) is a temperature-sensitive fatty acid-auxotrophic strain. Strain BL21(DE3) carries the structural gene for T7 RNA polymerase behind an IPTG-inducible promoter on a defective prophage (Studier & Moffat, 1986). Strain CE1433 was made by transferring the ΔpldA:km^r mutation of strain CE1347 (Brok *et al.*, 1994) to strain BL21(DE3) by P_1 transduction as described by Willetts and co-workers (Willetts *et al*., 1969). The following plasmids were used: pRB1, a pUC18 derivative carrying the *pldA* gene including the *pldA* promoter (Brok e*t al.*, 1994); pPN104, a pBR322 derivative, carrying a *pldA* allele with a silent mutation introducing an *Apa*I site (de Geus *et al*., 1986); and ppL7.5, a pT7-7 derivative containing the *pldA* gene without the signal sequence-encoding domain behind the T7Φ10 promoter (Dekker *et al.*, 1995).

DNA Techniques. Competent bacterial cells were transformed with plasmid DNA by using the $CaCl_2$ method (Sambrook *et al.*, 1989). Plasmid DNA was isolated by the boiling method (Holmes & Quigly, 1981) or by the alkaline extraction procedure (Birnboim & Doly, 1979), followed by anion-exchange chromatography on Qiagen pack 20 columns (Diagen, Düsseldorf, Germany). Oligonucleotides used in this study are described in Figure 1. PCRs were performed in 0.5 mL Eppendorf tubes with a Techne programmable Ori-block PHC-1. The PCR mixtures contained 7.5 pmol of both primers, 200 μM (each) deoxynucleoside triphosphate, approximately 350 ng of pPN104 as template DNA, *Taq* DNA polymerase buffer (Promega) or *Pfu* DNA polymerase buffer (Stratagene), and 1 unit of *Taq* DNA polymerase (Promega) or *Pfu* DNA polymerase (Stratagene) in a total volume of 25 μL. The solution was over layered with 25 μL of mineral oil. The thermal profile involved a first denaturing step at 94 °C for 3 min followed by 25 cycles of denaturing at 94 °C for 1 min, primer annealing at 48 °C (mutagenic primers B133 or B143) or 55 °C (mutagenic primers B144, B145, B146, B147, or B149) for 3 min, and extension at 65 °C (primer B133 or B143) or 72 °C (primers B144, B145, B146, B147, and B149) for 2 min (for 12 min in the last cycle).

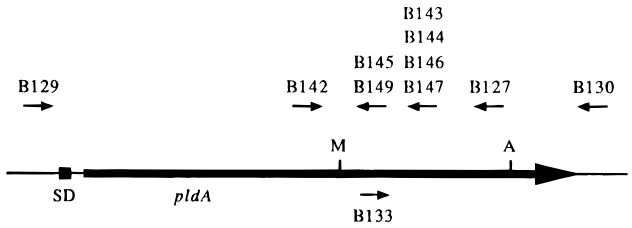

FIGURE 1: Position and orientation of synthetic oligonucleotides with respect to the *pldA* gene. The following oligonucleotides were used: B127 (18-mer), 5′-GTTCCAGTTGTACTGTCC-3′; B129 (24-mer), 5′-ATgaattCCTTATCAATAATTTCG-3′, nonhybridizing *Eco*RI site at 5′ end; B130 (23-mer), 5′-AGAagcttCAACCACTCAACCGT-3′, nonhybridizing *Hin*dIII site at 5′ end; B133 (18-mer), 5′-CCACGACdyTAACGGGCG-3′, introducing a S144A, S144V, S144F, S144T, or S144I mutation in OMPLA; B142 (18-mer), 5′-CGCAAAAATCCTGGTGGC-3′; B143 (18-mer), 5′-CAGCTGCGGkyGGTCGGG-3′, introducing a S152N, S152, S152D, or S152A mutation in OMPLA; B144, (19-mer), 5′-CCAGCTGCGGrcGGTCGGG-3′, introducing a S152A or S152V mutation in OMPLA; B145 (18-mer), 5′-CGCCCGTTAcAGTCGTGG-3′, introducing a S144C mutation in OMPLA; B146 (19-mer), 5′-CCAGCTGCGGcAGGTCGGG-3′, introducing a S152C mutation in OMPLA; B147 (21-mer), 5′-CCAGCTGCGtacGGTCGGGTC-3′, introducing a S152V mutation in OMPLA; B149 (18-mer), 5′-CGCCCGTTAGtGTCGTGG-3′, introducing a S144T mutation in OMPLA. Nucleotides indicated as lower case characters do not hybridize with the wild-type *pldA* gene. Degenerated positions in the oligonucleotides are indicated by a single character. Character d indicates either g, t, or a; character k indicates either g or t; character r indicates either g or a; character y indicates either t or c. SD, Shine−Dalgarno sequence; M, *Mun*I restriction site; A, *Afl*II restriction site.

Restriction endonucleases, T4 DNA polymerase, and T4 DNA ligase were obtained from Pharmacia LKB Biotechnology or New England Biolabs. DNA fragments were isolated from an agarose gel using the Qiaex DNA gel extraction kit (Diagen). DNA restriction fragments were subcloned into the *pldA* expression vector pRB1 (Brok *et al.*, 1994) and sequenced on double-stranded template DNA by the dideoxy chain termination method (Sanger *et al.*, 1977) with the T7 DNA polymerase deaza sequencing kit (Pharmacia LKB Biotechnology).

pldA Mutations. Mutations were introduced into *pldA* using the four- (Mikaelian & Sergeant, 1992) or the three-primer PCR method (Landt *et al.*, 1990). In both methods, two rounds of PCR are involved. In the four-primer PCR, round 1 consists of two separate PCRs in one of which primer B133 (Figure 1) was used as degenerated mutagenic primer. The products of the two separate PCRs were hybridized and used as template in a second round of PCR using the band-stab PCR technique (Bjourson & Cooper, 1992). After the second round of PCR, for which the primers B129 and B130 (Figure 1) were used, the resulting 930 bp DNA fragment was isolated from the gel, and the 329 bp *Mun*I−*Afl*II fragment obtained after double digestion was cloned into pRB1.

In the first round of the three-primer PCR, a so-called megaprimer was made for each desired mutation using either primer B143, B144, B145, B146, B147, or B149 as mutagenic primer in combination with primer B142 (Figure 1). After isolation from an agarose gel, the megaprimers were used in a second round of PCR together with primer B130

(Figure 1). Subsequently, the resulting 560 bp DNA fragments were amplified using the band-stab PCR technique (Bjourson & Cooper, 1992) and primers B142 and B130. Following this amplification, the resulting 560 bp DNA fragments were isolated from the agarose gel and cloned after *Mun*I−*Afl*II double digestion into pRB1. The construct pRB1-S152F was made by recloning the 329 bp DNA fragment obtained after *Mun*I−*Afl*II double digestion of a previously described recombinant plasmid that contains the allele for the S152F mutant OMPLA (Brok *et al.*, 1994) into pRB1. In all pRB1 derivatives, mutant OMPLA proteins are expressed under control of the authentic *pldA* promoter.

DNA fragments that contain the mutations coding for S144A, S144T, S144C, S152T, S152N, and S152C were also recloned into the *pldA* expression vector ppL7.5 (Dekker *et al.*, 1995) by exchange of *Mun*I−*Afl*II fragments. The resulting constructs encode the mutant OMPLA proteins with an N-terminal extension of seven amino acids (MARIRAP) instead of the signal sequence. These proteins are expressed under control of the T7Φ10 promoter.

Purification of Mutant OMPLA's. Mutant OMPLA's were overproduced in strain CE1433, containing the corresponding ppL7.5 derivative by induction with IPTG. Subsequent isolation of inclusion bodies and folding and purification of the mutant proteins were done as described for wild-type OMPLA (Dekker *et al.*, 1995) with minor modifications. Inclusion bodies (250−500 mg) were dissolved in a minimal volume of buffer containing 8 M urea and 50 mM glycine, pH 8.3. Refolding was done in a buffer containing 1.4 M urea, 1.6% (w/v) *n*-octylpoly(oxyethylene glycol), and 5 mM glycine pH 8.3, at a protein concentration of 0.8 mg/mL. In the cases of the S144C and S152C mutant proteins, 2.5 mM β-mercaptoethanol was present in all buffers to prevent the formation of intermolecular disulfide bridges. After the first DEAE-cellulose column (pH 8.3), suitable fractions containing heat-modifiable mutant OMPLA were pooled and dialyzed twice against a buffer containing 20 mM histidine, 2 mM EDTA, and 5 mM 12-SB, pH 6.5. After the second DEAE-cellulose column (pH 6.5), fractions containing pure, heat-modifiable mutant OMPLA were pooled and dialyzed twice against 20 mM Tris-HCl, 2 mM EDTA, and 2.5 mM 12-SB, pH 8.3. SDS−PAGE was used at all stages of the purification to assess the purity of the mutant proteins.

Gel Electrophoresis and Western Blot Analysis. Proteins in cell homogenates and purified mutant OMPLA's were separated by SDS−PAGE (Lugtenberg *et al.*, 1975). Samples were mixed with loading buffer (Sambrook *et al.*, 1989) and loaded either directly or after heating for 10 min at 100 °C on an 11% acrylamide gel (Lugtenberg *et al.*, 1975). Gels were stained with Coomassie Brilliant Blue (Sambrook *et al.*, 1989). Western blot analysis was performed using a mouse polyclonal anti-OMPLA serum raised against denatured OMPLA (Brok *et al.*, 1994). The specificity of the polyclonal antiserum was increased by affinity purification (Burke *et al.*, 1982). To denature all proteins after SDS−PAGE and prior to Western blotting, the gel glass plate sandwich was wrapped in aluminum foil, sealed into a plastic bag, and heated for 8 min in steam. Subsequently, the proteins were electroblotted onto a nitrocellulose filter. After blocking in a solution containing 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.05% Tween 20, and 0.5% dried milk (Protifar), the filter was incubated with the mouse anti-OMPLA serum and, subsequently, with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G as second antibody as described elsewhere (Sambrook *et al.*, 1989).

Outer Membrane Phospholipase Assays. A qualitative, *in vivo* assay for OMPLA activity was performed as described before (de Geus *et al.*, 1983). This assay is based on the release of fatty acids from colonies of cells expressing active OMPLA, which facilitates the growth at 42 °C of the fatty acid-auxotrophic strain CE1302 in a top layer. The latter strain lyses at temperatures above 40 °C when not supplied with unsaturated fatty acids. OMPLA activity was determined quantitatively in lysed cells and of purified proteins by using a chromogenic substrate (de Geus *et al.*, 1986; Horrevoets *et al.*, 1991). The substrate, 2-hexadecanoylthioethane-1-phosphocholine, was prepared as described (Aarsman *et al.*, 1976). Cells of an overnight culture were disrupted by sonication at 0 °C in 10 mL of buffer containing 50 mM Tris-HCl and 2 mM EDTA, pH 8.5. The assay buffer contained 50 mM Tris-HCl, 5 mM $CaCl_2$, 0.2 mM Triton X-100, 0.25 mM substrate, and 0.1 mM DTNB at pH 8.3. The activity was determined by following the increase of absorbance at 412 nm. To calculate specific activities, the total amount of protein in the cell homogenates was determined according to Bradford (1976); for the purified proteins, the concentration was calculated on the basis of the absorbance at 280 nm, using an $E^{1\%}$ of 29.2.

RESULTS

Construction and Expression of Mutant OMPLA's. The residues Ser144 and Ser152 were replaced one at a time by alanine, valine, phenylalanine, threonine, and cysteine residues by means of site-specific mutagenesis (see Materials and Methods). Similarly, a S152N substitution was created. DNA fragments carrying the mutations were subcloned in the vector pRB1, after which the nucleotide sequences were verified with the dideoxy chain termination sequencing method. The *pldA* alleles on the pRB1 plasmids were expected to be expressed constitutively in strain CE1348 from the authentic *pldA* promoter. Homogenates of harvested cells were analyzed for the presence of (mutant) OMPLA by Western blotting. The wild-type and all mutant OMPLA's appeared to be properly expressed and folded (Figure 2). In samples that were boiled before electrophoresis, the (mutant) proteins migrated as 30 kDa proteins. In samples that were not boiled before electrophoresis, a band was seen migrating as a 25 kDa protein. This heat-modifiable character of OMPLA (Nishijima *et al.*, 1977; Brok *et al.*, 1994) and of other outer membrane proteins (Heller, 1978) has been extensively described and is an indication of correct folding and assembly of the proteins in the outer membrane (Freudl *et al.*, 1986; Ried *et al.*, 1990). The heat-modifiability of all the mutant OMPLA's indicates that the mutant proteins are correctly folded and assembled into the outer membrane.

Activities of Mutant OMPLA's with Substitutions of Ser144. The activity of the mutant OMPLA's was determined qualitatively in an *in vivo* plate assay with colonies of CE1348 cells harboring the corresponding plasmids. Cells expressing wild-type OMPLA were clearly positive in this assay, whereas all mutants with a replacement of Ser144 scored negative (Table 1). The slight variation in the expression level of the various OMPLA's (Figure 2) cannot explain the lack of activity in these mutants. Moreover, the

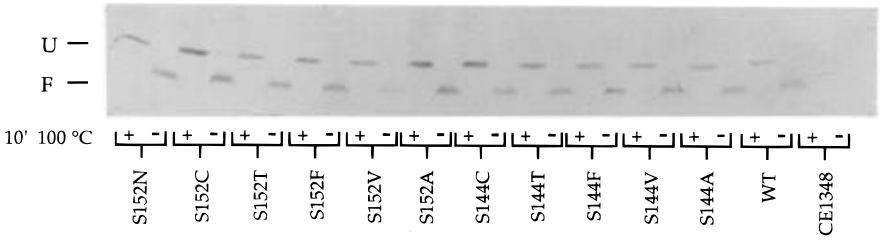

FIGURE 2: Expression and heat-modifiability of wild-type and mutant OMPLA's, demonstrated by Western blot analysis of cell homogenates with anti-OMPLA serum. To each lane was applied 15 μg of total cell protein of strain CE1348 expressing the following OMPLA's: S152N, S152C, S152T, S152F, S152V, S152A, S144C, S144T, S144F, S144V, S144A, or wild type (WT). A lysate of the *pldA* deletion strain CE1348 was also applied. The samples were either boiled for 10 min (+) or not boiled (−) before electrophoresis. Symbols: F, folded OMPLA; U, unfolded OMPLA.

Table 1: Enzymatic Activities of Wild-Type and Mutant OMPLA's[a]

(mutant) OMPLA	plate assay[b]	specific activity[c]: cell homogenates [milliunits·(mg of total cell protein)$^{-1}$]	specific activity[c]: purified OMPLA (units·mg^{-1})
no OMPLA[d]	−	3.6 ± 0.3	0
WT[e]	+	71.7 ± 4.2	92
S144A	−	3.5 ± 0.6	0.001
S144V	−	3.5 ± 0.2	ND
S144F	−	2.8 ± 0.4	ND
S144T	−	3.4 ± 0.3	0.002
S144C	−	4.3 ± 0.4	0.8
S152A	−	4.0 ± 0.2	ND
S152V	−	3.7 ± 0.6	ND
S152F	−	3.6 ± 0.2	ND
S152T	+	32.3 ± 1.7	37
S152C	−	2.8 ± 0.2	0.02
S152N	+	4.4 ± 0.1	2

[a] Activities were measured in *E. coli* strain CE1348 harboring pRB1-derived plasmids using two different assays. [b] A qualitative *in vivo* plate assay on whole CE1348 cells, where + or − indicates the presence or absence of detected phospholipase A activity. [c] A chromogenic assay with 2-hexadecanoylthioethane-1-phosphocholine as substrate. One unit is defined as 1 μmol of substrate converted per minute. The values are corrected for nonenzymatic hydrolysis which is about 6×10^{-6} μmol·min^{-1} in the assay buffer. The values for CE1348 homogenates are averages of triplicate measurements of a single culture (±standard deviation). [d] The *pldA* deletion mutant strain CE1348 was used. [e] WT stands for wild-type OMPLA expressed from the plasmid pRB1 in strain CE1348 or purified wild-type OMPLA. [f] ND, not determined.

assay is sensitive enough to detect OMPLA activity in cells carrying a single copy of the *pldA* gene on the chromosome (de Geus *et al.*, 1983). Since pRB1 is a pUC18 derivative having a high copy number, the absence of OMPLA activity in cells expressing the mutant proteins suggests that the activity of these mutant OMPLA's is very low, if not zero.

To obtain more quantitative data, the phospholipase A activity was determined in a chromogenic assay after sonication of the cells (Table 1). Although the strain CE1348 lacks OMPLA activity, homogenates of these cells contain sufficient alternative esterase activity to give a background value of approximately 3.5 milliunits/mg of total cell protein. Cell homogenates of all mutants with a replacement of Ser144 in OMPLA displayed enzymatic activities near this background level. Only in homogenates of cells expressing the S144C mutant OMPLA, activities slightly exceeding the background values seemed to be present (Table 1). Therefore, we decided to purify this S144C mutant protein, as well as S144A and S144T mutant proteins, to homogeneity. To this end, the *pldA* alleles coding for these mutant OMPLA's were cloned into the expression vector ppL7.5. After folding

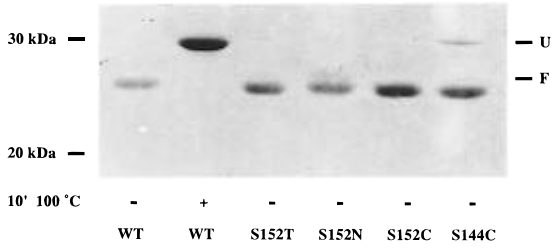

FIGURE 3: SDS−PAGE of the purified (mutant) OMPLA's. To each lane was applied 5 μg of purified protein: WT (wild-type *E. coli* OMPLA), S152T, S152N, S152C, or S144C; either boiled for 10 min (+) or not boiled (−) before electrophoresis. The positions of molecular mass standard proteins are indicated at the left (kDa). Symbols: F, folded OMPLA; U, denatured OMPLA. Proteins were stained with Coomassie Brilliant Blue.

of the proteins and purification on DEAE-ion exchange columns, the purity of the mutant OMPLA's was analyzed by SDS−PAGE. Densitometric tracings showed that the S144C mutant OMPLA was over 90% pure (Figure 3), whereas S144A and S144T showed only a single band (data not shown). Since the (major) band in all three cases displayed the same heat-modifiable character as wild-type OMPLA (results not shown), we concluded that the mutant OMPLA's were properly folded. The purified S144C mutant OMPLA exhibited a specific activity of approximately 1% of that of the wild-type OMPLA whereas the S144A and S144T mutant proteins were essentially inactive (Table 1). In conclusion, the lack of activity of the mutant OMPLA's S144A and S144T underscores the supposition that Ser144 is the catalytic center serine residue in OMPLA (Horrevoets *et al.*, 1991). The fact that a low residual activity is found for the S144C mutant protein does not contradict this conclusion, since in the literature several examples have been described of serine hydrolases with high residual activity after replacement of the catalytic center serine residue by a cysteine residue (see Discussion).

Activities of Mutant OMPLA's with Substitutions of Ser152. Substitution of Ser152 by Ala, Val, Phe, or Cys abolished all activity (Table 1). Again, the lack of enzymatic activity was not caused by a drastically reduced expression level (Figure 2). However, the mutations S152T and S152N scored positive, both in the *in vivo* plate assay and in the chromogenic assay (Table 1). Assuming identical expression levels, the S152T mutant OMPLA had approximately 42% residual activity compared with the wild-type enzyme, whereas the activity of the S152N mutant OMPLA was just above the background level. In order to measure their specific activities more accurately, the S152T and S152N mutant OMPLA's, as well as the S152C mutant OMPLA, were purified. The purity of all mutant OMPLA's was

analyzed by SDS−PAGE (Figure 3). Densitometric tracings showed that more than 90% of the protein in each sample was in the folded form. For all three mutant proteins, the specific activity was determined (Table 1). The S152T and S152N OMPLA's were clearly active, whereas the nearly isosteric S152C mutation virtually abolished activity. This observation raises the question which factors determine the effect of mutations at position 152. Steric hindrance may certainly play a role, especially for substitutions such as S152F, but the observation that S152C is inactive whereas S152T is active suggests that other factors may be more important. The latter conclusion is endorsed by the observation that the activities of the S152T and S152V mutant proteins, which contain nearly isosteric substitutions, differ widely. Thus, the presence of a hydroxyl group, capable of hydrogen bond formation, seems to be crucial for activity. Although the asparagine side chain can form strong hydrogen bonds, this side chain is longer than the serine side chain which might disturb the hydrogen bonding capacities and explain the low activity of this mutant. Apparently, the capacity of the sulfhydryl group in the S152C mutant to form a hydrogen bond is too low to support activity. We conclude that serine 152 has a structural role.

DISCUSSION

Previously, two serine residues have been identified that are important for enzymatic activity of OMPLA. Ser144 was identified as the catalytic center residue after modification of OMPLA with the inhibitor hexadecylsulfonyl fluoride (Horrevoets *et al.*, 1991). The second serine residue (Ser152) was identified after the finding of a phenylalanine residue at this position in the protein of a phospholipase-negative mutant strain (Brok *et al.*, 1994).

With one exception, *i.e.*, S144C mutant OMPLA, the mutant proteins with a substitution of Ser144 lacked enzymatic activity. The presence of residual activity in mutant serine hydrolases, where the catalytic center serine residue has been replaced by a cysteine residue, is not uncommon. Examples are subtilisin (Neet & Koshland, 1966), trypsin (Higaki *et al.*, 1989), acetylcholinesterase (Gibney *et al.*, 1990), rat mammary gland thioesterase II (Witkowski *et al.*, 1992), and F_1-ATPase of *E. coli* (Lee *et al.*, 1992), which had residual esterolytic activities ranging from 1% to 90% after replacement of the catalytic center serine residue by a cysteine residue. Therefore, the site-specific mutagenesis experiments described in this study support the identity of Ser144 as the nucleophile in catalysis.

Whereas the S144C mutant was scored negative in the *in vivo* assay, this mutant displayed residual activity in the *in vitro* chromogenic assay. After purification, the S144C mutant had 0.9% residual activity relative to wild type. It appears that activities lower than 1% are not detectable in the *in vivo* plate assay. We have studied whether the S144C mutation has reinforced the reported ability of OMPLA to use alcohols as acyl acceptor during phospholipid degradation (Doi *et al.*, 1972; Horrevoets *et al.*, 1991). Both for the serine hydrolase thioesterase II and for subtilisin, it has indeed been reported that replacement of the active site serine by a cysteine residue converts these enzymes from hydrolases into acyltransferases (Witkowski *et al.*, 1994; Chang *et al.*, 1994). However, since the measured activities of the mutant S144C OMPLA in the chromogenic assay were the same in the presence or absence of ethanol (data not shown), we conclude that the residual activity of S144C mutant OMPLA is not a result of increased acyltransferase activity.

The side chain of the amino acid residue present at position 152 has a major effect on OMPLA activity. Replacement of the Ser152 residue either by the bulky phenylalanine residue or by the smaller alanine residue resulted in complete loss of activity, even though overall folding and outer membrane assembly of these mutant OMPLA's appeared normal. Therefore, this loss of activity may be caused by a local distortion of the structure. Since the S152T mutant OMPLA has 40% residual activity compared with the wild-type level, it is clear that such a local distortion of the structure is not merely due to the size of the side chain. In this S152T mutant protein, like in the wild-type OMPLA, a hydroxyl group is present. The importance of this hydroxyl group is indicated by the fact that the isosteric substitution of threonine by valine on this position abolished enzymatic activity. Whereas threonine can form similar hydrogen bonds as serine, this is not the case with the asparagine side chain, which is longer. Consequently, a possible explanation for the low activity of the S152N mutant OMPLA could be distorted hydrogen bond foundation on this position. Therefore, our conclusion is that a hydrogen bond at position 152 is important for enzymatic activity.

The presence of hydroxyl amino acids, other than the active site serine, that are important for activity is not unique for OMPLA. In the lipase from *Penicillium camembertii* U-150, a serine was identified that may serve a similar role. Substitution of this serine by a glycine resulted in an inactive lipase (Yamaguchi *et al.*, 1992). *Rhizopus delamar* lipase contains a threonine that can be replaced by a serine, yielding a highly active enzyme, whereas substitution by alanine yields virtually inactive enzyme (Joerger & Haas, 1994). Thus, the presence of serines or threonines forming hydrogen bonds that are essential for activity might be a common feature of several serine hydrolases.

What could be the function of such a hydrogen bond? To answer this question, it is relevant to point out that in nearly all known serine protease structures (Meyer, 1992) a second serine residue has been identified that is hydrogen-bonded to the catalytic center aspartic acid. Moreover, in *Geotrichum candidum* lipase (Schrag & Cygler, 1993), where a Glu is present in the catalytic center instead of an Asp, such a serine residue has been identified as well. Although in these cases the hydrogen bond has been suggested to be important for the correct positioning of the acidic residue in the catalytic center, the relevance of the additional serine residue is not well understood, since in trypsin and in α-lytic protease replacement of this serine residue by an alanine yielded active enzyme (McGrath *et al.*, 1992; Epstein & Abeles, 1992). In OMPLA, Ser152 might serve a similar role as in the proteases by positioning a carboxylate group in the catalytic center (Figure 4B). On the other hand, unlike in the proteases described above, the Ser to Ala substitution in OMPLA blocks all activity, stressing the need for the presence of a hydrogen bond on position 152 for enzymatic activity. Hence, Ser152 might serve an alternative role, which is depicted in Figure 4C. We suggest that in OMPLA, Ser152 participates in the oxyanion hole. For several serine hydrolases, it has been suggested that backbone amides and conserved serine residues form the oxyanion hole, which is involved in stabilizing the tetrahedral intermediates that occur

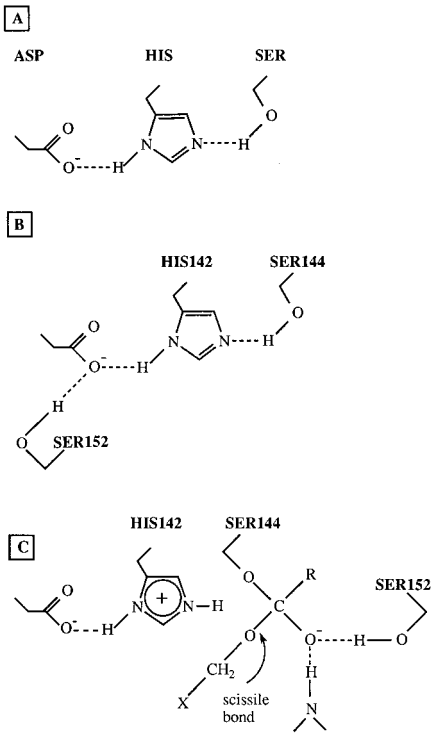

FIGURE 4: Model for possible hydrogen bonding patterns between catalytic center residues. (A) Generally accepted model for the catalytic center of serine hydrolases. Although the carboxylate is mostly donated by aspartic acid, a glutamic acid has been found in some cases (see text). In panels B and C, two possible roles of Ser152 of OMPLA are depicted. In both models, Ser144 (this study; Horrevoets *et al.*, 1991) and His142 (Brok *et al.*, 1995) represent active site residues of OMPLA. Moreover, the carboxylate of a yet unidentified Asp or Glu is proposed to play a similar role as the acid in the active site of serine hydrolases of known structure. In panel B, the role of Ser152 is to stabilize the carboxylate group. In panel C, a possible involvement of a backbone amide NH group and the hydroxyl function of Ser152 in the stabilization of the transition state is denoted. X represents the alcohol part of the substrate and R the acyl chain. Hydrogen bonds are denoted by dashes.

during the acylation and deacylation steps (Kraut, 1977; Derewenda, 1994; Jaeger *et al.*, 1994; Brzozowski *et al.*, 1991; Lawson *et al.*, 1994). This suggestion is endorsed by X-ray structures of lipases from the filamentous fungi *Rhizomucor miehei* (Brzozowski *et al.*, 1991) and *Humicola lanuginosa* (Lawson *et al.*, 1994) complexed with transition state inhibitors. Recently, a serine was identified in the lipolytic enzyme cutinase, which is directly involved in the stabilization of the oxyanion transition state (Nicolas *et al.*, 1996). The S42A mutation resulted in a drastic decrease in the activity (450-fold) without significant perturbation of the three-dimensional structure.

The final assessment of the catalytic center architecture of OMPLA has to await the X-ray structure analysis of this enzyme. In this respect, the S144C mutant OMPLA described in the present study may be helpful to create heavy-atom derivatives. The X-ray structure of an OMPLA−inhibitor complex will also be needed for the identification of residues that form the oxyanion hole.

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