
Structural investigations of the active-site mutant Asn156Ala of outer membrane phospholipase A: Function of the Asn–His interaction in the catalytic triad

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Abstract

Outer membrane phospholipase A (OMPLA) from *Escherichia coli* is an integral-membrane enzyme with a unique His–Ser–Asn catalytic triad. In serine proteases and serine esterases usually an Asp occurs in the catalytic triad; its role has been the subject of much debate. Here the role of the uncharged asparagine in the active site of OMPLA is investigated by structural characterization of the Asn156Ala mutant. Asparagine 156 is not involved in maintaining the overall active-site configuration and does not contribute significantly to the thermal stability of OMPLA. The active-site histidine retains an active conformation in the mutant notwithstanding the loss of the hydrogen bond to the asparagine side chain. Instead, stabilization of the correct tautomeric form of the histidine can account for the observed decrease in activity of the Asn156Ala mutant.

Keywords: Phospholipase; serine hydrolase; catalytic triad; active-site mutant; membrane protein; X-ray crystal structure; low-barrier hydrogen bond; histidine tautomer

Outer membrane phospholipase A (OMPLA; EC 3.1.1.32) is an integral-membrane enzyme present in the outer membrane of many Gram-negative bacteria. It catalyzes the hydrolysis of acyl ester bonds in phospholipids and lyso-phospholipids (Scandella and Kornberg 1971). The enzyme re-

quires calcium for activity, and depletion of Ca²⁺ renders the enzyme inactive (Nishijima et al. 1977; Horrevoets et al. 1989; Snijder et al. 2001). The activity of OMPLA is regulated by reversible dimerization in a calcium- and substrate-dependent fashion (Dekker et al. 1997, 1999; Ubarretxena-Belandia et al. 1999). Dimerization leads to the formation of productive substrate-binding pockets and facilitates binding of calcium in the active site (Snijder et al. 1999). In *Escherichia coli* the enzyme has a role in colicin secretion (Pugsley and Schwartz 1984; Van Der Wal et al. 1995). In *Campylobacter coli* and *Helicobacter pylori* OMPLA has been implicated in virulence and pathogenicity (Grant et al. 1997; Dorrell et al. 1999).

E. coli OMPLA has a 12-stranded antiparallel β -barrel fold with the active site located at the exterior of the barrel (Snijder et al. 1999). The catalytic residues, His142, Ser144,

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Abbreviations: Bis-Tris, bis-(2-hydroxyethyl)imino-tris hydroxymethylmethane; MPD, 2-methyl-2,4-pentanediol; OMPLA, outer membrane phospholipase A; LBHB, low-barrier hydrogen bond; β -OG, 1-O-*n*-octyl- β -D-glucopyranoside; Tris, tris(hydroxy-methyl)aminomethane; CD, circular dichroism.

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and Asn156, are arranged in a serine hydrolase-like constellation (Horrevoets et al. 1991; Brok et al. 1995, 1996; Snijder et al. 1999). The asparagine residue in this catalytic triad is unique among serine hydrolases, where commonly an Asp or Glu residue is found instead. In OMPLA, substitution of the Asn for an aspartate or a glutamine resulted in a 2-fold and a 40-fold decrease in activity, respectively (Kingma et al. 2000). Removal of the asparagine's functional moiety by an Asn156Ala mutation resulted in a 20-fold rate reduction, demonstrating that although the asparagine residue contributes to catalysis, it does not have an essential role (Kingma et al. 2000). In contrast, mutation of the catalytic aspartate in dipeptidyl-peptidase IV and subtilisin resulted in much more dramatic decreases in activity, 500-fold and 10^4 -fold, respectively (Carter and Wells 1988; David et al. 1993). A similar substitution in lipoprotein lipase completely abolished all activity (Faustinella et al. 1992).

To understand the role of the asparagine residue in the catalytic triad of OMPLA, we have determined crystal structures of the Asn156Ala mutant at various pHs and analyzed the thermal stability of wild-type and mutant protein. The structures show that no structural rearrangements or solvent molecules compensate for the loss of the stabilizing hydrogen bond between Asn156 and the catalytic histidine. Despite the loss of this hydrogen bond, the catalytic histidine retains its wild-type conformation. Instead, the catalytic importance of the asparagine can be explained by its ability to stabilize the correct tautomeric form of the histidine.

Results

3D structure of the mutant

The Asn156Ala mutant (pH 6.1) has an overall fold similar to that of the native protein (Fig. 1). The r.m.s. coordinate

difference between wild-type and mutant protein is as small as 0.3 Å for the common C $_{\alpha}$ atoms, and equals the estimated coordinate error in both structures (see Tables 1 and 2 for all statistics; Read 1986). The small r.m.s. coordinate difference of 0.65 Å for all 2090 common atoms stresses the overall similarity. The largest differences occur in loop regions and at the N terminus (Fig. 2) and correlate with the high crystallographic *B*-factors of these parts of the protein.

The active site of the mutant undergoes little structural rearrangement, owing to the rigid scaffold of the β -barrel to which the active-site residues are tethered. The mutated residue 156 lacks electron density after the C $_{\beta}$ atom, corroborating the successful mutation of this residue from an asparagine into an alanine (Fig. 3A). The mutation has not affected the position and orientation of nucleophilic serine 144. The conformation of histidine 142, however, has subtly changed. Its side chain has rotated $\sim 10^{\circ}$ about χ_1 and $\sim 40^{\circ}$ about χ_2 , away from Ser144, and occupies some of the space created by the mutation (Fig. 3B). As a result, the distance between the nucleophilic serine O $_{\gamma}$ atom and the histidine N $_{\epsilon}$ is increased from ~ 3.3 Å to 3.9 Å. However, this active-site constellation is similar to that observed in the inhibited dimeric structure (Fig. 3C), which we believe to be a catalytically relevant arrangement (Snijder et al. 1999).

Besides the movement of the imidazole ring, the space created by the mutation is partly filled by two water molecules. The first water molecule forms two hydrogen bonds, one with the hydroxyl group of tyrosine 140 and the other with the second water molecule. This second water molecule makes an additional hydrogen bond to the backbone amide of residue 181 in loop L4. None of these water molecules is at hydrogen-bonding distance from the N $_{\delta}$ atom of the histidine imidazole ring. Neither are other solvent molecules or protein atoms contacting His142. Although the hydroxyl group of tyrosine 140 has moved toward the imidazole side chain, it does not form a hydrogen bond as judged from the inadequate angle geometry and the large

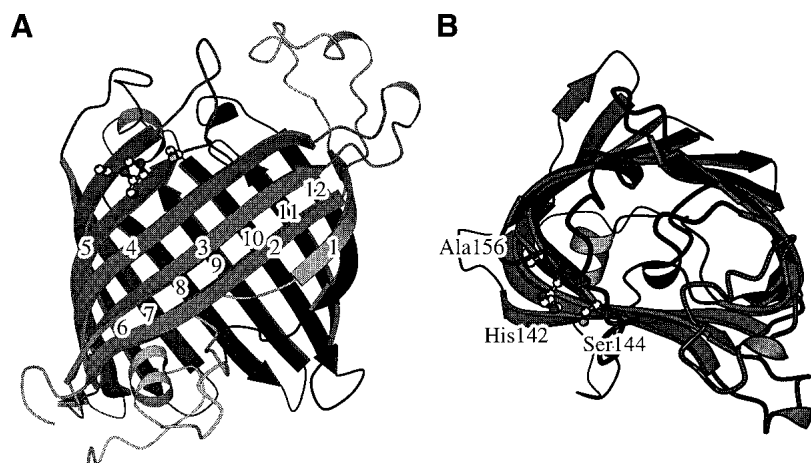


Fig. 1. Two orthogonal views of the structure of the Asn156Ala mutant of OMPLA at pH 8.3. β -strands are shown as arrows, α -helices as spirals, and active-site residues are shown in ball-and-stick format. The β -strands are numbered in the left panel; the active-site residues are named in the right panel. The figure was prepared using MOLSCRIPT (Kraulis 1991) and Raster3D (Merritt and Bacon 1997).

Table 1. Data collection statistics for the Asn156Ala mutant structures of *E. coli* OMPLA

Data set		pH 4.6	pH 6.1	pH 8.3
Wavelength	(Å)	1.5418	1.00	1.5418
Resolution	(Å)	20–2.8	31–2.5	21–2.98
		(2.85–2.8)	(2.54–2.5)	(3.03–2.98)
Cell dimensions	(Å) a	78.48	78.17	78.46
	(Å) b	78.48	78.17	78.46
	(Å) c	101.67	101.68	101.45
Observed reflections		98747	150605	106289
Unique reflections		9231 (453)	12728 (611)	7686 (372)
Completeness	(%)	99.3 (99.6)	98.8 (96.1)	99.6 (100.0)
R_{sym}^a	(%)	6.4 (34.3)	3.7 (11.3)	8.1 (34.0)
$\langle I \rangle / \langle \sigma \rangle$		25.4 (4.9)	39.2 (8.3)	24.5 (7.9)

The values in brackets present the highest resolution bins. The crystals belong to the trigonal spacegroup $P3_121$.

^a R_{sym} defined as $\sum_{hkl} \sum_i |I_i(hkl)| - \langle I(hkl) \rangle / \sum_{hkl} \sum_i I_i(hkl) * 100\%$.

distance. Thus, as a result of the mutation of Asn156, the histidine imidazole group has lost its hydrogen bond partner.

Heat-induced unfolding of OMPLA

To study the importance of the His–Asn interaction for protein stability, the heat-induced unfolding of wild-type and Asn156Ala OMPLA was followed using circular dichroism (CD). The CD spectra of both species show a strong negative signal at 219 nm and a positive signal at 195 nm (Fig. 4A), typical of β -sheet-containing proteins (OMPLA has 59.5% β -strand and 5.5% α -helix). Both wild-type and Asn156Ala OMPLA show an irreversible thermal transition from 83° to 95°C (Fig. 4B). The melting temperatures T_m , the temperature at the midpoint of the transition curves, are 91.5°C and 90°C for wild-type and Asn156Ala, respectively.

Influence of the pH

The mutant has a pH-activity profile with a broad optimum at pH 8.3. At acidic pH (around pH 4) the activity is virtu-

Table 2. Refinement statistics

Data set	pH 4.6	pH 6.1	pH 8.3
Resolution (Å)	20–2.8	31–2.5	21–2.98
R_{crys}^a (%)	21.1	22.2	22.6
R_{free}^b (%)	27.5	26.8	26.6
Protein residues	13–269	13–269	8–269
Protein atoms	2091	2091	2128
Non protein atoms	139	132	109
R.m.s.d. for			
Bond lengths (Å)	0.007	0.006	0.007
Bond angles (°)	1.5	1.2	1.5
Dihedral angles (°)	25.6	25.7	25.9
Average B -factor (Å ²)	49	53	46

^a $R_{\text{crys}} = \sum_{hkl, \text{work}} \|F_{\text{obs}}| - |k|F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}| * 100\%$.

^b $R_{\text{free}} = \sum_{hkl, \text{test}} \|F_{\text{obs}}| - |k|F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}| * 100\%$.

ally zero. In the very basic pH region (pH > 10) the activity levels off to ~75% of that at pH 8.3 (Kingma et al. 2000). We have investigated the pH dependence by examining crystal structures at three different pHs, at pH 4.6, 6.1, and 8.3, respectively. The crystal structures at pH 4.6 and pH 8.3 are very similar to the structure at pH 6.1. The r.m.s coordinate differences are all within the estimated coordinate error. Nevertheless, the decrease in distance between the Ser144 O_γ and the His142 N_ε atoms from ~4.0 Å at pH 4.6, via 3.9 Å at pH 6.1, to ~3.7 Å at pH 8.3 may be related to the deprotonation of the imidazole side chain at higher pH.

Interestingly, at pH 8.3 we observed interpretable density for residues 8–12. At the lower pH used previously (pH 6.1), only clear electron density was observed starting from residue 13. At pH 8.3 a stabilizing interaction is made possible between the His9 side chain and the main-chain peptide nitrogen atom of Asn85, most likely because His9 becomes deprotonated at this pH. Residues 8–12 form a short loop at the periplasmic side of the protein surface that points toward the center of the β -barrel. In our electron density maps we found no indication where residues 1–7 could be located, but electron microscopy projection maps of OMPLA at pH 7.5 indicate that they protrude away from the barrel (Boekema et al. 1998). The orientation of these residues away from the dimerization interface of the β -barrel domain indicates that they will neither conflict with nor be involved in dimerization.

Discussion

OMPLA is a phospholipase with a serine hydrolase-like catalytic triad. Instead of the usual carboxylic acid residue, OMPLA has a neutral asparagine in the triad together with a histidine and a serine. Although serine hydrolases have been reported with a neutral main-chain carbonyl oxygen atom (Wei et al. 1995) or a histidine residue (Chen et al. 1996; Qiu et al. 1996, 1997), the occurrence of an asparagine in a serine triad has never before been observed.

In OMPLA mutation of the active-site asparagine to an alanine resulted in a modest 20-fold reduction in the reaction rate (Kingma et al. 2000). A similar substitution in the cysteine protease papain, which contains a Cys–His–Asn catalytic triad, resulted in a decrease of the activity by two orders of magnitude (Vernet et al. 1995). For dipeptidyl-peptidase IV and subtilisin, such an Asp → Ala substitution decreased the activity even 500-fold and 10⁴-fold, respectively (Carter and Wells 1988; David et al. 1993), whereas in lipoprotein lipase it completely abolished all activity (Faustinella et al. 1992). Therefore, Asn156 in OMPLA is less strictly required for catalysis than the Asp/Asn in other hydrolases. Although the functions of the catalytic serine and histidine have been established beyond doubt as nucleophile and general base, respectively (Dodson and Wlodawer 1998), the role of the third residue in the triad is less clear.

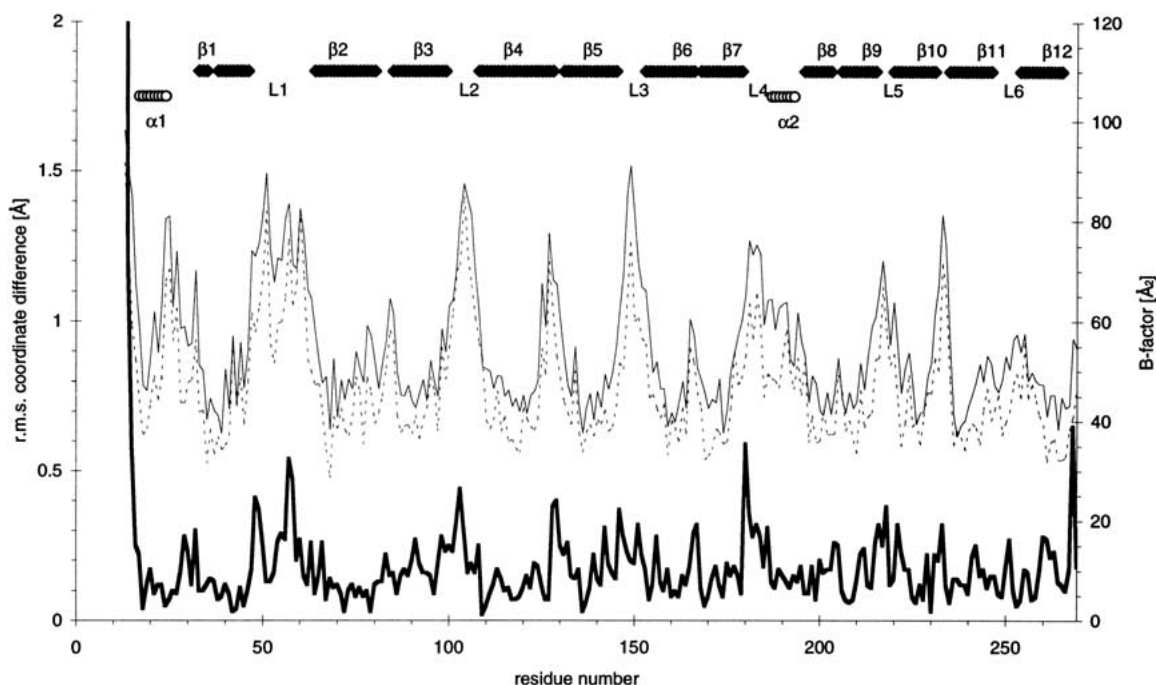


Fig. 2. Coordinate differences between Asn156Ala and wild-type OMPLA. The coordinate difference of the common C_{α} atoms (dark solid line); B values for mutant OMPLA (thin solid line) and wild-type OMPLA (dashed line). Secondary structure elements for residues in α -helices (open circles) and for residues in β -strands (filled diamonds).

Five roles have been proposed: (1) orientation of the histidine side chain, (2) structural stabilization, (3) activation of the histidine and stabilization of the transient positive charge on the histidine, (4) formation of a low-barrier hydrogen bond (LBHB), and (5) stabilization of the catalytic competent tautomer of the histidine.

Orientation of the histidine side chain

The correct positioning of the catalytic histidine in the active sites of serine and cysteine hydrolases is crucial for enzymatic catalysis. Disruption of the spatial arrangement of the His and Ser/Cys residues leads to inactivation (Sprang et al. 1987; Mhashilkar et al. 1993; Kagawa et al. 2000). However, in OMPLA the position of the catalytic histidine is largely unaffected by the Asn156Ala mutation; the histidine keeps its favorable *trans* conformation. The two other favorable conformations, namely, the *gauche*⁺ and the *gauche*⁻, are effectively blocked by the bulky side chain of tyrosine 140 and by the invariant proline 116, respectively. Therefore, in OMPLA, Asn156 does not contribute to a large extent to the correct positioning of the catalytic histidine in the active site. This is in contrast to trypsin, where the active-site histidine has an unfavorable conformation ($\chi_1 = 92^\circ$), and where mutation of the catalytic triad Asp results in the partitioning of the His between the native conformation and an alternative *trans* conformation (Sprang et al. 1987).

Structural stabilization

The His–Asp interaction (or His–Asn in papain) has been shown to be an important determinant for structural stability (Vernet et al. 1995; Quirk et al. 1998; Lau and Bruice 1999). Indeed, the His–Asn interaction in OMPLA is, to some extent, involved in structural stability, as illustrated by the slightly lower melting temperature of Asn156Ala OMPLA. The decrease in melting temperature is, however, much less than, for instance, for the Asp121Ala substitution in ribonuclease A (6°C; Quirk et al. 1998). The high melting temperatures of >90°C indicate the extraordinary stability of both the wild-type and Asn156Ala proteins. This exceptional thermal stability is a property of outer membrane β -barrel proteins in general (Heller 1978), wherein the many main-chain interactions between adjacent β -strands and the packing of side chains inside the β -barrel and the periplasmic turns are the principal factors for thermal stability (Koebnik 1999). As the loss of the Asn–His interaction in OMPLA has left the β -barrel and the periplasmic turns unperturbed, the paramount determinants for thermal stability are maintained, thus explaining the minor effect of the Asn156Ala mutation on the enzyme's thermostability.

Activation of the histidine and stabilization of the transient positive charge on the histidine

The acidic group in classical serine hydrolases functions in increasing the pK_a of the histidine, thereby increasing the

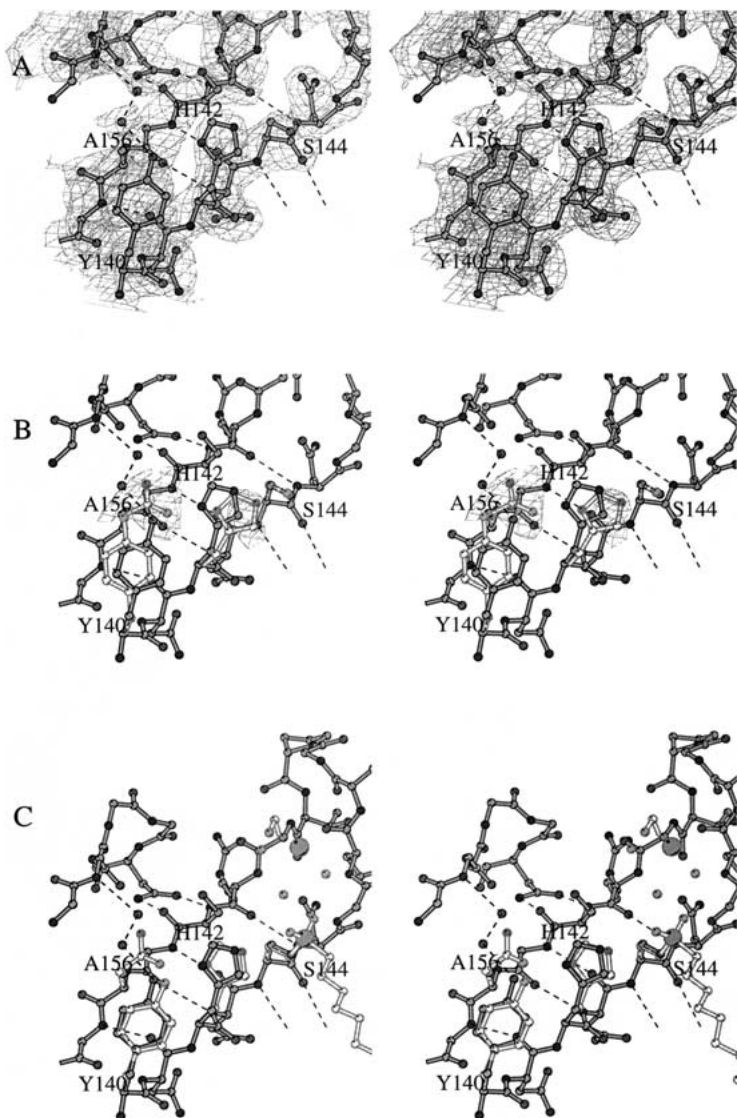


Fig. 3. Stereo diagrams of the active site of the Asn156Ala mutant at pH 6.1. (A) Final σ_A -weighted electron density of the active site. (B) Superimposition of Asn156Ala and wild-type monomeric OMPLA active sites. The wild-type active-site residues are depicted in light gray. Negative ($F_{\text{obs}(\text{mutant})} - F_{\text{obs}(\text{native})}$) difference electron density is shown in light gray; the contour level is -5σ . The two largest features in this difference density illustrate the successful mutation Asn156Ala and indicate the rotation of His142. (C) Superimposition of Asn156Ala and wild-type inhibited dimeric OMPLA. The wild-type active-site residues, the hexadecanesulfonyl inhibitor, and the catalytic calcium are depicted in light gray. Note that for some of the non-active-site residues the side chains have been omitted for clarity. This figure was constructed with BOBSCRIPT (Esnouf 1997).

nucleophilicity of the serine (Dodson and Wlodawer 1998). An Ala or Asn residue instead of an Asp may therefore compromise catalytic efficiency through a decrease in nucleophilicity of the serine. Conversely, the Asn156Asp mutant of OMPLA is significantly more active (although only at elevated pH; Kingma et al. 2000). Nevertheless, as wild-type OMPLA efficiently hydrolyzes phospholipids (with a specific activity of $\sim 40\text{--}50 \text{ sec}^{-1}$; Brok et al. 1996; Dekker et al. 1997), other factors must make up for a lowered pK_a of the active-site histidine and the accordingly less nucleophilic serine. We suggest that the nearby calcium ion in the active site of dimeric OMPLA contributes to competent catalysis (Snijder et al. 1999). The positive charge of the calcium, which is not compensated by its ligands, may directly increase the nucleophilicity of the active-site serine. Furthermore, mediated by water molecules, the calcium ion enhances the polarization of the carbonyl bond of the ester

substrate, thus creating a more electrophilic substrate center. In addition, in concert with three hydrogen-bond donors, the calcium ion stabilizes the oxyanion intermediates formed during the reaction (oxyanion hole). The increased polarization of the carbonyl function reduces the need for a strongly nucleophilic serine, and this may explain why OMPLA tolerates an asparagine and even an alanine residue in its catalytic triad. The same considerations are valid for the breakdown of the enzyme–acyl intermediate by the hydrolytic water molecule.

Formation of a low-barrier hydrogen bond (LBHB)

A much debated role of the Asp–His couple in serine proteases is the possible formation of a short and exceptionally strong hydrogen bond (low-barrier hydrogen bond, LBHB)

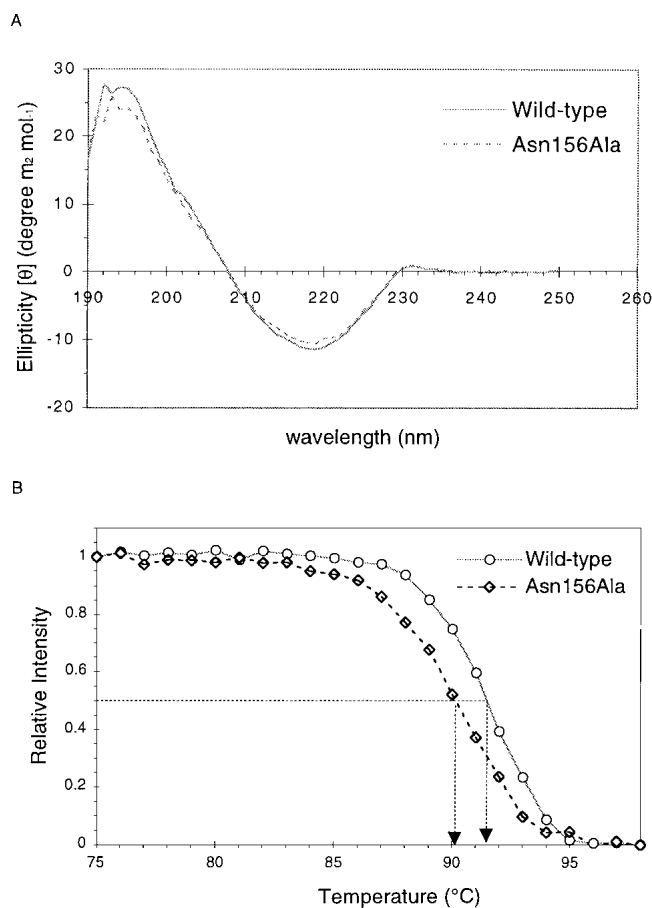


Fig. 4. Circular dichroism (CD) studies of OMPLA. (A) The CD spectra show a typical β -sheet spectrum with a minimum at 219 nm. The spectra are background and base-line corrected. (B) Heat-induced unfolding of OMPLA monitored by the CD signal at 219 nm. The difference in melting temperatures between mutant and wild-type protein is reproducible within 0.1°C.

during the transition state. It was proposed that such an LBHB could lower the energy of the transition state by as much as 20 kcal/mole, thereby facilitating efficient catalysis (Cleland and Kreevoy 1994; Frey et al. 1994). However, the existence and involvement of LBHBs in enzyme catalysis have been seriously questioned (Warshel et al. 1995; Ash et al. 1997). In the solvent-exposed active site of OMPLA, the presence of an LBHB between Asn156 and His142 is impossible, because the formation of an LBHB requires the absence of H-bonding solvents and matching of the pK_a values for the donor and acceptor (Cleland and Kreevoy 1994; Frey et al. 1994). Neither criterion is met, and OMPLA therefore presents an example of a serine hydrolase that functions efficiently without the need of an LBHB. Because a peptide bond is intrinsically less reactive than an ester bond, the activity of serine proteases might still require a strong hydrogen bond in the active site.

Stabilization of the catalytic competent tautomer of the histidine

Finally, the third residue of the triad selects the correct histidine tautomer to accept the serine hydroxyl proton during catalysis. In basic aqueous solutions, the N_ϵ -H tautomer dominates the imidazole form in free L-histidine, histidine derivatives, oligopeptides and polypeptides (Reynolds et al. 1973; Deslauriers et al. 1974), and proteins (Wilbur and Allerhand 1977; Bhattacharya et al. 1997). In Asn156Ala OMPLA, the His142 side chain is solvent-exposed and lacks specific protein and solvent interactions. Although the exact tautomeric state of His142 cannot be determined at the current resolution (2.5–3.0 Å), it is likely that in this mutant the His142 imidazole is substantially protonated on its N_ϵ atom, and cannot accept a proton from the nucleophilic serine. In wild-type enzyme the hydrogen-bond interaction of His142 with the carboxamide oxygen of Asn156 may shift the tautomeric equilibrium toward the catalytic competent form with the N_ϵ atom unprotonated. This is similar to what has been observed for other serine esterases (Sprang et al. 1987; Dodson and Wlodawer 1998). From the five roles proposed for the Asn/Asp residue in the classical catalytic triad, four functions (positioning of the histidine, contributing to structural stability, activation of the histidine, and formation of a LBHB) are hardly significant for OMPLA. Therefore, the catalytic importance of Asn156 in OMPLA seems mainly to stem from its role in stabilization of the correct tautomeric form of the active-site histidine. In α -lytic protease the Asp-His couple increases the amount of the correct histidine tautomer approximately 24-fold (Bachovchin and Roberts 1978). This change is of the same order as the decrease in activity observed for the Asn156Ala mutant, showing that, indeed, the lack of tautomer stabilization may account for the observed decrease in activity.

Materials and methods

The overexpression, refolding, and purification of the Asn156Ala mutant has been described earlier (Kingma et al. 2000). The mutant OMPLA was crystallized following a procedure similar to that of wild-type OMPLA (Blaauw et al. 1995). Hanging drops consisting of 3 μ L of protein solution and 2 μ L of reservoir solution were suspended over a 1-mL reservoir containing 27% (v/v) 2-methyl-2,4-pentanediol (MPD), 0.4–1.0 mM CaCl_2 , and 0.1 M Bis-Tris buffer at pH 6.0–6.3. The initial protein solution contained 10 mg/mL of Asn156Ala OMPLA, 10 mM KCl, 1% (w/v) 1-O-*n*-octyl- β -D-glucopyranoside (β -OG), and 0.2 mM Tris-HCl at pH 6.6. In these setups, one to six crystals grew to typical sizes of $0.5 \times 0.25 \times 0.15 \text{ mm}^3$ after 1 wk. Crystals were brought to pH 8.3 and pH 4.6 by 20-min soaks in four stabilizing mother liquors of increasing and decreasing pH, respectively.

Data were collected from cryocooled crystals at either the protein crystallography beamline 5.2 R of the ELETTRA synchrotron in Trieste (data set pH 6.1) using a 345 mm MarResearch image plate detector, or in-house with a Mac Science DIP2000 image

plate detector (Nonius) mounted on a Nonius FR591 rotating anode generator providing Cu K α radiation. All data were processed using DENZO, SCALEPACK (Otwinowski and Minor 1997), and TRUNCATE (French and Wilson 1978; Collaborative Computational Project Number 4 1994).

The structure of monomeric native OMPLA (pdb entry 1QD5; Snijder et al. 1999) without detergent and water molecules was used as a starting model for refinement. The active-site residues His142, Ser144, and Asn156 were replaced by alanine residues. The coordinates of all remaining atoms were given a random shift of maximally 0.5 Å using MOLEMAN (Kleywegt and Jones 1997) to reduce model bias. Refinement was performed with the CNS software suite (Brünger et al. 1998). A random set of 10% of the unique reflections was set apart to calculate a free *R*-factor (Brünger 1992). Care was taken to include all reflections in the free *R*-set that previously had been used for cross-validation of the refinement of native monomeric OMPLA. After rigid-body refinement, several rounds of positional and individual restrained *B*-factor refinement were performed, using maximum likelihood functions applied to amplitudes. Bulk solvent correction and anisotropic *B*-factor scaling were applied throughout the refinement. No σ cutoffs were used. After every refinement round, σ A-weighted $2F_{\text{obs}} - F_{\text{calc}}$ and $F_{\text{obs}} - F_{\text{calc}}$ electron density maps (Read 1986) were subjected to visual inspection using O (Jones et al. 1991). Water molecules were placed in spherical density, where there was substantial positive difference density. The side chains of the active-site residues His142 and Ser144 were added back to the model in the course of refinement, both showing clear electron density. Various ordered detergent and MPD molecules were identified and included in the models.

Heat-induced unfolding of wild-type and Asn156Ala OMPLA was studied by far-UV circular dichroism (CD). For these experiments 0.4 mg/mL protein was dialyzed for 6 d against a solution of 10 mM Tris-HCl at pH 6.6 and 1.1 % (w/v) β -OG. For the measurements the samples were diluted to a concentration of 0.1 mg/mL. All solutions were degassed prior to data collection. The CD experiments were performed on an Aviv 62A DS spectrometer equipped with a thermoelectric sample holder. Data were recorded in a 1-mm path length cell. Temperature scans between 25° and 98° C were performed in 1°C temperature steps with 30 sec of equilibration time per step. The unfolding of OMPLA was monitored by measuring the average CD signal at 219 nm for 20 sec.

The structures and experimental data of the Asn156Ala mutant of OMPLA have been deposited with the Protein Data Bank with accession codes 1ILD (pH 4.6), 1ILZ (pH 6.1), and 1IM0 (pH 8.3).

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