THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 24, pp. 17729–17737, June 15, 2007

Key Role of Amino Acid Residues in the Dimerization and Catalytic Activation of the Autolysin LytA, an Important Virulence Factor in *Streptococcus pneumoniae*[*]

Received for publication, December 26, 2006, and in revised form, February 22, 2007 Published, JBC Papers in Press, April 16, 2007, DOI 10.1074/jbc.M611795200

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LytA, the main autolysin of *Streptococcus pneumoniae*, was the first member of the bacterial *N*-acetylmuramoyl-L-alanine amidase (NAM-amidase) family of proteins to be well characterized. This autolysin degrades the peptidoglycan bonds of pneumococcal cell walls after anchoring to the choline residues of the cell wall teichoic acids via its choline-binding module (ChBM). The latter is composed of seven repeats (ChBRs) of ~20 amino acid residues. The translation product of the *lytA* gene is the low-activity E-form of LytA (a monomer), which can be "converted" (activated) *in vitro* by choline into the fully active C-form at low temperature. The C-form is a homodimer with a boomerang-like shape. To study the structural requirements for the monomer-to-dimer modification and to clarify whether "conversion" is synonymous with dimerization, the biochemical consequences of replacing four key amino acid residues of ChBR6 and ChBR7 (the repeats involved in dimer formation) were determined. The results obtained with a collection of 21 mutated NAM-amidases indicate that Ile-315 is a key amino acid residue in both LytA activity and folding. Amino acids with a marginal position in the solenoid structure of the ChBM were of minor influence in dimer stability; neither the size, polarity, nor aromatic nature of the replacement amino acids affected LytA activity. In contrast, truncated proteins were drastically impaired in their activity and conversion capacity. The results indicate that dimerization and conversion are different processes, but they do not answer the questions of whether conversion can only be achieved after a dimer formation step.

Streptococcus pneumoniae (pneumococcus) is a major human pathogen and the leading cause of pneumonia, bacteremia, and meningitis in adults, and of otitis media in children. The casualties due to pneumococcus are estimated to be over 1.6 million deaths per year, and most of these deaths occur in young children in developing countries (1).

Choline-binding proteins (ChBPs)[2] are among the most well known surface proteins of this bacterium. Ten ChBPs have been identified in R6 (2), a laboratory avirulent strain of *S. pneumoniae*, and in its parental strain D39 (3) in contrast with the 15 ChBPs that have been identified in TIGR4, a virulent isolate (4). Choline, which is essential for normal pneumococcal growth (5, 6), is a structural component of teichoic and lipoteichoic acids and serves to anchor ChBPs to the surface of *S. pneumoniae* (7, 8). Those ChBPs that hydrolyze the cell wall, such as the major autolysin LytA, also require the presence of choline-containing teichoic acids to initiate their activity (see Refs. 9 and 10 for recent reviews). These proteins are built by the fusion of different functional modules to a choline-binding module (ChBM) consisting of up to 18 repeats (ChBRs) of ~20 amino acid residues (10, 11).

LytA is a 36.5-kDa (318 amino acid residues) *N*-acetylmuramoyl-L-alanine amidase (NAM-amidase; EC 3.5.1.28) and is thought to be a major virulence factor in pneumococcus (12). The translation product of the *lytA* gene is the low-activity form (E-form) of the enzyme, which can be activated *in vitro* to the fully active form (C-form) in the presence of choline (or choline-containing pneumococcal cell walls) at low temperature (13, 14). This phenomenon, known as "conversion," was first described in 1971 by Tomasz and Westphal (14). This process cannot be reversed by dialysis. Early results obtained by gel filtration and sucrose gradient centrifugation analyses indicated that the E-form of the enzyme is a monomer (15).

LytA is a modular protein made up of an N-terminal catalytic module and a C-terminal ChBM containing seven ChBRs (16) (it was originally reported to contain six ChBRs plus a short C-terminal "tail" (17, 18). Usobiaga *et al.* (19) first reported that, in the presence of choline, LytA forms dimers through the interaction of the ChBMs of two monomers. Crystallographic studies have shown that the ChBM of LytA (C-LytA) folds in the form of a solenoid consisting exclusively of β-hairpins that stack to form a left-handed superhelix; this structure is maintained by choline molecules at the hydrophobic interface between consecutive hairpins (20, 21). The last two ChBRs (ChBR6 and ChBR7) in the solenoid are responsible for the formation of the catalytically active homodimer. This finding is in agreement with early results showing that, when lacking the

[*] This work was supported by grants from the Dirección General de Investigación Científica y Técnica (BMC2003-00074 and SAF2006-00390). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: ChBP, choline-binding protein; ChBM, choline-binding module; ChBR, choline-binding repeat; NAM-amidase, *N*-acetylmuramoyl-L-alanine amidase; E-form, low-activity form of LytA; C-form, fully active form of LytA; Doc, sodium deoxycholate.

C-terminal hairpin, LytA suffers a significant reduction (>90%) in catalytic efficiency (22) and becomes monomeric (17). In addition, it has recently been reported to possess a high-affinity binding site for choline located between ChBR5 and ChBR6 that appears to be responsible for the unusual stability of the dimer, even in 7.4 M guanidinium chloride (23).

Early work by Díaz *et al.* (24) shows that the NAM-amidase Ejl encoded by prophage EJ-1, a member of the LytA-like family of proteins (25), could be deconverted by a short dialysis treatment (24), giving rise mostly to monomers (26). Further, it has been recently shown that the substitution of Val-317 of LytA (one of the key residues involved in the hydrophobic interactions between the ChBR7s on the different monomers (21) by threonine (V317T) renders a NAM-amidase that can be reversibly deconverted by dialysis (27).

In summary, in all studies performed to date, the conversion of the E-form to the C-form of the enzyme has always been associated with dimerization, resulting in greater enzyme activity, whereas deconverted NAM-amidases have typically been found to show low enzymatic activity and to exist in a monomeric state. However, it is unclear whether conversion is synonymous with dimerization or whether dimerization is a prerequisite followed by additional structural and/or functional modifications for conversion to be completed. To gain insight into the structural requirements for this monomer-to-dimer modification and/or the conversion process, we have determined the biochemical consequences of replacing key amino acid residues in the ChBR6 and ChBR7 of LytA.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The *S. pneumoniae* strains used were the laboratory strain R6 (*lytA*$^+$) (28) and the Δ*lytA* mutant M31 (29). *Escherichia coli* DH10B (Invitrogen) was used as a host for recombinant plasmids and for the overexpression of mutated LytA enzymes. *E. coli* RB791 (pGL100) overexpressing $lytA_{R6}$ has been described elsewhere (30). Plasmid pRG2, a pLS1 derivative that harbors a 1,213-bp HindIII fragment containing the *lytA* allele of the R6 strain ($lytA_{R6}$), replicates in *S. pneumoniae* and has been previously described (31). Plasmid pIN-III (*lpp*^{p}*-5*)-A3 (32) was used as vector to achieve this overexpression. The construction of *E. coli* DH10B (pIN-lytA$_{V317T}$) (formerly known as pIN-lytA$_{R6(T)}$) overexpressing the mutated NAM-amidase LytA$_{V317T}$ has been previously described (27). *E. coli* was grown in Luria-Bertani (LB) medium (33) and pneumococci in Todd-Hewitt broth supplemented with 0.5% yeast extract or in C medium (34) supplemented with 0.08% of both yeast extract and bovine serum albumin. The procedures for genetic transformation of *S. pneumoniae* (24) and *E. coli* (33) have been previously described. Selection for pneumococcal or *E. coli* transformants was carried out using tetracycline (1 μg/ml) or ampicillin (100 μg/ml), respectively.

DNA Manipulations, PCR Amplification, Cloning, Nucleotide Sequencing, Plasmid Construction, and Site-directed Mutagenesis—Routine DNA manipulations were performed essentially as previously described (33). DNA fragments were purified using the High Pure PCR product purification kit (Roche Applied Science). The nucleotide sequence was determined using a PCR cycle sequencing method (BigDye™ Terminator v3.1 cycle sequencing kit) and employing an automated Abi Prism 3700™ DNA sequencer (Applied Biosystems). All primers for PCR amplification and nucleotide sequencing were synthesized in a Beckman Oligo 1000M synthesizer.

Mutations in Leu-314, Ile-315, and Val-317 were introduced into $lytA_{R6}$ by site-directed mutagenesis. For this, the $lytA_{R6}$ gene (from pGL100) was PCR-amplified using oligonucleotide lytA$_{100}$/X (containing an XbaI restriction site) (27) and a second oligonucleotide primer containing the required mutation and the BamHI restriction site (see Table 1). The amplification product was digested with XbaI and BamHI, ligated to pIN-III (*lpp*^{p}*-5*)-A3 previously treated with the same enzymes, and the ligation mixture used to transform *E. coli* DH10B. Clones harboring a recombinant pIN-lytA plasmid overexpressing the mutated NAM-amidases were selected from the ampicillin-resistant transformants. The accuracy of the construction was routinely checked by complete sequencing of the insert of the recombinant plasmid. Mutations in Tyr-294 or Pro-297 (see Table 1) were achieved using site-directed mutagenesis and overlap extension as described elsewhere (33). Mutant selection was performed as described above.

A recombinant plasmid (pRG2-2) expressing the LytA$_{V317T}$ NAM-amidase and able to replicate in *S. pneumoniae* was constructed as follows. Plasmid pRG2 was digested with HindIII, and the 4.3-kb fragment corresponding to the vector (pLS1) was isolated after electrophoresis in a 0.7% agarose gel. Afterward, this fragment was ligated to a HindIII fragment containing the $lytA_{V317T}$ allele that was amplified from pIN-lytA$_{V317T}$ using oligonucleotides lytA$_{V317T}$/H and lytA$_{100}$/H (Table 1). The ligation mixture was used to transform the M31 pneumococcal strain. Strain M51-T (M31 harboring pRG2-2) was selected among the tetracycline-resistant transformants using a filter technique previously described (35). The accuracy of the construction was confirmed by completely sequencing the insert of the recombinant plasmid.

Overproduction and Purification of NAM-amidases and Measurement of Enzyme Activity—NAM-amidase overproduction and purification were performed as previously described (27) with minor modifications. Briefly, *E. coli* DH10B cells harboring a recombinant plasmid encoding a mutant amidase were grown overnight with vigorous shaking at 37 °C in LB supplemented with ampicillin (100 μg/ml) and isopropyl-β-D-thiogalactopyranoside (50 μM). After centrifugation (10,000 × *g*, 10 min), the cells were suspended in 20 mM sodium phosphate NP buffer (20 mM sodium phosphate, pH 6.9) and broken open in a French pressure cell press, and the insoluble material obtained after centrifugation at 100,000 × *g* for 1 h at 4 °C was discarded. The supernatant was applied to a DEAE-cellulose column equilibrated in NP buffer and the contaminating proteins washed out using the same buffer containing 1.5 M NaCl. The amidases were eluted from the column with NP buffer containing 1.5 M NaCl and 2% choline chloride (36). Column fractions were analyzed by SDS-PAGE (37) and the LytA-containing fractions pooled and stored at −20 °C with or without a previous dialysis step involving NP buffer. Protein concentration was

TABLE 1
Oligonucleotides for performing SDM

Name	Sequence (5′ → 3′)[a]	Description/use[b]
$lytA_{100}$/X	GTTGTTTTAATTCTAGATAAGGAG	5′ of $lytA_{R6}$
$lytA_{100}$/B	GggATccTTATTTTACTGTAATCAAGCCATC	3′ of $lytA_{R6}$
$lytA_{V317T}$/B	GggaTCCATTATTATTTTGTTGTAATCAAGCC	3′ of $lytA_{V317T}$
$lytA_{100}$/H	GggaagcTTAATTCTAGATAAGGAGTAG	Construction of pRG2-2
$lytA_{V317T}$/H	cccaagcttATTATTATTTTGTTGTAATCAAGCC	Construction of pRG2-2
Y294.1a	GTACgAaCTCAAACCAGAC	Y294E
Y294.1b	GTCTGGTTTGAGtTcGTAC	Y294E
Y294.2a	GTACTtgCTCAAACCAGAC	Y294L
Y294.2b	GTCTGGTTTGAGcaAGTAC	Y294L
P297.1a	CTACCTCAAAggAGACGGAAC	P297G
P297.1b	GTTCCGTCTccTTTGAGGTAG	P297G
P297.2a	CTACCTCAAAgaAGACGGAAC	P297E
P297.2b	GTTCCGTCTtcTTTGAGGTAG	P297E
L314.1	GggaTCCATTATTTTACTGTAATt**ky**GCCATCTGG	L314K; L314A; L314T
L314.2	GggaTCCATTATTTTACTGTAATttcGCCATCTGG	L314E
I315.1	GggaTCCATTATTTTACTGT**wt**mCAAGCCATCTG	I315 E; I315Y; I315D; I315Stop
V317.1	GggaTCCATTATTATTTTt**h**TGTAATCAAGCC	V317K; V317E; V317Stop
V317.2	GggaTCCATTATTATTTc**mw**TGTAATCAAGCC	V317M; V317W; V317L
V317.3	GggaTCCATTATTATTTTGTAATCAAGCC	ΔVal-317

[a] Lower case letters indicate nucleotides introduced to construct appropriate restriction sites (these are underlined) or *lytA* mutations. Degenerate nucleotides are shown in bold: k may represent G or T; h may represent A, C, or T; m may represent A or C; w may represent A or T; and y may represent C or T. Nucleotides complementary to the TAA termination codon of *lytA* are indicated with a gray background.
[b] I315Stop and V317Stop indicate that the corresponding codon has been changed to a stop codon.

determined spectrophotometrically as previously described (17).

Conversion of the E-form of LytA and Measurement of NAM-amidase Activity—Pneumococcal R6 cell walls were radioactively labeled with [*methyl*-^{3}H]choline as previously described (38). NAM-amidase activity assays were performed according to standard conditions using labeled R6 cell walls as a substrate (15). LytA conversion was analyzed by incubating the enzyme at 0 °C for 5 min with either choline-containing pneumococcal cell walls or 2% choline chloride before raising the temperature to 37 °C. The activity of non-converted amidases was assayed by adding these enzymes to a suspension of radioactively labeled cell walls previously warmed to 37 °C. One unit of amidase activity was defined as the amount of enzyme that catalyzed the hydrolysis (solubilization) of 1 μg of cell wall material in 10 min.

Miscellaneous Techniques—A three-dimensional x-ray crystallographic model of C-LytA (Protein Data Bank accession number 1HCX) was used. The generated three-dimensional molecules of the mutated enzymes $LytA_{V317T}$ and $LytA_{V317W}$ were manually docked into possible sites on C-LytA using the software program FRODO (39). To prevent non-allowed contacts and to ensure proper geometry, the resulting working models were subjected to stereochemical and geometrical refinement by energy minimization using crystallography NMR software (40). Ribbon diagrams were prepared using MOLSCRIPT (41) and Raster3D software (42). Sedimentation equilibrium experiments with mutated NAM-amidases were performed using an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Inc.) as previously described (19), employing protein concentrations of ~1 mg/ml.

RESULTS

We have previously shown that the LytA dimer is formed by the tail-to-tail association of two monomers, in which ChBR6 and ChBR7 lie in an anti-parallel fashion (21). In both of these monomers, the aromatic and hydrophobic residues are directed internally, resulting in a hydrophobic core that provides the main driving force for dimerization. There are three major hydrophobic zones in this core. Zone I is formed by residues of hairpin 6 (Phe-283, Trp-292, and Tyr-294) from both monomers, whereas zone II is formed by residues of hairpin 7 (Pro-305, Phe-307, Val-309, Ile-315, and Val-317). Contact between the participating monomers is made possible by hydrophobic interactions between Tyr-294 and Ile-315. This dimeric interface is further supported by zone III, which consists of side-chain hydrophobic contacts between the Ser-286 and Tyr-293 of one monomer and the Leu-314 of the other in addition to three hydrogen bonds (Fig. 1).

To determine the degree of natural sequence variation among different LytA enzymes, the amino acid sequences of the two C-terminal ChBRs from the LytA NAM-amidases in the European Molecular Biology Laboratory data base (a total of seven, including

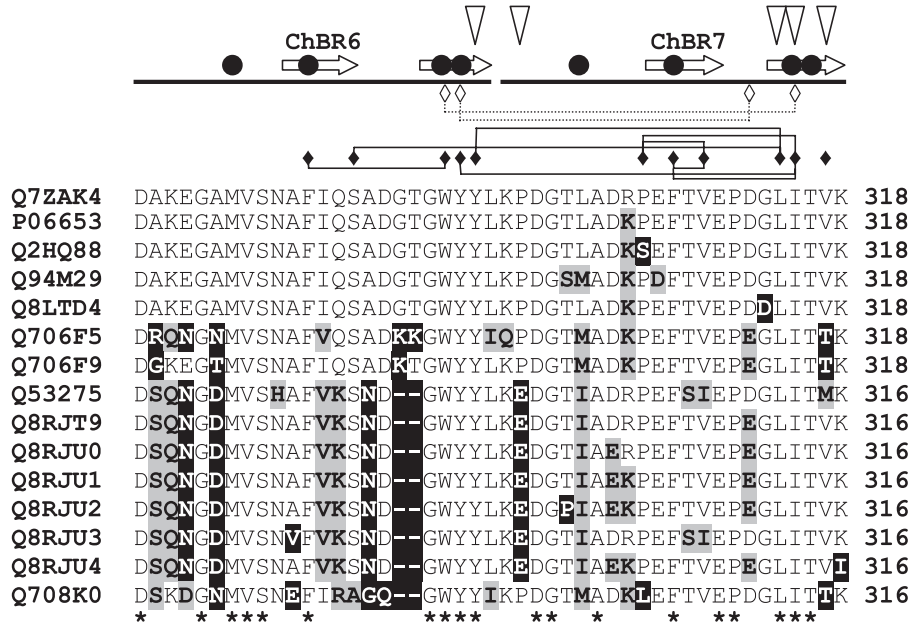

FIGURE 1. **Multiple alignment of the amino acid sequences of ChBR6 and ChBR7 in LytA and LytA-like NAM-amidases.** Amino acid residues conserved in all of the proteins are *boxed* and marked with an *asterisk*. Conserved and non-conserved amino acid substitutions are shown with *gray* and *black backgrounds*, respectively. *Circles* and *arrows* indicate choline-binding residues and the portions of the sequence that form the first and second strands of the hairpin, respectively. *Black* and *open diamonds* show, respectively, the amino acid residues that establish hydrophobic interactions (*solid lines*) and the hydrogen bonds (*dotted lines*) among them and/or between the corresponding residues of the neighboring monomer in the LytA dimer (21). *Inverted triangles* mark the amino acid residues substituted in this work. *Hyphens* represent amino acid residues absent from LytA-like proteins. Amino acid positions are indicated at the *right*. Each protein is identified by its accession number in the UniProtKB data base. Q7ZAK4, P06653, and Q2HQ88 correspond, respectively, to LytA from the R6 laboratory strain and the clinical pneumococcal isolates TIGR4 and ST942. Q94M29, Q8LTD4, Q706F5, and Q706F9 correspond to the lysines from pneumococcal prophages MM1 and V01 and to the lytic enzymes from the *S. mitis* temperate phages ϕB6 and ϕHER, respectively. Q53275, Q8RJT9, Q8RJU0, Q8RJU1, Q8RJU2, Q8RJU3, and Q8RJU4 correspond, respectively, to the *mitis* group streptococci strains 101/1987, 1078/1997, 1338/1996, 1283/1996, 1230/1996, 782/1996, and 10546/1994. Q708K0 corresponds to the lysine from the *S. mitis* prophage EJ-1.

four phage-encoded proteins; last date accessed, November 2, 2006) were aligned. The LytA-like enzymes from streptococci of the *mitis* group and of the phage EJ-1 (a total of eight proteins), which has a characteristic two-amino-acid deletion in ChBR6 (24, 41) (Fig. 1), were also aligned. With the noticeable exceptions of Pro-305 and Val-317, all of the other residues responsible for dimer formation were well conserved.

Val-317 plays an important role in conversion, as shown by the effects of replacing this residue by threonine ($LytA_{V317T}$) (27). Studies of the three-dimensional structure of C-LytA (20, 21) suggest that Val-317 might interact with Phe-283 and Tyr-294 of the same monomer to form a hydrophobic nucleus involved in the maintenance of the dimer (Fig. 2*A*). In sharp contrast to that reported for dialyzed $LytA_{R6}$, in which >80% of the molecules form dimers in the absence of choline (19), sedimentation equilibrium analyses have shown that $LytA_{V317T}$ remains mainly monomeric, although it auto-associates into a dimer as the choline concentration is increased (Fig. 3).

In an attempt to determine the biological consequences of the V317T mutation, the $lytA_{V317T}$ gene was used to construct pRG2-2 as described under "Experimental Procedures." This recombinant plasmid was introduced by genetic transformation into the M31 strain, a Δ*lytA* pneumococcal mutant. The behavior of the transformed strain (M51-T) was indistinguishable from that of the *S. pneumoniae* strain M51, an M31 transformant harboring pRG2 (31); *e.g.* M51-T grew at a normal growth rate as diplococci and lysed very rapidly either at the end of the exponential phase of growth or when treated with β-lactam antibiotics, three important properties that had been lost in the M31 strain (data not shown).

Enzymatic Properties of Mutant NAM-amidases—In addition to Val-317, three additional amino acid residues were chosen for site-directed mutagenesis: Tyr-294 (zone I), which is involved in the formation of the hydrophobic core as well as in the interaction with residues from the other monomer to maintain the dimeric form of the enzyme; and Leu-314 and Ile-315 (zone II), which are responsible for hydrophobic interactions that keep both monomers in contact. A non-conserved residue, Pro-297, which does not appear to be involved in dimerization due to its location outside the interaction zone, was selected as a control. It should be mentioned, however, that Pro-297 is located in the loop between ChBR6 and ChBR7 (Fig. 1). As expected, both $LytA_{P297G}$ and $LytA_{P297E}$ retained >50% of the activity of $LytA_{R6}$ (Table 2).

Tyrosine is a large, aromatic, hydrophobic amino acid with uncharged polar side groups at physiological pH levels. To establish the importance of these characteristics in the maintenance of enzyme activity, two mutations (Y294E and Y294L) were constructed and the corresponding NAM-amidases analyzed. $LytA_{Y294E}$ showed a marked reduction in specific activity (3.5% of that of $LytA_{R6}$), whereas $LytA_{Y294L}$ maintained ~80% of the activity of the wild type enzyme (Table 2).

The three-dimensional structure of C-LytA complexed with choline showed that Leu-314 is oriented outwards from the solenoid (Fig. 2*B*). This suggests that the modification of this residue should not lead to severe changes in the activity of the resulting enzyme. To test this hypothesis, four Leu-314 mutants were constructed. The above assumption appeared to be correct for three enzymes, $LytA_{L314T}$, $LytA_{L314E}$, and $LytA_{L314K}$, which showed no significant differences to the wild type enzyme in terms of specific activity (Table 2). However, the L314A mutation was less well tolerated, as it led to a nearly 50% loss of activity. During the process of constructing and sequencing the *lytA* mutants, a spontaneous mutation was identified that converted the TTG triplet encoding Leu-314 to a TAG stop codon, generating a truncated protein of 313 amino acids ($LytA_{L314Stop}$) with greatly reduced activity (Table 2). In addition, sedimentation equilibrium data obtained by analyti-

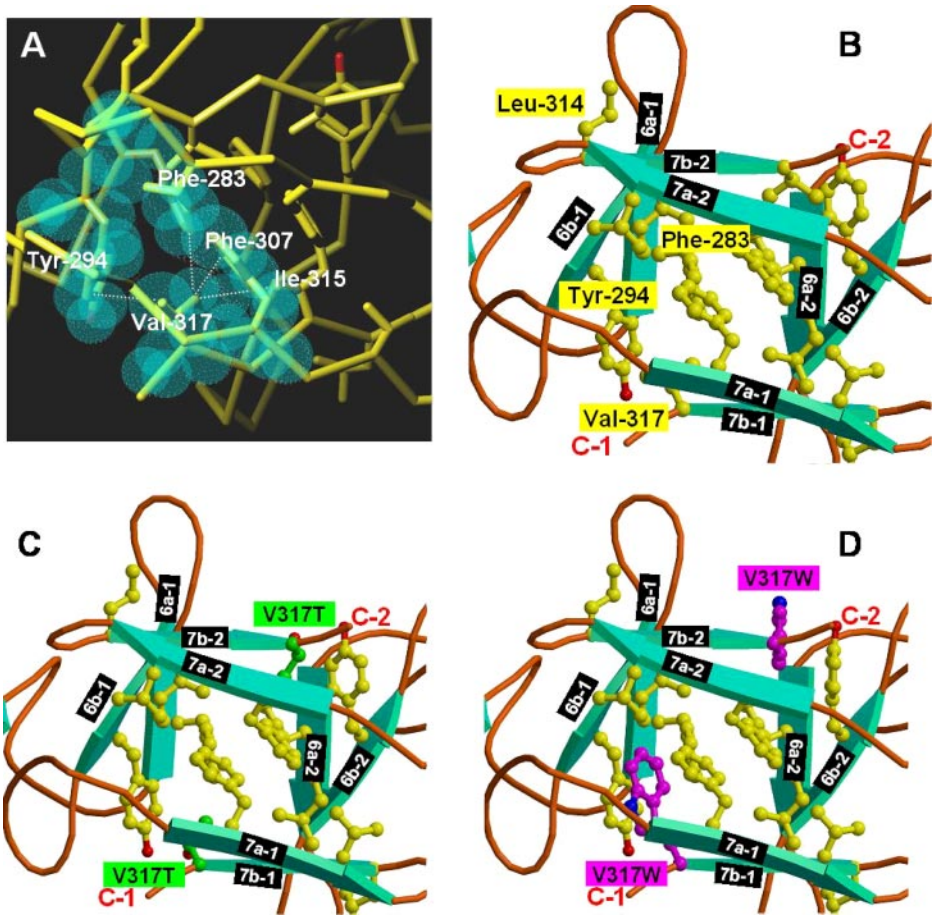

FIGURE 2. **Hydrophobic interactions and ribbon diagrams of the LytA homodimer interface.** Semitransparent spheres represent the van der Waals contacts involving Val-317 and its neighboring residues. Hydrophobic contacts defined when the center of mass of the distance between two hydrophobic side chains is <4 Å are indicated by *dotted lines* (*A*). Hairpins 6 and 7 are indicated for monomers 1 and 2. The structure of the wild type enzyme (21) is shown (*B*). Shown are the predicted three-dimensional structures of the $LytA_{V317T}$ (*C*) and $LytA_{V317W}$ (*D*) mutated enzymes. The locations of some important amino acid residues are *highlighted*. The Thr and Trp amino acid residues that replace Val-317 are labeled in *green* (*C*) and *magenta* (*D*), respectively. C-1 and C-2 indicate the C-terminal ends of both monomers.

cal ultracentrifugation showed that the size of this protein did not match that expected for either the monomeric or dimeric forms of NAM-amidase (Fig. 4).

Ile-315 is involved in several types of interaction involving zones I, II, and III at the LytA homodimer interface (Fig. 1), and together with Pro-305, Phe-307, Val-309, and Val-317 helps to produce a hydrophobic cavity (21). Four mutant NAM-amidases were constructed involving Ile-315 (Table 1) and, independent of the charge or polarity of the incorporated amino acid residue, a reduction in specific activity of at least 90% was seen in all cases (Table 2). $LytA_{I315D}$ was studied by sedimentation equilibrium analysis and found to be of a size that corresponded to neither the monomeric nor dimeric forms of NAM-amidase (Fig. 4). Taken together, these results indicate that Ile-315 is a key residue involved in both LytA activity and folding.

To gain further insight into the participation of Val-317 in the enzymatic activity of LytA, seven other mutants were produced and classified into three groups depending on their NAM-amidase activity. $LytA_{V317L}$, $LytA_{V317W}$, and $LytA_{V317T}$ formed a medium to high specific activity group (≥50% that of the wild type enzyme) (Table 2). It was anticipated that the substitution of valine by leucine would produce only minor biochemical alterations. However, in $LytA_{V317W}$, in which the valine residue was replaced by an aromatic amino acid containing an amino group, NAM-amidase activity was unexpectedly high (60% that of the wild type enzyme). A second group of mutant enzymes, $LytA_{V317E}$ and $LytA_{V317K}$, was produced in which Val-317 was replaced by a charged residue. These enzymes showed medium to low NAM-amidase activity (15–35% that of the wild type enzyme) (Table 2). The introduction of a charged amino acid at this position was expected to generate new electrostatic forces that would destabilize the hydrophobic core and its surroundings. The difference in the activities of these two mutant enzymes suggests that a positively charged amino acid (Lys) is better tolerated than a negatively charged residue (Glu).

The changes introduced in $LytA_{V317M}$, $LytA_{V317Stop}$, and $LytA_{\Delta V317}$ greatly impaired NAM-amidase activity (Table 2). In $LytA_{V317M}$, the substitution of a valine for a methionine residue naturally involved the introduction of a bulky sulfur atom; this likely reduced the distance between the methionine and neighboring amino acid residues. It is conceivable that this alteration brings about a slight collapse in the homodimer interface and, consequently, a destabilization of the whole structure. The marked reduction in the activity of the mutated NAM-amidases of $LytA_{V317Stop}$ and $LytA_{\Delta V317}$ clearly indicates that the introduction of a stop codon or a deletion at position 317 destabilizes the C-terminal end of ChBR7 and generates inactive proteins. Sedimentation equilibrium experiments were performed with both mutants in the presence and absence of choline, and the results showed that the size of the mutated NAM-amidases corresponded neither to a monomeric nor dimeric form but to an intermediate form. Both mutants seemed to have lost their capacity to form active homodimers.

Deconversion Capacity and Sensitivity to Detergents—The differences found in the specific activities of the mutated enzymes did not always correlate with their deconversion capacity. For example, among the nine mutations with >50% of the $LytA_{R6}$ NAM-amidase activity in the presence of choline (*i.e.* Y294L, P297G, P297E, L314E, L314K, L314T, V317T, V317W, and V317L), only L314K, V317L, and those affecting Pro-297 retained >50% of their original activity after dialysis (Table 2). In contrast, $LytA_{Y294L}$, $LytA_{L314T}$, and $LytA_{V317W}$

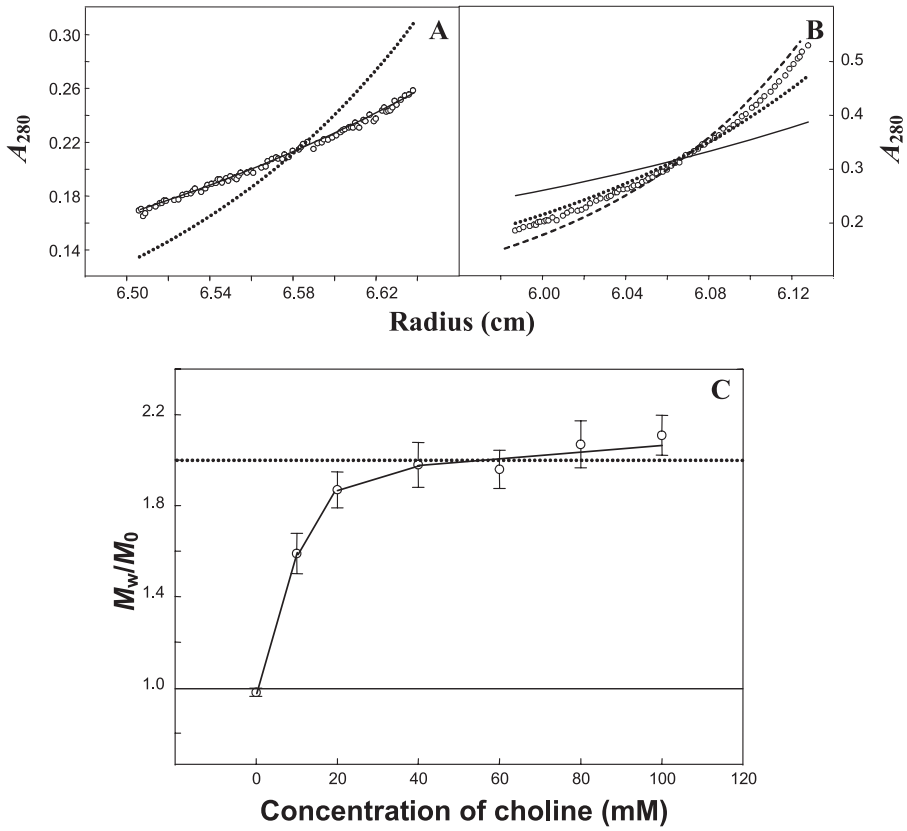

FIGURE 3. **Sedimentation equilibrium data and influence of choline binding on the relative apparent average molecular weight (M_w/M_0) of LytA$_{V317T}$.** *A* and *B* show, respectively, data for the mutated NAM-amidase in NP buffer and in NP buffer supplemented with 50 mM choline chloride. The auto-association of LytA$_{V317T}$ as a function of choline concentration in NP buffer is also shown (*C*). The mean ± S.D. of four independent determinations are shown. *Solid*, *dotted*, and *dashed lines* represent the theoretical fits with monomer, dimer, and trimer models, respectively.

became completely deconverted, as previously reported for LytA$_{V317T}$ (27). Interestingly, two other mutations affecting Leu-314 (L314E and L314A but not L314K) led to enzymes that behaved in a fashion similar to the wild type and that were moderately deconvertible. It should be noted that deconversion by dialysis was always reversible.[3]

Previous studies have shown that LytA-like NAM-amidases from phages or streptococci of the *mitis* group are partly inactivated by 1% sodium deoxycholate (Doc) (24, 25, 27). This property contrasts with the characteristic Doc-induced lysis of pneumococcal isolates widely accepted as an identification tool for *S. pneumoniae* (43). Although the susceptibility of the LytA-like enzymes to 1% Doc was initially attributed to a two-amino-acid deletion in ChBR6 (24), which is characteristic of this subfamily of proteins (44), it has been found that LytA$_{V317T}$ and the NAM-amidases from two *Streptococcus mitis* prophages are also sensitive to Doc, even though they have no such deletions (27). To investigate whether the inhibitory effect of the detergent was due to an alteration in the association state of the enzyme, the monomeric form of LytA$_{V317T}$ (prepared by dialysis) was dimerized by incubation with 50 mM choline chloride (Fig. 3*C*). Afterward, Doc was added to the enzyme at 0.1 or 1% (final concentration), the mixture was incubated for 10 min at room temperature, and then analyzed by sedimentation equilibrium in the analytical ultracentrifuge. The results clearly showed that Doc was unable to dissociate the NAM-amidase dimers.[3]

Table 2 shows that several of the mutants constructed were susceptible to Doc, although this was not the case for P297G, P297E, or L314K, in which the detergent had an evident stimulatory effect. Early observations made by other authors suggest that another detergent, Triton X-100, slightly stimulates LytA activity (by ~20%) (45). This was also true for most of the present mutated NAM-amidases, although in some cases (*e.g.* LytA$_{P297G}$, LytA$_{P297E}$, LytA$_{L314E}$, and LytA$_{L314K}$), their enzyme activity was stimulated by up to 3-fold (Table 2).

DISCUSSION

The LytA family of enzymes embraces a large number of bacterial and phage ChBPs with peptidoglycan-degrading, NAM-amidase activity. The peculiar three-dimensional folding of C-LytA has also recently been observed in the ChBM of the Cpl-1 lysozyme, a ChBP encoded by the pneumococcal phage Cp-1 (46), the ChBM of the pneumococcal Pce phosphocholine esterase (47), and the receptor-binding C-terminal repeats of *Clostridium difficile* toxin A (TcdA) (48, 49).

In the present work, most of the mutations introduced in the amino acid residues involved in the formation of the LytA dimers caused a strong reduction in the specific activity of the enzyme. This was particularly true for deletion mutant enzymes LytA$_{L314Stop}$, LytA$_{I315Stop}$, LytA$_{\Delta V317}$, and LytA$_{V317Stop}$ (Table 2). When studied in further detail, all deletion enzymes (even that with a single amino acid deletion (LytA$_{\Delta V317}$)) showed a nearly complete inability to form active dimers in the presence of choline (Fig. 4). These results fully confirm and extend previous physicochemical and biological studies on the role of ChBRs in the organization and functionality of *S. pneumoniae* LytA (17, 50, 51).

Quite surprisingly, some amino acid substitutions only produced minor changes in enzyme activity. In addition, the role in dimer formation of each of the amino acid residues studied in the different interaction zones appears to be different. In some cases, the size, polarity, or aromatic nature of the amino acid residue at position 314 seemed to be of little importance in dimer stability, possibly because of its relatively marginal location in the solenoid (Fig. 2*B*). For example, LytA$_{Y294L}$ showed as much as 80% of the activity of the wild type enzyme (Table 2), strongly suggesting that the substitution of the hydrophobic Tyr by Leu (a hydrophobic, although non-polar and non-aro-

[3] P. Romero, R. López, and E. García, unpublished observations.

TABLE 2
Comparison of the biochemical properties of mutant LytA NAM-amidases

Mutation	Sp. Act., U × 10^{-5} mg^{-1} of protein[a]	Activity after dialysis[b]	Activity after treatment[c] with:	
			1% Deoxycholate	1% Triton X-100
	%	%	%	%
None	7.3 (100)	100	105	120
Y294E	0.2 (3.4)	9	1	3
Y294L	5.9 (81)	17	79	188
P297G	4.8 (66)	64	94	152
P297E	4.2 (57.8)	67	102	144
L314E	7.0 (96)	39.5	61	182
L314K	6.0 (82)	84	263	207
L314A	3.2 (44)	34	23	58
L314T	7.2 (98.6)	23	51	136
L314Stop	0.6 (8.3)	60	4	13
I315E	0.6 (7.7)	26	3	11
I315Y	0.8 (10.9)	18	8	14
I315D	0.4 (5.2)	29	5.5	10
I315Stop	0.6 (8)	50	5	12
V317T	3.8 (51)	10	22	88
V317E	1.2 (16)	6	12	19
V317K	2.5 (34.4)	16	21	43
V317M	0.3 (4.7)	21	4	7
V317W	4.4 (60.1)	2.5	55	82
V317L	6.1 (84)	62	80	150
ΔV317	0.3 (4.5)	22	3	8
V317Stop	0.3 (4.1)	24	2	6

[a] Activity values are the averages of at least four independent determinations using pneumococcal cell walls as a substrate. The S.D. of the radioactive assay was always <15% of the mean value. Each enzyme was assayed in NP buffer. The percentage of $LytA_{R6}$ activity is shown in parentheses.
[b] Enzymes were dialyzed at low temperature (4 °C) against NP buffer and assayed with no conversion step. Percentages were calculated with respect to the activity of the corresponding non-dialyzed enzyme.
[c] Percentages were calculated with respect to that of the untreated $LytA_{R6}$.

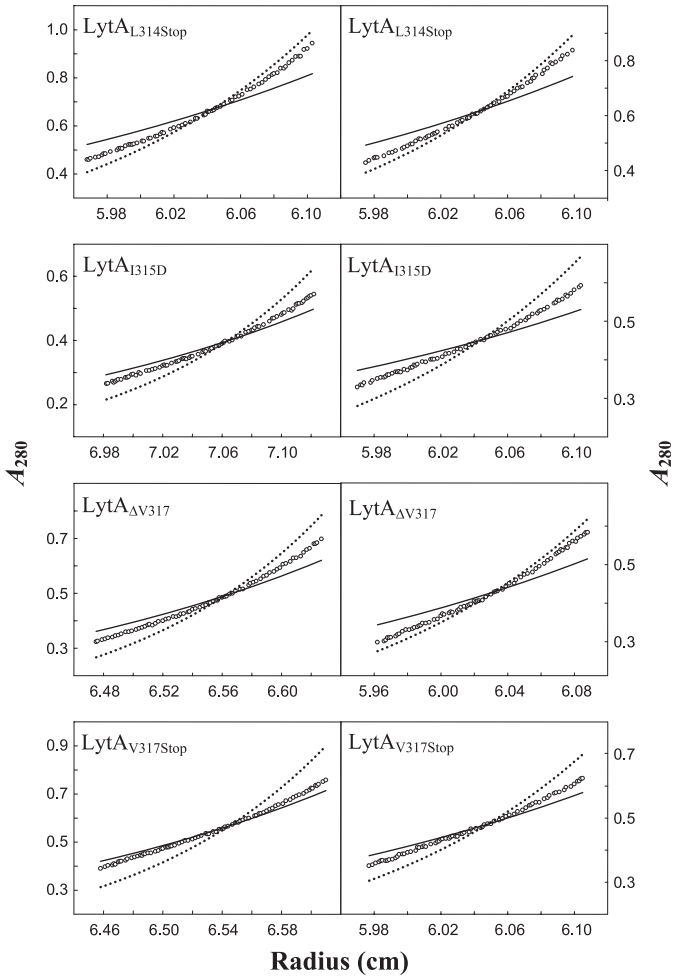

FIGURE 4. **Sedimentation equilibrium data for several LytA mutant enzymes.** The indicated enzymes were ultracentrifuged either in NP buffer (*left panels*) or in NP buffer containing 50 mM choline chloride (*right panels*). *Solid* and *dotted lines* represent the theoretical fits with monomer and dimer models, respectively.

matic residue) does not appreciably alter the interactions between monomers. In sharp contrast, the introduction at this position of a negatively charged Glu residue appeared to greatly modify the interactions between zones I and II. Ile-315 appears to play an important role(s) in the maintenance of many hydrophobic interactions as deduced from the finding that the enzyme is very sensitive to any changes at this position. In particular, the I315D mutation produced an enzyme with very reduced activity (Table 2), possibly because of a failure to form dimers in the presence of choline (Fig. 4). This may also be the case of other LytA enzymes harboring mutations affecting Ile-315.

Tyr-294, however, appears to be important for conversion, as the activity of $LytA_{Y294L}$ dropped by 80% after dialysis (Table 2). Similarly, $LytA_{L314T}$ was efficiently deconverted in the same way (Table 2). Several of the mutations introduced at Val-317 produced enzymes with >50% of the NAM-amidase activity of $LytA_{R6}$ (Table 2). In addition, $LytA_{V317L}$ showed activity similar to that of the wild type enzyme, and $LytA_{V317T}$ and $LytA_{V317W}$ could be completely deconverted. Although the behavior of $LytA_{V317T}$ has already been reported (27), that of $LytA_{V317W}$ was completely unexpected. Ribbon diagrams of the dimeric ChBD of $LytA_{V317T}$ and $LytA_{V317W}$ based on the structure of the C-LytA crystal (21) were created *in silico* (Fig. 2, *C* and *D*). These models indicate that the substitution of Val by Thr (or Trp) does not severely alter the hydrophobic core in which the valine takes part. Interestingly, the aromatic amino acid Trp fitted into the hydrophobic cavity, and therefore maintained the hydrophobic interaction between Phe-283 and Tyr-294. Presumably, however, this interaction is weaker than in the wild type NAM-amidase, as the enzyme was deconvertible by dialysis (Table 2).

The process of conversion has only been described so far for LytA (and LytA-like enzymes) and the NAM-amidase Pal encoded by the pneumococcal phage Dp-1 (52). Recently, Varea *et al.* (53) reported that choline-free Pal is mainly monomeric, whereas a dimer of Pal becomes the most abundant form of the choline-bound enzyme. The Cpl-1 lysozyme does not seem to require incubation at low temperatures with choline (or choline-containing cell walls) to be fully active. However, although it was originally assumed that the active form of Cpl-1 was monomeric, the dimerization of the Cpl-1 lysozyme in solution after choline binding was recently detected in sedimentation velocity experiments and small angle x-ray scattering analyses (54). Together, these results suggest that dimerization and conversion may be different processes, but the question of whether dimerization must always precede conversion remains to be answered. Preliminary ^1H NMR spectroscopic analyses of the decon-

verted form of the $LytA_{B6}$ NAM-amidase, the lysin of the *S. mitis* B6 phage (27), combined with analytical ultracentrifugation strongly suggest that incubating the enzyme for 5 min with 140 mM choline chloride at 37 °C allows self-association to occur. However, the conversion of $LytA_{B6}$ to the fully active form is only achieved after incubation of the enzyme with its ligand at low temperature (0 °C) as previously reported (14, 45).[3] Unfortunately, the monodimensional ^1H NMR spectra of $LytA_{B6}$ incubated with choline at low or high temperatures are indistinguishable, suggesting that conversion may entail very subtle, and largely unknown, structural modifications. These changes may represent intra- and intermodular interactions that can only be explored using sophisticated physicochemical techniques to determine the three-dimensional structure of LytA NAM-amidase in the deconverted state. Several of the mutants studied in this work could be of great help in such a study.

Acknowledgments—We thank A. Saville, M. Moscoso, J. M. Sanz, and P. García for helpful discussions and critical reading of the manuscript, A. Burton for correcting the English version, and E. Cano for skillful technical assistance. We also thank A. Romero for invaluable help with the modeling of C-LytA.

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Key Role of Amino Acid Residues in the Dimerization and Catalytic Activation of the Autolysin LytA, an Important Virulence Factor in *Streptococcus pneumoniae*

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J. Biol. Chem. 2007, 282:17729-17737.
doi: 10.1074/jbc.M611795200 originally published online April 16, 2007
