Structural Organization of the Major Autolysin from Streptococcus pneumoniae*

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LytA amidase is the best known bacterial autolysin. It breaks down the N-acetylmuramoyl-D-alanine bonds in the peptidoglycan backbone of Streptococcus pneumoniae and requires the presence of choline residues in the cell-wall teichoic acids for activity. Genetic experiments have supported the hypothesis that its 36-kDa chain has evolved by the fusion of two independent modules: the NH₂-terminal module, responsible for the catalytic activity, and the COOH-terminal module, involved in the attachment to the cell wall. The structural organization of LytA amidase and of its isolated COOH-terminal module (C-LytA) and the variations induced by choline binding have been examined by differential scanning calorimetry and analytical ultracentrifugation. Deconvolution of calorimetric curves have revealed a folding of the polypeptide chain in several independent or quasi-independent cooperative domains. Elementary transitions in C-LytA are close but not identical to those assigned to the COOH-terminal module in the complete amidase, particularly in the absence of choline. These results indicate that the NH₂-terminal region of the protein is important for attaining the native tertiary fold of the COOH terminus. Analytical ultracentrifugation studies have shown that LytA exhibits a monomer ↔ dimer association equilibrium, through the COOH-terminal part of the molecule. Dimerization is regulated by choline interaction and involves the preferential binding of two molecules of choline per dimer. Sedimentation velocity experiments give frictional ratios of 1.1 for C-LytA monomer and 1.4 for C-LytA and LytA dimers, values that deviated from that of globular rigid particles. When considered together, present results give evidence that LytA amidase might be described as an elongated molecule consisting of at least four domains per subunit (two per module) designated here in as N1, N2, C1, and C2. Intersubunit cooperative interactions through the C2 domain in LytA dimer occur under all experimental conditions, while C-LytA requires the saturation of low affinity choline binding sites. The relevance of the structural features deduced here for LytA amidase is examined in connection with its biological function.

Autolysins are enzymes that specifically degrade some bonds in the peptidoglycan backbone of the bacterial cell walls and eventually cause cell lysis (1). These enzymes have been involved in many important physiological functions (2). The best known bacterial autolysin is the N-acetylmuramoyl-D-alanine amidase of Streptococcus pneumoniae of 36.5 kDa, referred to here as LytA1 amidase (3). S. pneumoniae is one of the microorganisms that causes high mortality and morbidity in the world and it has been proposed that the autolytic process might be one of the factors responsible of its pathogenicity (4). Moreover, it has been shown that LytA amidase induces a protective response in mice and can be a potential antigen to prepare a pneumococcal vaccine (5). The pneumococcal lytA gene was the first autolytic gene that was cloned, sequenced, and overexpressed in an heterologous host (6). This fact provided the tools for a more detailed biochemical and genetic characterization of the enzyme (7). LytA amidase requires the presence of choline residues in the cell wall teichoic acids for activity. In this sense, S. pneumoniae is one of the rare microorganisms that contain choline in the cell envelope, and it does not autolyse when choline is replaced by ethanolamine in the culture medium (8). Genetic experiments have provided solid evidence that the enzyme has evolved by the fusion of two independent modules: the NH₂-terminal module, responsible for the catalytic activity, and the COOH-terminal module, involved in the attachment to the cell wall (9). It is generally accepted that the binding of the autolysin to its insoluble substrate (the cell wall) is an essential prerequisite for the hydrolysis of the covalent bonds. The COOH-terminal module of LytA, that is built by six repeated units of 20 amino acids, appears to play a double function, fixing the enzyme to the choline residues of cell wall and inducing its activation by a process called conversion (10). Interestingly, the COOH-terminal module of LytA (C-LytA) can be expressed and folded independently (11), a finding that has allowed the construction of fusion proteins that can be purified by affinity chromatography in a single-step process in matrices derived with DEAE, an analogue of choline (12).

Although genetic approaches have provided substantial evidence on the modular design of the LytA amidase, few data concerning its function have been obtained by biochemical and physicochemical techniques. Hence, we have studied the structural organization in cooperative domains of the LytA amidase and its COOH-terminal module using differential scanning calorimetry (DSC). The oligomerization state and the

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‡ The abbreviations used are: LytA, major autolysin from S. pneumoniae; C-LytA, COOH-terminal module of LytA; ΔH, calorimetric enthalpy change; ΔH°, van’t Hoff enthalpy change derived from deconvolution analysis; ΔH°, van’t Hoff enthalpy change calculated as 4RT, where ΔCp is the maximum of the excess heat capacity function, T, is the temperature of the maximum, and R is the gas constant; s, sedimentation coefficient; f, frictional ratio; R, Stokes’ radius of hydration.
molecular shape of both proteins have been characterized by analytical ultracentrifugation. In addition, we have determined the influence of choline as an analogue of the protein substrate on the thermal unfolding of LytA and C-LytA, as well as on their self-association equilibria. By combining results from thermal unfolding and ultracentrifugation a model for molecular organization of LytA is suggested. Finally, the structural characteristics of the amidase are examined in connection with its biological function.

MATERIALS AND METHODS

Protein Purification and Chemicals—LytA amidase and C-LytA protein were purified from Escherichia coli DH1[pG100] (13, 14) and E. coli DH1[pCE17] (11) cells, respectively, using an affinity chromatographic procedure (12). Fractions showing the maximum LytA amidase activity or the maximum C-LytA concentration were pooled and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). Samples for calorimetric and ultracentrifugation analyses were extensively dialyzed against the appropriate buffer at 4°C and then centrifuged at 13,000 rpm in a MSE microcentrifuge for 5 min. LytA and C-LytA concentrations were determined spectrophotometrically using the molar absorption coefficients at 280 nm of 113,750 and 62,540 M⁻¹ cm⁻¹, respectively (6, 11). Choline concentration was measured by differential refractometry in a L13 interferometer (Zeiss Jena), using a double sector cell of 1 cm optical path and a specific differential refraction index of 0.021 M⁻¹ in 20 mM phosphate buffer, pH 6.9.

Assay of Cell Wall Lytic Activity—Enzymatic assays were carried out using [methyl-3H]choline-labeled pneumococcal cell walls as described previously (16). One unit of enzymatic activity was defined as the amount of enzyme required for the solubilization of 1 μg of cell walls in 10 min.

Differential Scanning Calorimetry—DSC measurements were performed using a Microcal MC-2 instrument (Microcal, Inc. Northampton, MA) at a heating rate of 0.33 K min⁻¹, unless otherwise stated, and under an extra constant pressure of 2 atm. The standard DA-2, Cpcalc, and MAH-1 optima were used for data acquisition and analysis. Reversibility of thermal transitions was checked by reheating the samples after a first scan. The influence of scanning rate on the calorimetric irreversible profiles was checked running samples at 0.2, 0.75, and 1 K min⁻¹. The excess heat capacity functions were obtained after subtraction of buffer baseline.

Sedimentation Equilibrium—Sedimentation equilibrium experiments of C-LytA and LytA were performed by centrifugation of 80-μl samples at 25°C and 15,000 and 20,000 rpm, respectively, in an Optima XL-A analytical ultracentrifuge (Beckman Instruments), employing 12-mm double sector epon-charcoal centerpieces. Radial scans were taken at 4-h intervals at 280 nm until the equilibrium condition was reached. Base-line offsets were determined taking a radial scan at equilibrium speed, after running the samples 5 h at 42,000 rpm. Conservation of mass in the cell was checked in all the experiments.

Apparent weight-averaged molecular weights were obtained by fitting individual data sets to a sedimentation equilibrium model for single species, using the signal conservation algorithm (17) from EQASOC and XLAEQ programs. The equilibrium dimerization constants, K₂, were calculated by fitting experimental data to a monomer ↔ dimer sedimentation equilibrium model using the ORIGIN version of the NONLIN algorithm (18). Similar results were obtained using the signal conservation algorithm (17). The monomer molecular masses (in daltons) were constrained to the following: 36,530 for LytA and 16,500 for C-LytA. The partial specific volumes for C-LytA and LytA calculated from amino acid composition were 0.725 and 0.722 ml g⁻¹ (19). The specific volume of choline measured with a DM2 Paar precision density meter was 0.885 ml g⁻¹. The variations in the calculated specific volumes of both proteins assuming 2 mol of bound ligand monomer (see below) were less than 0.3%.

Sedimentation Velocity—The experiments were performed at 25°C and 80,000 rpm, using protein concentrations of about 0.5 mg ml⁻¹. Radial scans at 280 nm were taken every 10 min. The sedimentation coefficients were calculated from the movement of the sedimentation boundary using the XLAVE program (Beckman). The experimental values were converted to standard conditions (S₂₀,ₐₚ) and the frictional ratio, Stokes' radii of hydration, and axial ratios were derived as described by Waxman et al. (20), using the molecular weight determined by sedimentation equilibrium under the same experimental conditions. The specific hydration grade of both proteins was assumed to be 0.28 g of H₂O/g of protein (21).

RESULTS

Differential Scanning Calorimetry

Thermal Stability of LytA Amidase—Fig. 1 (bottom trace) shows the typical DSC scan of thermal denaturation of LytA in 20 mM phosphate buffer, pH 6.9. The thermogram is characterized by a broad peak centered at about 45°C, followed by a sharper one with a maximum at 58°C. The reversibility of the overall endotherm is temperature-dependent and indicates the existence of different processes. Nevertheless, DSC curves recorded at different scanning rates did not show significant variations either in the form or in the enthalpy of the endotherm (data not shown), excluding kinetic control in any of the processes (22).

Considering the complexity of the endotherm associated to LytA thermal denaturation, the interaction with choline, a specific ligand of the COOH-terminal region, was studied (Fig. 1). Choline binding has a clear influence on the peak at highest temperature, as reflected by the strong shift induced in the Tm value. This result suggests that at least that peak should correspond to the melting of the COOH-terminal module of LytA. On the other hand, above 80 mM choline, thermal denaturation of LytA proceeds in two well resolved peaks. The reversibility of the thermal-induced transitions was unaffected by choline interaction. Increasing NaCl concentration up to 0.28 M did not modify the thermal denaturation profile of LytA, discarding ionic strength effects.

The detailed analysis of the calorimetric curves (see Fig. 1) shows that denaturation of LytA can be resolved into three independent two-state transitions and a final asymmetric peak characterized by a ΔH°ₐₚ/ΔH ratio of 1.4 (Fig. 1 and Table I), suggesting the existence of intermolecular interactions (23).
Thermodynamic Organization of LytA

The asymmetry of the peak and the value of \( \Delta H^c_{\text{cal}}/\Delta H \) would be consistent with a dimer which dissociates upon denaturation. The transitions centered near 41°C and 51°C are rather choline-independent up to about 80 mM ligand concentration, supporting the notion that they should correspond to the unfolding of the NH2-terminal region of LytA. However, above such concentration, the low temperature calorimetric peak cannot be deconvoluted into two elementary transitions, revealing a higher interdependence. The final steps, 3 and 4, are stabilized by increasing concentrations of ligand, suggesting that both transitions correspond to the melting of the COOH-terminal region of the protein.

Thermal Stability of C-LytA—The denaturation profile of the COOH-terminal module of LytA in phosphate buffer at pH 6.9 is shown in Fig. 2 (trace A). Its thermal unfolding is characterized by a major peak with a maximum at 61°C and a small broad shoulder centered at about 40°C. Heating C-LytA up to 50°C results in the irreversible disappearance of the shoulder (Fig. 2, curve B). The peak centered at 61°C is about 50% reversible (Fig. 2, trace C) and scan rate-independent (data not shown). The value of the \( \Delta H^c_{\text{cal}}/\Delta H \) ratio for this peak is 0.67, revealing that it is not a two-state process.

Fig. 3 shows the melting curves of C-LytA at increasing concentrations of choline. The influence of ligand binding on the thermal stability of C-LytA is complex and exhibits a biphasic character. At the lowest ligand concentration assayed, C-LytA shows a broad heat absorption curve and the total enthalpy change increases from 162 kcal mol\(^{-1}\), in the absence of ligand, up to 220 kcal mol\(^{-1}\), at 5 mM choline. Within this ligand concentration range, the intensity of the small shoulder observed in the free protein increases and its transition temperature is shifted to higher values. The major peak is also shifted up to 63.6°C, and its cooperativity becomes strongly protein-concentration dependent (data not shown). Above 10 mM choline, a single asymmetric peak is observed (see Fig. 3) and the calorimetric profile is slightly dependent on protein concentration (data not shown). Under these conditions, the ratio \( \Delta H^c_{\text{cal}}/\Delta H \) is 0.60, indicating the existence of intermediates. Reversibility of C-LytA thermal denaturation is dependent on choline concentration. Above 40 mM choline, 80% reversibility is achieved, while at lower concentrations, renaturation never exceeds 50%.

The analysis of the calorimetric profiles at low ligand and protein concentrations shows that C-LytA denaturation can be described by three independent transitions (see Fig. 3 and Table II). The calorimetric enthalpy changes of the two first endotherms increase with choline concentration up to 52 and 75 kcal mol\(^{-1}\), respectively, without changes in their van't Hoff enthalpies. On the contrary, the highest temperature transition is characterized by a constant enthalpy change of 115 kcal mol\(^{-1}\) and a \( \Delta H^c_{\text{cal}}/\Delta H \) ratio of 0.95 (see Table II). Above 10 mM choline, thermal denaturation of C-LytA can be resolved into two transitions, stabilized by increasing concentrations of ligand (Table II). The former is a two-state process with a calorimetric enthalpy change of 75 kcal mol\(^{-1}\), while \( \Delta H^c_{\text{cal}}/\Delta H \) for the latter endotherm is about 1.4 times that of \( \Delta H \) (150 kcal mol\(^{-1}\)), indicating, as in LytA amidas, intermolecular interactions (23).

The choline-induced variations observed in the thermal denaturation process of C-LytA suggest the existence of at least two types of binding sites with different affinity. At low choline concentration, ligand binding favors the refolding of the COOH-terminal module into a native-like tertiary structure, reducing the fraction of C-LytA molecules containing a region that does not undergo a cooperative unfolding transition in the free protein. Since calorimetric data are normalized to the total protein concentration, this effect would result in a reduced calorimetric enthalpy for the observed transition. At high cho-
line concentrations, ligand binding seems to trigger intermolecular cooperativity.

On the other hand, the similarity of behavior of C-LytA transitions 2 and 3 with that of transitions 3 and 4 of LytA agrees with their previous assignments to the unfolding of the COOH-terminal module. Differences in molecular cooperativity, particularly at low choline concentration, reveals the influence of the NH2-terminal module on the final organization and stability of the complete polypeptide chain.

Analytical Ultracentrifugation

Sedimentation Equilibria of C-LytA and LytA—The self-association behavior of C-LytA and LytA was investigated by sedimentation equilibrium measurements in the absence and in the presence of increasing concentrations of choline. C-LytA sedimented as a monomer in 20 mM phosphate buffer at pH 8.0, but dimers are present at equilibrium at pH 6.9 (Fig. 4, panels A and B). LytA forms dimers at both pH values, and the equilibrium constant is about 10 times that of C-LytA at pH 6.9 (Table III; Fig. 4, panel C).

Choline binding strongly enhances the dimerization of both proteins. Other oligomeric forms were not observed, even at the highest concentrations of protein and choline assayed. Table III summarizes the dimerization equilibrium constants under different experimental conditions. The slopes of Wyman plots (24) of choline-induced dimerization are 1.93 and 1.96 for LytA and C-LytA, respectively, and essentially indicate the preferential interaction or binding of 2 choline molecules/formed dimer.

Sedimentation Velocity—Characterization of the hydrodynamic behavior of both proteins was performed by sedimentation velocity. The sedimentation coefficients, $s_{20,w}$, of monomeric and dimeric C-LytA are 2.1 S (20 mM phosphate, pH 8.0) and 2.5 S (20 mM phosphate, 140 mM choline, pH 6.9), respectively. No correction for protein concentration dependence of $s$ was performed since the LytA and C-LytA concentrations employed were low enough to assume no significant effect. Taking into account the molecular masses of both C-LytA species, these $s_{20,w}$ correspond to $f/f_0$ of 1.1 (monomer) and 1.4 (dimer). These values are consistent with protate ellipsoids of $15 \times 31$ Å for the monomer and $10 \times 130$ Å for the dimer; indicating that the global hydrodynamic behavior of both species deviates from that of a globular rigid particle (25), being more pronounced in the case of the dimer.

The sedimentation coefficient of LytA dimer is 4.2 S (10 mM choline), which corresponds to a $f/f_0$ of 1.4. This value is consistent with a protate ellipsoid of $13 \times 190$ Å. The impossibility of having 100% of LytA monomer hampered its hydrodynamic characterization.

C-LytA Aggregation State During Thermal Unfolding—The effect of temperature on the aggregation state of C-LytA was analyzed by analytical ultracentrifugation. Protein samples were heated for 30 min at different temperatures before running the ultracentrifugation experiments at 25 °C. Heating C-LytA at 50 °C in the presence of 3 mM choline does not modify its dimerization constant at 25 °C. At this ligand concentration, high order aggregates are observed by increasing protein concentration when the heating is performed at 85 °C. In contrast, at 140 mM choline only C-LytA dimers are present after heating at the same temperature. This set of experiments allows the following conclusions. (i) The irreversible low temperature shoulder cannot be assigned to dimer dissociation since the quaternary structure is preserved at low temperature after heating; (ii) the enhanced cooperativity of the last transition observed by increasing protein concentration at intermediate choline saturation is due to the aggregation of the thermally denatured state, since the native protein under similar conditions exists always as a dimer; (iii) the irreversibility of C-LytA...
displays a biphasic character. Above 10 mM choline, at the intact. The effect induced by choline on DSC thermograms toward the state in which the tertiary structure contacts are equilibrium between the different forms of C-LytA is shifted (data not shown). Upon saturation of choline binding sites, theiation could be observed in the corresponding DSC profiles with a different degree of cooperativity in their tertiary structure in the absence of choline. In spite of the differences in results show the presence of two populations of C-LytA molecules with a different degree of cooperativity in their tertiary structure in the absence of choline. In spite of the differences in aggregation found between pH 8.0 and 7.0, no significant variation could be observed in the corresponding DSC profiles (data not shown). Upon saturation of choline binding sites, the equilibrium between the different forms of C-LytA is shifted toward the state in which the tertiary structure contacts are intact. The effect induced by choline on DSC thermograms displays a biphasic character. Above 10 mM choline, at the protein concentration used on DSC experiments, C-LytA is a dimer. Its thermal denaturation is more than 80% reversible and can be theoretically described assuming the existence of two cooperative domains (Fig. 5). Two identical independent domains, C1, at the edges of the dimer; and a cooperative dimeric block, (C2)2, which dissociates upon denaturation (C2)2 ↔ 2(U2). The low temperature transition in DSC thermograms (ΔH1 = 75 kcal mol−1) corresponds to the melting of C1 domains, while the asymmetric high temperature transition (ΔH3 = 2 × 144 kcal (mol of dimer)−1) corresponds to the (C2)2 domain (Fig. 3 and Table II). Below 10 mM choline, the first transition observed (Tm1) could be compatible with the existence either of a third domain per monomer or of an unfolding intermediate of C2. Given the low molecular weight of C-LytA, the latter seems the more plausible explanation. The destabilization of the possible intermediate of C2 at high choline concentration would explain the disappearance of the first transition and the concomitant increase of ΔH3 in 30 kcal mol−1. Furthermore, this event is simultaneous to the disappearance of high order aggregates of the denatured form.

The biphasic character of choline effects brings about the existence of two different classes of binding sites. The Wyman plot shows that a single molecule per monomer is involved in the ligand-induced dimerization at low choline concentration. However, the slope of the van’t Hoff plot (26, 27) for the Tm values as a function of ligand concentration (data not shown) results in a stoichiometry of 4.4 molecules of choline bound/dimer (2.2/monomer). This second molecule of choline could be responsible for the intermediate destabilization, the induction of intersubunit cooperativity, and the reversibility increase.

Structural Organization of LytA Protein—Opposite to C-LytA, more than 80% of LytA forms dimers at 1 mg ml−1 in the absence of ligand. DSC profiles are consistent with the existence of two cooperative domains involving the NH2-terminal module of the protein, N1 and N2 (four per dimer), and domains C1 and (C2)2 previously described in C-LytA (Fig. 5). As in C-LytA, the coupling between the two-state unfolding of (C2)2 and LytA dimer subunit dissociation accounts for the asymmetry of the highest temperature transition (Fig. 1). The thermodynamic parameters derived for the different transitions are reported in Table I. Transitions 1 and 2 correspond to the melting of the NH2-terminal domains, while 3 and 4 can be assigned to the unfolding of the COOH-terminal domains.

The comparison of the thermodynamic parameters of transitions 3 and 4 of LytA with those of transitions 2 and 3 in C-LytA establishes a dependence between the NH2- and the COOH-terminal modules. In this sense, suppression of the NH2-terminal region results in a loosening of the structure of the isolated COOH-terminal module. In addition, cooperativity between monomers through the C2 domain is always observed in LytA, while in C-LytA saturation of the lower affinity binding sites is required. Communication between LytA modules is also derived from the observed interdependence between N1 and N2 domains at high choline concentration, despite the ligand specificity for the COOH-terminal module. On the other hand, the lower reversibility of LytA thermal denaturation suggests that the unfolded NH2-terminal region blocks the renaturation of
the whole polypeptide.

In spite of the homology in the sequences of the COOH-terminal modules of LytA amidase and CPL1 lysozyme (28), there are great differences between their structural organization in cooperative domains (29). Although both choline-binding modules are designed to improve the attachment of the enzyme to the cell wall, it is obvious that a host-encoded lytic enzyme should exhibit a more efficient regulatory mechanism. In this sense, CPL1 lysozyme is encoded by the bacteriophage Cp1 and its biological regulation should be restricted to the phage cycle. In contrast, LytA amidase is a host-encoded protein involved in cell division of the S. pneumoniae whose activity should be under time and spatial regulation.

Biological Relevance—The choline-modulated dimerization of LytA can be physiologically significant, considering the local concentration of the amidase along the septum (30), and may of LytA can be physiologically significant, considering the local concentration of the amidase along the septum (30), and may play a role in the enzymatic efficiency by increasing the affinity toward the substrate. A lower limit for the free energy of linkage² between dimerization and choline binding of -2.7 kcal mol⁻¹ can be roughly estimated using the dimerization constants of free and choline-bound amidase reported in Table III (see Scheme I). Furthermore, upon binding to its natural polymeric substrate, subsequent saturation of free sites after binding the first choline molecule would be an intramolecular process. Therefore, dimerization would further improve the affinity toward the bacterial cell wall, by reducing the loss of entropy associated to ligand binding. Thus, the overall equilibrium constant for dimer binding should be significantly higher than the product of equilibrium constants for binding of two monomers to the same sites. In the in vitro experiments, where choline molecules are discrete entities, this effect cannot operate. However, the local concentration of choline on the cell wall surface, about 16 residues/pneumococcal teichoic acid (31, 32), is high enough to allow simultaneous multiple interactions.

LytA dimer can be modeled as a prolate ellipsoid of about 13 × 190 Å, which can be visualized as a stalk, made of the COOH-terminal modules, bearing at each end a catalytic site (Fig. 5). This shape would facilitate the diffusion of the molecule through the highly cross-linked framework of the cell wall and increase the number of accessible hydrolyzable bonds per attachment site. On the other hand, dimerization can also facilitate the concomitant interaction with two teichoic acids and the motion of the amidase along the septum by means of a step binding-release mechanism, using the COOH-terminal modules of both subunits, without completely detaching from the cell wall.

The finding of two classes of binding sites, with different affinity, per monomer of LytA or C-LytA is in agreement with previous COOH-terminal deletion studies (33, 34). Deletion of the terminal tail of 11 amino acids, responsible for the conversion process, dramatically reduces the catalytic activity. The loss of choline recognition occurs upon additional suppression of P6, P5, and P4 motives, while intermediate deletions have no significant effect. Recently, Markiewicz and Tomasz (35) have reported the presence of a single mole of choline bound per mole of LytA activated by affinity chromatography purification, which dissociates very slowly. This observation is not incompatible with the existence of other types of binding sites, since any molecule of choline bound in fast equilibrium would dissociate after choline depletion of the sample buffer (35). The retained choline molecule might be that involved in LytA dimerization, explaining the higher degree of dimerization found in LytA when compared with C-LytA. Nevertheless, there is no experimental evidence supporting a slow monomer ↔ dimer equilibrium. Moreover, the enzyme purified by the procedure described under “Experimental Procedures” is retained again in DEAE-cellulose, after exhaustive dialysis. The previous finding that the activated enzyme was no longer retained by choline-Sepharose columns (35), even after extensive dialysis, may be ascribed to a different local concentration of binding residues in both matrices. The results here reported provide new insights on the molecular structure of pneumococcal autolysins. They establish the absence of a straightforward correspondence between module and structural cooperative domain, as demonstrated by the complex organization of LytA amidase. The NH₂- and COOH-terminal modules are organized into independent domains but the influence of choline on LytA stability argues in favor of the existence of communication between modules. The stepwise acquisition of repeating units of the COOH-terminal module may represent an evolution-dictated enzyme advantage related to the existence of several choline-binding sites, which in turn would improve the affinity toward the substrate, as also does the protein dimerization. Finally, the molecular shape of the tail-to-tail dimer would also confer on the system notable properties, by means of a facilitated diffusion and a catalytic site distance constraint.

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\begin{align*}
\text{Scheme I.} \\
2 \text{LytA} + 2nS & \rightarrow 2 \text{LytA.S}_n \\
K_2 & \rightarrow K_1^2 \\
\frac{K_1^2}{K_2} & \rightarrow \frac{K_B^{2n}}{K_D^{2n}} \\
\text{(LytA)}_2 + 2nS & \rightarrow (\text{LytA})_2.S_n \\
\end{align*}
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