Structural Characterization of the Unliganded and Choline-bound Forms of the Major Pneumococcal Autolysin LytA Amidase

CONFORMATIONAL TRANSITIONS INDUCED BY TEMPERATURE*

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The secondary and tertiary structures of the choline-dependent major pneumococcal autolysin LytA amidase and of its COOH-terminal domain, C-LytA, have been investigated by circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy. Deconvolution analysis shows that the far-UV CD spectrum of both proteins is governed by chiral contributions, ascribed to aromatic residue clusters contained in the COOH-terminal module. The secondary structure of LytA, determined from the FTIR spectral features of the amide I band, results in 19% of α-helix and tight loops, 47% of β-sheets, 23% of turns, and 11% of irregular structures. Similar values are obtained for C-LytA. The addition of choline significantly modifies the far- and near-UV CD spectra of LytA and C-LytA. These changes are attributed to alterations in the environment of their aromatic clusters, since the FTIR spectra indicate that the secondary structure is essentially unaffected. CD choline titration curves at different wavelengths show the existence of two types of binding sites/subunit. Data analysis assuming protein dimerization upon saturation of the high affinity sites reveals positive cooperativity between the low affinity sites. Thermal denaturation of both proteins occurs with the formation of unfolding intermediates and the presence of residual secondary structure in the final denatured state. The irreversibility of the thermal denaturation of LytA and C-LytA results from the collapse of the polypeptide chain into intermolecular extended structures. At saturating concentrations, choline prevents the formation of these structures in the isolated COOH-terminal module.

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despite of the subarachnoid space and the lung is largely induced by the pneumococcal cell wall during pneumococcal infection (4). Hence, the autolytic process that causes the release of cell wall fragments might well be considered a potential pathogenic factor (5). For this reason, the major pneumococcal autolysin LytA amidase, which induces a protective response in mice (6), can be envisioned as a possible candidate in the preparation of pneumococcal vaccine.

Genetic approaches have demonstrated that LytA1 amidase has evolved from the fusion of two independent modules: the NH2-terminal module, responsible for the catalytic activity, and the COOH-terminal module, involved in the recognition and attachment to the cell wall (7, 8). The latter is constructed by six repeated units of 20 or 21 amino acids and a tail of 11 residues, which generates a protein, C-LytA, that specifically binds the choline residues contained in the teichoic and lipoteichoic acids of the pneumococcal cell envelope (8).

The 36-kDa polypeptide chain of LytA is organized into four cooperative domains: N1 and N2 (assigned to the NH2-module) and C1 and C2 (comprising the COOH-terminal region) (9). In addition, analytical ultracentrifugation has revealed the self-association capacity of LytA in a tail-to-tail dimer that involves C2-C2 domain interactions. This dimer is strongly stabilized by choline binding and can be modeled based on hydrodynamic studies as a prolate ellipsoid, bearing at each end a catalytic site (9). All of these biophysical studies have opened new insights on the evolution-dictated regulation and catalytic efficiency improvement of autolysins. However, the conformational aspects of this enzyme and its choline-stabilized dimer are still unknown. The aim of this paper is to characterize the following by means of circular dichroism (CD) and Fourier transform infrared (FTIR) techniques: (i) the secondary structure composition; (ii) the structural and thermodynamic basis for choline interaction; and (iii) the structural basis of thermal denaturation irreversibility of LytA and its COOH-terminal module (C-LytA). Combination of both CD and FTIR spectroscopies is a very valuable approach to characterizing protein structures, particularly in those cases where the far-UV CD spectrum interpretation may be hampered by a high content of aromatic residues and β-structures (10).

MATERIALS AND METHODS

Protein Purification and Chemicals—LytA amidase and C-LytA protein were purified from Escherichia coli DH1 (pGL100) (11) and E. coli

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Spectroscopic Features of LytA Amidase

Assay of Amidase Activity—LytA amidase activity was determined using [methyl-3H]-choline-labeled pneumococcal cell walls as reported previously (14). One unit was defined as the amount of enzyme required for the solubilization of 1 μg of cell walls in 10 min (700 cpm/μg of cell wall).

Circular Dichroism Spectra—Circular dichroism spectra were recorded in a JASCO J-720 spectropolarimeter, fitted with a thermostat-

Fourier Transform Infrared Spectra—Fourier transform infrared spectra were recorded in a JASCO J-720 spectropolarimeter, fitted with a thermostat-

RESULTS

Spectroscopic Characterization of LytA Amidase and C-LytA

Circular Dichroism Spectroscopy—The spectrum in the far-UV region of LytA amidase and C-LytA contains bands at 210 and 230 nm (Fig. 1A, curve 1). The low absolute value of the ellipticity at 230 nm and the negative band at 230 nm suggest a high contribution of aromatic amino acid side chains. The CD spectrum of C-LytA in this region is characterized by a large positive band at 224 nm, a minimum at 195 nm, and two shoulders centered at 233 and 209 nm (Fig. 1A, curve 2). Deconvolution of experimental spectra according to the convex constraint analysis method (16–18) results in six pure components shown in Fig. 1C and the theoretical spectra represented in Fig. 1D. Reduction of the lowest component number to five significantly increases the standard deviation between the experimental and calculated curves (data not shown). The percentage contributed by each component and the tentative in-

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terpretation in terms of secondary structure elements are listed in Table I. The far-UV CD spectra of LytA and C-LytA are dominated by contributions of components II and IV attributable to the chiral contribution of aromatic side chains (20–22). Component IV reproduces the CD spectrum of t-tryptophan derivatives in water solutions (22), while component II closely resembles the shape of the exciton couplet associated with the tryptophan B$_0$ transition (23). The pure components III and V can undoubtedly be assigned to unordered and α-helical structures, respectively (20, 24, 25), while component I can be correlated with β-sheets and turns producing a type C spectra (20, 25, 26). Component VI can be assigned to antiparallel β-sheets and/or type B β-turns (20, 25, 26). The high percentage contributed by the chiral component to the total spectrum of both proteins prevents the secondary structure characterization on the exclusive basis of CD spectra.

The CD spectra in the near-UV region of LytA and C-LytA are shown in Fig. 1B (curves 1 and 2, respectively). The spectrum of LytA presents two positive bands at 265 and 292 nm and two small shoulders at 284 and 300 nm. The C-LytA spectrum also exhibits a maximum at 265 nm, two small positive bands at 293 and 305 nm, and two minima at about 286 and 297 nm. The similarity of both spectra suggests that the spectroscopic properties of LytA in the near-UV are governed by the dichroic behavior of its COOH-terminal module. However, the interpretation of the spectra in terms of individual contributions is hampered by the high content of aromatic residues of LytA (22 Tyr, 15 Trp, and 10 Phe) and C-LytA (12 Tyr, 9 Trp, and 6 Phe) as well as the peculiar arrangement of some of them in WYY clusters (15).

**FTIR Spectroscopy**—The conformational sensitive amide I’ regions of the FTIR spectrum of LytA and C-LytA in D$_2$O buffer and after subtraction of the contributions originated by residue side chains are depicted in Fig. 2, A and C. The broad amide I’ band contour exhibits in both proteins a maximum at 1635 cm$^{-1}$ and a shoulder at 1654 cm$^{-1}$, characteristic of β-sheet and α-helix structures, respectively. The result of curve analysis in terms of secondary structure composition is shown under the evolutions in Fig. 2, whereas the band positions, their relative areas, and their tentative assignments are listed in Table II. Bands maximizing in the 1639–1620 cm$^{-1}$ frequency range are associated with β-sheet structures (19, 27, 28). The band observed at 1648 ± 1 cm$^{-1}$ can be assigned to irregular structures (19, 29). The band near 1636 cm$^{-1}$ can be attributed to α-helix structures and solvent-shielded peptide bonds (19, 29, 30). Those bands with a maximum in the 1662.5–1682.5 cm$^{-1}$ frequency range are mainly due to β-turn structures, since the expected contribution from the in phase vibration of amide groups in antiparallel β-sheets is rather slight (31, 32). Considering the previous assignments, the secondary structure composition of LytA results in 19% of α-helix and tight loops, 47% of β-sheet (10% of low frequency β-sheet), 23% of turns, and 11% of unordered segments (Table III), while that of C-LytA results in 17% of α-helix and tight loops, 51% of β-sheet (16% of low frequency β-sheet), 20% of turns, and 12% of unordered segments (Table III). It should be mentioned that the fraction of secondary structure elements derived from the convex constraint analysis upon normalization to the absence of components II and IV is in reasonable agreement with the composition determined by FTIR.

**Protein-Choline Interaction: Thermodynamics and Structural Variations Induced in LytA and C-LytA Proteins**

**CD Spectroscopy**—The far- and near-UV CD spectra of both proteins recorded in the presence of 20 mM choline are shown in Fig. 1 (curves 3 and 4 in panels A and B), together with theoretical curves derived from the deconvolution analysis of the spectra in the far-UV region. At the ligand concentrations employed, the ionic strength effect is negligible, and choline has no dichroic behavior but prevents the accurate registration of spectra below 200 nm. Minor differences in the component contributions are noted between the choline-bound and unligated forms of both proteins (Table I). Ligand addition to LytA induces a positive band at 224 nm, a red shift in the small negative band at 230 nm, and an increase in the negative ellipticity at 210 nm (Fig. 1). In contrast, two positive bands centered at 204 and 224 nm are observed in the difference spectra of C-LytA (see Fig. 3A). In the near-UV spectrum of both proteins, the ellipticity at 290 nm decreases, and two negative bands appear at 286 and 295 nm (Fig. 1B). The difference spectra of C-LytA at increasing choline concentration are depicted in Fig. 3, A and B. The analysis of the relative variations in $\Theta_{222}$ and $\Theta_{295}$ values of both proteins and of $\Theta_{260}$ and $\Theta_{240}$ in C-LytA as functions of choline concentration (Fig. 3, C and D) reveals the existence of two types of sites, with different ligand affinity, as was previously suggested on the
Saturating concentrations of choline.

The renaturation of C-LytA at 20°C results in a partial recovery of LytA, which increases up to 80–90% under these conditions. In contrast, LytA is not recovered after cooling from 75°C. In contrast, the negative ellipticity at 224 nm (Fig. 4). The minimum at 210 nm also decreases in intensity and shifts to 206 nm above 45°C, appearing as a new minimum at 215 nm at temperatures above 60°C. The most significant deviation observed in the presence of 20 mM choline was the enlargement of the 224-nm maximum in the 40–60°C temperature range. Above this temperature, the peak falls sharply and finally disappears at 70°C (Fig. 4B). In addition, the band at 210 nm decreases, is initially blue-shifted to 203 at 52°C, and is then red-shifted to 206 nm at 71°C. Above this temperature a new minimum appears at 217 nm.

In the absence of choline, the variations with temperature in the CD spectrum of C-LytA proceed in two well separated steps, revealing the existence of a stable intermediate between 45 and 55°C (Fig. 5A). As the temperature increases, the positive band at 224 nm decreases bi-phasically, reaching an intermediate plateau at 50°C and disappearing above 70°C. In addition, the negative band at 194 nm increases and shifts to 204 nm above 47°C. After cooling from 55 to 20°C, the original spectrum of C-LytA is not recovered, in agreement with the irreversibility of the low temperature transition detected by DSC (9). At 20 mM choline, the ellipticity at 224 nm remains nearly constant up to 50°C, and then the intensity of the whole spectrum decreases and a new minimum at 205 nm is observed (Fig. 5B).

Fig. 6 summarizes the θ_{204} and θ_{224} relative variations induced by temperature in LytA and C-LytA, both in the absence and presence of choline, whereas Table V summarizes the contributions of the different elementary transitions to the ellipticity variations at each wavelength, as obtained from the analysis of CD transition curves using Equation 5. The ellipticity change at 204 nm in both free and choline-bound C-LytA proceeds as a single step (Fig. 6, A and C) that can be correlated with transition 1 observed by DSC. On the contrary, the variations in θ_{224} can be resolved in two steps (Fig. 6, B and D), corresponding to calorimetric transitions 1 and 3 of C-LytA, in the absence of choline, and to transitions 1' and 2' in 20 mM choline (Table V).

In agreement with the more complex behavior of LytA, the relative variations in θ_{204} and θ_{224} with temperature cannot be explained in terms of a single elementary transition. In the unligated amidase, the unfolding of the four cooperative domains needs to be considered in order to explain the θ_{204} change with temperature (Fig. 6A). At 20 mM choline, the experimental curve can be resolved in terms of the contribution arising from the thermal denaturation of the NH₂-terminal module and the highest temperature transition of the COOH-terminal region (Fig. 6C). The largest variation in θ_{224} occurs at a higher temperature (T_m = 61.1°C) than that reported for the loss of tertiary structure monitored by DSC, particularly in the absence of choline (Fig. 6, B and D). The increase in θ_{224} toward more positive values is coupled to the thermal denaturation of the N2 domain (Table V).

**TABLE I**

Secondary structure and aromatic chiral contributions to the far-UV CD spectra of LytA and C-LytA

| Component | Assignment | LytA | LytA/choline | C-LytA | C-LytA/choline |
|-----------|------------|------|--------------|--------|----------------|
| I | Parallel β-sheet/type C β-turn | 13.6 | 15.3 | 21.3 | 13.5 |
| II | Chiral/230 | 14.3 | 8.6 | 11.7 | 8.0 | 12.9 | 18.5 |
| III | Random | 2.9 | 47.0 | 3.5 | 34.0 | 3.8 | 29.0 | 6.3 | 25.3 |
| IV | Chiral/224 | 47.9 | 11.0 | 47.6 | 19.0 | 67.8 | 28.2 | 76.9 | 30.9 |
| V | α-Helix | 17.8 | 18.2 | 12.7 | 12.2 | 15.2 | 18.8 | 6.8 | 20.0 |
| VI | Antiparallel β-sheet/type B β-turn | 3.6 | 0.0 | 3.3 | 13.3 | 2.3 | 7.4 | 0.3 | 25.3 |

Note: N and D refer to the native and denatured states, respectively.

*a* 20 mM choline.
The variations of the contributions of components I–VI with the temperature in LytA and C-LytA are shown in Figs. 4C and 5C, respectively. As the temperature increases, the main changes correspond to the decrease in the chiral component IV, the increase in the percentage of unordered structures, and the enhancement of component VI fraction (antiparallel β-sheet and type B β-turn) after protein denaturation. The content in component I also goes up with temperature in LytA amidase. No significant variations were observed in the fraction of α-helix, since the slight increase observed at higher temperatures could mainly reflect a more accurate determination of the α-helix content due to the decrease in the contribution of the aromatic components.

TABLE II

| Band frequencies, fractional areas, and proposed structural assignments of the components obtained in the curve-fitting analysis of the amide I’ band of LytA and C-LytA in the absence and presence of 140 mM choline |
|---------------------------------------------------------------|
| LytA                  | LytA/choline | C-LytA                  | C-LytA/choline |
| Band frequency (cm⁻¹) | Area %       | Band frequency (cm⁻¹) | Area %       | Band frequency (cm⁻¹) | Area %       | Band frequency (cm⁻¹) | Area %       |
| 1681                  | 4            | 1680                    | 7            | 1678                    | 10           | 1681                    | 5            |
| 1672                  | 9            | 1671                    | 10           | 1670                    | 5            | 1671                    | 11           |
| 1663                  | 10           | 1662                    | 12           | 1663                    | 5            | 1662                    | 10           |
| 1656                  | 19           | 1656                    | 17           | 1656                    | 17           | 1656                    | 17           |
| 1647                  | 11           | 1647                    | 12           | 1648                    | 12           | 1647                    | 16           |
| 1638                  | 18           | 1639                    | 16           | 1639                    | 14           | 1638                    | 15           |
| 1632                  | 19           | 1633                    | 15           | 1631                    | 21           | 1632                    | 14           |
| 1624                  | 10           | 1627                    | 11           | 1625                    | 16           | 1625                    | 12           |

Assignment:
- Turn
- α-Helix, tight loops
- β-Sheet
- Unordered
- Low frequency β-sheet

TABLE III

| Secondary structure composition of LytA and C-LytA proteins in the presence and absence of 140 mM choline at 25 °C |
|---------------------------------------------------------------------------------------------------------------|
| Percentage values are averaged to the close integer and represent the result of three independent analyses. |
| Secondary structure | LytA | LytA + 140 mM choline | C-LytA | C-LytA + 140 mM choline |
| α-Helix (%)         | 19   | 17                    | 17     | 17                      |
| β-Sheet (%)         | 47   | 42                    | 51     | 41                      |
| Turns (%)           | 23   | 29                    | 20     | 26                      |
| Unordered (%)       | 11   | 12                    | 12     | 16                      |

*Values for α-helix signature account for α-helix and closed or unchangeable loops as mentioned under “Results.”

FIG. 3. Effect of choline on the CD spectra of C-LytA and LytA. Panels A and B depict the CD difference spectra of C-LytA in the far- and near-UV regions, respectively, at the choline concentrations specified in the curve labels (mM units). Panels C and D represent the relative variations in the ellipticity values of LytA (top) and C-LytA (bottom) at 224 (■), 240 (○), 280 (●) and 295 nm (∆), as a function of ligand concentration. The experimental values were normalized to the variations observed at saturating concentration of ligand, except for data at 280 nm, which were normalized to the variation of ellipticity induced by saturation of the higher affinity sites (right hand side scale). Solid traces correspond to the best fitting curves calculated using the spectroscopic and thermodynamic parameters reported in Table IV.

Temperature Dependence of the FTIR Spectrum of LytA Amidase and C-LytA Protein

The temperature dependence of the Fourier self-deconvoluted amide I’ band of LytA is shown in Fig. 7. The thermally induced unfolding is irreversible as inferred from the spectra obtained after cooling from the highest temperature to 19 °C. The deconvoluted amide I’ band evolution of LytA amidase remains virtually unchanged up to 39 °C (Fig. 7A). Above this temperature, the well defined peaks representing the native β-structures and α-helices are replaced by a strong band around 1614 cm⁻¹, a weaker band at 1683 cm⁻¹, and a broad band at 1645 cm⁻¹. The latter feature is characteristic of un-
ordered structures, whereas the other two bands can undoubtedly be assigned to intermolecular hydrogen-bonded extended structures that accompany thermal and solvent-induced protein unfolding (33, 34). Such structures could represent collapsed unfolding intermediates that impair the productive refolding, explaining the irreversibility observed by DSC. The variations of the intensity at 1635 cm$^{-1}$ and at 1614 cm$^{-1}$, characteristic of native and unfolded structures, as a function of temperature (Fig. 8) show a single broad transition with $T_{1/2}$ of 47 $\pm$ 1°C. On the contrary, the variation of the amide II/amide I $\nu$ area ratio as a function of temperature reveals a complex behavior, with a first transition occurring at 30°C and a second near 50°C (data not shown). This magnitude is related to the peptide bonds’ degree of exposure to the solvent and, consequently, to the changes in the tertiary structure.

The thermal denaturation of LytA in the presence of 140 mM choline shows two well-defined transitions, characterized again by a decrease in the intensity at 1635 cm$^{-1}$ and a simultaneous increase in the intensity at 1614 cm$^{-1}$ (Fig. 8B). The $T_{1/2}$ of the transitions are 47 $\pm$ 1 and 73 $\pm$ 1°C. According to DSC data (9), the first process could be attributed to the unfolding of the NH$_2$-terminal module, while the second one should be related to the thermal denaturation of the COOH-terminal module. Data on hydrogen-deuterium exchange in the presence of choline are unreliable, due to the high contribution of methyl-bending vibrations of the -N(CH$_3$)$_3$ group found at 1490 and 1480 cm$^{-1}$ that hampers its accurate subtraction.

The temperature dependence of the Fourier self-deconvo-
luted amide I band of C-LytA is shown in Fig. 7. The band contour remains constant up to 51°C. Above this temperature, the thermally unfolded signatures of the 1616–1683 cm\(^{-1}\) band doublet appear. Variation of the intensities at 1635 and 1614 cm\(^{-1}\) with temperature show a single transition centered at about 52 ± 1°C (Fig. 8A). Choline binding results in a marked stabilization of the native secondary structure up to 78°C (Fig. 2D). At 140 mM choline, the thermally denatured state is characterized by a broad band centered at about 1645 cm\(^{-1}\) that corresponds to unordered structures. Furthermore, after cooling from 87°C, the native secondary structure features are regained, indicating the reversibility of the unfolding process. Therefore, choline binding prevents the collapse of the unfolded state. Ligand concentrations below 140 mM stabilize the mole-

**FIG. 5.** Effect of temperature on the far-UV CD spectrum of C-LytA in the absence (A) and presence of 20 mM choline (B). The spectra were registered in 10 mM phosphate buffer, pH 6.9, at the following temperatures: A, 19°C (thick solid line); 33.0°C (– – –); 38°C (– – –); 56.6°C (– – –); 61.5°C (– – –); 71°C (– – –); 75.5°C (– – –); and 23.5°C (thin solid line; sample cooled from 80.0°C). C and D represent the temperature-induced changes in the contributions of components I (■), III (▲), IV (△), and VI (○) to the CD spectra of C-LytA in the absence (C) and presence of 20 mM choline (D).

**FIG. 6.** Relative changes in the molar ellipticity of C-LytA (■) and LytA (○) at 204 and 224 nm as a function of temperature. A and C depict the CD denaturation curves monitored at 204 nm in 10 mM phosphate buffer, pH 6.9, in the absence and presence of 20 mM choline, respectively. B and D depict the CD denaturation curves followed at 224 nm in 10 mM phosphate buffer, pH 6.9, in the absence and presence of 20 mM choline, respectively. Solid lines represent the theoretical curves calculated using Equation 5 with the parameters reported in Table V. The dotted line in B is the theoretical curve calculated using the calorimetric \(T_m\) value for the last transition of LytA.
cule, but the unfolding process is only partially reversible, being accompanied by the appearance of bands at 1683 and 1614 cm

2

1 (data not shown). The apparent discrepancy in the choline concentration range needed for reversibility in CD and FTIR spectroscopy can easily be explained in terms of the different protein concentration ranges employed.

DISCUSSION

Secondary Structure of LytA and C-LytA Proteins—In the absence of structural information at atomic resolution, the secondary structures of LytA amidase and of its COOH-terminal module have been studied by two complementary spectroscopic techniques, CD and FTIR. The far-UV CD spectrum of both proteins is strongly influenced by chiral contributions of aromatic residues (components II and IV) that account for 60% of the total spectrum of LytA and 81% of the total spectrum of C-LytA. Both components, in particular that maximizing at 224 nm, are responsible for the unusual negative band at 227 nm in LytA amidase and for the large positive maximum of C-LytA spectrum. It is worth noting the great similarity of the pure components IV and II, directly derived from the deconvolution analysis of experimental spectra, to the CD spectra of L-tryptophan (22) and the exciton couplet associated with the B\textsubscript{1} transition of this chromophore (23), respectively. The prom-

TABLE V

Contributions of the different transitions to the temperature-induced variations of ellipticity at 204 and 224 nm in LytA and C-LytA

| Choline Concentration (mM) | LytA | C-LytA |
|---------------------------|------|--------|
| Tm (°C) | ΔH\textsubscript{i} | Δθ\textsubscript{i} / Δθ\textsubscript{max} | Tm (°C) | ΔH\textsubscript{i} | Δθ\textsubscript{i} / Δθ\textsubscript{max} |
|                |      |        |                |      |        |
| 0              | 40.4 | 0.44   | 39.2           | 0.23 |
| 54.8           | 51.5 | 0.32   | 51.5           | 0.17 |
| 61.1           | 58.8 | 0.28   | 61.1           | 1.15 |
| 20             | 64.2 | 0.35   | 64.2           | 0.18 |
| 69.4           | 65.1 | 0.65   | 65.1           | 0.52 |
| 100            | 61.1 | 1.30   | 61.1           | 1.2  |

* The disappearance of the CD band centered at 224 nm proceeds with a T\textsubscript{m} value of 61.1 °C, whereas the corresponding calorimetric transition occurs at 58 °C.

FIG. 7. Temperature-dependent changes in the Fourier self-deconvoluted amide I band of LytA (A and B) and C-LytA (C and D) in the absence (A and C) and presence of 140 mM choline (B and D). The spectra were recorded in D\textsubscript{2}O-prepared 10 mM phosphate buffer, pH 6.9, after 3 h of hydrogen-deuterium exchange at room temperature, at the following temperatures (from bottom to top): 19.9, 25.2, 29.2, 33.4, 38.1, 42.3, 46.7, 51.1, 55.6, 60.2, 64.3, 68.6, 73.2, 77.6, 81.9, and 86.6 and after cooling at 20 °C from the highest temperature.

FIG. 8. Thermal denaturation of LytA (open symbols) and C-LytA (solid symbols) in the absence (A) and presence of 140 mM choline (B) monitored by the intensity at different wave numbers. Intensities were measured from the spectra depicted in Fig. 7 at 1635 cm

2

1 (●), 1614 cm

2

1 (●), signatures of native and denatured protein states, respectively.
inert chiral contribution observed in both proteins can be explained by the high number of aromatic amino acids and their clustering in the sequence that allows additional coupling processes. In this sense, LytA amidase and other choline-binding proteins contain six repeated units in the COOH-terminal region with the consensus sequence GVVKIDGWYFDFNS-GAMATN (15). FTIR spectroscopy shows that both LytA and its COOH-terminal module are αβ proteins, with a very high content of β-sheets (47–51%) and turns (23–20%) and also a lower fraction of unordered segments (about 10%). The similarity in the secondary structure composition of LytA and C-LytA suggests that the NH2-terminal region should also exhibit a similar secondary structure composition.

**C-LytA and LytA-Choline Interaction**—Choline binding modifies the UV CD spectra of LytA and C-LytA, mainly at 224, 286, and 295 nm, resulting in emerging bands in the difference spectra. Choline-induced changes near the N-UV region follow a similar pattern in LytA and in C-LytA; therefore, they can be attributed to modifications in the environment of those tryptophan and tyrosine residues located in the COOH-terminal module. The similarity between θ224 and θ294 variations with ligand concentration reveals that the modifications in the far-UV CD spectra are mainly due to the same molecular event. The lack of a large conformational change affecting the secondary structure of LytA or of its COOH-terminal region upon choline binding is further confirmed by FTIR. Notwithstanding, the slight changes found in the amide I band upon ligand binding indicate a certain increase in the β-turn content at expenses of β-sheet. In this sense, deconvolution of the far-UV CD spectra also suggests a partial conversion of the β-structure associated with component I in that associated with component VI. The above results suggest that cation-π interactions between choline and the side chains of the highly conserved aromatic residues of the repeated sequence motif of the COOH-terminal module could account for the ligand recognition process, as has been previously described for the acetylcholine receptor (35, 36).

Choline titration curves for LytA and C-LytA demonstrate the existence of two types of binding sites/COOH-terminal module, in agreement with previous findings (9). Saturation of high affinity sites is involved in LytA dimerization and in the subsequent enhancement of affinity toward the substrate (9). On the contrary, saturation of the low affinity sites requires choline concentrations similar to those resulting in amidase inhibition in in vitro competition experiments (37). However, such correlation should be considered cautiously, since the apparent affinity of the inhibitor would depend on the substrate concentration present in the activity assays and be particularly difficult to estimate in the case of the bacterial cell wall, which constitutes an insoluble, polymeric, and heterogeneous substrate.

The free energy changes derived from the equilibrium constants reported in Table IV show that choline binding to the high affinity sites of LytA or C-LytA dimers will be favored, on average, by −2.8 to −1.6 kcal·mol⁻¹, over the monomer. The values derived for the stepwise equilibrium constants Kc₁ and Kc₂ indicate that the two high affinity binding sites are approximately equivalents, whereas those of Kc₃ and Kc₄ show the existence of positive cooperativity between the low affinity binding sites; therefore, dimeric species with a single low affinity saturated site will be imperceptibly populated.

**Structural Changes in LytA and C-LytA Induced by Temperature**—The conformational changes induced by temperature in the secondary structure of LytA and C-LytA show the formation of unfolding intermediates, consistent with the complexity of their DSC profiles (9). The analysis of the CD thermal transitions in terms of the parameters derived by DSC reveals a simultaneous variation in secondary and tertiary structures, with the exception of the final transition centered at 224 nm in LytA amidase. On the other hand, the presence of residual secondary structures in the final thermally denatured forms is also deduced from both CD and FTIR spectroscopies.

Irreversible thermal denaturation of LytA and C-LytA occurs with the appearance of the 1683–1616 cm⁻¹ doublet in the FTIR spectrum, which has been correlated with the formation of aggregated extended structures. This change is concomitant with an increase in the contribution of component VI (assigned to antiparallel β-sheet structures) to the CD spectrum and with the formation of high order aggregates, as proved by analytical ultracentrifugation experiments (9). Under reversible unfolding conditions (C-LytA at saturating choline concentrations) none of the previous features are observed. Therefore, the irreversibility of thermal transitions of LytA amidase and of its COOH-terminal module has its origin in the collapse of the polypeptide chain into extended structures characterized by a hydrogen-bonding pattern that resembles that of native antiparallel β-sheets involving intermolecular interactions. The capacity of choline to prevent the formation of such structures in the isolated COOH-terminal region suggests an important role of aromatic side chains in the maintenance of the intermolecular aggregates. It should, however, be mentioned that choline inhibition of the irreversible process operates only in the absence of an aggregated core such as that of the denatured NH2-terminal module of LytA.

Choline is not found in the cell walls of pathogens other than pneumococcus, and this amino alcohol appears to be a critical determinant of the inflammatory activity of the pneumococcal teichoic and lipoteichoic acids (4). Recent studies have demonstrated that choline is also involved in the anchor of S. pneumoniae to activated human cells, mimicking the effect of platelet-activating factor (38). Moreover, genetic approaches have demonstrated that choline has directed the evolution of a family of proteins. In this sense, the presence of a specific choline-binding domain provides the cell wall lytic enzymes with an essential property to recognize its target. Thus, choline-protein interaction keeps the enzyme attached to its polymeric insoluble substrate, therefore increasing the catalytic efficiency. The presence of two choline sites/subunit as well as LytA dimerization may play an important role in the amidase mode of action. Both processes may enhance LytA affinity to its cellular target by preferential location of the amidase near the site of cell wall lysis and by reducing the entropy loss associated with ligand binding (9). This latter effect has been shown to operate in many situations where molecular recognition is important (39). The results reported in the current study provide the thermodynamic and structural bases of choline-LytA interaction. They also provide a further characterization of the pneumococcal autolysin structure and of its COOH-terminal domain. Assuming the substantial role that this enzyme plays in cell division and pneumococcal infection, these studies open new insights into the possibility of considering the choline-binding domain of LytA amidase as a potential target to develop new antibiotics.

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