Differential scanning calorimetry (DSC) has been employed to characterize the thermal denaturation of CPL1 lysozyme and its isolated fragments in the absence and presence of choline. The heat capacity function of CPL1 lysozyme shows two peaks with $T_m$ values of 43.5 and 51.4 °C. At saturating concentrations of choline the second transition disappears, and the $T_m$ is shifted to higher temperatures. The DSC thermogram of the C-CPL1 protein corresponding to the carboxy-terminal domain of CPL1 lysozyme has a single peak with a $T_m$ of 42.9 °C. The effect of choline is very similar to that observed for the whole CPL1 lysozyme. The NH$_2$-terminal fragment obtained by proteolytic digestion shows a $T_m$ of 52 °C, close to that of 51.4 °C found for the second transition of CPL1, and choline does not affect the $T_m$ nor the denaturation enthalpy. These data suggest that choline is bound to the COOH-terminal domain of the protein. Deconvolution of the excess heat capacity curve of the CPL1 lysozyme shows that the data can be fitted to two two-state independent transitions. The analysis of the DSC curves showed that the NH$_2$-terminal unfolding enthalpy steadily decreases with increasing concentrations of choline. These results indicate that, under saturating concentrations of choline, whole CPL1 could unfold as a single cooperative unit.

The probability that a polypeptide chain with some arbitrary amino acid sequence could fold into a compact cooperative structure is so low that the evolution of new proteins is most likely favored by the use of structural blocks whose folding technology is already perfectly worked out. It appears that domains present in large proteins are descendents of formerly independent pre-proteins which still retain the spatial structure and folding technology of their ancestors (1). The cell wall lytic enzymes of *Streptococcus pneumoniae* and its bacteriophages (2) represent one of the best characterized systems that have contributed to experimentally support this hypothesis, which describes the central concept of the modular theory of evolution (3). One member of the pneumococcal cell wall lytic system is the CPL1 lysozyme encoded by the bacteriophage Cp-1 (4). This protein belongs to the lysozyme group that was preliminary exemplified by the lysozyme of the fungus *Chalaropsis* (5). The number of proteins of this group has recently increased due to the characterization of new lysozymes (6). In contrast with other lysozyme types, no tertiary structure of a member of the *Chalaropsis* group has so far been determined. Nowadays, structural data can be obtained using only physicochemical techniques together with the powerful assistance of genetic engineering. These approaches have allowed us to demonstrate that the CPL1 lysozyme is composed of two modules, the NH$_2$-terminal region that contains the catalytic center (7) and the COOH-terminal region, responsible for the binding of the protein to specific components of the cell wall (8). One of these components is the amino alcohol choline, present only in the teichoic acids of the cell wall of pneumococcus (9) and of a very limited number of bacteria (10, 11). It is worthwhile to note that the presence of choline in the pneumococcal cell wall is needed for CPL1 lysozyme activity, since the substitution of choline by its analogous ethanolamine renders the cell wall resistant to hydrolysis by this lysozyme (12). To demonstrate that the CPL1 lysozyme was built from at least two independent domains we have independently cloned and expressed both segments of the protein (7, 8) and have used them to construct chimeric enzymes (13, 14), showing that the proposed domains retain their assigned functionalities. In addition, we have demonstrated that the NH$_2$-terminal module of the CPL1 lysozyme can be isolated after proteolytic digestion (7). When combined with a demonstration of the structural integrity of the domains through studies of their folding-unfolding processes, these types of experiments are considered as the ultimate proof of the existence of independently folded domains. Hence, we have studied the melting behavior of the polypeptide chain of the CPL1 lysozyme using differential scanning calorimetry. In addition, we have determined the thermodynamic parameters of choline binding and the influence of this ligand on the thermal unfolding of CPL1.

**MATERIALS AND METHODS**

Protein Purification—CPL1 lysozyme (molecular mass 39.1 kDa) and the C-CPL1 carboxyl-terminal domain (molecular mass 18.7 kDa) were purified from cell extracts of *Escherichia coli* DH1 (pCIP100) and DH1 (pCM1), respectively, by affinity chromatography on DEAE-cellulose according to the method of Sanz et al. (15). The 24-kDa F1 fragment, which corresponds to the NH$_2$-terminal domain of CPL1 lysozyme was prepared by limited proteolysis as described earlier (9). The purity of the proteins was analyzed by SDS-polyacrylamide gel electrophoresis (16). The concentration of the protein solutions was determined spectrophotometrically using the following values of $E_{max}$: 113,322 for CPL1 lysozyme (17); 48,923 for F1 fragment (7); and 81,076 for C-CPL1 fragment (8). The $E_{max}$ values were calculated using the parameters of Fasman (18), according to the amino acid sequences of the proteins.

*Differential Scanning Calorimetry—*Caibroimetric measurements.
were carried out with a Microcal MC-2 DSC¹ (Microcal, Inc, Northampton, MA) at a heating rate of 0.5 K min⁻¹, unless otherwise stated, and under an extra constant pressure of 2 atm. The standard DA-2, Cpcalc, and Dynacp software packages were used for data acquisition and analysis. The excess heat capacity functions were obtained after base line subtraction and correction of the instrument response time effect, as described previously (19). Unless otherwise indicated the calorimetric measurements were carried out in 20 mM phosphate buffer, pH 7.0, and at a protein concentration of 1 mg ml⁻¹.

Isothermal Flow Calorimetry—The enthalpy of binding of choline to CPL1 lysozyme was determined in a LKB 10700-1 flow microcalorimeter at 25 °C. Electrical and chemical calibrations were performed according to the method previously described (20). All measurements were made using 20 mM phosphate buffer, pH 7.0, at a flow rate of 8 ml h⁻¹ and a protein concentration of 1 mg ml⁻¹.

Analytical Ultracentrifugation—The sedimentation coefficients were determined at 25 °C, in an Analytical Centrifuge Beckman model E at 60,000 rpm and a protein concentration of 1 mg ml⁻¹.

RESULTS

Thermal Denaturation of CPL1—A tracing of the DSC curve for a typical scan with CPL1 lysozyme is shown in Fig. 1. The thermogram exhibits two peaks with transition temperatures, Tm, at 43 and 50 °C, respectively. The reversibility of the thermal transitions was checked by reheating the solution in the calorimeter cell, after cooling from the first run. The first transition was irreversible under all experimental conditions, while the reversibility of the second endotherm depends on both the final temperature reached during the first run and the heating rate. At a scanning rate of 1.5 °C min⁻¹ and a final temperature of 46 °C, the heat absorbed (Fig. 1, curve B) was 80% of the value calculated for the second transition in the first scan (Fig. 1, curve A). This transition was still 45% reversible when the first heating of the sample was stopped at 60 °C (Fig. 1, curve C). Above this temperature, no signal was detected during the second scan. These results suggest the existence of at least two independent calorimetric transitions.

The heat capacity curves recorded at different scanning rates (0.5, 1, and 1.5 °C min⁻¹) did not show significant variations either in the form or in the enthalpy of the endotherm, suggesting that the thermal denaturation of the CPL1 lysozyme was not kinetically controlled. Thus, application of equilibrium thermodynamics to the experimental traces would not lead to significant errors (21, 22). The deconvolution of the excess heat capacity curve (23–25) shows that the thermal transition can be fitted to two independent two-state transitions (see Fig. 2). The thermodynamic parameters for both transitions are presented in Table I.

Thermal Denaturation of the COOH-terminal Module of CPL1 Lysozyme—Fig. 3 shows the excess Cp profile of the C-CPL1 protein (curve A), which corresponds to the COOH-terminal module of the CPL1 lysozyme. The thermal transition of the C-CPL1 protein shows a single peak with a Tm of 42.9 °C. This value is very close to that of the first transition of CPL1 lysozyme (curve B), suggesting that it could correspond to the thermal denaturation of the COOH-terminal domain. A second rescan of the sample shows that the transition is completely irreversible. The thermodynamic parameters of this transition are summarized in Table I.

Thermal Denaturation of the NH₂-terminal Fragment of CPL1 Lysozyme—To study the melting behavior of the NH₂-terminal part of the CPL1 lysozyme, the F1 fragment was isolated by trypsin digestion of the whole enzyme as described above. The melting curve of the fragment (Fig. 3, curve C) shows a peak with a Tm of 52 °C, close to that of 51.4 °C found for the second transition of CPL1. The thermodynamic parameters are presented in Table I.

It should be remarked that, despite the fact that the reversibility of thermal transitions is important for its thermodynamics treatment (1, 27), the observation that the irreversible DSC thermograms of CPL1 are insensitive to scan rate suggests that the experimental heat capacity values should not depart significantly from those that would be observed for the folding ⇔ unfolding equilibrium. Thus, as it has been recently proposed (21, 22), application of equilibrium thermodynamics to these calorimetrically irreversible transitions would be permissible.

Thermal Denaturation of CPL1 Lysozyme and Its Isolated Modules in the Presence of Choline—We have previously observed by circular dichroism that the presence of choline induced a conformational change in the protein (17), as well as in the C-CPL1 fragment (8). Fig. 4 shows the effect of different concentrations of choline on the thermally induced transitions of CPL1. The binding of the ligand increases the enthalpy of denaturation and induces a shift in the Tm of the

¹The abbreviations used are: DSC, differential scanning calorimetry; H_v, van't Hoff enthalpy change calculated as 4RT_e² C_p/cal/mg•°H.
FIG. 2. Deconvolution of the excess heat capacity function of CPL1 lysozyme. The continuous line corresponds to the experimental curve while the dashed line represents the best fit to two independent two-state transitions (dotted lines).

TABLE I
Thermodynamics of the thermal unfolding of CPL-1, C-CPL1, and F1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Choline</th>
<th>Tm</th>
<th>ΔH</th>
<th>ΔHrel</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPL1</td>
<td>0</td>
<td>43.5</td>
<td>894</td>
<td>418</td>
</tr>
<tr>
<td>First transition</td>
<td>0</td>
<td>43.5</td>
<td>564</td>
<td>618</td>
</tr>
<tr>
<td>Second transition</td>
<td>0</td>
<td>51.4</td>
<td>309</td>
<td>359</td>
</tr>
<tr>
<td>C-CPL1</td>
<td>0</td>
<td>42.9</td>
<td>455</td>
<td>968*</td>
</tr>
<tr>
<td>F1</td>
<td>0</td>
<td>52.0</td>
<td>725</td>
<td>504*</td>
</tr>
<tr>
<td>CPL1</td>
<td>10</td>
<td>51.5</td>
<td>994</td>
<td>1398*</td>
</tr>
<tr>
<td>C-CPL1</td>
<td>10</td>
<td>50.9</td>
<td>614</td>
<td>1212*</td>
</tr>
<tr>
<td>CPL1</td>
<td>22</td>
<td>53.3</td>
<td>1209</td>
<td>1669*</td>
</tr>
</tbody>
</table>

*ΔHrel calculated as 4RTm² Cpentrant/ΔH.

first transition to higher temperatures (Fig. 5). The second peak apparently disappears or is shifted to lower temperatures, and, above 8 mM choline, a single peak is observed which becomes increasingly asymmetric as the concentration of choline is further increased. The effect of the choline binding in the Tm is shown in Fig. 5. The experimental thermodynamic parameters of CPL1 unfolding at saturating choline concentrations are summarized in Table I. Substitution of 0.2 M NaCl for choline did not produce any alterations on the profile of the CPL1 lysozyme (data not shown), indicating that the effect of choline was not due to a simple increase in the ionic strength. The thermal denaturation of CPL1 in the presence of choline was partially reversible when the heating was stopped at temperatures lower than 55 °C. However, the Tm values and the shapes of the traces were scan-rate independent, suggesting that the irreversible process would not proceed at a significant rate within the transition range (21, 22).

The influence of choline on the ΔCp profiles of the C-CPL1 protein was very similar to that observed for the first transition of the CPL1 lysozyme (Fig. 6). These results support the above hypothesis that this transition corresponds to the unfolding of the COOH-terminal domain of the lysozyme. As expected, the addition of choline to the F1 fragment did not produce a significant change of the thermal unfolding of the fragment.

Deconvolution of the DSC curves into the sum of two independent two-state steps showed that the NH₂-terminal unfolding enthalpy steadily decreases with increasing concen-

FIG. 3. Calorimetric traces of the C-CPL1 protein (curve A), NH₂-terminal fragment (curve C) and the whole CPL1 lysozyme (curve B).
Thermal Denaturation of Free and Choline-bound CPL1 Lysozyme

FIG. 4. Effect of choline concentration on the DSC transitions corresponding to the thermal unfolding of the CPL1 lysozyme. The numbers alongside the transitions indicate the choline concentration in mM.

FIG. 5. Dependence of $T_m$ on the concentration of choline. The $T_m$ value was that of the first peak at ligand concentrations at which the two endotherms were still observed.

FIG. 6. Effect of choline binding (10 mM) on the DSC profiles of CPL1 lysozyme (solid line) and C-CPL1 protein (double-dotted line).
tions of choline. These results indicate that under saturating concentrations of choline, whole CPL1 could unfold as a single cooperative unit. This conclusion would be also supported by the similitude of the DSC curves obtained above 10 mM choline for the C-CPL1 protein and the whole polypeptide chain (Fig. 6). A possible source of the asymmetry seen at high concentrations of choline would be the dissociation of an oligomer. The fact that the ratio between $\Delta H_m/\Delta H$ increases to 1.3, and the sedimentation coefficient of the enzyme changes from 3.2 to 4.4 suggest that the ligand induces some degree of intermolecular cooperation between monomers of CPL1. To check this supposition the experimental curves were fitted to a single two-state transition involving dissociation of a dimer on unfolding (26). This model did not lead to a standard deviation significantly lower than that obtained from fitting of the data to a single two-state transition assuming intermolecular cooperation. Actually, the unfolding of CPL1 could have an intermediate behavior between those expected from the last two models and at high temperatures the association-dissociation process could probably become predominant.

The value of $T_{1/2}$ increases with the total ligand concentration, as predicted by the van't Hoff equation (Fig. 7). The number of choline molecules bound to the cooperative subunit, $m$, was estimated from the slope of this plot ($\Delta H_m = -S R m$; where $S$ is the slope of the plot) (27, 28). A value of $m$ of around 6.5 has to be assumed in order that the ratio of $\Delta H_m$ values at $T_m$ from both the plot and calorimetry is equal to 1. From this value, 5 binding sites per monomer of CPL1 are obtained if a degree of intermolecular cooperation of 1.3 is assumed.

**Enthalpy of Choline Binding to CPL1 Lysozyme**—Fig. 8 shows the enthalpy of choline binding to CPL1 lysozyme as a function of the ligand concentration at 25 °C. The continuous line corresponds to the theoretical fit of the experimental data assuming one class of binding sites, an enthalpy change of $-160$ kJ per mol of CPL1 and a value of $2.3 \times 10^{-3}$ M for the dissociation constant. The binding of choline is enthalpically driven and would proceed with a free energy change of $-14.5$ kJ mol$^{-1}$ and an entropy change of $-57$ J K$^{-1}$ per mol of choline, if the number of choline binding sites in the CPL1 lysozyme is assumed to be 5 (as estimated from the van't Hoff plot).

FIG. 7. Van't Hoff plot for DSC experiments of CPL1 in the presence of various concentrations of choline. The logarithm of the ligand concentration is plotted as a function of $T_{1/2}$, the temperature of half completion of the transition. The value of the slope is 26,600 K.

**DISCUSSION**

The genetic and biochemical analyses which had been carried out with the CPL1 lysozyme indicated that this protein may probably have evolved by the fusion of two preexisting modules. These analyses supported the hypothesis that the NH$_2$- and COOH-terminal fragments of the protein can be correctly and independently folded when they are cloned individually. However, in the absence of crystallographic data, we could not ascertain that both fragments represent independent folded domains in the native lysozyme. The thermodynamic analysis of the unfolding of the CPL1 lysozyme and its fragments presented here is in good agreement with the previous hypothesis on the modular structure of the cell wall lytic enzymes of *S. pneumoniae* and its bacteriophages. The heat capacity function shows two peaks corresponding to two different transitions. The first one was apparently irreversible under all conditions employed while the second one was partially reversible depending on the scanning conditions. However, the irreversible processes would not proceed at significant rates within the transition temperature range, since neither the shape of the transitions nor the $T_m$ values were scan-rate dependent. The analysis of the thermograms allowed us to deconvolute the melting curves into two independent two-state transitions with $T_m$ values of 43.5 and 51.4 °C, respectively. The ratio between the van't Hoff and calorimetric enthalpies is 1.1 for both transitions.

The comparison between the DSC curves of the CPL1 lysozyme and its isolated fragments allowed us to assign the first transition to the COOH-terminal region. The reasons for this assignment were (i) its $T_m$ value is very similar to that of the C-CPL1 protein and (ii) it is specifically and strongly influenced by the addition of choline to the CPL1 lysozyme, which is also found for the C-CPL1 protein. We have previously demonstrated by biochemical methods and circular dichroism that choline strongly interacts with the C-CPL1 protein but not with the NH$_2$-terminal fragment (8, 17). The second peak was assigned to the NH$_2$-terminal region of lysozyme because of the proximity of the $T_m$ values of their respective transitions. The specific enthalpies of denaturation of both the C-CPL1 protein and the F1 fragment are of the same order as literature values for many globular proteins at the same temperature (29). The linking of both modules in CPL1 reduces the value of the specific enthalpy for the NH$_2$-terminal transition by a factor of 2 without a remarkable change in $T_m$. This results could indicate that the presence of the COOH-terminal region enhances the microstability of the native structure of the NH$_2$-terminal domain (30). On the other hand, the independence of both domains for their folding-unfolding transitions indicates that few hydrophobic contacts are established between each domain. This conclusion is in agreement with the finding that chimeric enzymes constructed by interchanging these domains with different NH$_2$-or COOH-terminal modules of other pneumococcal enzymes were as efficient as the parent enzymes (13, 14). This result would be expected if the efficiency of the enzymes was not dependent on the presence of strong interdomain interactions.

As pointed out above the CPL1 lysozyme depends on the presence of choline for activity (4). Choline seems to play a relevant role in the recognition of the pneumococcal cell wall by the enzyme (8). As occurs in other enzymes that act on polymeric substrates the binding of the enzyme to the substrate is a fundamental step that can contribute to maintain an effective concentration of substrate in the vicinity of the enzyme, increasing its catalytic efficiency (31). We have previously observed that the addition of choline to the assay medium produces an inhibition of the hydrolytic activity of CPL1 lysozyme by competition with the choline present in
the teichoic of the pneumococcal cell wall (4). This inhibition was accompanied by a drastic change in the fluorescence (not shown) and circular dichroism spectra of the protein (8, 17). In all cases the half change was observed at around 2–3 mM choline. Similar results were obtained with the C-CPL1 protein (8), but no changes were observed in the circular dichroism spectrum of the F1 fragment in the presence or in the absence of choline (7). In addition, choline reduces the rate of proteolytic digestion of the COOH-terminal domain of the CPL1 lysozyme. However, in spite of these observations, more conclusive evidence that choline could affect the folding of the protein was not found. The binding of choline affects both the thermal stability of the COOH-terminal domain and, probably, the independence of the two structural domains observed in unbound CPL1. As discussed above, the single peak observed in the presence of saturating concentrations of choline indicates a cooperative melting of the whole molecule under such conditions, suggesting that the number of hydrophobic contacts between the NH2- and the COOH-terminal domains is enhanced by the ligand. The ratio between ΔHν and ΔH indicates that the protein is somewhat associated in the presence of choline. This fact is also supported by the observed increase in the sedimentation coefficient. The value of T1/2 changes with the ligand concentration in the manner predicted by the van’t Hoff equation, as expected if the ligand is dissociated on denaturation (27). The difficulties in finding an accurate estimation of ΔCp (∆H = (the change in heat capacity from the native to denatured state)) prevent the determination that the difference found in the enthalpies of denaturation of the protein in the presence and absence of the ligand is a result of choline binding.

According to the deduced amino acid sequence of the CPL1 lysozyme the COOH-terminal domain was made from 6 repeated sequences of about 20 amino acids, P1 to P6. This repeated sequence contains a highly conserved hydrophobic core composed of three aromatic amino acids that could be involved in the interaction with choline. The 5 binding sites for choline estimated from the van’t Hoff plot (Fig. 7) fit with the modular structure of the COOH-terminal domain. The dissociation constant calculated from isothermal calorimetry agrees with those obtained from the changes in the fluorescence and circular dichroism spectra, by titration with choline (8). The existence of several binding sites in CPL1 could explain the strong retention by DEAE-cellulose columns (15, 17) despite the fact of the low apparent binding constant for choline.

Acknowledgments—We thank M. V. López Moyano for excellent technical assistance and Dr. H. Lindsell for linguistic revision.

REFERENCES


FIG. 8. Calorimetric titration curve of CPL1 with choline at 25 °C. The solid line corresponds to the theoretical fit of the experimental data using the thermodynamic parameters indicated in the text.