Structural and Thermodynamic Characterization of Pal, a Phage Natural Chimeric Lysin Active against Pneumococci*

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Pal amidase, encoded by pneumococcal bacteriophage Dp-1, represents one step beyond in the modular evolution of pneumococcal murein hydrolases. It exhibits the choline-binding module attaching pneumococcal lysins to the cell wall, but the catalytic module is different from those present in the amidases coded by the host or other pneumococcal phages. Pal is also an effective antimicrobial agent against Streptococcus pneumoniae that may constitute an alternative to antibiotic prophylaxis. The structural implications of Pal singular structure and their effect on the choline-amidase interactions have been examined by means of several techniques. Pal stability is maximum around pH 8.0 (T_m = 50.2 °C, ΔH_m = 183 ± 4 kcal mol⁻¹), and its constituting modules fold as two tight interacting cooperative units whose denaturation merges into a single process in the free amidase but may proceed as two well resolved events in the choline-bound state. Choline titration curves reflect low energy ligand-protein interactions and are compatible with two sets of sites. Choline binding strongly stabilizes the cell wall binding module, and the conformational stabilization is transmitted to the catalytic region. Moreover, the high proportion of aggregates formed by the unbound amidase together with choline preferential interaction with Pal dimers suggest the existence of marginally stable regions that would become stabilized through choline-protein interactions without significantly modifying Pal secondary structure. This structural rearrangement may underlie in vitro "conversion" of Pal from the low to the full activity form triggered by choline. The Pal catalytic module secondary structure could denote folding conservation within pneumococcal lytic amidases, but the number of functional choline binding sites is reduced (2–3 sites per monomer) when compared with pneumococcal LytA amidase (4–5 sites per monomer) and displays different intermolecular interactions.

Dp-1, the first described pneumococcal bacteriophage, belongs to the Siphoviridae family (1). Its peptidoglycan-degrading enzyme, Pal, was biochemically characterized as a choline-dependent amidase (2) synthesized as a low activity form that requires in vitro incubation with choline or choline-containing cell walls to achieve full activity (3), a process designated as "conversion." Pal shows the modular organization characteristic of pneumococcal cell wall lysins (3), but represents one step beyond in the modular evolution of pneumococcal murein hydrolases. Its N-terminal region has no similarity with the amidases coded by the host or other pneumococcal phages (4, 5),2 but is highly similar to the murein hydrolase coded by Lactococcus phage BK5-T.3 The C-terminal region comprises a choline binding module (ChBM) homologous to that found in the pneumococcal lytic system4 (6) that attaches the enzyme to choline residues present in pneumococcal envelope (7). As in most pneumococcal lysins so far known, six repeats of about 20 amino acids (p1–p6) and a short C-terminal tail form the ChBM of Pal (3). Up to now, only the structures of Cpl-1 (8), the lysozyme coded by Cp-1 bacteriophage, and C-LytA (9), the ChBM of LytA, have been solved at atomic resolution. Their choline-binding repeats have the same secondary structure (a symmetrical β-hairpin connected to the following repeat by a loop and a coiled region), but the global fold varies despite being 53% identical in sequence. Since choline-binding loci are located at the interface of two consecutive repeats, the number of functional sites depends on both conservation of the aromatic residues involved in choline binding and the global fold of the ChBM (8, 9).

The capabilities of the Pal amidase to kill pneumococcus and to prevent and eliminate the nasopharyngeal colonization in mice (10), as well as its protective effect against bacteremia in a murine sepsis model (11) have been recently proved. Moreover, oral streptococcal strains without phosphorylcholine in their bacterial envelope are unaffected. Intraperitoneal inoculation of Cpl-1 lysozyme also protected mice against septicemia caused by pneumococci (12). These findings strongly indicate that bacteriophage lytic enzymes can constitute effective antimicrobial agents (enzymbiotics) by hydrolyzing the bacterial peptidoglycan. Since the asymptomatic carrier state is the major reservoir of Streptococcus pneumoniae, Pal

1 The abbreviations used are: Pal, amidase encoded by pneumococcal Dp-1 bacteriophage; LytA, major autolysin of S. pneumoniae; ChBM, pneumococcal choline binding module; ChBPs, pneumococcal choline-binding proteins; C-LytA, ChBM of LytA; Pfam, protein domain database; CD, circular dichroism; IR, Fourier transform infrared spectroscopy; DSC, differential scanning calorimetry; T_m, temperature of the maximum 1734 in the heat capacity curve; ΔH, calorimetric enthalpy change; ΔH_f, van’t Hoff enthalpy change; <ΔH(T)>; excess enthalpy change at temperature T<; <ΔC_p(T)>; excess heat capacity change at T<; K_b, ligand concentration inducing half variation in the monitored property.

2 Pfam Data Base: Pfam accession number PF01510.

3 Pfam Data Base: Pfam accession number PF05382.

4 Pfam Data Base: Pfam accession number PF01473.

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and other pneumococcal lysins may also constitute a new strategy alternative to antibiotic prophylaxis. This is of primary interest because of the high morbidity and mortality caused by pneumococcal diseases, and to the increasing prevalence of antibiotic resistant strains and the suboptimal efficacy of available vaccines (13, 14). Despite its singular structural features and potential biomedial relevance, the conformational stability of ChBPs, and their interaction with the cell wall remain almost unknown. In this study we address this issue from structural and thermodynamic standpoints in order to characterize Pal conformational stability and choline binding capability, the structural relationship of its catalytic module with that of the other pneumococcal lytic amidases, and how the nature of the catalytic and cell wall binding modules can affect the transmission of conformational information between them. Current results could also facilitate the optimization of Pal antimicrobial capability in its use against pneumococcal-caused diseases.

EXPERIMENTAL PROCEDURES

Protein Purification and Chemicals—Pal amidas (34,453 Da) was purified from Escherichia coli DH5α (pMSP1) cells (3) by affinity chromatography on DEAE-cellulose taking advantage of the capacity of ChBPs to bind diethylaminoethyl groups (15), and the purity of the isolated protein samples was routinely analyzed by SDS-PAGE (16). Before use, protein samples were extensively dialyzed at 4°C against the appropriate buffer (20 mM phosphate, with or without choline) and then centrifuged at 11,600 × g for 5 min. Pal concentration was determined spectrophotometrically using a molar absorption coefficient of 280 nm in phosphate buffer of 117,500 M−1 cm−1 measured as described (17). Choline concentration was measured by differential refractometry (18). All reagents (Sigma) were of analytical grade.

Circular Dichroism Spectra—CD spectra were recorded in a JASCO J-510 spectropolarimeter equipped with a Peltier type cell holder (Jasco Corp.), sing protein concentrations of 5 μM (1-mm pathlength; far-UV region) and 8 μM (1-cm pathlength; near-UV region). Each spectrum (0.2-mm intervals) was the average of 3–4 measurements with different samples (each of them the average of 4 scans) performed at a rate of 20 nm min−1 using a response time of 2 s and a bandwidth of 1 nm. The temperature was kept constant at 20°C. The buffer contribution to the CD spectrum was subtracted from the experimental data, and the corrected ellipticities were converted to mean residue ellipticities using an average molecular mass per residue of 116.4. The secondary structure composition was estimated from the far-UV spectra using the following programs: CONTIN (17 protein spectra data set) that implements the ridge regression algorithm of Provencher and Glockner (19); CDNN (33 protein spectra data set) based in the use of precompiled neural networks (20), and SELCON (17 protein spectra data set) that incorporates a self-consistent method together with the singular value decomposition algorithm (21).

Infrared Spectroscopy—The IR spectrum was the average of four independent scans recorded at 20°C in a Nicolet Magna II 550 FTIR spectrometer equipped with a MCT detector (Nicolet Corp.), following the procedure previously described (22). Pal was initially prepared at 18.5 μM in 10 mM phosphate buffer, pH 8.0 with choline. Aliquots containing about 300 μg of protein were dried and rehydrated by adding 25 μl of D2O and allowed to undergo proton-deuterium exchange until the amide band was invariant with time. Data treatment and band decomposition of the original amide I have been described elsewhere (23, 24). In D2O (25), bands centered around 1679 cm−1 can be assigned to the high frequency component of β-sheets, whose intensity would be less than one-tenth of the low frequency component that appears around 1630 cm−1, and bands centered near 1671 cm−1 have been associated to β-turns. The band about 1652 cm−1 is usually produced by d-amino acids (26), with bands originated from large loops have also been described around this frequency (26, 27). Finally, bands centered at about 1645 cm−1 are characteristic of unordered segments.

Prediction Methods—Secondary structure prediction for each isolated module was performed by three independent methods: PSIPred (28), SPSsore (29), and PROF (30) using the program servers. Their prediction accuracy for the residue (PSIPred: 8.0%; SPSsore: 74.3%; PROF: 75.1%) is weekly and automatically evaluated by EVA server (31).

Sedimentation Equilibrium—Sedimentation equilibrium experiments were performed by centrifugation of 80-μl samples at 15,000 × g and 25°C in an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Inc.), using 12-mm double-sector six-channel epon-charcoal, L-shaped anodes (AN50Ti rotor). Radial scans were taken at 12, 16, and 18 h with the same results. Baseline offsets were determined from absorbance near to the meniscus of a radial scan taken after running the samples for 8 h at 200,000 × g. Conservation of integral absorbance in the cell was checked in all the experiments. Data in the absence and in the presence of 140 mM choline are the average of five independent measurements. The apparent weight-average molecular mass, Mr, was obtained fitting a sedimentation equilibrium model for single species to individual data sets, using the conservation of signal algorithm (32) from EQASSOC & XLAEQ programs as reported elsewhere (33). The partial specific volume of Pal calculated from amino acid composition was 0.71 ml g−1 (34).

RESULTS

Choline Recognition by the ChBM Stabilizes the Catalytic Module of Pal Amidase

Thermal Stability: Dependence on Choline Binding—The DSC thermal denaturation profile of Pal amidas in phosphate buffer, pH 8.0 (Fig. 1A) displays a single peak centered at 50.2°C (23.8 μl Pal; v = 2.0 °C h−1) and denaturation proceeds with an enthalpy change, ΔHd, of 178 ± 7 kcal mol−1. The transition temperature was pH-dependent (Fig. 1A, inset) with maximum stability seen around pH 8.0. In the presence of choline (Fig. 1B), the endotherm shifts upward and becomes broader as the ligand concentration increases, showing two well separated peaks at around 40 mM choline and above. At 140 mM choline the heat capacity profile shows two endotherms centered on 54.7 and 65.6°C at a scan rate of 20 °C h−1, and characterized, respectively, by calorimetric enthalpy changes of 115 ± 10 and 147 ± 15 kcal mol−1. Supplementation of phosphate buffer with 140 mM NaCl did not affect Pal denaturation (Table I), demonstrating the specificity of choline-mediated stabilization. Pal denaturation was irreversible under all the conditions assayed and its DSC transitions were scanning rate-dependent both in the absence and in the presence of choline (Fig. 2A and Table II). It was, therefore, kinetically controlled, although at high choline concentrations the first endotherm presented a weaker dependence on the scanning rate than the high temperature peak. On the other hand, the transition temperature of the unbound amidas increased, while the high temperature peak was protein concentration-independent (Fig. 3C).

Assignment of Thermal Transitions to Pal-constituting Modules—Thermal denaturation in the presence of ε-Ala, the amino acid moiety involved in the amide bond hydrolyzed by the amida, was used to check the implication of the catalytic module in the transitions observed at high choline concentration. As it is shown in Fig. 1B (upper trace), supplementation of...
Denaturation enthalpy changes and transition temperatures of Pal amidase

The temperature scan rate was 20 °C h⁻¹ (23.8 μM Pal; 20 mM phosphate buffer, pH 8.0). The estimated errors are 10% in ΔH and ±0.1 °C in Tm.

| [Choline] | Tn | ΔH1 | Tm | ΔH2 | ΔH* |
|-----------|----|-----|----|-----|-----|
| mM        | °C | kcal mol⁻¹ | °C | kcal mol⁻¹ | kcal mol⁻¹ |
| 0         | 50.2 | 178 | 178 |
| 5         | 51.4 | 193 | 193 |
| 11        | 53.4 | 190 | 190 |
| 40        | 60.8 | 127 | 236 |
| 85        | 64.4 | 133 | 238 |
| 140       | 65.6 | 147 | 261 |
| 140 mM NaCl | 50.4 | 169 | 169 |

ΔH* is the total enthalpy change.

The 140 mM choline-phosphate buffer with 100 mM L-Ala stabilizes the protein region giving rise to the low temperature peak without affecting the high temperature peak. This result strongly supports the implication of the catalytic module in the low temperature endotherm, and the assignment of the peak centered at 65.6 °C (ν = 20 °C h⁻¹) to denaturation of the ChBM. The strong influence of choline binding on the low temperature peak reveals the transmission of the conformational stabilization promoted by choline binding to the catalytic module of Pal and, therefore, the presence of tight intermodular interactions between both modules (36, 37). Since the N- and C-terminal modules have been acquired from independent evolutive sources, this feature did not probably appear in the first event of fusion and strongly suggests that new stabilizing interactions between the constituting modules would have been gained as the resulting protein evolved.

Denaturation of the Unbound Pal Amidase Can Be Described in Terms of a Three-state Model (N ↔ D → I)—The simplest model to explain Pal denaturation profiles can be represented by Scheme 1,

\[ K_D \frac{k}{N} = D \rightarrow I \]

where N, D, and I are, respectively, the native, the reversibly denatured, and the final irreversibly denatured states of the protein, and the D state undergoes a rate controlled conversion to the final state I following an order-n kinetic to account for the stability dependence on Pal concentration. According to this model (see Appendix), at constant temperature and protein concentration, C, the dependence of \( (1 - \frac{<\Delta H(T)>}{\Delta H_0})^n \) with 1/ν, being \( <\Delta H(T)> \) the excess enthalpy change, follows Equation 1, and its plot should result in a straight line (38) whose ordinate intercept and slope contain, respectively, the excess enthalpy contributed by the states in equilibrium, \( <\Delta H_{eq}(T)> \), and the kinetic information about the irreversible formation of the I state (\( F(T) = f_{k_{app}}dT \)).

\[
1 - (<\Delta H(T)>/\Delta H_0)^n = [1 - (<\Delta H_{eq}(T)>/\Delta H_0)^n]^{-1} + C_i n F(T)(n - 1)/ν \tag{1}
\]

The dependence of \( (1 - \frac{<\Delta H(T)>/\Delta H_0})^n \) on the scan rate was found to be linear for n around 1.2 (Fig. 2C), and the clear deviation of the ordinate intercept from unity indicated the presence of equilibrium intermediates. As it is shown in Fig. 2C (inset), the temperature dependence of F(T) can be described assuming that \( k_{app} \) changes with temperature according to the Arrhenius equation in terms of Equation 2 (see Appendix),

\[
F(T) = \frac{RT_0}{E_{app}} \exp \left( \frac{E_{app}}{RT_0} \right) \tag{2}
\]

where \( E_{app} \) is the activation energy, \( T^* \) the temperature where \( k_{app} = 1 \), and \( T_m \) is the experimental transition temperature. The least square fitting of Equation 2 to F(T) data yields values of \( E_{app} = 318 \pm 4 \) kcal mol⁻¹ and \( T^* = 46.0 \pm 0.2 \) °C. The experimental excess \( \Delta C_P \) profiles of Pal were then, analyzed in terms of Equation 3,

\[
<\Delta C_P> = (\Delta H_0 y_D - \Delta H_D) \frac{dx_{eq}}{dT} + x_{eq} \Delta H_D \frac{dy_D}{dT} \tag{3}
\]

where \( y_D = C_D/(C_N + C_D) \), \( \Delta H_D \) is the transition enthalpy change for the equilibrium step, and \( x_{eq} \) is the total fraction of protein in equilibrium (see Appendix). The continuous lines in Fig. 2A–B show the theoretical curves fitted to the experimen-
Denaturation Mechanism of the Choline-bound Amidase—The low temperature transition of Pal in the presence of 140 mM choline can be also described in terms of the three-state model used for the unbound amidase. The plots of \(1 - \frac{\Delta H(T)}{\Delta H_i}\) as a function of \(1/T\) at constant temperature and Pal concentration were found to be linear for \(n\) around 2 (data not shown) and consistent with the presence of an intermediate. As in the free amidase, the dependence of \(F(T)\) with temperature can be described assuming that \(k_{\text{app}}\) changed with temperature according to the Arrhenius equation, and the best fitting parameters describing the irreversible process were \(E_{\text{app}} = 126 \pm 2\) kcal mol\(^{-1}\) and \(T^* = 33.9 \pm 0.3\) °C (data not shown). On the other hand, the temperature dependence of \(\langle \Delta H_i(T) \rangle\) values derived from the ordinate intercept of the linear plots of \(1 - \langle \Delta H(T)/\Delta H_i \rangle\) versus \(1/T\) can be described in terms of Equation 4 (see Appendix),

\[
\langle \Delta H_i(T) \rangle = \frac{\Delta H^*_D}{1 + \exp[-\Delta H^*_D/(R T D)]) - \exp(-\Delta H^*_D/R T_D T)]}
\]

where \(\Delta H^*_D\) and \(T_D\) are, respectively, the van’t Hoff enthalpy change for the \(N \rightleftharpoons D\) step and the temperature at which \(K_D = 1\), and \(r\) is the ratio between the van’t Hoff and the calorimetric enthalpy changes (\(\Delta H_{\text{app}}/\Delta H_i\)). The best least-square fitting of Equation 4 to the experimental data yielded values of \(\Delta H_{\text{app}} = 257 \pm 14\) kcal mol\(^{-1}\), \(T_D = 56.9 \pm 0.6\) °C and \(\Delta H_{\text{app}}/\Delta H_i = 2\). The finding that the van’t Hoff enthalpy change for the intermediate formation was twice the calorimetric enthalpy change suggested that the cooperative denaturation unit in the choline bound state might be a dimer.

The presence of intermediates during the second transition of Pal at 140 mM choline was checked by plotting the variation of \(1 - \langle \Delta H(T)/\Delta H_i \rangle\) with the scan rate at constant temperature in terms of Equation 5, assuming that irreversible denaturation of the ChBM follows a first order kinetics (38), since it was protein concentration-independent \((n = 1\); see Appendix).

\[
\ln[1 - \langle \Delta H(T)/\Delta H_i \rangle] = \ln[1 - \langle \Delta H(T)/\Delta H_i \rangle] - F(T)/v
\]
Fig. 3. Influence of the scanning rate and protein concentration on thermal denaturation of the choline-bound Pal amidase. A depicts the dependence of ln1−<ΔH(T)/ΔHf> on 1/v, for the highest temperature transition, at varying temperatures (63.47 °C; ▲, 64.17 °C; □, 64.55 °C; ♦, 64.92 °C; ○, 65.12 °C; ●, 65.30 °C; ▪, 65.49 °C; ■, 65.68 °C; △, 65.87 °C; and ★, 66.06 °C). Experimental conditions are as in Fig. 2. B shows F(T) and <ΔHm(α)/ΔHf> values (solid and open squares, respectively) derived from A as a function of temperature, and their theoretical fit using, respectively, Equations 2 and 4 (Eapp = 135 kcal mol−1, Tm = 62.2 °C, ΔHm = 229 kcal mol−1, Tm = 66.4 °C, and ΔHm = ΔHf = 2). C shows the heat capacity profiles calculated according to the three-state model (first transition: xMo, xMo, xMo; second transition: xMo, xMo, xMo) are shown in D. The profiles were calculated as in Fig. 2 using the parameters given in Table III (9.5 µM Pal; 20.5 °C).

The experimental curves at two protein concentrations and the calculated profiles (solid line) using the parameters reported in Table III. As it is shown in Fig. 3D, the population of the intermediate in equilibrium with the native catalytic module is significantly larger than the intermediate fraction formed during denaturation of the ChBM. This will explain the lower dependence of the first transition on the heating rate. It is worth noting the agreement between the parameters derived from Equations 2 and 4 and those derived from direct fitting of the excess heat capacity curves.

**Estimation of the Number of Choline Binding Sites—**Since the reversibly denatured state D of the ChBM was a very minor fraction of the total protein at high choline concentration (Fig. 4A), the high temperature transition primarily reflects the irreversible formation of state I. Therefore, the choline concentration dependence of its Tm, at constant scan rate, can be approximated by the equation derived for irreversible two-state transitions with simultaneous dissociation of bound ligand (38) in Equation 6,

\[
\ln[L] = -\frac{mE_{app}}{s} \frac{1}{R \ T_m} + \text{constant}
\]

where L is the ligand concentration and s is the number of binding sites of the cooperative unit consisting of m subunits. The number of choline binding sites per ChBM, s/m, estimated from the slope of the plot in Fig. 4A was 2.7 ± 0.3 using the Eapp reported in Table III. A somewhat lower ratio (s/m = 2.2 ± 0.2) was obtained when the dependence of Tm with choline concentration was analyzed in terms of the equation derived for systems denaturing under equilibrium conditions (39) (data not shown). These results are consistent with the presence of 2–3 molecules of choline bound per monomer of Pal.

**Pal Self-association Is Regulated by Choline**

Fig. 4B illustrates the variation of the relative apparent weight-average molecular mass, Mw/Mp, of Pal with choline concentration. The unbound amidase sediments with an appa-

**Table III**

**Pal denaturation parameters in the absence and in the presence of choline**

The values are the average of 5–12 independent measurements at different Pal concentrations and temperature scan rates.

| Parameter | Experimental conditions | 140 mM choline | Phosphate buffer pH 8.0 |
|-----------|------------------------|----------------|------------------------|
| Eapp | kcal mol−1 | 273 ± 7 | 112 ± 5 | 174 ± 4 |
| Tm | °C | 45.5 ± 0.1 | 33.2 ± 0.7 | 62.4 ± 0.2 |
| ΔHm | kcal mol−1 | 112 ± 5 | 209 ± 6 | 285 ± 10 |
| Tm | °C | 52.1 ± 0.5 | 55.9 ± 0.3 | 66.7 ± 0.3 |
| n | | 1.31 ± 0.03 | 1.98 ± 0.04 | 1.0 ± 0.01 |
| ΔH | kcal mol−1 | 183 ± 4 | 97 ± 4 | 138 ± 4 |

a Eapp is the apparent activation energy of h app, the apparent constant of the rate limiting step.

b Tm is the temperature where h app = 1 in the specified units.

c ΔHm and Tm are the enthropy change and the transition temperature for the equilibrium step (N ≈ D).

d The fitting of <ΔHm(T)> and the thermal transitions suggest that the cooperative unit in 140 mM choline was a dimer; accordingly, the value of ΔHm would be in kcal mol−1 dimer−1, whereas ΔH is in kcal mol−1 monomer−1.

e n is the order of the reaction leading to formation of the irreversible final state I.

<ΔHm(T)>/ΔHf functions derived from the linear plots shown in Fig. 3A can be described, respectively, in terms of Equations 2 and 4, and the best fitting parameters were Eapp = 135 ± 4 kcal mol−1 and Tm = 62.2 ± 0.1 °C for the irreversible process, and ΔHm = 339 ± 10 kcal mol−1, Tm = 66.4 ± 0.2 °C for the equilibrium step. As in the first transition, the best fitting value for the ΔHm/ΔHf ratio was 2, supporting the notion that the cooperative denaturation units involved in the transitions displayed by the choline-bound amidase have a dimeric nature.

The experimental heat capacity profile of each peak was, then, analyzed in terms of Equation 3 using the expressions derived in the Appendix for n ≠ 1 (first peak; protein concentration-dependent) or n = 1 (second peak). Fig. 3C shows the
Fig. 4. Titration of Pal amidase by choline. A illustrates the dependence of Tm, for the high temperature transition of Pal as a function of ligand concentration. B depicts the variation of the relative apparent weight-average molecular mass, Mw/M0, with choline concentration (M0 is the monomer molecular mass); the arrow indicates the value of Mw/M0 in the absence of choline. C shows the CD titration curve generated from the ellipticity changes induced by choline binding to Pal (λ, 290 nm; □, 222 nm; ○, 220 nm; △, 213 nm).

The far-UV spectra of Pal in the absence and presence of choline are shown in Fig. 6A. Choline binding strongly modifies the spectrum; the minimum at 200 nm disappears, the positive peak centered at 235 nm slightly downshifts, increasing its intensity by a factor of 2.5, and a high positive peak appears around 140 nm. This strongly contrasted with the rather local changes induced in LytA spectra (18) that take place in the region dominated by the chiral contribution of aromatic side chains (Fig. 6, A and B). Fig. 4C shows the choline titration curve of Pal generated by monitoring the relative ellipticity changes as a function of choline concentration at several wavelengths. The curve has a biphasic character, which can be correlated with the variations found in the apparent molecular weight-average molecular mass of 54,000 ± 2,000, which is one-and-a-half-fold the monomer molecular mass, M0. The Mw/M0 ratio decreases as choline concentration increases, reaching a minimum of about 1.2 around 5 mM choline. Beyond this point, Mw/M0 increases in a rather cooperative way up to 2.0 at saturating choline concentrations. In contrast, supplementation of phosphate buffer with NaCl between 0.1 and 140 mM scarcely modified the Mw value (57,200 ± 600; average of eight measurements), revealing the specificity of the choline effect on Pal self-association equilibria. The conservation of the signal was above 95% both in the absence and in the presence of choline, and this indicated that Pal did not form high order aggregates. The distribution of states was investigated by sedimentation Pal at 200,000 × g. The distribution of the sedimentation coefficients c(s*) showed a major peak with a s20,w of 3.3 ± 0.1 S corresponding to the monomer (M0 = 34,453), a shoulder around 4.5 ± 0.3 S (M0 = 76,000 ± 3,000) and two smaller peaks with sedimentation coefficients around 7.4 S (M0 = 160-103) and 12.5 S (M0 = 314-103) (Fig. 5). In the presence of 140 mM choline, a peak with a s20,w of 4.2 ± 0.1 (M0 = 74,000 ± 5,000) was the major species according to the c(s*) distribution, although minor forms with sedimentation coefficients around 6.7 ± 0.3 (M0 = 155-103) and 10.0 ± 0.2 (M0 = 300-100) were also present. These results clearly show that the dimer of Pal (4.2 ± 0.1 S) becomes the more populated form of the choline-bound amidase under these conditions, as expected from DSC results. This contrasted with the large fraction of aggregates (probably dimers, tetramers and octamers) in equilibrium with the monomer (3.3 ± 0.1 S) exhibited by the free amidase in this concentration range, which account for the observed increase of Mw/M0 to 1.6. The high proportion of aggregates formed by the unbound amidase, their dissociation upon choline binding, and the strong influence of choline on Pal thermal stability could reflect the stabilization, mediated by the ligand binding, of low stability regions present in the conformations that constitute the free native-state of Pal. The exposure of hydrophobic regions in marginally stable regions could account for aggregate formation.

To check whether irreversibility of Pal denaturation could involve protein aggregation, the sedimentation coefficients of samples heated for 30 min at increasing temperatures were measured at 20 °C. At 5 µM Pal, the sedimentation coefficient of the major species increased from 3.3 S up to 22.5 S upon heating to 51.5 °C. Additional peaks (35 and 67 S) were observed at 21 µM Pal. The sedimentation coefficient was invariant below 52 °C at 140 mM choline, but the protein sedimented at very short centrifugation times as the first transition became completed, showing that irreversible aggregation follows denaturation of Pal catalytic module.

Variations in Pal CD Spectra Promoted by Choline Binding

The far-UV spectra of Pal in the absence and presence of choline are shown in Fig. 6A. Choline binding strongly modifies the spectrum; the minimum at 200 nm disappears, the positive peak centered at 235 nm slightly downshifts, increasing its intensity by a factor of 2.5, and a high positive peak appears around 224 nm. This strongly contrasted with the rather local changes induced in LytA spectra (18) that take place in the region dominated by the chiral contribution of aromatic side chains (Fig. 6, A and B). Fig. 4C shows the choline titration curve of Pal generated by monitoring the relative ellipticity changes as a function of choline concentration at several wavelengths. The curve has a biphasic character, which can be correlated with the variations found in the apparent molecular
weight (Fig. 4B). The interaction with choline and the dissociation of Pal aggregates that takes place below 5 mM choline occurred with a rather low variation in ellipticity (less than 25% of Δθ/Δθ_max), and is followed by a highly cooperative increase in Δθ/Δθ_max (K_a = 10 mM) as the population of dimers stabilized by choline increased (K_a = 7.8 mM). The simplest explanation for the biphasic character of M/M and CD titration curves would be the presence of two sets of binding sites as in LytA amidase (18). Saturation of the high affinity sites would be responsible for dissociation of Pal aggregates and a rather low variation in ellipticity, while choline binding to the low affinity loci would account for Pal dimerization and the large ellipticity changes accompanying saturation of choline binding sites.

Choline-mediated Structural Changes Do Not Extend to Pal Secondary Structure

CD and IR Spectroscopic Estimation—Deconvolution of the far-UV CD spectra of Pal using different methods (CONTIN, CDNN, SELCON) yielded average values of 7% α-helix, 45% β-strands (4% parallel), 21% β-turns and 30% non-periodical structures for secondary structure content. Similar values were derived for the choline-bound amidase (4% α-helix, 46% anti-parallel β-strands, 8% parallel β-strands, 18% β-turns, and 24% unordered structures). Solid lines in Fig. 6A are the theoretical spectra derived by CONTIN. Because of the significant chiral contribution of aromatic side chains to the Pal spectra, secondary structure was also estimated from the IR spectra of Pal at choline-saturating concentration (Fig. 6C). The position of the amide I components, their assignment and their relative contribution to the envelope are shown in Table IV. As it can be seen, IR and CD estimations fairly agreed for β-sheet and turns, while the percentage of the amide I component at 1653 cm⁻¹ is higher than expected from the CD α-helix estimation. However, the band at 1653 cm⁻¹, usually ascribed to α-helix, can also include contributions from the long loops (26, 27) predicted in Pal structure (see below), and, in fact, it was also present in the IR spectra of the all-β protein C-LytA (18).

Prediction Methods—The estimations derived from sequence-based prediction methods (PSIpred, SSPro, and PROF) gave reasonably consistent results with average values of 10% α-helix, 39% β-strands, and 51% other structures. The distribution of the predicted secondary structure over the amino acid sequence (Fig. 7) shows that α-helices alternated with β-strands at the N-terminal moiety of the catalytic domain, whereas its C-terminal part and the ChBM would consist of short β-strands connected by loops. The prediction for the catalytic module agrees with the results obtained for other related murein hydrolases (data not shown) such as the Lactococcus phage BK5-T (41% identity) or Staphylococcus thermophilus phage O1205 (35% identity). The β-strand location in the ChBM fairly agreed with the distribution found in the 3D structures of Cpl-1 (8) and C-LytA (9), suggesting that each repeat contains a β-hairpin followed by a coiled region.

The agreement between IR and CD estimations of β-structure (sheets and turns) as well as the correlation found between

![Fig. 6. Spectroscopic characterization of Pal. A and B show, respectively, the far- and near-UV CD spectra of Pal in the absence (Δ) and in the presence of 140 mM choline (●). The spectra of LytA with (○) and without 140 mM choline (□) are also included for comparison. C depicts the decomposition of the amide I band of Pal in D₂O at saturating concentration of choline. The parameters derived for the components are shown in Table IV.

![Fig. 7. Predicted secondary structure of Pal. Arrows, cylinders, and thick traces depict, respectively, β-strand, α-helix, and loops in the catalytic module and the ChBM. Below the amino acid sequence, E (extended), H (helix), and L (loop) indicate the conformations predicted with accuracy higher than 85.7% (bold letters) by at least two of the three methods employed or with lower reliability (nonbold letters) by one of the three methods. Gray-highlighted amino acids in the ChBM show the conserved residues potentially involved in choline binding according to Cpl-1and C-LytA structures.

| Band | Assignment                      | Area |
|------|---------------------------------|------|
| 1669 | High frequency β-sheet          | 4    |
| 1669 | Turn                           | 24   |
| 1653 | α-Helix and long loops          | 23   |
| 1636 | Low frequency β-sheet           | 46   |
| 1614 | Other structures                | 3    |

*Bands around this value in native proteins arise from amino acid side chains.*
becomes apparent through the transmission of choline-induced stabilization to the catalytic module, is another feature only shared by Pal and Cpl-1 (43).

**Structural Stability of Pal- and Choline-induced Conformational Changes**—The stability of Pal is maximum around pH 8.0 and protein concentration-dependent. Pal denaturation was essentially irreversible due to association of the unfolded protein in large aggregates, which is primarily mediated by the denatured catalytic module as shows the aggregation dependence on temperature. In the free enzyme, denaturation of both modules occurs simultaneously and the theoretical analysis of the thermograms is consistent with a denaturation mechanism in three steps \( N \leftrightarrow D \rightarrow I \). The influence of choline binding on Pal thermal stability highlights the modular nature of the amidase and its fold into two interdependent cooperative regions comprising, respectively, the catalytic and the cell wall anchoring modules. This assignment is supported by the influence of L-Ala in the low temperature peak, and the non-saturating effect of choline on the high temperature transition. Denaturation of each module in the choline-bound amidase can also be explained in terms of a three-state process \( N \leftrightarrow D \rightarrow I \), although the fraction of the intermediate leading to the irreversibly denatured form of the ChBM is rather low and the process approaches the behavior expected for a first-order irreversible two-state transition. The unexpected independence on protein concentration of this transition can be accounted for by the aggregated nature of Pal upon denaturation of the catalytic module that makes invariant the effective protein concentration of the ChBM upon denaturation.

The strong stabilization of the catalytic module induced by choline binding to Pal ChBM reflects the interactions between both domains and the transmission of conformational effects between them (36, 37). Because choline-protein interactions occur at specific sites and involve only a small number of residues (8, 9), propagation of binding signals to distal locations within the protein structure implies a chain of cooperative interactions. Under native conditions, proteins are characterized by local unfolding reactions scattered throughout their entire structures (44), and this collection of states that defines the native state ensemble together with the uneven distribution of stability may reflect specific functional requirements. Indeed, the presence of low stability regions in binding sites facilitates the occurrence of ligand-induced conformational changes. The high proportion of aggregates in equilibrium with monomers of the unbound amidase, the redistribution triggered by choline that preferentially binds to the dimeric form of the amidase, and the large spectroscopic changes observed in Pal CD spectra indicate that this might be the situation of Pal amidase. If choline loci exhibit low structural stability in the unbound amidase, the distribution of states will be shifted by choline selective interaction with the binding-competent conformations of Pal that will become, in this way, additionally stabilized. In doing so, choline may stabilize specific contacts with adjacent regions that will result in the observed conformational changes and, eventually, in stabilization of the catalytic module and the high activity conformation of Pal. Indeed, these changes could be related with the requirement of Pal for in vitro incubation with choline or choline-containing cell walls to achieve full activity (3).

**Choline-Pal Interactions**—The attachment of Pal to pneumococcal cell wall seems to involve a network of low energy interactions, as denoted the rather high choline concentrations needed for saturation of the ligand binding sites. These multiple binding contacts (2–3 sites per monomer), facilitated through Pal dimerization, are weak enough to permit the amidase diffusion across the surface without detaching from the
cell wall. As in LytA, the biphasic character of choline titration curves (Fig. 4, B and C) could be easily explained assuming that the choline-binding sites of Pal displayed different affinity toward its target. However, the complexity of the events accompanying Pal-choline interactions do not allow completely discarding the possibility that Pal conformational changes triggered by the first molecule of ligand bound to its ChBM could account for it. According to the three-dimensional structures so far known, the ChBMs of pneumococcal lysins seem to exhibit long and rather open structures characterized by a low number of contacts between distant regions of the polypeptide chain (8, 9). Their stability would, therefore, denote a subtle balance between stabilizing and destabilizing forces that, as mentioned above, can result into marginally stable regions in the unbound ChBM of Pal. This feature could facilitate the recognition of the pneumococcal cell wall as well as the proper disposition of the ChBM and the catalytic module in order to improve the orientation of the peptidoglycan bond to be hydrolyzed within the catalytic cavity. Such effect could explain why the catalytic activities of modular pneumococcal lysins are orders of magnitude higher than those displayed by their isolated catalytic modules (45, 46) whereas substrate recognition domains in modular glycoside hydrolases typically increase the catalytic efficiency by 2–5-fold (47, 48).

On the other hand, the idea of exploiting phage lytic enzymes that specifically recognize choline has been successfully used to protect mice from pneumococcal caused bacteremia and death. Moreover, the simultaneous attack of the pneumococcal peptidoglycan by the lysozyme Cpl-1 and the amidase Pal led to a remarkable synergic effect through enhanced destruction of the bacterial cell wall, improving the therapeutic effect (12). In this sense, the biochemical and physicochemical properties of Pal here reported are useful to optimize the formulation stability of the ChBM and the catalytic module in order to improve the orienta-

The compar-

Variability of the Pneumococcal Lytic Family—The comparison of current results with those reported for LytA (18, 22), Cpl-1 (8, 43), and the amidase coded by the Ej-1 bacteriophage (50) shows that they all share common features such as forming multiple choline-protein interactions, choline-dependent self-association equilibria or a modular structural organization. Indeed, Pal and LytA might present a similar folding of their unrelated catalytic modules. However, the number of functional choline binding sites, the quaternary structure of the unbound native states, or the structural stability show drastic variations even in enzymes sharing a high percentage of identity between either one or both modules. This variability also extends to the conformational changes promoted by choline recognition and communication between modules, quite strong in Pal amidase and rather limited in LytA since choline binding does not affect the catalytic module stability (22). This diversity of behaviors strongly supports the notion that, in addition to modular interchange, co-evolution of the catalytic and the ChBM might have determined the specific features of the proteins of the pneumococcal lytic family. Thus, evolutionary pressure may have preferentially selected in each case a distribution of structural stability through a given lysin polypeptide chain that optimizes the substrate recognition through communication between the cell wall binding module and the catalytic region, and regulation of lysin activity, since the end product of the folding process should be a native structure in which the relative balance and location of stabilizing forces was also dictated by functional requirements.

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Theoretical Analysis of DSC Curves

Three-state Denaturation Mechanism Including Irreversibility: Dependence of State Distribution on Temperature and Scan Rate—The simplest form of the Lumry and Eyring model (51) can be represented by Scheme A1,

\[ K_0 \quad k \]

\[ N \ \Rightarrow \ D \rightarrow I \]

**SCHEME A1**

where \( N, D, \) and \( I \) are the native, the reversibly denatured, and the final (irreversibly denatured) states of the protein. If the intermediate in equilibrium with the native state is significantly populated during the thermally induced denaturation and undergoes an irreversible and rate-controlled conversion to the final state \( I, \) the concentration of the states can be expressed as Equation A1,

\[ C_i = C_n + C_f \quad \text{(Eq. A1)} \]

where \( C_i \) is the total protein concentration, \( C_f \) is the concentration of the final state \( I, \) and \( C_n = (C_N + C_D) \) is the total concentration of states in equilibrium. The fraction of state \( x_i \) \((i = N, D, \) or \( I)\) is given by Equation A2,

\[ x_i = \frac{C_i}{C_n + C_f} \quad \text{(Eq. A2)} \]

A second set of fractions \((y_N \) and \( y_D))\) including only the states in equilibrium (52) can be also defined as Equation A3,

\[ y_N = \frac{C_N}{C_n}; \]

\[ y_D = \frac{C_D}{C_n} = \frac{k_D}{1 + k_D} \quad \text{(Eq. A3)} \]

which are related to the \( x_i \) fractions by Equation A4,

\[ y_i = x_i/(1 - x_i) \quad \text{(Eq. A4)} \]

Assuming that the rate of formation of the final state is given by Equation A5,

\[ \frac{dC_f}{dt} = \frac{dC_n}{dt} = -k_{app} C_n^e \]

where \( n \) is the reaction order and \( k_{app} \) the apparent \( n \)-order rate constant, for a DSC experiment at constant heating rate \((v)\) in Equation A6,

\[ \frac{dC_n}{dT} = \frac{1}{v} k_{app} C_n^e \]

When the reaction order is \( n = 1, \) separation of variables in Equation A6 followed by integration from a temperature, \( T_p, \) so low that the reaction rate is negligible, and all the protein is in the native state, leads to Equation A7,

\[ x_n(T) = \exp[-F(T)/v] \quad \text{(Eq. A7)} \]

where \( F(T) \) is the integral in Equation A8,
where $T_D$ is the temperature at which $K_D = 1$, and $\Delta H^\text{ch}_{T_D}$ is the van’t Hoff enthalpy change accounting for the temperature dependence of $K_D$. Accordingly, expression of Equation A13 in terms of Equation A18 leads to Equation A19,

$$<\Delta H_{eq}(T)> = \frac{\Delta H^\text{ch}_{T_D}}{r \left[ 1 + \exp(-\Delta H^\text{ch}_{T_D}/RT_D) \right]}$$

and fitting of Equation A19 to $<\Delta H_{eq}(T)>$ profiles allows estimating $\Delta H^\text{ch}_{T_D}$, $T_D$, and the ratio $r = \Delta H^\text{ch}_{T_D}/\Delta H_D$, which would indicate the size of the cooperative unit.

On the other hand, if $k_{app}$ changes with temperature according to the Arrhenius Equation A20,

$$k_{app} = \exp[A_{app}(T^*)] \exp[-E_{app}/RT]$$

where $k_{app} = 1$ at $T^*$ and $E_{app}$ is the activation energy, the integral $F(T)$ can be approximated as Equation A21,

$$F(T) = \left( RT_D^*/A_{app} \right) \exp[A_{app}(T - T^*)/RT_D^*] \exp[A_{app}DT_D^*/RT_D^*] \exp[A_{app}(T - T^*)/RT_D^*]$$

taking into account that within the temperature range of the DSC transition/1$T$ can be expressed as $1/T = 1/T_m - \Delta T/T_m^e$, where $T_m$ is the temperature of the maximum in the heat capacity curve and $\Delta T = T_m - T_m^e$ (38). Fitting of Equation A21 to the $F(T)$ values derived by using Equation A16 ($n = 1$) or Equation A17 ($n \neq 1$) allows estimation of the values of $T^*$ and $E_{app}$, when the dependence of $k_{app}$ conforms to the Arrhenius equation.

Temperature Dependence of the Excess Heat Capacity Function—The apparent excess heat capacity is given by Equation A22,

$$<\Delta C_P> = \frac{d <\Delta H>}{dT} \left[ \frac{\exp[\Delta H/RT]}{\exp[\Delta H/RT]-1} \right]$$

and differentiation of Equation A14 versus temperature yields Equation A23,

$$<\Delta C_P> = \frac{d x_{eq}}{d T} + (1 - x_{eq}) \frac{d <\Delta \Delta H(T)>}{dT}$$

that can be easily transformed into Equation A24.

$$<\Delta C_P> = (\gamma_D \Delta H_0 - \Delta H_1) \frac{d x_{eq}}{d T} + x_{eq} \frac{d y_D}{d T} \Delta H_0$$

Differentiation of $y_D$ and $x_{eq}$ using the expressions given in Equation A3, and Equations A7 ($n = 1$) or A10 ($n \neq 1$), leads to Equations A25–A27.

$$\frac{d y_D}{d T} = \frac{\Delta H^\text{ch}_{T_D}}{RT^* (1 + K_D)^n}$$

$$\frac{d x_{eq}}{d T} = \frac{k_{app}}{v} \left[ \frac{\exp[-F(T)/v]}{\exp[-F(T)/v]-1} \right]$$

Thus, the substitution of $y_D$, $x_{eq}$, and their temperature derivatives ($dy_{eq}/dT$ and $dx_{eq}/dT$) in Equation A24 by the corresponding equations, and using the expressions given in Equation A18 and Equation A21 for $K_D$ and $F(T)$, respectively, allows a non-linear least-square analysis of the excess heat capacity curve based on Equation A24, using as fitting parameters $n$ (for $n \neq 1$), $E_{app}$, $T^*$, $\Delta H^\text{ch}_{T_D}$, and $T_D$. These parameters can be previously estimated from the temperature dependence of the $F(T)$ and $<\Delta H_{eq}(T)>$ functions generated from the DSC transitions.
curves recorded at different scan rates by means of Equations A16 (n = 1) or A17 (n ≠ 1).

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