Role of Asp-9 and Glu-36 in the Active Site of the Pneumococcal CPL1 Lysozyme: An Evolutionary Perspective of Lysozyme Mechanism†

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ABSTRACT: The role of carboxylic amino acids Asp-9 and Glu-36 in the activity of CPL1 lysozyme was investigated by site-directed mutagenesis. The enzymatic activity of the single mutants D9E, D9N, D9H, D9K, D9A, E36D, E36Q, E36K, and E36A and of the double mutant D9A–E36A was analyzed using a highly sensitive radioactive assay. All mutants but D6K showed detectable activities. Interestingly, the mutants E36D and E36Q retained 67% and 37% activity, respectively. Amino acid replacements at position 9 turned out to be more critical for activity than at position 36. In analogy to the mechanism described for hen egg-white lysozyme, where the proton donor play a central role, we propose that, in the CPL1 lysozyme, Asp-9 might act as the proton donor for activation of the substrate, and Glu-36 could help in the stabilization of the intermediate oxocarbocation. The residual activity of lysozyme mutants lacking one or two of the acidic amino acids may be explained by the participation of a water molecule as proton donor and/or to electrostatic contributions in the active center stabilizing the transition state of the reaction. Our results are in agreement with the hypothesis that enzymes have been optimized during evolution from an ancestral protein able to bind more tightly the transition state of the substrate than the substrate itself, by the acquisition of amino acids serving a function in catalysis.

The name lysozyme (EC 3.2.1.17) is given to a wide variety of different enzymes distributed throughout the animal and plant kingdoms, found in the tissues and secretions of many vertebrates and invertebrates, as well as in bacteria and bacteriophages (Jollès & Jollès, 1984). These enzymes have long served as model systems for the study of many aspects of protein structure and function (Jollès & Jollès, 1961, 1984; Blake et al., 1965, 1967; Phillips, 1966, 1967; Prager & Wilson, 1971; Dunn & Bruice, 1973; Schindler et al., 1977; Ibrahimi et al., 1979; Teshima et al., 1980; Kuharu et al., 1982; Smith-Gill et al., 1984; Weaver et al., 1985; Johnson et al., 1988; Malcolm et al., 1990). Early chemical modification studies (Parsons & Raftery, 1969) together with crystallographic analyses (Blake et al., 1965, 1967; Phillips, 1966, 1967) had already implicated the essential presence of two acidic amino acids, Asp and Glu, in the catalytic mechanism of lysozymes, and the involvement of such residues in catalysis by lysozymes has become almost axiomatic (Jollès & Jollès, 1984). It has been proposed that Glu-35 and Asp-52 participate in the catalytic process of HEWL[1] (c-type lysozyme), the former as the proton donor to the GlcNAc residue leaving group and the latter stabilizing, by ion-pairing, the oxocarbocation arising in the MurNAc residue of the substrate until a water molecule reacts with such an intermediate. On the basis of sequence homology and X-ray diffraction studies, equivalent acidic residues have been implicated in the active center of GEWL (g-type lysozyme) (Grütter et al., 1983; Jollès & Jollès, 1984) or T4L (Anderson et al., 1981; Jollès & Jollès, 1984). Finally, in CHL (ch-type lysozyme), which represents the first studied example of the group of bacterial and fungal lysozymes, Asp-6 and Glu-33 have been suggested to be responsible for catalytic activity (Jollès & Jollès, 1984; Fouche & Hash, 1978). This proposal has recently received strong support since all lysozymes of the ch-type sequenced so far retain these two acidic amino acids in equivalent positions and show a remarkably similarity in their respective surrounding regions (Flech et al., 1975; García et al., 1988, 1990; Boizet et al., 1990; Lichenstein et al., 1990; Croux & García, 1991) (Figure 1). Although the lysozymes of *Chalara* and *Streptomyces erythraeus* have been crystallized (Harada et al., 1981; Lyne et al., 1990), the present low resolution of the structure has not allowed precise location of the two acidic amino acids in the active center.

In the present work we have investigated, by site-directed mutagenesis, the role of Asp-9 and Glu-36, the two conserved amino acid residues of the ch-type CPL1 lysozyme thought to be involved in catalysis (García et al., 1987, 1990). Different genetic and biochemical studies have suggested that CPL1 lysozyme, coded by the pneumococcal phage Cp-1, has evolved from the fusion of two independent domains (modules): the N-terminal domain, containing the catalytic center, and the C-terminal domain, which is responsible of the recognition of choline residues present in the teichoic acids of the pneumococcal cell wall (García et al., 1988, 1990; Sánchez-Puelles et al., 1990; Díaz et al., 1990, 1991). The data presented here revealed a new evolutionary perspective on lysozyme structure–function relationships.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Escherichia coli strains used were DH1 [F⁻ *recA1 endA1 gyrA1 thi1 hsdR17* (r_K^- m_K^- *supE44*] (Sambrook et al., 1989) and TG1 [(*lac–pro*) *supE thi hsdD5*/F′ *traD56 proA*⁺ *B*⁺ *lacI*^{q} *lacZ* M15)] (Amersham Corp.). The latter strain was used as a host for phage M13tg130 (Amersham Corp.). The *Streptococcus pneumoniae* strain used was M31 (*lytA*) (Sánchez-

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[1] Abbreviations: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; HEWL, hen egg-white lysozyme; GEWL, goose egg-white lysozyme; T4L, T4 lysozyme; CHL, *Chalara* lysozyme; SDS, sodium dodecyl sulfate.

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CPL   VKKNDLFVDVSSHNGYDITGILEQMGTTNTIIKISESTTYLNPCLSAQVE
CAC      MKGIDIYSGQGSVDFNAVKESGVEVVYIKATEGLTYTDSTYKDFYD
SGL   DTSGVQGIDVSHWQGSINWSSVKSAGMSFAYIKATEGTNYKDDRFSANYT
CHA      TVQGFDISSYQPSVNFAGAYSAGARFVIIKATEGTSYTNPSFSSQYN
MV1    TKT-YGVDVAVYQP-IDLAAYHKAGASFAIVKLTEGVDYVNRRGPSRWT
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FIGURE 1: Comparison of the N-terminal amino acid sequences of several lysozymes. CPL, lysozyme from the pneumococcal phage Cp-1 (García et al., 1988); CAC, from *Clostridium acetobutylicum* (Croux & García, 1991); SGL, from *Streptomyces globisporus* (Lichenstein et al., 1990); CHA, from the fungus *Chalara* (Fouche & Hash, 1975); MV1, from the phage mv1 of *Lactobacillus bulgaricus* (Boizet et al., 1990). Most conserved residues are shown italicized and underlined, whereas the amino acids proposed to be involved in the enzymatic mechanism are shown in boldface and indicated with an asterisk.

Puelles et al., 1986). Plasmids used were pClP100 (Ap^R *lacI*^{q} *cpl1*) (Sanz & García, 1990) and pUC4K (Ap^R Km^R) (Pharmacia Fine Chemicals). *E. coli* was grown in LB medium at 37 °C with shaking and *S. pneumoniae* in C medium (Tomasz, 1970) supplemented with yeast extract (0.8 mg/mL, Difco Laboratories) (C + Y medium) at 37 °C without shaking. Growth of *S. pneumoniae* was monitored with a Coleman nephelometer.

Plasmid Isolation and Transformation. Plasmid DNA was prepared by the rapid alkaline method (Sambrook et al., 1989). Transformation of *E. coli* was carried out using the RbCl method (Sambrook et al., 1989).

Oligonucleotide Site-Directed Mutagenesis. Mutagenesis was performed by using the mutagenesis system of Amersham International according to the procedure recommended by the supplier, using oligonucleotide primers synthesized in a Pharmacia LKB Gene Assembler Plus DNA synthesizer.

DNA Sequence Analysis. DNA sequencing was carried out by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase kit from U.S. Biochemicals.

Purification of Lysozyme Mutants. Mutant lysozymes were purified from extracts of *E. coli* DH1 recombinant cells grown in the presence of 2% lactose by affinity chromatography using the single-step procedure described elsewhere (Sanz et al., 1988). Extracts were prepared by using a French pressure cell press (American Instruments Co.) operating at 1100 psi. Apparent M_r values were determined by SDS–polyacrylamide gel electrophoresis. Protein concentration was determined spectrophotometrically using an ϵ_{280} value of 113 322 M^{-1} cm^{-1} (Sanz & García, 1990).

Assay for Cell Wall Lytic Activity. Assays for cell wall lytic activity were carried out according to the previously described standard method (Höltje & Tomasz, 1976), using choline-containing pneumococcal cell walls labeled with [*methyl*-^{3}H]choline as substrate.

Circular Dichroism. Circular dichroism spectra were recorded in a Roussell–Jouan Dichrograph II. Cells of 0.05-cm optical path length were used and the instrument operated at a sensitivity of 2×10^{-6} A/mm, as previously described (Sanz & García, 1990).

RESULTS

Construction, Expression, and Purification of Mutant Lysozymes. Construction of mutant lysozyme genes was performed by the method described in Figure 2. To reduce the possibility that the mutagenesis procedure could introduce undesirable mutations in the *cpl1* gene, we subcloned in the M13 phage vector the 384-bp *Xba*I–*Sph*I fragment of plasmid pClP100 which contains the region to be mutagenized. This fragment was completely sequenced in every case to ensure that only the desired nucleotide changes had been introduced.

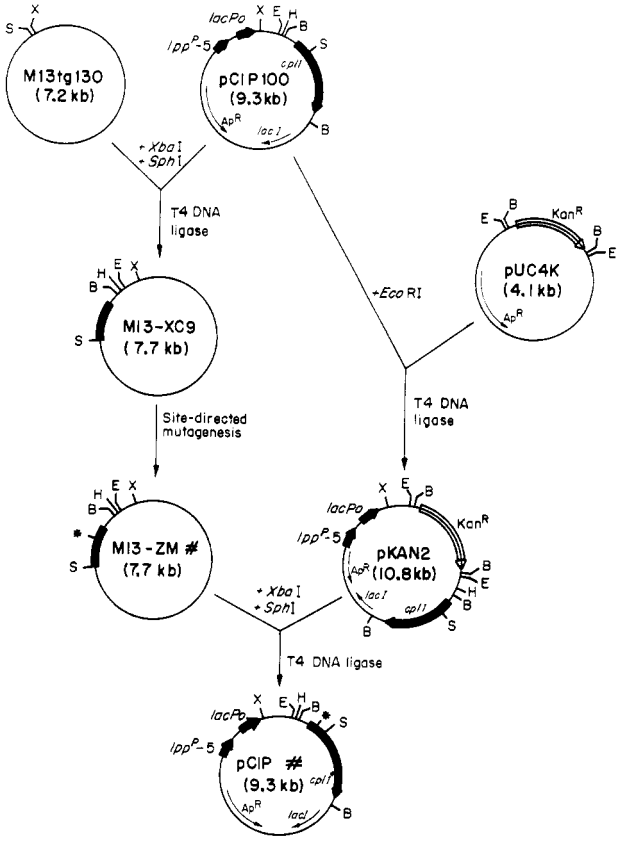

FIGURE 2: Construction of plasmids coding for mutant CPL1 lysozymes. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sph*I; X, *Xba*I. Ap^R and Kan^R, genes that code for resistance to ampicillin and kanamycin, respectively; *lacI*, gene of the lactose operon repressor; *lpp*^{p}-5, modified lipoprotein promoter; *lacPO*, lactose operon promoter–operator. The asterisk denotes the mutation in the *cpl1* gene. (#) symbolizes the presence of plasmids with the same construction but containing different mutations, which are described in the text.

Table I: Oligonucleotides Synthesized for Site-Directed Mutagenesis of CPL1 Lysozyme

oligonucleotide	mutation
O1: 5'-GATTTATTTGTA [A/G/C] A [A/C] GTTTCAAGTCAC-3'	Asp-9→ Asp, Asn, Glu, Gln, His, Lys
O2: 5'-CATTAAAATTTCT [A/G/C] A [A/C] AGTACGACC-3'	Glu-36→ Asp, Asn, Glu, Gln ,His, Lys
O3: 5'-TATTTGTA GCT GTTTCAAG-3'	Asp-9→ Ala
O4: 5'-AAATTTCT GCA AGTACGAC-3'	Glu-36→ Ala

Plasmid pKAN2 was constructed to allow the direct selection of new recombinant plasmids carrying the mutant genes, since the introduction in pKAN2 of the mutated *Xba*I–*Sph*I fragments eliminates the resistance to kanamycin. The use of pKAN2 instead of pClP100 eliminates the possibility of reconstructing a wild-type CPL1 lysozyme by a putative contamination with the wild-type *Xba*I–*Sph*I fragment.

Site-directed mutagenesis was carried out using the oligonucleotides shown in Table I. The oligonucleotides O1 and O2 allowed the substitution, in a single experiment, of the amino acids D9 and E36 by six different polar amino acids, namely, Asp, Glu, Lys, His, Asn, and Gln. Using this procedure we constructed the mutant CPL1 lysozymes D9E, D9K, D9N, D9H, E36D, E36Q, and E36K. Oligonucleotides

Table II: In Vitro Enzymatic Activity of Mutant CPL1 Lysozymes

mutant	amino acid 9	amino acid 36	activity[a] (%)
wild type	Asp	Glu	48.0×10^6 (100)
101 (D9E)	Glu	Glu	0.8×10^6 (1.7)
111 (D9K)	Lys	Glu	nd[b]
113 (D9N)	Asn	Glu	1.1×10^6 (2.2)
130 (D9H)	His	Glu	0.5×10^3 (0.001)
314 (D9A)	Ala	Glu	59.4×10^3 (0.1)
205 (E36D)	Asp	Asp	17.2×10^6 (37)
213 (E36Q)	Asp	Gln	30.1×10^6 (67)
217 (E36K)	Asp	Lys	1.6×10^3 (0.003)
304 (E36A)	Asp	Ala	2.3×10^6 (5.0)
308 (D9A, E36A)	Ala	Ala	0.1 × 103 (0.0002)

[a] Radioactivity released in the assay, given in cpm·min^{-1}·(mg of protein)$^{-1}$, with the percent activity relative to wild type in parentheses. Results are the average of four independent experiments. The standard deviation of the radioactive assay was always lower than 15% of the mean value. [b] nd: no activity above the background could be detected. Non-enzymatic hydrolysis (background activity) was determined by measuring the radioactivity released by the cell walls in the absence of enzyme, at 0 min (190 ± 19 cpm) and after 24 h of incubation at 37 °C (177 ± 23 cpm).

O3 and O4 were used to study the influence of the replacement of Asp-9 and Glu-36 by an apolar amino acid on the CPL1 lysozyme activity. Three new lysozymes were produced, the single mutants D9A and E36A and the double mutant D9A–E36A.

The strong promoter *lpp-lac* allowed the overexpression of the lysozymes after induction of the *E. coli* cultures with IPTG or lactose. Since all mutant lysozymes conserved unaltered their choline-binding domain, the resulting proteins were purified in a single step by affinity chromatography on DEAE-cellulose, as described for the wild type (Sanz & García, 1990).

Functional and Structural Analyses of the Mutant Lysozymes. The specific activities of the purified mutant lysozymes (Table II) were determined using radioactively labeled pneumococcal cell walls as substrate, a procedure that is remarkably more sensitive than the conventional spectrophotometric procedures used to measure the activity of HEWL lysozyme on cell walls as such that of *Micrococcus luteus*. This method allowed us to detect the activity of enzymes that otherwise could be considered inactive by conventional assay procedures. According to the results shown in Table II, the only lysozyme that did not display a detectable activity was the mutant D9K. Hence, the D9K lysozyme constituted an excellent negative control for the assay of activity, especially for the mutants that showed very low, but detectable, activity. The activity of the D9E and D9N mutants was considerably higher, i.e., about 2% that of the wild-type CPL1 enzyme. Surprisingly, the mutants E36D and E36Q retained 37% and 67% of the initial activity, respectively.

Table II also shows that the substitution of the acidic amino acids by alanine did not eliminate completely the activity of the mutant lysozymes. The single mutants E36A and D9A retained 5% and 0.1% activity, respectively, whereas the activity of the double mutant D9A–E36A still showed a very low but detectable activity. This residual activity cannot be ascribed to a nonenzymatic reaction, since no significant release of radioactivity could be measured after incubation of cell walls for 24 h at 37 °C in the absence of enzyme (Table II). Furthermore, the lower activity displayed by the mutants when compared with wild-type CPL1 lysozyme cannot be ascribed to a change in the optimum pH for activity, which was determined to be 5.0 for all mutants.

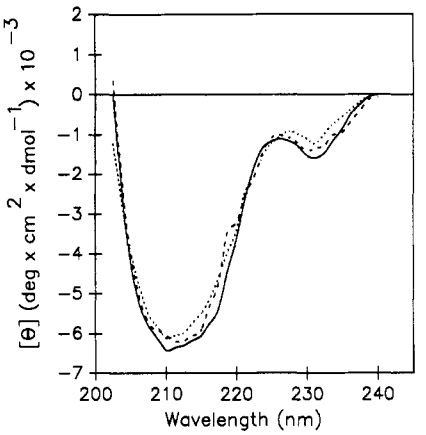

FIGURE 3: Far-UV circular dichroism spectra of wild-type (—), D9K (- - -), and E36K (···) CPL1 lysozymes. The θ values represent the arithmetic mean of at least four independent runs, calculated at 1-nm intervals.

To determine the influence of amino acid substitutions in the secondary structure of the proteins, the purified lysozymes were analyzed by CD. Only the mutants D9K and E36K showed significantly different spectra when compared to that of wild type (Figure 3). In this sense, the curve of D9K showed a shoulder at 220 nm that was not observed in the wild-type spectrum and the ellipticities of the bands at 210 and 230 nm of the E36K spectrum were clearly altered. The other mutants presented CD spectra that were within the confidence limits of the wild-type curve. These results indicate that the conformation of those mutants had been slightly altered and might explain the absence of activity of the mutant E36K and the rather low activity shown by the mutant D9K.

The most active mutant lysozymes E36D and E36Q were used to induce the lysis of the pneumococcal strain M31, which has a complete deletion of the autolytic gene *lytA* and is unable to autolyse at the end of the stationary phase of growth (Sánchez-Puelles et al., 1986). Autolysis of this strain (phenotypic curing) is used as an in vivo test for the activity of pneumococcal murein hydrolases (Díaz et al., 1990, 1991; Sanz & García, 1990). The lysozymes E36Q and E36D induced the lysis of M31 strain but with efficiencies of 15% and 5% that of the wild-type enzyme, respectively (data not shown). These values are lower than those expected taking into account their relative activities of 67% and 37% determined in vitro, indicating that the mutant enzymes are less efficient in vivo than in vitro. Nevertheless, these results allow us to conclude that the mutant lysozymes are also active in vivo and demonstrate that the activity of the mutant lysozymes was not an artifact produced by the in vitro assay. Other mutant enzymes were not tested since it would have required huge quantities of protein, which could affect the bacterial growth behavior.

DISCUSSION

Although evidence has been presented supporting the participation of two acidic amino acids in the catalytic mechanism of lysozymes (Phillips, 1966, 1967), some experiments cannot be explained by this model. Sinnott (1987) considered that, in the case of many glycosidases, the idea that the glycosyl enzyme intermediate is an ion pair (Blake et al., 1967) is untenable, proposing a covalent intermediate for these enzymes. However, recent studies on the structure of a HEWL–(MurNAc–GlcNAc–MurNAc) complex seem to support the original hypothesis (Strynadka & James, 1991). Furthermore, Malcolm et al. (1989) have attributed the

residual activity of the HEWL mutants D52N and E35Q to the presence of a small class of hyperlabile linkages in the natural substrate. However, an alternative explanation might be that electrostatic interactions may play a more important role in the enzymatic mechanism than has generally been thought (Dao-Pin et al., 1989). From these experiments, it has been suggested that the net charge of Asp-52 is not essential for catalytic activity. In this sense, the comparison of the three-dimensional structures of the HEWL, GEWL, and T4L lysozymes showed that neither Asp-86 nor Asp-97 of the GEWL is located at a position comparable to the corresponding Asp-52 of HEWL and Asp-20 of T4L (Weaver et al., 1985). In addition, the activity shown by the T4L mutants, D20N and D20E, suggests that this residue is not absolutely required for catalytic activity (Anand et al., 1988).

The process of protonation of the leaving group ascribed to Glu-35 in HEWL is also subject to controversy (Imoto et al., 1972). An analysis of the sequence of the lysozyme from *Bacillus subtilis* YT-25 revealed that there was no residue equivalent to Glu-35 (Kamei et al., 1988). Furthermore, the role played by Glu-11 in T4L is also controversial, since this amino acid is located 5 Å away from the glycosidic oxygen and forms an ion pair with Arg-145 (Anderson et al., 1981) and its replacement by Asp, which increases the distance between the putative proton donor and the substrate, allowed the enzyme to retain 16% activity (Anand et al., 1988).

It is important to keep in mind that some glycosidases can catalyze the hydrolysis of glycosylpyridinium compounds, a process in which protonation of the leaving group is impossible (Sinnott, 1987). On the other hand, it has been postulated that the mechanism of cellulases could resemble that of the lysozymes, with the participation of acidic groups (Tomme & Claeyssens, 1989; Rouvinen et al., 1990). The role of acidic residues of *Trichoderma reesei* cellobiohydrolases I and II has been investigated by site-directed mutagenesis (Mitsuishi et al., 1990; Rouvinen et al., 1990) but the results of these experiments do not allow to consider unequivocally these residues as essential for activity.

As pointed out above, Fouche and Hash (1978) showed that Asp-6 and Glu-33 were protected from chemical modification when analogs of substrates were bound to CHL. These results suggested that at least one of these residues was essential for activity, but not necessary both, as the authors claimed. Hence, the role of the two acidic amino acids in the catalytic mechanism of ch-type lysozymes remained unclear. Although the data presented in Table II do not allow us to establish unambiguously the role of Asp-9 and Glu-36 in the mechanism of the CPL1 lysozyme, some conclusions can be drawn. The residue Asp-9 of the CPL1 lysozyme seems to play a more critical role in the catalytic process than Glu-36, since its substitution remarkably reduced the activity of the enzyme, and only the mutants D9N and D9E conserved 2% activity. According to the experiments of Malcolm et al. (1989) with HEWL, substitution of the proton donor residue should cause the main decrease in activity. Assuming that the catalytic mechanism of CPL1 lysozyme is similar to that proposed for HEWL, Asp-9 should probably correspond to the residue that acts as the proton donor, whereas Glu-36 would correspond to the charged residue which stabilizes the carbonium ion. Nevertheless, our data also indicate that the presence of both acidic amino acids, although important, is not absolutely essential for catalytic activity. We consider that the presence of "hyperlabile linkages" (Malcolm et al., 1989) cannot satisfactory explain our data, since all mutants would be expected to show identical residual activity according to that model. Moreover, the activity of E36Q and E36D is high enough to rule out the possibility of nonspecific hydrolysis of the cell walls. Taking into account that in the D9N mutant, which still remains 2% activity, the asparagine residue cannot transfer a proton, the most attractive possibility is that a water molecule acts as the proton donor.

As pointed out above, the contribution of Glu-36 to the stabilization of the carbonium ion does not seem to be indispensable for the hydrolysis of the cell walls, since the glutamine residue in the mutant E36Q cannot carry the charge required to stabilize the carbocation. In this case, the most satisfactory assumption is that the electric field of the active site, and not the charge of the acidic amino acid, is responsible for the stabilization of the carbonium ion (Dao-Pin et al., 1989). Nevertheless, it should be also considered that the amide group can accelerate glycoside hydrolysis by a nucleophilic participation mechanism (Dunn & Bruice, 1973). On the other hand, it has been clearly shown that the electrostatic stabilization attributed to Asp-52 in HEWL was worth about 50–200-fold in catalytic acceleration (Dunn & Bruice, 1973), a result that is in the range of activity shown by the E36A mutant.

Although we cannot rule out the possibility that amino acid substitutions at positions 9 and 36 produce local conformational changes that might alter the structure of the active site and reduce the efficiency of the catalytic process, our CD data indicate that detectable changes in the secondary structure of the protein are observed only in mutants carrying the most radical substitutions.

On the other hand, it is generally accepted that site-directed mutagenesis is one of the most powerful and precise approaches to investigate the influence of individual amino acid residues in protein structure and function. However, the fidelity of biological information (Parker, 1989) and the effect of post-translational modifications of amino acids (Wright, 1991) are usually not taken into account in the evaluation of experimental results. Hence, it is important to keep in mind the possibility that hydrolytic activities lower than 10^{-4} of the wild-type one could be due to the substitution of "noncatalytic" residues by "catalytic" ones. In this case, several CPL1 mutants that do not contain catalytic amino acids still retain a hydrolytic activity significantly higher than that which could be attributed to misreading or deamidation. Where no "catalytic" residues exist, catalysis has been explained in terms of substrate transition-state stabilization (Fersht, 1985; Kraut, 1988). Construction of catalytic antibodies appears to support this hypothesis (Schultz, 1988). Moreover, Carter and Wells (1988) have replaced the three amino acids involved in the catalysis of subtilisin by alanine, producing a triple mutant that retains a very small, although detectable and significant, hydrolytic activity (1000-fold higher than the nonenzymatic one). These authors suggested that involvement of specific amino acids in catalysis could have been an evolutionarily late event. The activity found in some CPL1 mutants as D9A, E36A, or D9A–E36A allows us to speculate, following Carter and Wells (1988), that these proteins could resemble primitive ancestors of the lysozyme. The acquisition of catalytic amino acids, Asp-9 and Glu-36, would then have brought about a considerable evolutionary advantage for individuals requiring a lysozyme with higher catalytic efficiency. The absence of at least one of these amino acids in some lysozymes (Kamei et al., 1988) and glycosidases (Rouvinen et al., 1990) and the different levels of activity shown by several of these enzymes after mutation of their active-site residues seem to support this hypothesis. The data presented here might represent an

experimental contribution to support the hypothesis that the presence of two acidic amino acids in the active center of lysozymes or glycosidases is the consequence of the convergent evolution of these enzymes in the acquisition of a more efficient hydrolytic mechanism.

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Registry No. Asp, 56-84-8; Glu, 56-86-0; lysozyme, 9001-63-2.