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Site-directed mutagenesis of the catalytic residues Asp-52 and Glu-35 of chicken egg white lysozyme

(active site)

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ABSTRACT **The roles of the catalytic active-site residues aspartic acid-52 and glutamic acid-35 of chicken lysozyme (EC 3.2.1.17) have been investigated by separate *in vitro* mutagenesis of each residue to its corresponding amide (denoted as D52N and E35Q, respectively). The mutant enzyme D52N exhibits ≈5% of the wild-type lytic activity against *Micrococcus luteus* cell walls, while there is no measurable activity associated with E35Q (0.1% ± 0.1%). The measured dissociation constants for the chitotriose–enzyme complexes are 4.1 μM (D52N) and 13.4 μM (E35Q) vs. 8.6 μM for wild type, indicating that the alterations in catalytic properties may be due in part to binding effects as well as to direct catalytic participation of these residues. The mutant lysozymes have been expressed in and secreted from yeast and obtained at a level of ≈5 mg per liter of culture by high-salt elution from the cell walls.**

Chicken egg white lysozyme (CEWL; EC 3.2.1.17) has a distinguished history in the field of mechanistic enzymology. It was the first enzyme for which an atomic resolution x-ray structure was published (1), and the essence of the presently accepted catalytic mechanism was proposed almost exclusively from the structural information (2). The enzyme has additionally served as an important paradigm for studies in (*i*) the physical biochemistry of polydentate liganding, whereby the individual contributions to the several binding subsites have been separately evaluated (3); (*ii*) molecular evolution investigations (4, 5); and (*iii*) immunochemical studies (6). The enzyme is thus an attractive candidate for modification by site-directed mutagenesis, because the availability of specifically mutated constructs will have the potential to contribute significantly to all of the above investigations, as well as to studies on protein folding and stability. We report here the relatively high-efficiency expression of cloned CEWL in yeast and results of studies of the effects of individual mutations of the catalytically important residues Asp-52 and Glu-35 to their corresponding amides. (The mutant proteins are designated D52N and E35Q, respectively.)

MATERIALS AND METHODS

Materials. $(GlcNAc)_3$ (chitotriose), *Micrococcus luteus*, and lysozyme for use as a standard were purchased from Sigma. $(GlcNAc)_5$ was a generous gift from S. Smith-Gill (National Institutes of Health). Glycol chitin was obtained from Seikagaku Kogyo (Tokyo). 4-Methylumbelliferyl *N*-acetyl-chitotrioside was purchased from Calbiochem. CM-Sepharose Fast Flow was from Pharmacia. Sequenase sequencing kits were purchased from United States Biochemical. Other chemicals were reagent grade or better.

Strains. Plasmids were propagated in HB101 grown in LB medium (7). M13mp18 subclones were grown in JM103 and in RZ1032 for mutagenesis (8). *Saccharomyces cerevisiae* strain GRF180 is *leu2-3,-112, ura3-52, his4-580, can* (cir°) and was derived from GRF18 by curing this strain of its endogenous 2-μm circle (9, 10). *Escherichia coli* and *S. cerevisiae* transformations were carried out as described (11, 12).

Plasmids. Plasmid pAB24 is a yeast–*E. coli* shuttle vector derived from pBR322 and pJDB219 (13). It contains the complete 2-μm circle and the *LEU2-D* and *URA3* genes for selection and replication in yeast and pBR322 sequences and sequences for selection and replication in *E. coli* (14). Plasmid pAGAP1 is a derivative of pPGAP1 (15) that has the GAPDH-491 promoter replaced with a hybrid ADH2–GAPDH promoter fusion (14). Plasmid pLS1023 containing a CEWL cDNA clone (16) was the generous gift of G. Schutz (University of Cologne). DNA sequencing revealed two discrepancies between pLS1023 and the published sequence, which were repaired by site-directed mutagenesis.

Site-Directed Mutagenesis of the Active Site. Two 21-base synthetic primers were used in separate experiments to change bases 237 (G to A) and 186 (G to C), converting aspartic acid-52 in lysozyme to asparagine and glutamic acid-35 to glutamine, respectively. Sequenced mutant cassettes were subcloned into pAB24 as described in Fig. 1 and transformed into *S. cerevisiae* strain GRF180. Transformants were selected on leucine-selective plates.

Growth and Purification of Recombinant Lysozyme. A single colony from the transformation plate was typically inoculated into 2 ml of minimal medium without leucine containing 8% glucose and incubated with vigorous shaking for 48 hr at 30°C. This seed culture was subcultured (2% inoculum) into 50 ml of the same medium for an additional 48 hr. A 20-ml aliquot of this culture was used to inoculate a 1-liter fermentation of yeast extract/peptone (YEP) medium with 8% glucose (grown at 30°C with shaking). The 1-liter culture was harvested by centrifugation after 5 days. Cell pellets were washed with 100 ml of 1 M ammonium acetate (pH 9.0) to remove the secreted lysozyme, most of which is adsorbed to the yeast cell walls. The cell wash was spun at 29,000 × *g* for 20 min to remove residual cells and other debris. The supernatant was diluted with 9 vol of water and loaded onto a 5-ml column of CM-Sepharose Fast Flow (Pharmacia) previously equilibrated with 0.1 M ammonium acetate (pH 9.0). The column was washed with 5–10 vol of the same buffer and the lysozyme was eluted with 3 column vol of 0.5 M ammonium acetate (pH 9.0). Fractions with high lysozyme content (by activity assays and/or native gel electrophoresis) were concentrated using Centricon-10s

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Abbreviation: CEWL, chicken egg white lysozyme.

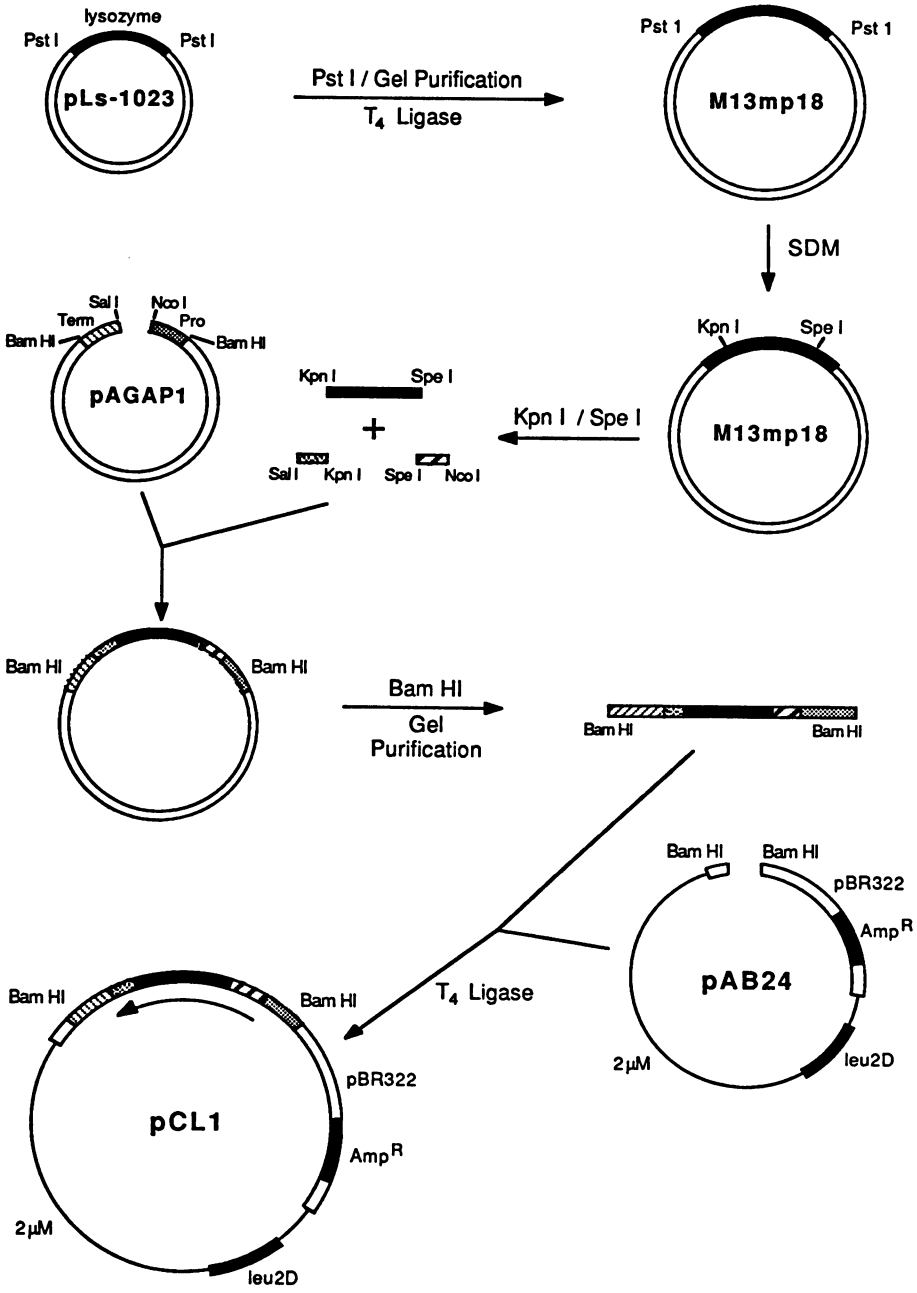

FIG. 1. Construction of lysozyme expression plasmid pCL1. A cDNA clone of chicken lysozyme (15) was subcloned into the *Pst* I site of M13mp18 in the nonexpressible orientation. Silent substitutions were introduced at amino acids 6 and 7 in the lysozyme signal peptide to generate a unique *Spe* I site. Synthetic adapters were used to reconstruct the 5′ and 3′ ends of the gene, converting it to an *Nco* I/*Sal* I fragment for insertion into plasmid pAGAP1 (14). The resulting 2.4-kilobase *Bam*HI cassette containing the promoter, cDNA, and terminator was then subcloned into M13mp18 in the nonexpressible orientation for sequence verification and subsequently subcloned into the unique *Bam*HI site of pAB24 to generate plasmid pCL1.

(Amicon) into 66 mM potassium phosphate (pH 6.2) for activity assays, or 10 mM Mes, pH 6.6/0.1 M NaCl for fluorescence measurements.

Assays. Bacteriolytic activity of lysozyme was assayed by a modification of the method of Shugar (17). Lysozyme (0.1–10 units in 100 μl) was added to a 2.5-ml suspension of *M. luteus* in 66 mM potassium phosphate buffer (pH 6.2). Absorbance at 450 nm was followed for at least 2 min on a Shimadzu UV-160 or Cary 118 spectrophotometer against a water blank. Reaction mixtures in which <1 unit of activity was present were monitored for at least 6 min. One unit is defined as the amount of lysozyme that causes a decrease in absorbance at 450 nm of 0.001 per min under these conditions. All activity assays were performed in duplicate. Lysozyme-catalyzed hydrolysis of $(GlcNAc)_5$ was followed by the reducing-sugar method of Park and Johnson (18). $(GlcNAc)_5$ was characterized by thin-layer chromatography and by comparison of reducing-sugar contents before and after total acid hydrolysis. Wild-type lysozyme (5–50 μM) and D52N (400 μM) were assayed at 40°C for 2–24 hr with 2 mM $(GlcNAc)_5$ in 10 mM sodium acetate buffer (pH 5.2) adjusted to 0.1 ionic strength with NaCl. Protein was removed before the reducing-sugar assay by centrifugation after addition of a small amount of CM-Sepharose Fast Flow resin. Glycol chitin assays were performed as described by Yamada and Imoto (19), except that the wild-type enzyme concentration was varied from 2 to 20 nM. D52N was assayed at 400 nM. This level of protein was found not to interfere in the assay used. Activity against the fluorogenic substrate 4-methylumbelliferyl *N*-acetyl-chitotrioside was assayed as described by Yang and Hamaguchi (20). D52N was assayed at 6.1 μM against this substrate.

Electrophoresis. Denaturing gels were run in 20% polyacrylamide on samples denatured in Laemmli buffer (21). Native polyacrylamide gel electrophoresis was performed in 10% gels in 0.1 M Tris/50 mM glycine, pH 8.6. The activity overlay was a separate 7% polyacrylamide gel containing suspended *M. luteus* cells at 1 mg/ml in 50 mM potassium phosphate (pH 6.2). Overlays were developed according to Hammer *et al.* (22).

Fluorimetry. Fluorescence titrations were performed on wild-type and each mutant lysozyme with $(GlcNAc)_3$ (23). Ligand depletion due to formation of the bound complex was negligible under the conditions used (see Table 2, legend). The values of the dissociation constants were obtained from nonlinear regression on Eq. **1**,

$$F_{obs} = F_0 + (C_1)(L)/(L + K_d), \quad [1]$$

where F_{obs} is the observed fluorescence (corrected for volume), F_0 is the fitted initial fluorescence of the enzyme alone, C_1 is the maximum change in fluorescence due to ligand binding, and L is the concentration of $(GlcNAc)_3$. Analysis of the residuals showed some upward deviation from the simple hyperbolic fit predicted from Eq. **1**, indicating a second binding site for $(GlcNAc)_3$ with a much larger dissociation constant. A small improvement in the residuals was seen on fitting the same data to Eq. **2**,

$$F_{obs} = F_0 + [(C_1)(L)(K_2) + (C_2)(L^2)]/[(L)(K_2) + (K_1)(K_2) + L^2], \quad [2]$$

where C_2 is the difference in fluorescence between free and doubly bound enzyme, and K_1 and K_2 are the two dissociation constants. Holler *et al.* (24) demonstrated a second binding site for $(GlcNAc)_3$ with a K_2 of 15 mM by dye displacement. The highest concentration of $(GlcNAc)_3$ used in the present experiment was 300 μM. Thus, binding to the low-affinity site had a very small effect on the fitted value of K_d from Eq. **1**.

RESULTS

Purification of Lysozyme. The distribution of lysozyme among the intracellular, cell-wall-associated, and extracellular fractions was found to vary widely from one expression culture to another (25). Only the cell-wall-associated fraction was purified for these studies. The recovery of this lysozyme component amounted to ≈1% of the soluble cellular protein, representing 10–75% of the total lysozyme activity. The yield was as high as 5 mg per liter of culture, which is a considerable improvement over the 200 μg/liter reported by Kumagai *et al.* (26). The purified and concentrated protein exhibited a major band comigrating with commercial lysozyme in both native and denaturing gel electrophoresis. Native gels also revealed two minor bands of lower mobility, amounting in aggregate to 10–15% of the purified protein. UV and visible spectral analysis indicates that one major remaining contaminant is probably yeast cytochrome *c*. This is present at ≈10% of total protein, according to the ratios of peak heights at 280 and 410 nm.

Electrophoretic Behavior of Mutants. Native polyacrylamide gel electrophoresis at pH 8.6 shows a clear electrophoretic difference between yeast-derived wild-type lysozyme and the D52N and E35Q mutants (Fig. 2), consistent with a net loss of one negative charge in each mutant. In contrast, the yeast-derived wild-type lysozyme and the commercial chicken enzyme show identical mobilities. The same pattern is observed in the overlay gel (Fig. 3), in which the residual

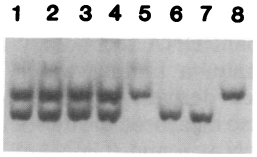

FIG. 2. Native polyacrylamide gel electrophoresis of wild-type and mutant lysozymes. Mutant lysozymes E35Q (lane 7) and D52N (lane 6) were mixed, respectively, with wild-type lysozyme from yeast (lanes 3 and 4) and with commercial chicken lysozyme (lanes 1 and 2). Lane 8, yeast-derived wild-type enzyme; lane 5, commercial lysozyme alone. Two micrograms of protein was loaded in each lane.

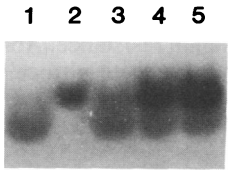

FIG. 3. Activity overlay applied to a native gel. Various amounts of wild-type and D52N mutant lysozymes were loaded onto a 10% polyacrylamide gel. An activity overlay containing *M. luteus* cells was used to detect lysozyme activity as described in the text. Lanes contained the following amounts of lysozyme(s): 1, 3.6 μg of D52N; 2, 15 ng of commercial wild type; 3, 2.6 μg of D52N; 4, 15 ng of commercial wild type and 3.6 μg of D52N; 5, 10 ng of yeast-derived wild type and 3.6 μg of D52N.

activity of the mutant D52N protein has been amplified by the loading of ≈30-fold more protein than in the wild-type lanes. The bacteriolytic activity of D52N is thus seen to be associated with a protein that is electrophoretically distinct from wild type.

Bacteriolytic Activity of Yeast-Derived Lysozymes. Specific activities of the purified lysozymes from yeast are presented in Table 1. Upon addition of sufficient D52N to measure activity, the A_{450} of the *M. luteus* suspension was seen to increase for ≈30 sec prior to the enzyme-catalyzed decrease in absorbance. Maurel and Douzou (27) observed an increase in A_{450} upon addition of CEWL to a *M. luteus* suspension in a water/methanol mixture (6:4) at −30°C, conditions under which lysozyme does not catalyze bacteriolysis (28). Polylysine was shown to cause a similar increase, while poly(glutamic acid) did not, indicating that the effect may be due to cellular aggregation induced by high concentrations of any highly cationic polypeptide. The result of adding large amounts of the slightly active mutant lysozyme to the suspension may be analogous to that of the polylysine experiment.

Assays of the D52N mutant exhibit apparent biphasic kinetics, with a fairly rapid period of lysis corresponding to ≈5% of the wild-type specific activity followed after ≈15 min by a "steady state" in which virtually constant activity (≈10 times lower) is observed. The onset of the second phase could be slowed by lowering the concentration of the enzyme in the assay solution. The specific activity of D52N was determined from the initial rates at concentrations of 1–6 nM enzyme, over which range the activity is proportional to enzyme concentration. This observed activity is unlikely to be due to deamidation of a portion of the mutant protein to form wild type, because such activity would have appeared in the wild-type position on the activity gel, and because contaminating wild-type enzyme would exhibit activity in the lysozyme assays performed with other substrates (see below). The E35Q mutant, in contrast with D52N, exhibits no measurable activity (0.1% ± 0.1%) in the *M. luteus* assay.

One of the nine preparations of E35Q exhibited 1% of wild-type activity in the *M. luteus* assay, while the eight others had <0.1% activity. It was therefore important to determine whether this was due to wild-type protein contamination or to a small amount of wild-type plasmid in the mutant plasmid preparation. The purported E35Q DNA as

Table 1. Specific activities of wild-type and mutant lysozymes

Enzyme	Specific activity, % of yeast wild type
Yeast-derived wild type	100
Commercial lysozyme	116 ± 2
Asn-52	5.5 ± 2.5
Gln-35	0.1 ± 0.1

Activities were measured as described using *M. luteus* cell suspensions in 66 mM potassium phosphate (pH 6.2) at 25°C.

well as E35 (wild type) DNA were transformed into *E. coli* and selected for ampicillin resistance. About 1000 colonies of each were plated and duplicate nitrocellulose filter lifts of each were made. Two synthetic oligomers (17-mers) corresponding to the wild-type and mutant sequences

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                Glu
wild-type:  CAAAATTCGAGAGTAAC
                Gln
mutant:     CAAAATTCCAGAGTAAC
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were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and each hybridized to one copy of each filter. The filters were washed in 3.0 M tetramethylammonium chloride at 52°C as described by Wood *et al.* (29). There was no evidence for wild-type DNA contamination of the E35Q preparation, or vice versa, as judged by autoradiography, so that there is <0.1% cross-contamination between the plasmid preparations. Thus, the 1% activity in the single E35Q preparation was most likely due to wild-type protein contamination or assay artifact.

Other Lysozyme Assays. Since the D52N enzyme exhibits unusual kinetics in the *M. luteus* assay, and since this substrate is chemically heterogeneous, this mutant enzyme was also tested against three other lysozyme substrates. The rates of reaction of D52N measured against glycol chitin, $(GlcNAc)_5$, and 4-methylumbelliferyl *N*-acetyl-chitotrioside were <0.025% ($P < 0.01$), <0.1% ($P < 0.01$), and <2% of the wild-type enzyme rates, respectively.

$(GlcNAc)_3$ Dissociation Constants of the Mutant Lysozymes. Values of the dissociation constants for $(GlcNAc)_3$–lysozyme complexes are given in Table 2.

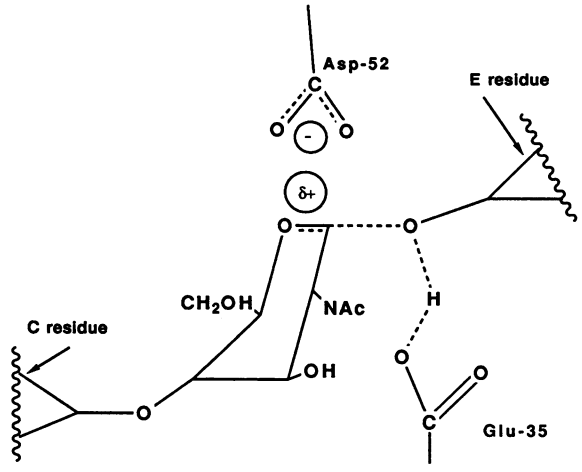
Table 2. Dissociation constants (μM) of lysozyme–$(GlcNAc)_3$ complexes

Enzyme	This work	Kuroki *et al.*	Schindler *et al.*
Commercial lysozyme	8.6 ± 1.0	17	8.6
Wild-type lysozyme (yeast)	7.5 ± 0.4		
Asn-52 mutant	4.1 ± 0.4	40	
Gln-35 mutant	13.4 ± 0.9	44	

The K_d values reported under "This work" were determined by fluorimetric titration. Fluorescence (excitation at 280 nm, emission at 316 nm) was measured as a function of $(GlcNAc)_3$ concentration on an SLM4800S spectrofluorimeter using a 1-cm cell at 25°C in a buffer containing 10 mM Mes (pH 6.6), 0.1 M NaCl. Enzymes were at 0.1–0.2 μM and $(GlcNAc)_3$ was varied from 1 to 300 μM. Kuroki *et al.* (30) measured K_ds by difference spectroscopy at 40°C in 0.1 M acetate (pH 5.5). Their mutant proteins were prepared by chemical derivatization. Schindler *et al.* (23) measured K_d in a buffer identical to ours at 22°C.

DISCUSSION

The catalytic mechanism for CEWL was deduced almost *de novo* from an examination of the crystal structure (1). Model building from the CEWL–chitotriose complex suggested that Asp-52 and Glu-35 would operate in concert to help form and to stabilize transiently an oxocarbocation on the D ring of the hexasaccharide occupying subsites A–F (cf. Scheme I; hypothetical lysozyme transition state).



Scheme I

Model building further suggested that it would be difficult to fit the monosaccharide unit occupying the D position in its most stable chair conformation into the protein superstructure without distorting the ring toward the half chair favored by the sp^2 transition state. Thus, the enzyme was postulated to act in part by means of a substrate distortion mechanism, whereby part of the substrate intrinsic binding energy is converted to an acceleration of the rate constant (31). The S_N1 nature of the transition state was subsequently confirmed by secondary hydrogen isotope effects (32, 33) and by oxygen-18 leaving group kinetic isotope effects (34). The latter group concluded that the transition state for the reaction resembled a nearly fully formed oxocarbocation. Kuhara *et al.* (3) have determined by means of a computer optimization technique that the free-energy cost of fitting a GlcNAc residue into the D subsite is +4.5 kcal/mol (1 cal = 4.18 J). This aspect of the catalytic mechanism is still somewhat controversial as Kelly *et al.* (35) report that the trisaccharide MurNAc-GlcNAc-MurNAc (MurNAc, *N*-acetylmuramic acid) binds to the BCD subsites without ring distortion. Ikeda and Hamaguchi (36) earlier determined that the free energy of binding to subsite D may be negative for any GlcNAc oligomer that does not bind across the C–D junction. Molecular dynamics simulations over a 55-psec time span also suggest that the protein conformation relaxes so that a GlcNAc residue can be accommodated without distortion in the D subsite (37).

Amino acid residues corresponding to positions 52 and 35 in CEWL have been identified by homology in other C-class type lysozymes (38) including the cloned human and T4 enzymes, but almost no investigations of the catalytic mechanism of the latter enzymes have appeared, and thus it is most valuable to probe the catalytic functions of these residues in CEWL. This can be achieved most directly by converting these residues into other amino acids. A number of chemical transformations have been reported. Asp-52 has been specifically esterified (39), converted to homoserine (40), and treated with carbodiimide (41). The derivatives were generally considered to be inactive, with the residual activity being attributed to unreacted wild-type enzyme. Recently, Kuroki *et al.* (30) used chemical methods to convert Asp-52 and Glu-35 to their corresponding amides in independent experiments. Both derivatives exhibited a small amount of residual activity against the substrate glycol chitin, but the authors were unable to determine whether that activity was true or due to contamination by native enzyme. The mutants prepared by recombinant DNA technology exhibit no activity against this substrate, a finding which suggests that the chemically prepared derivatives were not fully modified. Kuroki *et al.* (30) also measured the dissociation constants for the binding of $(GlcNAc)_3$ and $(GlcNAc)_6$ to these modified species. Their results with $(GlcNAc)_3$ are compared with the corresponding enzymes prepared in this work in Table 2. The present determinations of the $(GlcNAc)_3$ dissociation constants (Table 2) are all considerably lower than those reported by Kuroki *et al.* (30). This may be due in part to the different buffers and temperatures used; but a qualitative difference is

observed as well, in that Kuroki *et al.* report a higher K_d for the D52N–$(GlcNAc)_3$ complex than for the wild-type complex, while we observe the reverse order. Since the previous workers used chemical means to create their mutant proteins, it is not clear whether this reversal of binding affinities is due to differences in titration conditions or to side effects of the chemical procedures. The present wild-type $(GlcNAc)_3$ dissociation constants of 7.5 μM (recombinant yeast derived) and 8.6 μM (commercial, chicken) are in good agreement with the value of 8.6 μM at 22°C reported by Schindler *et al.* (23).

In the CEWL–$(GlcNAc)_3$ complex, the β- and γ-carboxylates of Asp-52 and Gln-35 are, respectively, 3.6 Å and 6.1 Å from the nearest sugar residue atom in the C subsite. This inhibitor is uncharged, yet the data in Table 2 show that the carboxylic acid to carboxamide mutations influence the CEWL–$(GlcNAc)_3$ dissociation constant. The D52N protein binds $(GlcNAc)_3$ 2 times more tightly than does wild type, but an opposite 2-fold effect is seen with the mutant E35Q. The qualitative difference of the results indicates that the effect is not due to the influence of the mutation on the pK_a of a single residue in the A–C subsites—for example, Asp-101—but may be a consequence of small transmitted conformational changes.

Neither D52N nor E35Q enzyme exhibits detectable catalytic activity against any of the three synthetically defined substrates investigated, indicating that the normal β-glycosidic linkages of *N*-acetylglusosamine derivatives are resistant to enzymes so modified. The 5% initial activity shown by D52N, which decays to a much smaller residual rate in the cell wall lysis assay, is suggestive of a small class of hyperlabile linkages in the natural substrate, which are susceptible to scission by D52N.

The analogous residue in T4 lysozyme to D52 in CEWL is D20 (42), which has been mutated to asparagine by Perry *et al.* (43). They report little or no activity for the mutant with the *M. luteus* substrate. In earlier work, the mutation to glutamic acid at this position (D20E) was generated by genetic-selection techniques and observed to have 7% activity with this substrate (44). These studies indicate the importance of this aspartate residue in the related enzyme. However, wild-type T4 lysozyme does not hydrolyze glycol chitin or the substrates of smaller molecular weights used in the present study.

Site-directed mutagenesis of cloned enzymes has already provided highly incisive probes into the roles of individual residues in enzyme mechanisms (45–47). The present experiments confirm the earlier suggestion of the importance of Glu-35 for catalytic activity measured against any substrate, while the mutant D52N is inactive for the scission of all but an apparently highly susceptible class of glycosidic bonds found in *M. luteus* cell walls.

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