Is Aspartate 52 Essential for Catalysis by Chicken Egg White Lysozyme? The Role of Natural Substrate-Assisted Hydrolysis†

Ichiro Matsumura‡ and Jack F. Kirsch*

Department of Molecular and Cell Biology, 229 Stanley Hall, University of California, Berkeley, California 94720

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ABSTRACT: The chicken and goose egg white lysozymes (ChEWL and GoEWL) are homologues, but differ in substrate specificity. ChEWL catalyzes the hydrolysis of the glycosidic bonds of bacterial peptidoglycans and chitin-derived substrates, while GoEWL is specific for bacterial peptidoglycans. The active-site aspartate 52 residue of ChEWL, which is postulated to stabilize the oxocarbenium ion intermediate, has no counterpart in GoEWL. The substrate specificity of the D52A ChEWL mutant was compared with those of wild-type ChEWL and GoEWL. D52A ChEWL retains approximately 4% of the wild-type catalytic activity in reactions with three different bacterial cell suspensions. Asp52 therefore is not essential to the catalytic mechanism, accounting for only a 2 kcal/mol decrease in $\Delta G^{\ddagger}$. The function of Asp52 in D52A ChEWL- and GoEWL-catalyzed cleavage of (carboxymethyl)chitin may be partially fulfilled by an appropriately positioned carboxyl group on the substrate (substrate-assisted catalysis). D52A ChEWL and GoEWL, unlike wild-type ChEWL, exhibit biphasic kinetics in the clearing of *Micrococcus luteus* cell suspensions, suggesting preferences for subsets of the linkages in the *M. luteus* peptidoglycan. These subsets do not exist in the peptidoglycans of *Escherichia coli* or *Sarcina lutea*, since neither D52A ChEWL nor GoEWL exhibits initial bursts in reactions with suspensions of these bacteria. We propose that substrate-assisted catalysis occurs in reactions of D52A ChEWL and GoEWL with *M. luteus* peptidoglycans, with the glycine carboxyl group of un-cross-linked peptides attached to *N*-acetylmuramic acid partially substituting the function of the missing Asp52.

Amino acids critical to catalysis are usually conserved among homologous enzymes. All chicken-type lysozymes, for example, have aspartate at position 52. This residue is postulated to stabilize the metastable oxocarbenium intermediate through charge−charge interactions in the classical Vernon−Phillips mechanism for chicken egg white lysozyme (ChEWL)[1] (Scheme 1; Phillips, 1966; Vernon, 1967). This mechanism has received substantial experimental support from kinetic isotope effect measurements, which provide evidence for an sp^2 transition state (Smith et al., 1973; Rosenberg & Kirsch, 1981). A recent NAM-NAG-NAM/ChEWL cocrystal structure shows that Asp52 has a second role in catalysis: it interacts with the active-site NAM substrate so as to help strain that pyranose ring into the more reactive half-chair conformation (Strynadka & James, 1991).

Not all lysozymes, however, have an equivalent to Asp52. All are thought to have diverged from a common ancestor into the chicken, goose, and phage types (or c-, g-, and p-types). They have sequence identity only with homologues of the same type, but certain features of the active sites are universally conserved; all lysozymes have a counterpart to Glu35 of ChEWL (Weaver et al., 1985, 1995; Grütter et al., 1983), which is believed to act as a general acid in catalysis (Phillips, 1966; Vernon, 1967). None of the g-type lysozymes has an equivalent to Asp52 (Weaver et al., 1995). Some of the p-types, including the bacteriophage ϕ29 lysozyme (Garvey et al., 1986) as well as the distantly related *Escherichia coli* soluble lytic transglycosylase (Thunnissen et al., 1994), also lack an Asp52 equivalent.

Certain Asp52 ChEWL mutants catalyze the clearing of *Micrococcus luteus* cell suspensions at an unexpectedly fast rate. D52N ChEWL exhibits biphasic kinetics, with fast and slow phase velocities equal to 5% and 0.5% of the wild-type rate, respectively (Malcolm et al., 1989; Corey, 1990). The mutation of the counterpart of Asp52 in human lysozyme to Glu also yields an enzyme that exhibits biphasic kinetics (Muraki et al., 1991). D52S ChEWL exhibits ≤1% of the catalytic activity of the wild type (Lumb et al., 1992). Asp52 apparently is thus not *essential* for catalysis, even in c-type lysozymes. It is not known, however, whether Asn52 or Ser52 plays a catalytic role in these mutants.

The g-type lysozymes also differ from the c-types in that they are much more specific for peptide-substituted substrates (Arnheim et al., 1973). Embden goose egg white lysozyme (GoEWL), for example, is a poor catalyst of the hydrolysis of chitin-derived substrates (Charlemagne & Jollès, 1967; Fukamizo et al., 1983). The peptide portion of the peptidoglycan provides additional free energy of association to this enzyme. GoEWL complexes with NAG oligomers have higher K_i values than the corresponding ChEWL complexes

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* Corresponding author: telephone, (510) 642-6368; fax, (510) 642-6368; e-mail address, jkirsch@mendel.berkeley.edu.

‡ Present address: Department of Chemistry, Indiana University, Bloomington, IN 47405.

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[1] Abbreviations: ChEWL, chicken egg white lysozyme; DAP, diaminopimelic acid; EDTA, ethylenediaminetetraacetate; GoEWL, goose egg white lysozyme; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; NAM-NAG-NAM, β(1−4)-linked trisaccharide *N*-acetylmuramic acid-*N*-acetylglucosamine-*N*-acetylmuramic acid.

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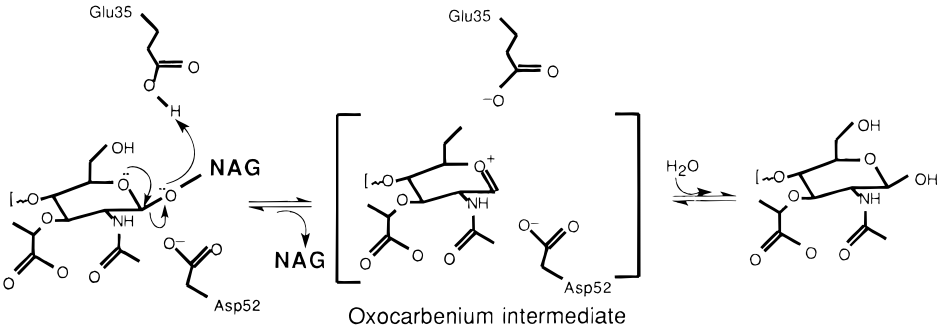
Scheme 1: Vernon−Phillips Mechanism for Chicken Egg White Lysozyme (ChEWL) Catalyzed Hydrolysis of Bacterial Peptidoglycans (Vernon, 1967; Phillips, 1966; Blake et al., 1967a,b)[a]



[a] The NAM residue (shown above) is distorted into the half-chair conformation when it binds in the D-subsite, thereby weakening the glycosidic bond. Glu35 accelerates the cleavage of the bond by donating a proton to the exocyclic oxygen atom. The positive charge of the unstable oxocarbenium intermedate is stabilized through charge−charge interactions. A water molecule attacks the intermediate at the C1 position and regenerates the protonated form of Glu35.

(Jollès et al., 1968; McKelvy et al., 1970), but the opposite is true for peptide-substituted oligosaccharides (Allen & Neuberger, 1971). GoEWL also utilizes the free energy of association with the peptide to strain the D-subsite NAM unit into the more reactive half-chair conformation (Weaver et al., 1995).

Thus, the wild-type chicken and goose lysozymes differ in that the latter lacks a second catalytic carboxylate functionality and is more dependent upon the interactions of the enzyme with the peptide portion of the peptidoglycan substrate for the catalyzed reaction to occur. This study shows how these differences, particularly the former, can account for the differences in substrate specificity. The relative rates of the D52A ChEWL, GoEWL, and wild-type ChEWL reactions with the cell suspensions of *M. luteus*, *E. coli*, and *Sarcina lutea* are compared. These bacterial species differ in the peptide component of their peptidoglycans, both in the sequence and in the extent of cross-linking. In addition, the lysozymes are assayed for catalytic activity in reactions with two substrates lacking a peptide portion, (ethylene glycol)chitin and (carboxymethyl)chitin. The substrate specificity differences lead to a proposal for a substrate-assisted mechanism for the linkages most susceptible to D52A ChEWL- and GoEWL-catalyzed hydrolysis.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized by the Core Facility of the Department of Molecular and Cell Biology, University of California at Berkeley. Expression vector pAB24 (Barr et al., 1987) and *Saccharomyces cerevisiae* strain GRF180 (Donahue et al., 1983) were donated by the Chiron Corporation (Emeryville, CA). Plasmid Bluescript II SK(−) and helper phage R408 were from the Strategene Corp. (La Jolla, CA). Shuttle vector pRS425 (Christianson et al., 1992) was a gift from Dr. Thomas Christianson. *E. coli* strain DH5α F′*lac* I^q was from Gibco-BRL (Gaithersburg, MD). DNA purification columns were purchased from Qiagen (Chatsworth, CA), CM-Sepharose column resin was from Pharmacia Biotech (Uppsala, Sweden), and Centricon ultrafiltration units from were Amicon (Beverly, MA). Frozen Embden goose egg whites were a gift from Dr. Ellen Prager. The wild-type ChEWL and lyophilized *M. luteus* (ATCC no. 4698) and *E. coli* cells (strain B, ATCC no. 11303) used in lysozyme assays were purchased from Sigma Chemicals (St. Louis, MO). *S. lutea* (ATCC no. 272) was obtained from the ATCC (Rockville, MD). (Ethylene glycol)chitin was purchased from Seikagaku America (Rockville, MD) and (carboxymethyl)chitin was a gift from Mr. Michael Kim. The M13 clone containing the E35Q ChEWL gene was made by Ms. Karen Fu.

Site-Directed Mutagenesis. All mutants were prepared by a modification of the method described by Kunkel et al. (1987). The pCL-1 vector (Malcolm et al., 1989; Shih et al., 1993) was digested with *Bam*HI. The 2.5 kb fragment containing the wild-type ChEWL gene and yeast regulatory elements was subcloned into Bluescript II SK(−). The single-stranded uridine-labeled template version of this plasmid was made with helper phage R408 (Russel et al., 1986) and purified using Qiagen Tip-100 columns. The D52A mutant was made with the oligonucleotide 5′-d(GGGAGTACCGCCTACGGAATCCTACA)-3′. The mutagenesis reactions were carried out with T7 DNA polymerase (Zhou et al., 1990) and subsequently electroporated into *E. coli* DH5α F′*lac* I^q as described by Dower et al. (1988). Plasmid DNA was purified from overnight cultures by using Qiagen spin-prep columns, and the lysozyme gene was double-strand-sequenced (Hsiao, 1991) to confirm its mutant identity.

Protein Expression. The vector pAB24 was digested with *Spe*I. The fragment containing the complete 2 μm circle (and selectable markers) was subcloned into the *Spe*I sites of pRS425 to form shuttle vector pABS. The plasmid containing the D52A ChEWL gene described earlier was digested with *Bam*HI, and the 2.5 kb fragment was ligated into pABS by using standard methods. The resulting expression vector was transformed into *S. cerevisiae* strain GRF180 by the spheroplast transformation method (Burgers & Percival, 1987). Yeast transformants were grown in a corn steep liquor medium.

For the E35Q mutant, the gene contained in M13 was sequenced, and the 2.5 kb *Bam*HI insert from the corresponding replicating form DNA was purified and ligated into yeast shuttle vector pAB24 as described by Shih et al. (1993). Yeast strain GRF180 was transformed with the construct (Burgers & Percival, 1987) and grown for 5 days in a 50 L fermentor culture of YPD^+ medium [1% yeast extract, 2% peptone, 1.5 g/L $MgSO_4$, 11.5 g/L KH_2PO_4, 8% glucose, plus 1× histidine, trace elements, and vitamins as described by Rothblatt and Schekman (1989)]. Ethanol was added to 1% every 24 h.

Protein Purification. D52A ChEWL was separated from the supernatant by CM-Sepharose chromatography as described by Shih et al. (1993), except that it was further purified by a hydroxylapatite chromatography step. The active fractions from the CM-Sepharose columns were diluted and loaded onto a hydroxylapatite column, which was subsequently eluted with a 0.01−1 M potassium phosphate (pH 6.6) gradient. The active fractions were concentrated to ≥500 μM by Centricon-10 ultrafiltration as described by the manufacturer.

The YPD$^+$ medium supernatant containing the E35Q lysozyme was diluted 4-fold, and the enzyme was concentrated on a 8 × 3.5 cm CM-Sepharose column, washed with 4 L of 66 mM potassium phosphate (pH 6.6), and eluted with buffer containing 0.5 M NaCl. The fractions containing lysozyme activity were pooled, diluted, and purified on a second 5 × 1.5 cm CM-Sepharose column. The column was washed with 500 mL of phosphate buffer and eluted with a 50 mL gradient of 0−0.75 M NaCl in the same buffer. The active fractions were dialyzed and concentrated to ≥500 μM with Centriprep-10 ultra-filtration units.

GoEWL was purified from frozen Embden goose egg whites as described by Canfield and McMurry (1967), except that CM-Sepharose was substituted for CM-cellulose and ultrafiltration with a Centriprep-30 unit replaced gel filtration chromatography. All enzymes used in this study were shown to be >99% pure by denaturing polyacrylamide gel electrophoresis (Laemmli, 1970; data not shown). Protein concentrations were determined by the Bio-Rad protein assay (Hercules, CA), with wild-type ChEWL as a standard. The concentration determined for the GoEWL preparation was adjusted for the number of arginine residues per molecule, since the assay is most sensitive for the presence of that side chain (Compton & Jones, 1985).

Analysis of the Kinetics of Reactions Catalyzed by Mutant Lysozymes. The rates of clearing of *M. luteus* cell suspensions were followed at 450 nm as described by Shugar (1952), except as noted. Lyophilized cells were suspended at 2 mg/mL in 66 mM potassium phosphate (pH 6.6), allowed to soak overnight at room temperature, distributed in aliquots, and stored frozen to eliminate the problem of substrate variability. The cells were thawed and diluted 10-fold in buffer prior to the addition of lysozyme. Wild-type ChEWL-catalyzed clearing of *M. luteus* cells follows a linear decrease in A_{450} per unit time for the first 20% of the reaction. The D52A ChEWL- and GoEWL-catalyzed reactions, however, exhibit biphasic kinetics. The time dependence of A_{450} for the first 20% of the reactions catalyzed by the latter two lysozymes is satisfactorily described by

$$[S] = [S_0] - [S_{FP}](1 - e^{-k_1[E]t}) - (k_2[E]t) \qquad (1)$$

where [S] is A_{450} at any given time. $[S_0]$ is the A_{450} at time zero, $[S_{FP}]$ is the amplitude (in units of A_{450}) of the initial fast phase, k_1 is the rate constant of the initial fast phase, [E] is the enzyme concentration, and k_2 is the rate constant for the initial part of the slower phase. The data were poorly accommodated by either simple exponential or linear decay models.

S. lutea was grown in Difco Nutrient Broth overnight at 30 °C. The cells were centrifuged, washed in deionized water, and freeze-dried. The lyophilized cells were resuspended at 4.4 mg/mL in 66 mM potassium phosphate (pH 6.6), incubated overnight at room temperature, and frozen in aliquots. The cells were thawed, diluted 10-fold in buffer, and assayed under the same conditions described earlier for *M. luteus*.

Methods originally employed for spheroplast formation were developed into an *E. coli*-based lysozyme assay (Birdsell & Cota-Robles, 1967). Lyophilized cells were suspended at a concentration of 5 mg/mL in deionized water and incubated at room temperature overnight. The cells were distributed in aliquots and stored frozen. Prior to assay, cells were thawed and diluted 10-fold in 10 mM ammonium phosphate, pH 8.0, 25 °C. The presence of the ammonium phosphate both increased the rate of clearing and made it roughly proportional to the enzyme concentration. EDTA (1 mM) was added to the cells for 2 min prior to enzyme addition. EDTA is believed to disrupt the outer membrane of *E. coli* so that lysozymes gain access to the peptidoglycan layer (Birdsell & Cota-Robles, 1967). Upon enzyme addition, clearing was followed at 600 nm with a 0.5 mg/mL suspension corresponding to an initial A_{600} reading of about 0.7.

The first 20% of the reactions of wild-type and D52A ChEWL with *S. lutea* cell wall suspensions fit a simple linear model. Clearing of *S. lutea* by GoEWL and of *E. coli* by all three lysozymes is adequately described by the simple exponential decay model:

$$[S] = [S_0] - [S_{amp}](1 - e^{-k_1[E]t}) \qquad (2)$$

where [S] is the absorbance (at 450 nm for *S. lutea* assays and at 600 nm for *E. coli* assays) at any given time, $[S_{amp}]$ is the amplitude (in units of absorbance) of the reaction, and $[S_0]$ is the initial absorbance. For the *E. coli* digests, the data were corrected for "background" clearing attributable to EDTA alone, which accounted for up to 20% of the reaction rate in some reactions, and for substrate aggregation observed when large concentrations of protein were added.

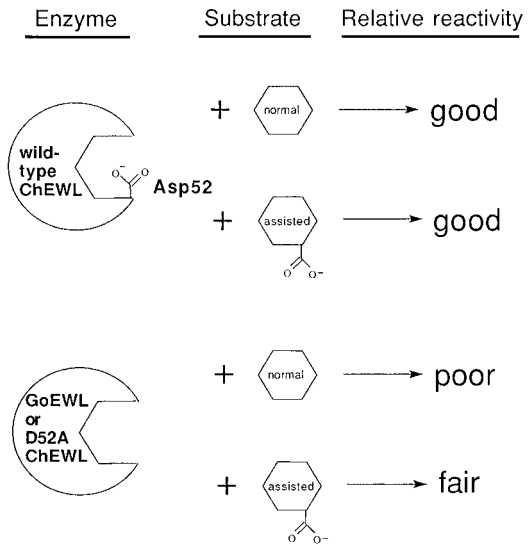
(Ethylene glycol)chitin assays were performed as described by Yamada and Imoto (1981), except that 0.1% (ethylene glycol)chitin in 66 mM potassium phosphate (pH 5.5, 42 °C) was reacted with 5 nM−1 μM GoEWL, E35Q,D52A ChEWL, or wild-type ChEWL for 1−5 h. Aliquots were removed periodically, added to 1 vol of color reagent, and incubated at 100 °C for 15 min. (Carboxymethyl)chitin assays were performed in the same way, except that 0.2% solutions were used.

RESULTS

Rationale. Some of the differences in substrate specificity between wild-type ChEWL and GoEWL might be explained by the substrate-assisted catalysis model shown in Scheme 2. This model postulates that some of the catalytic functionality in enzymes lacking Asp52 may be substituted by appropriately positioned carboxylate residues of certain substrates. The experiments described here test the model with a series of kinetic investigations of wild-type ChEWL, D52A ChEWL, and GoEWL. These enzymes are reacted with a variety of substrates, some with and some without appropriately positioned carboxylate groups.

Several lines of evidence support the purity of the D52A ChEWL preparations. Mutation of the other active-site carboxyl group Glu35 to Gln (E35Q) yields a protein that

Scheme 2: Substrate-Assisted Catalysis Model[a]



[a] Wild-type ChEWL has Asp52, while D52A ChEWL and GoEWL do not. The kinetics of each enzyme variant with two broad classes of substrates were investigated. "Assisting" substrates, such as (carboxymethyl)chitin and a subset of the *M. luteus* glycosidic bonds, contain a carboxylate group that partially complements Asp52 when this functionality is missing naturally or is deleted by site-directed mutagenesis. "Normal" substrates, including (ethylene glycol)chitin, most *M. luteus* glycosidic linkages, and the *E. coli* peptidoglycan, lack this functionality. The relative rate for each reaction is listed in Table 1.

has no detectable activity against any of the substrates (Malcolm et al., 1989), even when assayed at concentrations 200-fold higher than those used for wild-type ChEWL in the experiments described here (data not shown). These results show that the purification procedure excludes impurities that catalyze the hydrolysis of any of the substrates and that high concentrations of lysozyme-like protein do not interfere with any of the assays. Furthermore, D52A ChEWL from three independent preparations behaved the same way in the assays described here, ruling out the possibility of cross-contamination with strains expressing wild-type ChEWL. Finally, D52A ChEWL differs from the wild type in substrate specificity (see the following), demonstrating that the observed catalytic activity cannot be attributed to wild-type contamination.

Chitin Analogues. (Ethylene glycol)chitin contains no carboxylate groups that might functionally substitute for Asp52. The wild-type ChEWL-, D52A ChEWL-, and GoEWL-catalyzed hydrolyses of 1 mg/mL of this substrate in 66 mM potassium phosphate (pH 5.5, 42 °C) exhibit linear kinetics (Figure 1A). D52A ChEWL and GoEWL catalyze the cleavage of (ethylene glycol)chitin at rates equal to 2.4% and 0.4% of that of the wild-type ChEWL, respectively (Table 1). (Carboxymethyl)chitin, an analogous substrate, has a carboxyl group at the C6 position of some of the NAG subunits (Chart 1) that might partially substitute for Asp52 (Scheme 2). D52A ChEWL and GoEWL catalyze the hydrolysis of 2 mg/mL (carboxymethyl)chitin at velocities equal to 5.9% and 1.3% of the wild-type ChEWL value, respectively. These values are about 3-fold higher than the comparable ones observed for (ethylene glycol)chitin hydrolysis (Table 1), consistent with this hypothesis.

M. luteus. We find, in common with previous investigators (Shugar, 1952), that the wild-type ChEWL-catalyzed clearing of an *M. luteus* cell suspension in 66 mM potassium

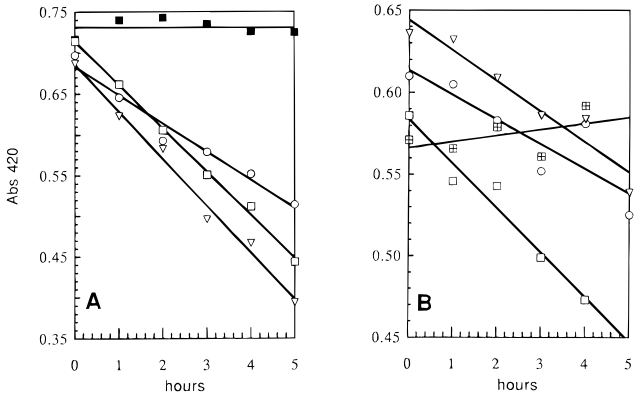


FIGURE 1: Lysozyme activity against chitin derivatives. (A) Solutions of 0.1% (ethylene glycol)chitin in 66 mM potassium phosphate (pH 5.5, 42 °C) were incubated with no enzyme (■), 1 μM GoEWL (○), 5 nM wild-type ChEWL (□), or 250 nM D52A ChEWL (▽) for the times indicated. Aliquots were removed and the reducing sugar content was assayed as described in the Materials and Methods. (B) Similar to (A), except that 0.2% (carboxymethyl)-chitin was reacted with 100 nM D52A ChEWL (▽), 500 nM GoEWL (○), 500 nM E35Q (crossed square) or 15 nM wild-type ChEWL (□).

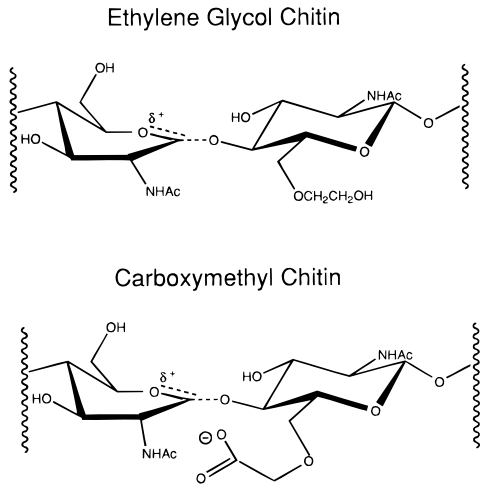
Table 1: Relative Rates of Lysozyme-Catalyzed Reactions[a]

substrate	D52A ChEWL (%)	GoEWL (%)
M. luteus fast phase amplitude[b]	2.7 ± 0.6	2.7 ± 0.5
M. luteus fast phase velocity[c]	22.1 ± 1.0	204 ± 14
M. luteus slow phase velocity[c]	4.1 ± 0.1	142 ± 13
S. lutea velocity[d]	3.5 ± 0.6	202 ± 20
E. coli velocity[e]	5.2 ± 0.1	9.4 ± 0.4
(ethylene glycol)chitin velocity[f]	2.4 ± 0.2	0.4 ± 0.1
(carboxymethyl)chitin velocity[f]	5.9 ± 1.4	1.3 ± 0.1

[a] Except for the first row (see footnote *b*), the velocities of D52A ChEWL- and GoEWL-catalyzed reactions are expressed as percentages relative to the 100% value for wild-type ChEWL acting on the given substrate. All values are averages of at least three independent determinations. [b] Fraction of the total light scattering of a 0.2 mg/mL suspension of *M. luteus* cells in 66 mM potassium phosphate (pH 6.6, 25 °C) cleared in the fast phases of D52A ChEWL- and GoEWL-catalyzed reactions. [c] The rate of the wild-type ChEWL-catalyzed clearing of an *M. luteus* suspension is linear with time for at least 20% of the total reaction (see text). Since no fast phase is exhibited, the initial velocity of the wild-type-catalyzed reaction is the standard of comparison for both the fast and slow phases of the D52A ChEWL- and GoEWL-catalyzed reactions. [d] Relative initial rates of lysozyme-catalyzed clearing of 0.44 mg/mL *S. lutea* cell suspensions in 66 mM potassium phosphate (pH 6.6, 25 °C). [e] 10 mM ammonium phosphate buffer (pH 7.9), 1 mM EDTA, 25 °C. [f] 0.1% (ethylene glycol)chitin or 0.2% (carboxymethyl)chitin in 66 mM potassium phosphate buffer (pH 5.5, 42 °C).

phosphate (pH 6.6, 25 °C) is nearly linear with respect to time for the first 20% decrease in light scattering measured at 450 nm (Figure 2A). In contrast, D52A ChEWL depletes about 3% of the total A_{450}, with a relative velocity that is ~20% of that exhibited by the wild-type enzyme (Figure 2A,B; see the Discussion). This fast phase is followed by a linear slow one that proceeds at 4.1% of the wild-type rate (Figure 2A,B, Table 1). Similarly, GoEWL exhibits biphasic kinetics with a fast phase that also accounts for ~3% of the light scattering. Its fast and slow phase velocities are 204% and 142% of the wild-type ChEWL velocity, respectively (Figure 3A,B, Table 1). We tentatively assign the fast phases of the D52A and GoEWL reactions to that fraction of the peptidoglycan having a carboxylate group in position to substitute functionally for Asp52 (Scheme 2), while the slow

Chart 1: Transition States for the Cleavage of (Ethylene glycol)chitin and (Carboxymethyl)chitin[a]



[a] The extent of substitution at the C6 position of the latter is dependent upon the synthesis protocol. Substitution at the secondary alcohol in the C3 position (not shown) is less extensive. The structures are shown in the orientation that would be expected if the left and right saccharide units were bound in the D- and E-subsites of ChEWL, respectively (Miyazaki & Matsushima, 1968).

phase is analogous to the reactions with (ethylene glycol)-chitin.

The biphasic kinetics exhibited in the reactions of D52A ChEWL and GoEWL with *M. luteus* cell suspensions could also be the result of enzyme instability or of the accumulation of inhibitors released early in the assay. The enzyme instability hypothesis can be ruled out because the addition of fresh D52A ChEWL to substrate already digested by this mutant does not result in an additional burst of clearing (Figure 4A). Fresh substrate added to a digest, however, is hydrolyzed with the same velocity as by fresh enzyme (Figure 4C). These results are consistent only with the explanation that D52A ChEWL readily catalyzes the hydrolysis of a subset of the bonds susceptible to wild-type ChEWL (i.e., containing glycine carboxylate groups of un-cross-linked peptides; see the Discussion), while the remainder are relatively resistant to attack. Similar experiments led to the same conclusion for GoEWL (Figure 4B,D).

E. coli. The peptidoglycan of *E. coli* lacks the glycine group that may be responsible for the fast phase reactions shown by D52A ChEWL and GoEWL in reactions with *M. luteus* cell suspensions (Chart 2). Traces of lysozyme-catalyzed clearing of *E. coli* cell suspensions are shown in Figure 5A. The first 20% of each reaction follows single-exponential decay kinetics for all three lysozymes, with similar amplitudes (Figure 5A and other data not shown). Supernatant from an extended *E. coli* digest inhibits the clearing of fresh cells by lysozyme (data not shown), indicating that the nonlinear kinetics are due at least in part to the accumulation of inhibitors during cell lysis. The initial velocities of D52A ChEWL and GoEWL against *E. coli* cell suspensions are 5.2% and 9.4% of the value for wild-type ChEWL (Figure 5B, Table 1), reminiscent of the slow phases of reactions with *M. luteus* suspensions where substrate assistance does not occur.

S. lutea. The peptidoglycan of *S. lutea* differs from that of *M. luteus*, in that it contains very few un-cross-linked NAM-associated peptides (Table 2; see the Discussion). If substrate assistance requires such structures, D52A ChEWL

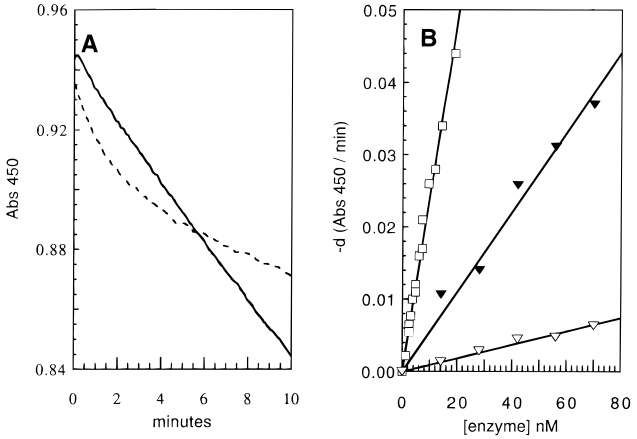


FIGURE 2: Biphasic kinetics exhibited in the D52A ChEWL-catalyzed clearing of an *M. luteus* cell suspension. (A) 0.2 mg/mL *M. luteus* cells in 66 mM potassium phosphate (pH 6.6, 25 °C) were reacted with 20 nM D52A ChEWL (dashed line) or 2 nM wild-type ChEWL (solid line). The data for the D52A ChEWL reaction are superimposable on the fit to eq 1 (the minimizing parameters are $[S_{FP}] = 0.032\ A_{450}$ units, $k_1 = 5.2 \times 10^5\ M^{-1}\ s^{-1}$, and $k_2 = 2700\ A_{450}\ M^{-1}\ s^{-1}$). (B) The D52A ChEWL fast (▼) and slow (▽) phases and wild-type ChEWL initial (□) reaction velocities, expressed as the decrease in A_{450} units per minute, plotted against enzyme concentration.

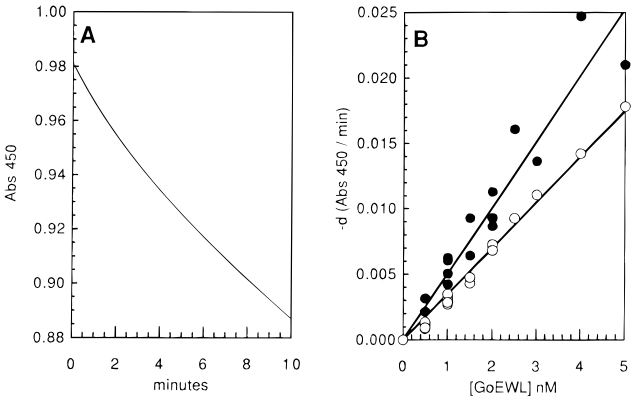


FIGURE 3: GoEWL-catalyzed hydrolysis of an *M. luteus* cell suspension exhibits biphasic kinetics. (A) 2 nM GoEWL was reacted with the cells, and the data were fit to eq 1 as described for Figure 2A. The data and the calculated line are difficult to distinguish within the resolution of the figure. The minimizing parameters are $[S_{FP}] = 0.028\ A_{450}$ units, $k_1 = 2.7 \times 10^6\ M^{-1}\ s^{-1}$, and $k_2 = 5.7 \times 10^4\ A_{450}\ M^{-1}\ s^{-1}$. (B) Dependence of GoEWL fast (●) and slow (○) phase reaction velocities on enzyme concentration.

and GoEWL kinetics would not be expected to exhibit initial bursts against *S. lutea* cell suspensions. Wild-type ChEWL digestion of this substrate shows linear decreases in A_{450} with time under the standard *M. luteus* assay conditions (Figure 6A), with an initial rate of clearing of about half that for *M. luteus* suspensions of similar initial absorbance. A mixing artifact causes a rapid decrease of about 0.01 A_{450} units during the first minute of any *S. lutea* assay (see the Figure 6 legend). Neither the kinetics of D52A ChEWL nor GoEWL exhibits a true burst against this substrate, implying that substrate assistance does not occur. The linear ($[S] > K_M$) D52A ChEWL-catalyzed rate of clearing of *S. lutea* cell suspensions is equal to 3.5% of that of the wild type (Figure 6B and Table 1). In contrast, the reaction kinetics of GoEWL with this substrate shows single-exponential decay kinetics ($[S] < K_M$, Figure 6), with an initial rate that is twice that of wild-type ChEWL (Table 1).

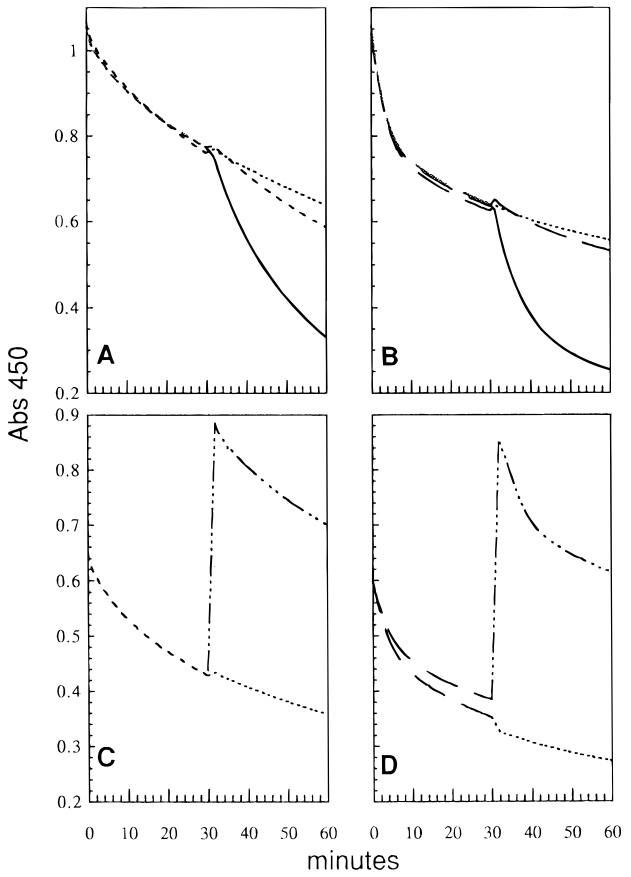


FIGURE 4: Enzyme or substrate addition following D52A ChEWL or GoEWL predigestion of *M. luteus* cell suspensions. (A) A 0.2 mg/mL cell suspension was treated with 200 nM D52A ChEWL (dashed line) for 30 min under the conditions described for Figure 2 prior to the addition of 200 nM additional D52A ChEWL (dashed line), or 20 nM wild-type ChEWL (solid line). (B) As described for part A, except that 20 nM GoEWL (long-dashed line) replaced D52A ChEWL prior to the addition of 20 nM additional GoEWL (long-dashed line) or 20 nM wild-type ChEWL (solid line). (C) A 0.1 mg/mL *M. luteus* suspension was treated with 200 nM D52A ChEWL (dashed line) prior to the addition of fresh substrate to 0.2 mg/mL (dashed−dot line). (D) Similar to part C, except that 20 nM GoEWL (long-dashed line) replaced D52A ChEWL prior to substrate addition (dash−dot line). Control digestions with no further additions are shown (dotted lines) in parts A−D.

DISCUSSION

Substrate-Assisted Catalysis

Two important roles have previously been suggested for Asp52 in ChEWL catalysis: providing electrostatic stabilization to the transition state (Phillips, 1966; Vernon, 1967) and helping to strain the substrate into a more reactive conformation (Strynadka & James, 1991). The substrate specificity of the D52A ChEWL mutant is compared with those of wild-type ChEWL and GoEWL in the present study. The latter is a naturally occurring lysozyme that lacks a counterpart to the ChEWL Asp52 (Weaver et al., 1995). The results are consistent with a model in which the catalytic activities of D52A ChEWL and GoEWL are partially complemented by correctly positioned carboxylate groups of certain substrates (Scheme 2). Correlations are made in the following section between the kinetic behavior of the lysozymes and the known structures of the substrates.

Chitin-Derived Substrates. D52A ChEWL and GoEWL catalyze the cleavage of (ethylene glycol)chitin at velocities equal to 2.4% and 0.4% of that of wild-type ChEWL,

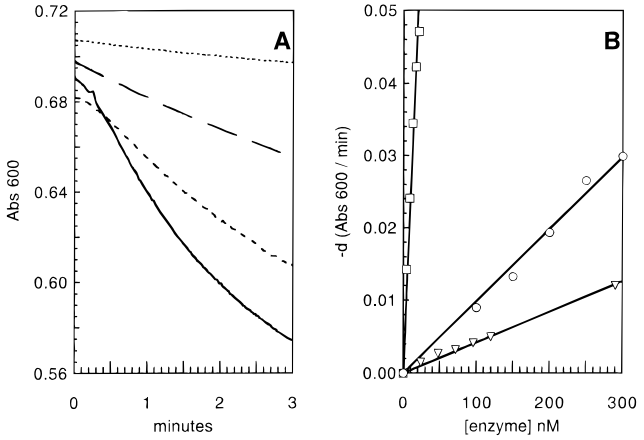
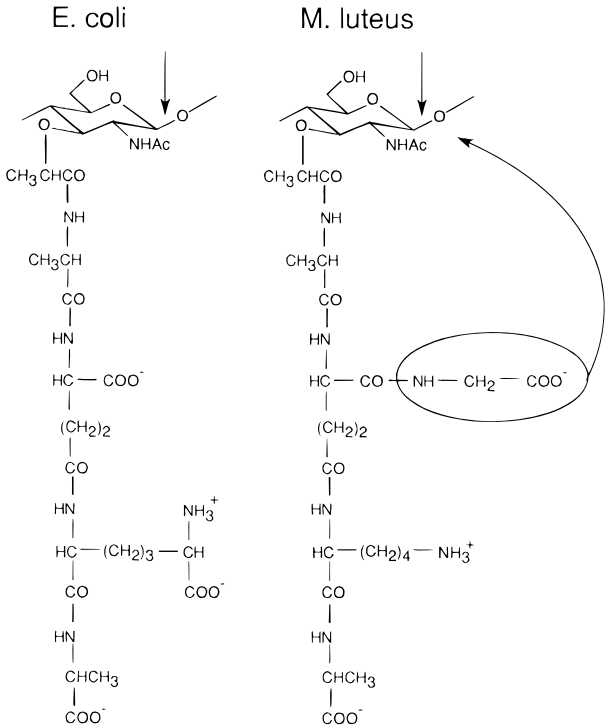


FIGURE 5: Lysozyme-catalyzed clearing of *E. coli* cell suspensions. (A) Cell suspensions (0.5 mg/mL, $A_{600} \approx 0.6$) in 10 mM ammonium phosphate (pH 7.9, 25 °C), were treated with 1 mM EDTA for 2 min (not shown), prior to addition of no enzyme (dotted line), 110 nM GoEWL (long-dashed line), 1 μM D52A ChEWL (short-dashed line) or 40 nM wild-type ChEWL (solid line). (B) Dependence of initial velocities of wild-type ChEWL (□), GoEWL (○), and D52A ChEWL (▽) catalyzed clearing of *E. coli* suspensions, expressed as the decrease in A_{600} units per minute upon enzyme concentration. Data from the first 20% of each *E. coli* digest were fit to a single-exponential decay model (eq 2).

Chart 2: Primary Structures of the NAM-Associated Peptides of *E. coli* and *M. luteus* (Ghuysen, 1968)[a]



[a] The primary structure of the *S. lutea* peptide is identical to that of *M. luteus*, but the peptidoglycan differs in that a much smaller proportion of the pentapeptide units are un-cross-linked as shown above and in Chart 3B (Table 2; Campbell et al., 1969).

respectively. The relative rates of their respective reactions with (carboxymethyl)chitin are greater at 5.9% and 1.3% of the wild-type ChEWL value, respectively (Table 1). A 6-*O*-(carboxymethyl)NAG subunit of (carboxymethyl)chitin fits into the E-subsite of wild-type ChEWL (Chart 1; Miyazaki and Matsushima, 1968). Model building shows that this substrate carboxylate group can be rotated into the location that is normally occupied by the carboxyl group of Asp52.

Table 2: Proportions of NAM residue Types in Bacterial Cell Walls

species	no peptide[a] (%)	un-cross-linked[b] (%)	dimer[c] (%)	bridged[d] (%)
M. luteus[e]	50	5	4	30
S. lutea[f]	∼30	∼0	∼0	∼70
E. coli[g]	0	33	66	0

[a] These NAM residues have free lactyl carboxylate groups (Chart 3A). [b] The lactyl group forms an amide bond with the peptide unit (Chart 3B). [c] Directly cross-linked to another NAM (Chart 3C). [d] Cross-linked to another NAM residue through one or more "bridge" peptides (Chart 3D). [e] Proportion of unsubstituted NAM from Leyh-Bouille et al. (1966) and Muñoz et al. (1966). Other percentages were calculated from Ghuysen et al. (1968). [f] Values estimated from Campbell et al. (1969). [g] From Wiedel and Pelzer (1964) and Takabe (1964). The sequence of the peptide unit in the *E. coli* cell wall is L-Ala-D-γ-Glu-*meso*-DAP-D-Ala (Chart 2). Cross-linking occurs between the terminal D-Ala of one peptide and the *meso*-DAP of another, resulting in a structure analogous to the dimer (Chart 3C).

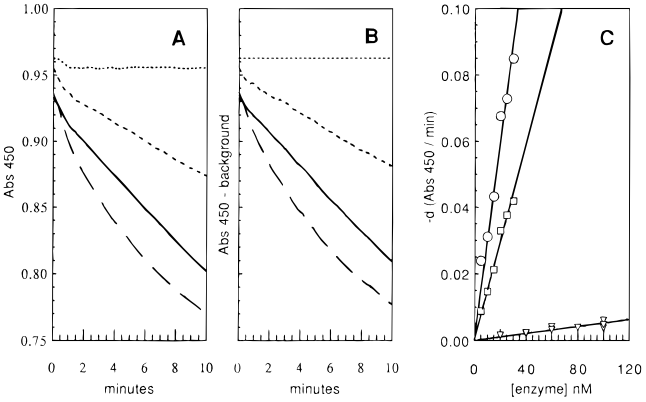
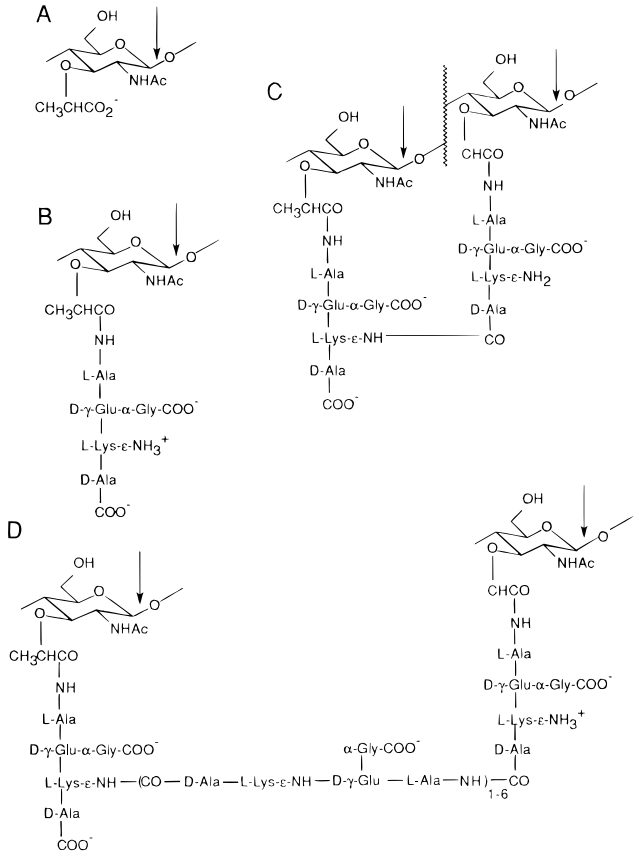


FIGURE 6: Lysozyme-catalyzed clearing of *S. lutea* cell wall suspensions. (A) 0.44 mg/mL *S. lutea* cell suspensions ($A_{450} \approx 1.0$) in 66 mM potassium phosphate (pH 6.6, 25 °C) were incubated with no enzyme (dotted line), 2.5 nM GoEWL (long-dashed line), 200 nM D52A ChEWL (short-dashed line) or 5 nM wild-type ChEWL (solid line). The slight decrease in light scattering from $t = 0$ to $t = 1$ min appears to be a mixing artifact as it is observed in the absence of protein (dotted line). (B) Same as (A), except that the data were corrected for the mixing artifact. (C) Initial velocities of wild-type ChEWL (□), GoEWL (○), and D52A ChEWL (▽) catalyzed clearing of *S. lutea* suspensions, expressed as the decrease in A_{450} units per minute, plotted against enzyme concentration. Data from *S. lutea* digests were fit to a linear model for the ChEWLs or to the single-exponential decay model (eq 2) for GoEWL. The velocities were determined from portions of the curve following the mixing artifact.

Since the carboxylate group is the only difference between (ethylene glycol)chitin and (carboxymethyl)chitin, the substrate assistance model (Chart 1) provides a feasible explanation for the 2−3-fold faster relative rates of reaction of the latter substrate with D52A ChEWL and GoEWL. Such substrate-assisted catalysis has been demonstrated in reactions of a catalytically compromised site-directed subtilisin mutant (Carter et al., 1991).

M. luteus. D52A ChEWL and GoEWL, unlike wild-type ChEWL, exhibit nonlinear kinetic behavior in the clearing of *M. luteus* cell suspensions. The data fit well to a biphasic model (eq 1), in which these lysozymes catalyze the cleavage of two classes of substrates with different rate constants (Figures 2A and 3A). The more labile class of bonds is present at concentrations below K_M, and its hydrolysis therefore follows single-exponential decay kinetics. The fast phases of D52A ChEWL- and GoEWL-catalyzed reactions

Chart 3: Classes of NAM Linkages in the *M. luteus* Peptidoglycan[a]



[a] All NAM residues are covalently bound to NAG residues on either side as part of a chain of alternating NAG-NAM residues. Lysozyme cleaves the bonds indicated by arrows. Structures are reproduced from Ghuysen et al. (1968). (A) Unsubstituted NAM residue. (B) NAM residues substituted with an un-cross-linked peptide. (C) NAM residues connected through a dimer of cross-linked peptides. (D) NAM linkages connected through one or more bridge peptides. Wild-type ChEWL, while exhibiting preference for structures B−D, is known to catalyze the hydrolysis of all four types of linkages (Ghuysen, 1974). GoEWL is thought to cleave only the B−D-type linkages (Arnheim et al., 1973). The model presented in the text is that the fast phase of D52A ChEWL *M. luteus* cell wall hydrolysis represents the cleavage of class B linkages only.

are experimentally identical 2.7% decreases in light scattering that may indicate substrate-assisted catalysis for this small subset (Figures 2A and 3A). The second rate constant of the biphasic model pertains to the cleavage of less labile bonds, whose initial concentration in the assay is well above K_M. The assay is carried out at 0.2 mg/mL *M. luteus* cells, which is about 10 times the K_M value of the complex with wild-type ChEWL (Matsumura & Kirsch, 1996); therefore, the rates of the slow phase of the reaction are independent of substrate concentration for that fraction of the clearing reaction assayed (≤20%).

The kinetic results can be correlated with the distribution of substrate types within the *M. luteus* peptidoglycan to suggest the identities of the linkages cleaved during the fast phase of the D52A ChEWL-catalyzed reaction. These linkages hereafter will be referred to as "D52A ChEWL hyperlabile". The NAM residues in the *M. luteus* peptidoglycan can be grouped into the four subclasses shown in Chart 3 (Ghuysen et al., 1968). Subclasses B, C, and D all bear free carboxyl groups at the glycine positions indicated and therefore are candidates for providing substrate-assisted

catalysis. As in the case for the (carboxymethyl)chitin discussed earlier, this carboxyl group can be model built into the Asp52 position. The conformational freedom of cross-linked peptides (Chart 3C,D) is restricted, so that the most likely candidate for the D52A hyperlabile linkage is that shown in Chart 3B. The 2.7% burst is reasonably close to the assigned population of 5% of the total glycan population (Table 2; Ghuysen et al., 1968). An exact agreement might not be expected given that a precise correlation between the decrease in light scattering and linkage type has not been established.

Non-Hyperlabile Cell Wall Substrates

D52A ChEWL. Kinetic results from D52A ChEWL-catalyzed reactions with other cell wall substrates are consistent with the hypothesis that un-cross-linked peptides in the *M. luteus* peptidoglycan assist in catalysis (Chart 2). The D52A ChEWL-catalyzed clearing of *E. coli* or *S. lutea* cell suspensions exhibits the same kinetics as the corresponding wild-type ChEWL-catalyzed reactions (Figures 5A and 6A), but at one-twentieth of the rate (Table 1). These D52A ChEWL-catalyzed reactions therefore are essentially the same as the slow phase of the reaction of this enzyme with an *M. luteus* suspension (Figure 2A, Table 1), where substrate assistance does not occur.

The absence of fast phases in the D52A ChEWL-catalyzed clearing of *E. coli* and *S. lutea* suspensions correlates with the lack of the un-cross-linked peptide shown in Charts 2 and 3B. The peptidoglycan of *E. coli* cell walls has a high proportion of NAM residues substituted with un-cross-linked peptides (Table 2), but the peptides lack the glycine residue that might functionally replace Asp52 (Chart 2). In contrast with the *M. luteus* peptide, it is not possible by using molecular models with planar peptide bonds to position the *E. coli* peptide such that a carboxyl group is near the C_1 glycosidic bond. The peptidoglycans of *S. lutea* and *M. luteus* contain peptides of the same sequence, but virtually all are cross-linked in the former (Chart 3C,D, Table 2) and therefore cannot assist in catalysis. The evidence for this is that digestion of *M. luteus* with lysozyme or the F1 *endo-N*-acetylmuramidase releases detectable amounts of NAG-NAM disaccharide with a un-cross-linked peptide (Ghuysen et al., 1968), while similar digests of *S. lutea* cells do not (Campbell et al., 1969).

The catalytic activity of D52A ChEWL is also of interest because it further deflates the importance of Asp52 in the wild-type mechanism. The mutant enzyme retains ~4% and 2.4% of the catalytic activity of the wild-type in unassisted reactions with cell wall substrates and (ethylene glycol)chitin, respectively (Table 1). The D52N and D52S mutations result in enzymes that are significantly poorer catalysts than the D52A mutant (Malcolm et al., 1989; Corey, 1990; Lumb et al., 1992). The larger Asn52 and Ser52 side chains therefore must interfere with the catalytic mechanism. Weaver et al. (1995) proposed that the equivalent to Glu35 in GoEWL acts both as a general acid catalyst and to stabilize the oxocarbenium intermediate. The redundancy of the latter role in wild-type ChEWL may be the reason Asp52 is not essential to the mechanism.

Asp52 of ChEWL and Asp20 in the phage T_4 lysozyme occupy similar positions within their respective crystal structures (Weaver et al., 1985). Mutation studies of the latter residue show that it also is not essential for catalytic activity. The D20C mutant of T_4 lysozyme catalyzes the hydrolysis of *E. coli* cell suspensions at a rate equal to 77% of that of the wild type (Hardy & Poteete, 1991). Furthermore, D20A T_4 lysozyme is also apparently catalytically active. P22 phage in which the P22 lysozyme gene is replaced by a D20A T_4 lysozyme gene formed plaques when plated on a lawn of *Salmonella typhimurium* cells. Control experiments showed that plaque formation requires at least 3% of the total lysozyme activity produced by the "wild-type" hybrid phage (Rennell et al., 1991). Taken together, these results suggest that c-, g-, and p-type lysozymes do not require a second carboxylate group for catalysis.

GoEWL. The data and rationale described earlier provide a consistent explanation for the initial bursts exhibited by D52A ChEWL and GoEWL in reactions with *M. luteus* suspensions. The substrate-assisted catalysis model (Scheme 2) is not intended to explain other aspects of the substrate specificity displayed by GoEWL. The initial velocities of GoEWL-catalyzed clearing of *E. coli* and *S. lutea* cell suspensions are equal to 9.4% and 202%, respectively, of the comparable wild-type ChEWL values (Table 1). The catalytic activity of GoEWL, relative to that of D52A ChEWL, is far more dependent upon binding interactions with the peptide portion of the substrate (Arnheim et al., 1973). The dependence of these binding interactions upon peptidoglycan structure remains unclear.

CONCLUSIONS

The results of this study are consistent with the substrate assistance model, in which reactions catalyzed by lysozymes lacking a counterpart to Asp52 of wild-type ChEWL are partially complemented by the carboxyl groups of certain substrates. D52A ChEWL and GoEWL have more catalytic activity, relative to the wild-type ChEWL, in reactions with (carboxymethyl)chitin than in reactions with (ethylene glycol)chitin. D52A ChEWL and GoEWL are apparently the most reactive with a subset of linkages in the *M. luteus* cell wall. We suggest that these linkages are the glycosidic bonds following NAM residues substituted with un-cross-linked peptides (Chart 3B) and that the glycine α-carboxylate groups on the peptide assist in catalysis. This study also confirms that Asp52 is less important to ChEWL catalysis than was once generally believed (Phillips, 1966; Vernon, 1967) and further defines the substrate specificity of GoEWL.

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