Synergistic Contributions of Asparagine 46 and Aspartate 52 to the Catalytic Mechanism of Chicken Egg White Lysozyme†

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ABSTRACT: The X-ray structure of a chicken egg white lysozyme (ChEWL) complex with a peptidoglycan-derived inhibitor suggests that interactions of Asn46 and Asp52 with the D-subsite *N*-acetylmuramic acid residue help to distort that pyranose ring into the reactive half-chair conformation and that a hydrogen bond is formed between Asn46 and Asp52 [Strynadka, N. C. J., & James, M. N. G. (1991) *J. Mol. Biol. 220,* 401−424]. These hypotheses were investigated through the D52A, N46A, and D52A/N46A mutants of ChEWL. The Michaelis constants of the D52A and D52A/N46A ChEWL complexes with *Micrococcus luteus* cells are 3- and 4-fold higher, respectively, than the wild-type K_M; the corresponding k_{cat} values are 25- and 50-fold lower, respectively, than the wild-type k_{cat}. These results support the proposal of Strynadka and James. The velocities of reactions catalyzed by the N46A and D52A mutants are approximately equal to each other for all classes of substrate, suggesting that the respective roles of Asn46 and Asp52 in transition state stabilization do not vary. The mutation of either Asn46 or Asp52 to Ala apparently disrupts the interactions of the other (nonmutated) residue with the substrate, supporting the crystallographic evidence of a hydrogen-bond interaction between the two residues. The mutations do not change the values of the dissociation constants of complexes with (carboxymethyl)chitin complexes, suggesting that ground state complexes of ChEWL with chitin-derived substrates differ in conformation from complexes with bacterial peptidoglycans.

Chicken egg white lysozyme (ChEWL)[1] catalyzes the hydrolysis of the glycosidic bond between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) in bacterial cell walls, as well as bonds between adjoining NAG units of chitin. The generally accepted catalytic mechanism is based upon the structure of ChEWL cocrystallized with the inhibitor chitotriose. According to this model, Glu35 acts as a general acid by contributing a proton to the glycosidic bond oxygen between the saccharide residues in subsites D and E. The resulting cleavage of the glycosidic bond leads to a metastable oxocarbenium intermediate, which is stabilized through charge−charge interactions by the ionized Asp52 side chain [Phillips, 1966; Vernon, 1967; see Scheme 1 of Matsumura and Kirsch (1996)].

The site-directed D52A mutant of ChEWL, however, retains ∼4% of the catalytic activity of the wild-type in reactions with *Micrococcus luteus*, *Sarcina lutea*, and *Escherichia coli* cell suspensions. This result shows that Asp52 accounts for only a 2 kcal/mol decrease in $\Delta G^{\ddagger}$ and therefore is not essential to the catalytic mechanism (Matsumura & Kirsch, 1996). The function of this residue, therefore, deserves further study. High-resolution crystal structures of native enzyme (Kundrot & Richards, 1987) and of a lysozyme complex with the peptidoglycan-derived inhibitor, NAM-NAG-NAM (Figure 1; Strynadka & James, 1991), show that Asp52(OD1) and Asn46(ND2) are 2.9 Å apart, suggesting a hydrogen-bond interaction. This bond probably stabilizes the negative charge on Asp52 and helps to hold that residue in the proper orientation to stabilize the positive charge on the oxocarbenium intermediate. The precise position of the ionized carboxyl group is important, as the D52E mutant is a weaker catalyst relative to the wild type (Corey, 1990) than is D52A ChEWL for all substrates tested (Matsumura & Kirsch, 1996).

Asn46 and Asp52 of the wild-type ChEWL may also be important in straining the NAM residue bound in the D-subsite into the reactive half-chair conformation. This conformation helps to stabilize the oxocarbenium intermediate by allowing the positive charge to be shared by two members of the pyranose ring (Phillips, 1966; Vernon, 1967). The NAM-NAG-NAM/lysozyme cocrystal structure is assumed to be representative of complexes with the more extended bacterial peptidoglycan substrates. That structure shows numerous van der Waals interactions between atoms of Asp52, Asn46, and the NAM residue bound in the D-subsite. In addition, Asp52(OD2) and Asn46(OD1) each form hydrogen bonds with the acetamido nitrogen atom of the D-subsite NAM (Figure 1). These specific interactions can only be made when the pyranose ring is in the half-chair conformation; therefore, they partially compensate for the energetic cost of distortion (Strynadka & James, 1991).

Comparisons of the dissociation constants of lysozyme complexes with various oligosaccharides suggest that NAG,

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[1] Abbreviations: ChEWL, chicken egg white lysozyme; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; NAM-NAG-NAM, $\beta(1-4)$-linked trisaccharide, *N*-acetylmuramic acid-*N*-acetylglucosamine-*N*-acetylmuramic acid.

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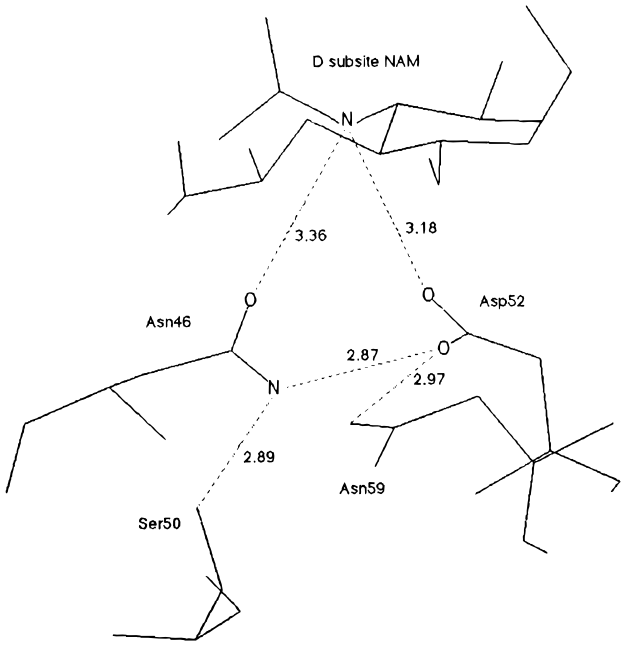

FIGURE 1: View of the lower half of the lysozyme active site. The hydrogen-bond (dashed lines) distances are in angstroms. The figure is drawn from coordinates provided by N. Strynadka (Strynadka & James, 1991).

unlike NAM, residues bind in the D-subsite without being distorted (Schindler et al., 1977). The interactions of Asn46 with chitin-derived substrates were previously studied through the N46D mutant of ChEWL (Inoue et al., 1992). The k_{cat} and K_M values derived from N46D ChEWL reactions with (ethylene glycol)chitin are both 25% of the corresponding wild-type values; the k_{cat}/K_M therefore is unaffected. These workers concluded that Asn46 in wild-type ChEWL "is involved in the initial substrate binding but not in the transition state at all". It is, however, possible that Asn46 and the mutant Asp46 contribute equally to transition state stabilization and that the extra free energy seen in the Asp46 contribution to the stabilization of the enzyme−substrate complex is lost in the transition state. These two positions can be decided by the investigation of additional Asn46 mutants.

In this study, the roles of Asn46 and Asp52 in substrate association and catalysis are investigated by mutation analysis. The catalytic efficiencies of the N46A, D52A, and N46A/D52A mutants in reactions with a variety of substrates are compared with those of the wild type. The results show that Asn46 and Asp52 act cooperatively in the wild-type enzyme, supporting the crystallographic evidence for a hydrogen bond between these two residues. The Michaelis constants for complexes of the mutant and wild-type lysozymes with the *M. luteus* cells, and the dissociation constants for the corresponding lysozyme complexes with (carboxymethyl)chitin, are determined. Our data suggest that interactions of Asn46 and Asp52 with the substrate contribute to the stabilities of complexes with cell wall substrates, but not to those formed with chitin derivatives. The results also imply that interactions of the two residues with both types of substrate contribute to the stabilization of each of the corresponding transition states.

MATERIALS AND METHODS

Materials. Oligonucleotides for mutagenesis were synthesized commercially by GenSet, Inc. (La Jolla, CA) or by the Core Facility of the Department of Molecular and Cell Biology, University of California at Berkeley. Chitotriose was from Seikagaku America (Rockville, MD). All other materials were obtained as previously described (Matsumura & Kirsch, 1996).

Production of Mutants. A single-stranded uridine-labeled template was prepared from the D52A ChEWL phagemid previously described (Matsumura & Kirsch, 1996). The oligonucleotide 5′-d(TACAAACCGTGCCACCGATGGGAG)-3′ was annealed to this template, or to the wild-type ChEWL template, to generate the N46A and N46A/D52A mutants. Sequencing, subcloning, yeast transformation, expression, and purification were carried out as previously described for D52A ChEWL (Matsumura & Kirsch, 1996). All enzymes characterized in this study were ≥99% pure as judged by SDS−PAGE analysis (Laemmli, 1970; data not shown). Protein concentrations were determined by the Bio-Rad protein assay (Hercules, CA) as described by the manufacturer.

Cell Wall Substrates. The catalytic activities of N46A and N46A/D52A ChEWL in reactions with *M. luteus*, *S. lutea*, and *E. coli* cell suspensions were assayed, and the data were analyzed as previously described (Matsumura & Kirsch, 1996), except as noted. The K_M value for the wild-type ChEWL complex with *M. luteus* cell walls was determined as described by Maurel and Douzou (1976), with differences noted in the text. Those of the corresponding D52A and N46A/D52A ChEWL complexes were derived from the velocities of the slow phases of the clearing reactions catalyzed by each enzyme. The small initial bursts were not evaluated because they represent the cleavage of a small minority (≤5%) of linkages. The rate of the slow phase for each reaction was obtained from the fit of the data to the biphasic model (Matsumura & Kirsch, 1996). These rates were subsequently fit to the Michaelis−Menten equation.

Chitin Derivatives. The relative rates of N46A and N46A/D52A ChEWL-catalyzed cleavage of (ethylene glycol)chitin and (carboxymethyl)chitin were assayed as previously described in Matsumura and Kirsch (1996). The dissociation constants of the various lysozyme complexes with (carboxymethyl)chitin and chitotriose were based upon the observed decrease in the fluorescence intensity of the protein upon binding (Schindler et al., 1977). Solutions (250 nM) of each lysozyme in 66 mM potassium phosphate (pH 5.5, 25 °C) were excited at 280 nm, and the emissions at 365 nm were followed in a Perkin-Elmer LS-50B spectrofluorimeter with increasing concentrations of chitotriose or (carboxymethyl)chitin. Measurements were taken after a 10 min incubation time. Little hydrolysis of (carboxymethyl)-chitin occurs within the time scale of the binding assay for the mutant lysozymes [Figure 1 of Matsumura and Kirsch (1996)]. The determined values of the dissociation constants of wild-type ChEWL complexes with (carboxymethyl)chitin were independent of the incubation time, which was either 5−10 s or 10 min.

The fluorimetric titration data were corrected for the minor contributions of the substrate and buffer, as well as for the volume of the added substrate, and fit to

$$F_{obs} = F_o - (\Delta F[L])/([L] + K_d) \quad (1)$$

where F_{obs} is the volume- and background-corrected fluo-

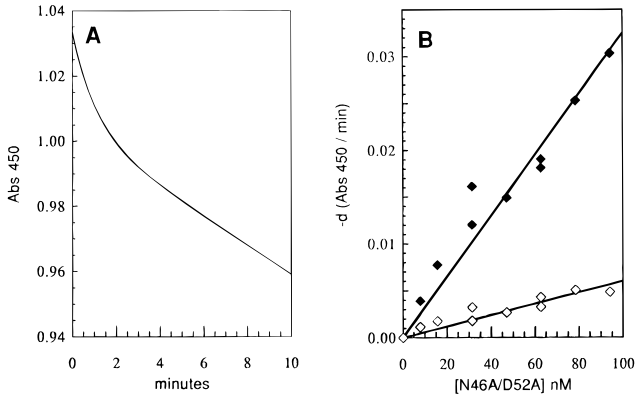

FIGURE 2: N46A/D52A ChEWL-catalyzed hydrolysis of *M. luteus* cell suspensions showing biphasic kinetics. (A) 120 nM N46A/D52A ChEWL was reacted with a 0.2 mg/mL *M. luteus* suspension in 66 mM potassium phosphate (pH 6.6, 25 °C). The reaction trace was fit to the biphasic model (Matsumura & Kirsch, 1996). The data are superimposable on the calculated line within the resolution of the figure. The minimizing parameters for this reaction are $[S_{FP}] = 0.0299\ A_{450}$ units, $k_1 = 1.30 \times 10^5\ M^{-1}\ s^{-1}$, and $k_2 = 620\ A_{450}\ M^{-1}\ s^{-1}$. (B) Velocities, expressed as the decrease in A_{450} units per minute, of the fast (◆) and slow (◇) phases of N46A/D52A ChEWL-catalyzed reactions plotted against enzyme concentration. The slopes are equal to 11.0% and 1.7% of that derived from the initial velocities of wild-type ChEWL-catalyzed reactions (Table 1).

rescence intensity, F_o is the fitted intrinsic fluorescence of enzyme alone, ΔF is the fitted change in fluorescence upon association of ligand with enzyme, and [L] is the molar concentration of chitotriose or (carboxymethyl)chitin in mg/mL.

RESULTS

Catalytic Activities

M. luteus. The interdependence of the contributions of Asn46 and Asp52 to the ChEWL catalytic mechanism was explored by mutation analysis. The D52A mutant, unlike the wild-type enzyme, shows biphasic kinetics in reactions with *M. luteus* cell suspensions, indicating a preference for a subset of glycosidic bonds in the peptidoglycan (Matsumura & Kirsch, 1996). Superposition of the additional N46A mutation on the D52A ChEWL framework (N46A/D52A) yields a construct exhibiting biphasic kinetics that is qualitatively identical to that exhibited by the D52A mutant. The reactions of both the D52A and N46A/D52A mutants show initial bursts that rapidly decrease the light scattering of the suspension by 2.7%, followed by linear slow phases (Matsumura & Kirsch, 1996; Figure 2A). The quantitative differences, listed in Table 1, are as follows: the fast and slow phase velocities of the N46A/D52A-catalyzed reaction are equal to 11.0% and 1.7% of the wild-type initial rate, respectively, (Figure 2B, Table 1), while the corresponding values for D52A ChEWL are 22.1% and 4.1%, respectively (Matsumura & Kirsch, 1996). The contribution of Asn46 in the D52A ChEWL mutant therefore accounts for only a 2−3-fold rate acceleration over that of the double mutant, with no qualitative change in the shape of the kinetic trace.

The kinetics exhibited by the N46A mutant in reactions with *M. luteus* cell suspensions is apparently biphasic, suggesting that it also has a preference for a subset of the glycosidic bonds in the *M. luteus* peptidoglycan. The fast and slow phases, however, are more difficult to distinguish

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

substrate	N46A ChEWL (%)	D52A ChEWL[b] (%)	N46A/D52A ChEWL (%)
M. luteus fast phase amplitude[c]	6.8 ± 1.3	2.7 ± 0.6	2.7 ± 0.5
M. luteus fast phase velocity[d]	9.1 ± 0.2	22.1 ± 1.0	11.0 ± 0.2
M. luteus slow phase velocity[d]	5.7 ± 0.1	4.1 ± 0.1	1.7 ± 0.1
E. coli velocity[e]	8.0 ± 0.2	5.2 ± 0.1	1.7 ± 0.1
(ethylene glycol)chitin velocity[f]	0.40 ± 0.04	2.4 ± 0.2	1.0 ± 0.2
(carboxymethyl)chitin velocity[f]	2.0 ± 0.5	5.9 ± 1.4	4.1 ± 0.8

[a] Except for the first row (see footnote *c*), the velocities of N46A ChEWL-, D52A ChEWL-, and N46A/D52A ChEWL-catalyzed reactions are expressed as percentages relative to the 100% value for wild-type ChEWL acting on the given substrate. All values are the averages of at least three independent determinations. [b] The values in this column are from Matsumura and Kirsch (1996). [c] 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 the N46A, D52A, and N46A/D52A ChEWL-catalyzed reactions. [d] 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 N46A, D52A, and N46A/D52A ChEWL-catalyzed reactions. [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).

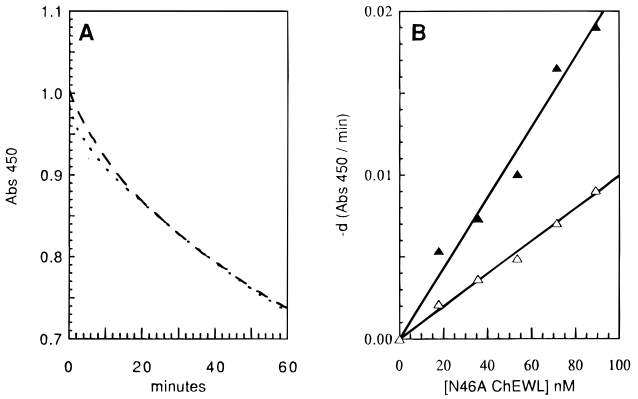

FIGURE 3: Biphasic kinetics of the N46A ChEWL-catalyzed clearing of *M. luteus* suspensions. (A) 40 nM N46A (dashed line) or 200 nM N46A/D52A (dotted line) ChEWL was reacted with the *M. luteus* substrate as described in Figure 2A. The superposition of the traces shows that N46A ChEWL exhibits the same kinetics as N46A/D52A ChEWL, except for a more gradual transition from the fast to the slow phase. Note that the scale of the *y*-axis is larger than that of Figure 2A, so that the fast phase of the D52A ChEWL reaction is lost. (B) N46A ChEWL traces were fit as described in Figure 2B, and the minimizing parameters for the fast (▲) and slow (△) phase rates were plotted against enzyme concentration. These slopes are equal to 9.1% and 5.7% of that derived from the initial velocities of reactions catalyzed by wild-type ChEWL (Table 1).

than those of the D52A and N46A/D52A ChEWL-catalyzed reactions (Figure 3A). Predigestion of the *M. luteus* substrate with the N46A mutant eliminates the initial burst when either D52A or N46A/D52A ChEWL is subsequently added (Figure 4). This result demonstrates that the N46A ChEWL specificity subsumes at least that subset of D52A ChEWL hyperlabile linkages (Matsumura & Kirsch, 1996). In quantitative terms, the rate of the N46A ChEWL-catalyzed clearing of an *M. luteus* suspension is not much faster than those of the corresponding N46A/D52A ChEWL reaction. The fast and slow phase velocities of the N46A ChEWL-catalyzed reaction are equal to 9.1% and 5.7% of the wild-type ChEWL initial reaction rate, respectively (Figure 3B,

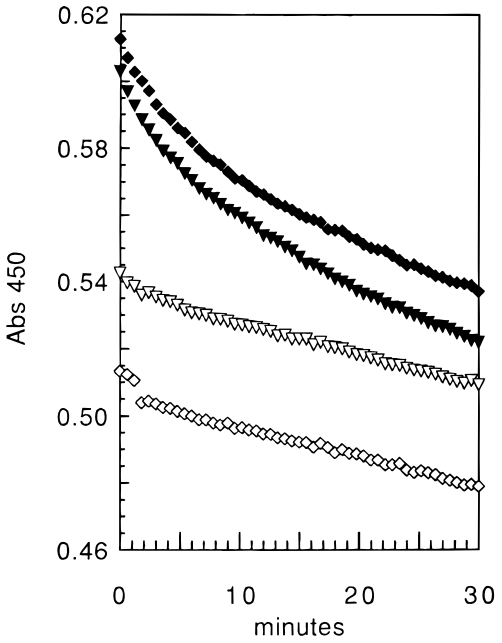

FIGURE 4: Reactions of 20 nM D52A (▼, ▽) or 40 nM N46A/D52A ChEWL (◆, ◇) with 0.1 mg/mL *M. luteus* cell suspensions that were fresh (▼, ◆) or pre-reacted for 30 min with 10 nM N46A ChEWL (△, ◇). The latter traces were corrected for the residual catalytic activity attributable to the original N46A ChEWL aliquot. Quantitatively, predigestion for the specified time reduces the burst amplitude (i.e., the hyperlabile subset) by 80%, while only 12% of the total A_{450} is consumed.

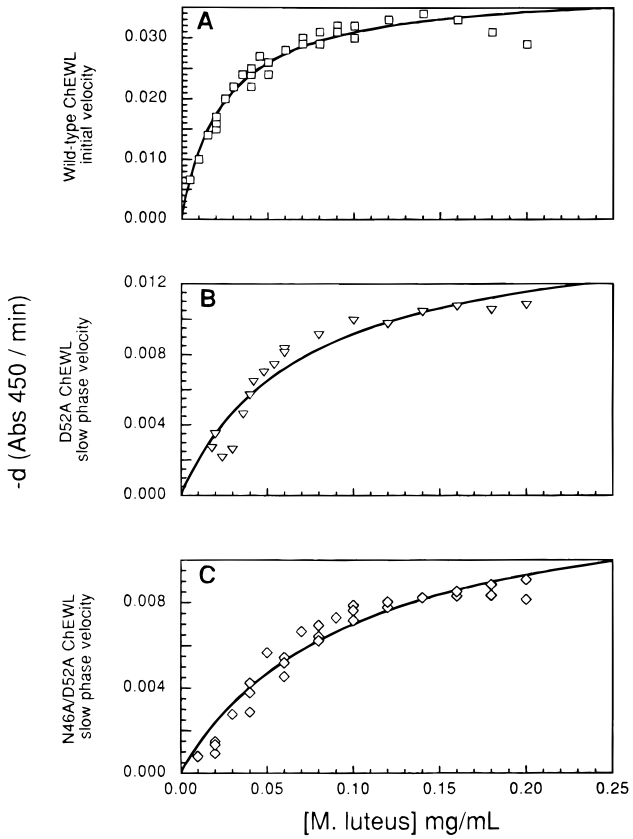

FIGURE 5: Determination of the Michaelis constants of lysozyme complexes with *M. luteus* cells. (A) The initial velocities of 10 nM wild-type ChEWL (□) in reactions with varying concentrations of cells, expressed as the decrease in A_{450} units/minute, plotted against substrate concentration. The data were fit to the Michaelis−Menten equation, where $K_M = 0.024 \pm 0.002$ mg/mL. (B) Cell suspensions were digested with 200 nM D52A ChEWL (▽) for 10 min. The data were fit to the biphasic model (Matsumura & Kirsch, 1996), and the slow phase velocities were plotted against *M. luteus* concentration. The K_M value of the slow phase is 0.070 ± 0.01 mg/mL. (C) Like (B), except that N46A/D52A (◇) was used and the K_M value is 0.098 ± 0.02 mg/mL.

Table 1). Thus, both Asn46 and Asp52 are necessary for the expression of wild-type activity and substrate specificity in reactions with *M. luteus* suspensions.

E. coli. The N46A, D52A, and N46A/D52A mutants exhibit kinetics in reactions with *E. coli* suspensions that are satisfactorily accommodated by the single-exponential decay model used for the wild-type enzyme (Matsumura & Kirsch, 1996). This implies that all of the lysozymes react with the same subset of glycosidic linkages, thereby simplifying the interpretation of the data. The specific rate constants are decreased by the factors shown in Table 1. The rate constant for the double mutant is less than that of either of the singles, but is greater than their product. That is, $0.017 > 0.052 \times 0.080 = 0.004$, indicating catalytic contributions of a cooperative, rather than an additive, nature.

Chitin-Derived Substrates. The catalytic activities of the lysozymes in reactions with (ethylene glycol)chitin and (carboxymethyl)chitin show that the conclusions about the cooperative behavior of Asn46 and Asp52 can be extended to chitin-derived substrates. The velocities of the N46A, D52A, and N46A/D52A ChEWL-catalyzed cleavage of (ethylene glycol)chitin are equal to 0.4%, 2.4%, and 1.0% of that of the wild type, respectively. (Carboxymethyl)chitin was found to be a substrate for all three mutants and was used to study the interactions of lysozyme with chitin-derived substrates, as described in the following. The velocities of the N46A, D52A, and N46A/D52A ChEWL-catalyzed reactions with (carboxymethyl)chitin are equal to 2.0%, 5.9%, and 4.1% of that of the wild-type enzyme, respectively (Table 1).

Michaelis and Dissociation Constants

M. luteus. The Michaelis constants of the wild-type and mutant ChEWL complexes with *M. luteus* cells in suspension were determined to help separate the observed effects of the mutations on the kinetics into catalytic and dissociation constants. The K_M value of the wild-type lysozyme complex in units of cell concentration is 0.024 ± 0.002 mg/mL in 66 mM potassium phosphate buffer (pH 6.6, 25 °C) (Figure 5A, Table 2). Determination of the K_M values for the mutant ChEWL complexes is more complicated. In the D52A and N46A/D52A ChEWL-catalyzed clearing of *M. luteus* suspensions, the fast phase of each reaction accounts for only 2.7% of the overall reaction. Each clearing trace was therefore fit to the biphasic model (Matsumura & Kirsch, 1996), and the slow phase velocity at each substrate concentration was used for the K_M determination. The K_M values of the D52A and N46A/D52A ChEWL complexes with *M. luteus* cells are 0.070 ± 0.014 and 0.098 ± 0.016 mg/mL, respectively (Figure 5B,C, Table 2). The corresponding value for the N46A ChEWL cannot be determined accurately, because the fast and slow phases of each reaction are more difficult to differentiate. The higher K_M values of the D52A and N46A/D52A complexes with *M. luteus* cells are sufficient to show that both Asn46 and Asp52 contribute to the stability of the enzyme−substrate complex for the wild-type enzyme, assuming that the K_M values derived from

Table 2: K_d and K_M Values of Lysozyme−Ligand Complexes

lysozyme	chitotriose[a] K_d (μM)	(carboxymethyl)-chitin[b] K_d (μg/mL)	*M. luteus*[c] K_M (mg/mL)
wild-type ChEWL	13 ± 1	4.1 ± 0.8	0.024 ± 0.002
N46A ChEWL	10 ± 2	2.6 ± 0.5	ND
D52A ChEWL	14 ± 4	4.2 ± 0.8	0.070 ± 0.014
N46A/D52A ChEWL	14 ± 3	3.4 ± 0.4	0.098 ± 0.016
E35Q ChEWL	ND	2.0 ± 0.7	ND

[a] Determined from the changes in fluorescence (λ_{ex} = 280 nm, λ_{em} = 365 nm) of 250 nM lysozyme in 66 mM potassium phosphate (pH 5.5, 25 °C) upon the addition of 0.4−200 μM chitotriose (1 μM chitotriose = 0.63 μg/mL). The data are shown in Figure 7B. Values are based a fit of eq 1 to 13 points, and the standard error of the fit is given. [b] Similar to the K_d determinations for the lysozyme complexes with chitotriose, except that 0.4−200 μg/mL (carboxymethyl)chitin was added to each lysozyme variant (Figure 7A). For the mutant enzymes, the amount of (carboxymethyl)chitin hydrolyzed during the binding assay is small [Figure 1B of Matsumura and Kirsch (1996)]. For assays with wild-type ChEWL, each addition of (carboxymethyl)chitin was to fresh enzyme with minimal (5−10 s) incubations. Each of the values is an average of three independent determinations. [c] The values are based upon the fit of the Michaelis−Menten equation using at least 18 points, as described in Figure 5.

the velocities of the slow phases of the reactions are true dissociation constants.

Chitin Derivatives. The dissociation constants of the wild-type and mutant ChEWL complexes with (carboxymethyl)-chitin were determined with an assay based on the observed quenching of fluorescence associated with the formation of the complex (Figure 6; Nakatani & Hiromi, 1974; Schindler et al., 1977). Attempts to determine the K_d values of lysozyme complexes with (ethylene glycol)chitin proved to be impractical due to substrate aggregation at the concentrations employed (data not shown). The K_d values for the complexes of wild-type, N46A, D52A, and N46A/D52A ChEWL with (carboxymethyl)chitin are all approximately 3 μg/mL (Figure 7A, Table 2). The value for the E35Q mutant, which has no detectable activity against this substrate (Matsumura & Kirsch, 1996), is similar (Table 2). Since none of the mutations affect the K_d value, it is unlikely that the Asn46, Asp52, or Glu35 side chain interacts with (carboxymethyl)chitin in the complex. This is in contrast to the observed effect of the same mutations upon the K_M value of the complex with *M. luteus* cells.

Chitotriose associates with subsites A−C (Blake et al., 1967a,b; Imoto et al., 1972). The dissociation constant should be insensitive to the mutations under consideration here since they are all in the D-subsite. The results are in accord with this expectation: the K_d values of the chitotriose complexes with the wild-type and mutant lysozymes are all approximately 12 μM or 8 μg/mL (Figure 7B, Table 2). Previous workers have found that the D-subsite mutations, E35Q and D52N, affect chitotriose binding (Corey, 1990; Malcolm et al., 1989), but the ones employed in this study do not.

DISCUSSION

Crystal structures of the chitotriose and NAM-NAG-NAM complexes with ChEWL suggest that Asn46 and Asp52 play several important roles in catalysis. The ionized Asp52 stabilizes the oxocarbenium intermediate by charge−charge interaction (Phillips, 1966; Vernon, 1967). Asn46 and Asp52 make specific van der Waals and hydrogen-bond interactions

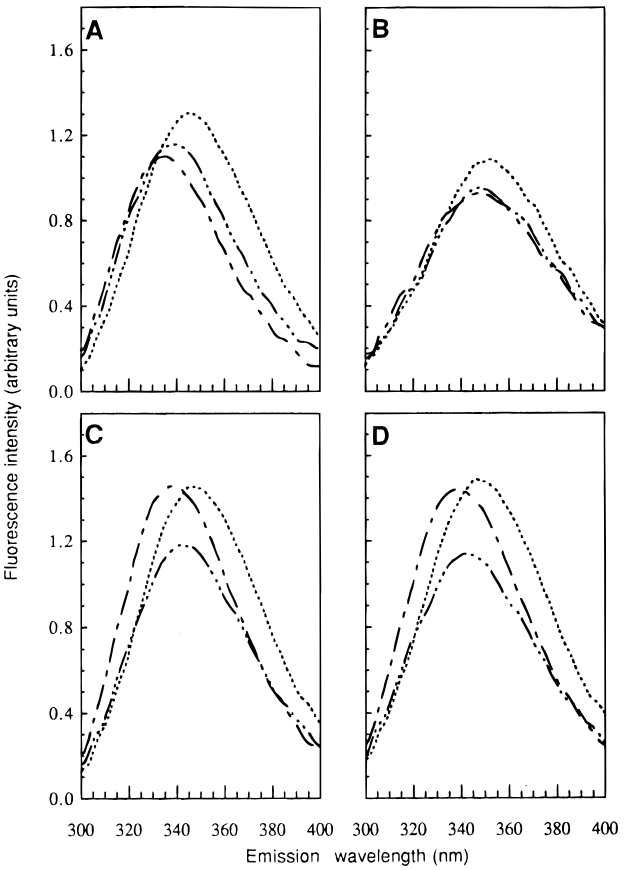

FIGURE 6: Fluorescence emission changes in the spectra of wild-type and mutant ChEWLs in the presence of chitotriose or (carboxymethyl)chitin. Emission spectra (λ_{ex} = 280 nm) of 250 nM (A) wild-type, (B) N46A, (C) D52A, or (D) N46A/D52A ChEWL in 66 mM potassium phosphate (pH 5.5, 25 °C): enzyme alone (dotted line), with 140 μM chitotriose (long-dashed−short dashed line), or with 30 μg/mL (carboxymethyl)chitin (long-dashed−dotted line). The concentrations of the chitin derivatives are 10-fold higher than the K_d values of their respective complexes with wild-type ChEWL. The data were corrected for background fluorescence and dilution and normalized to a value of 1 for 250 nM wild-type ChEWL when λ_{em} = 365 nm.

with the D-subsite NAM residue only when the pyranose ring is in the reactive half-chair conformation. A hydrogen bond formed between Asn46(ND2) and Asp52(OD1) holds both residues in the proper position for the enzyme−substrate interactions to occur (Figure 1; Strynadka & James, 1991). The contributions to ligand association and catalyzed hydrolysis of these residues in the wild-type enzyme are explored here through the N46A, D52A, and N46A/D52A mutants.

Cell Wall Substrates

Biphasic Kinetics. The D52A and N46A/D52A mutants exhibit qualitatively identical biphasic kinetics in reactions with *M. luteus* suspensions (Figure 2A; Matsumura & Kirsch, 1996), most likely for the same reasons. The D52A mutant expresses a preference for a "hyperlabile" subset of the linkages in this substrate. It was proposed that the mutant exhibits a greater preference for the subset of glycosidic bonds associated with carboxyl groups in the correct orientation to replace Asp52 functionally. The fast phase of the D52A ChEWL-catalyzed reaction is limited, because the hyperlabile subset only comprises approximately 5% of the *M. luteus* peptidoglycan. The slow phase of the reaction

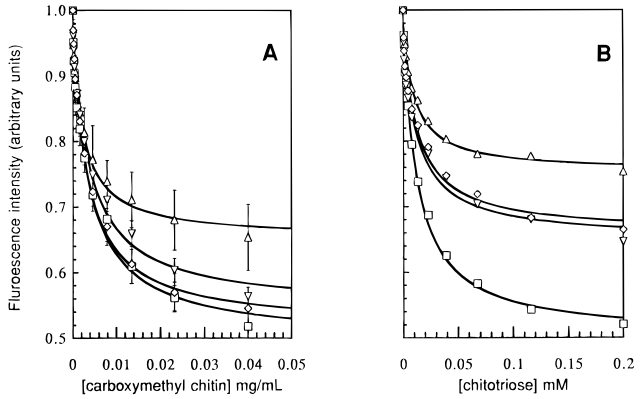

FIGURE 7: Determination of the dissociation constants of lysozyme complexes with chitin derivatives by fluorescence (λ_{ex} = 280 nm, λ_{em} = 365 nm). (A) The fluorescence intensity of 250 nM wild-type (□), N46A (△), D52A (▽), or N46A/D52A (◇) ChEWL in 66 mM potassium phosphate (pH 5.5, 25 °C), corrected for background fluorescence and dilution, plotted against the concentration of (carboxymethyl)chitin. Each point represents the average value of three independent determinations. The calculated curves are least-squares fits to eq 1 with the K_d values given in Table 2. (B) Similar to (A), except that chitotriose replaced (carboxymethyl)-chitin, with each point representing a single trial.

represents the cleavage of other bonds; its existence shows that Asp52 is not essential for catalysis (Matsumura & Kirsch, 1996).

N46A ChEWL also exhibits biphasic kinetics in reactions with *M. luteus* suspensions, but the initial burst differs in character from those of the D52A or N46A/D52A mutants (Figure 3A). All three mutants show linear kinetics during the slow phases of their reactions with *M. luteus* suspensions (Figures 2A and 3A; Matsumura & Kirsch, 1996). The velocities of these latter reactions reflect the catalytic activity of the mutants in reactions with ordinary (not hyperlabile) cell wall substrates where substrate assistance plays no role. The nonlinear kinetic traces initially exhibited by the N46A mutant in reactions with *M. luteus*, unlike the corresponding traces of the D52A and N46A/D52A mutants, cannot be readily related to the known structure of the *M. luteus* peptidoglycan.

Catalytic Activity. The slow phase velocities of the N46A, D52A, and N46A/D52A ChEWL-catalyzed reactions with *M. luteus* suspensions are equal to 5.7%, 4.1%, and 1.7% of the wild-type initial velocity, respectively (Table 1). These values are similar to the initial velocities of the reactions of these mutants with *E. coli* suspensions, which are 8.0%, 5.2%, and 1.7% of the corresponding wild-type value, respectively. In the D52A mutant, the contribution of the Asn46 side chain to catalysis accounts for only a 2−3-fold rate acceleration over the double mutant. The N46A mutant is generally as poor a catalyst as D52A ChEWL. The N46A mutation apparently weakens the contribution of Asp52, so that the rate of reaction is only 3−5-fold faster than that of the double mutant. The cooperative behavior of Asn46 and Asp52 in the wild-type enzyme is correlated with crystal structures later in this section.

Substrate Distortion. The K_M values of the D52A and N46A/D52A ChEWL complexes with *M. luteus* are 3- and 4-fold higher than that of the wild-type complex, respectively (Table 2). The reactions of the D52A and N46A/D52A ChEWL mutants have k_{cat} values that are 25- and 50-fold less than that of the wild-type, respectively (Table 1). These data are consistent with the model that the N46A and D52A mutations disrupt specific enzyme−ligand interactions that contribute to substrate distortion. Such interactions are seen in the ChEWL/NAM-NAG-NAM cocrystal structure (Figure 1) and are assumed to persist in complexes with the more extended peptidoglycan substrates. Asn46(OD1) and Asp52-(OD2) form direct hydrogen bonds with the acetamido nitrogen of the D-subsite NAM residue. Five atoms of that NAM residue make van der Waals contact with atoms of Asn46 or Asp52; these two amino acids, along with Asn59, Ser50, and Asp48, constitute a hydrogen-bonded "platform" that contributes to ring distortion by packing against the D-subsite NAM. None of these interactions occurs when the pyranose ring is in the chair conformation (Strynadka & James, 1991).

The cooperative nature of the contributions of Asn46 and Asp52 to catalysis is based upon the hydrogen-bond interaction between Asn46(ND2) and Asp52(OD1) (Strynadka & James, 1991). The mutation of either of these residues eliminates the hydrogen bond and disrupts the interaction of the remaining one with the substrate. The equivalent of Asp52 in human lysozyme was mutated to Glu, resulting in an enzyme with 1% of the catalytic activity of the wild type (Muraki et al., 1987). The crystal structure of the latter mutant is nearly identical to that of the wild type, except that the mutant residue and the chicken lysozyme equivalents of Asn46 and Asn59 are moved out of place (Harata et al., 1992).

Chitin Derivatives

Catalytic Activity. The velocities of the N46A, D52A, and N46A/D52A ChEWL-catalyzed cleavage of (ethylene glycol)chitin are equal to 0.4%, 2.4%, and 1.0% of the wild-type value, respectively. The relative rates of the corresponding reactions with (carboxymethyl)chitin are higher (Table 1), possibly because the carboxyl group of (carboxymethyl)NAG bound in the E-subsite assists in catalysis (Matsumura & Kirsch, 1996). (Ethylene glycol)chitin kinetic data are an unambiguous measure of catalytic activity in the absence of substrate assistance. The N46A and D52A mutations influence the catalysis of chitin-derived and cell wall substrates in similar ways; the structure of the transition state is most likely S_N1-like for all of the reactions considered here (Capon, 1969; Sinnott, 1990).

Dissociation Constants. The K_d values of the N46A, D52A, N46A/D52A, and wild-type ChEWL complexes with (carboxymethyl)chitin were all determined by fluorometric titration to be approximately 3 μg/mL. This suggests that interactions between Asn46 or Asp52 and the substrate do not contribute significantly to the stability of the enzyme−substrate complex. By contrast, the K_M values for the reactions with cell wall substrates do vary (see above). The K_d values for the wild-type and mutant ChEWL complexes with the inhibitor chitotriose, which binds in subsites A−C (Blake et al., 1967a,b; Imoto et al., 1972), are all about 12 μM or 8 μg/mL (Table 2). The latter results also show that the mutations do not affect subsites A−C.

The (carboxymethyl)chitin binding data are consistent with other evidence that ChEWL complexes with chitin-derived substrates differ in conformation from complexes with bacterial peptidoglycans. Comparisons of the dissociation constants of lysozyme complexes with NAG-NAM-NAG,

NAG-NAM-NAG-NAG, and NAG-NAM-NAG-NAM show that NAG, but not NAM, is capable of binding in the D-subsite without being distorted (Schindler et al., 1977). Three binding processes for lysozyme and chitohexose are kinetically distinguishable: the α−process, in which three NAG residues bind in subsites A−C, forming a nonproductive complex; the β-process, in which six NAG residues bind in subsites A−F, forming an intermediate on the reaction pathway; and the γ-process, a slow rearrangement resulting in a productive complex. There is evidence that the β- and γ-processes result in complexes in which the enzyme−substrate interactions in subsites D−F are not fully developed (Holler et al., 1975b; Banerjee et al., 1975).

The conformation of the lysozyme complex with (carboxymethyl)chitin may be similar to those of the β- or γ−complexes with chitohexose described earlier. The N46A and D52A mutations do not change the value of the dissociation constant for the lysozyme/(carboxymethyl)chitin complex, suggesting that the D-subsite interactions are partially or completely undeveloped in the ground state. The polymeric nature of (carboxymethyl)chitin makes it unlikely that lysozyme association is limited to interactions with the three saccharide units at the reducing end. The nonproductive A−C binding mode of chitohexose has a dissociation constant 3 times lower than that of the productive binding mode (Holler et al., 1975a). The concentration of reducing ends in the polymer is much less than one-third that of interior binding sites.

Inoue et al. (1992) found that the k_{cat} and K_M values derived from the reactions of N46D (COOH form) ChEWL with (ethylene glycol)chitin are both 25% of the corresponding wild-type values. The K_M values are assumed to be true dissociation constants, as is the case for the substrate chitohexose (Banerjee et al., 1973). The k_{cat} values of N46A ChEWL reactions with (ethylene glycol)chitin and (carboxymethyl)chitin are 250- and 50-fold lower than the corresponding values for wild-type ChEWL (Table 1). The K_d values for the wild-type and N46A ChEWL complexes with (carboxymethyl)chitin are identical (Table 2). The combined data of Inoue et al. (1992) and the present study can be accommodated by the following model. The mutant Asp46 residue contributes to the stabilization of the enzyme−substrate complex, while its counterparts in the N46A and wild-type enzymes do not ($K_d^{N46D} < K_d^{wild\text{-}type} = K_d^{N46A}$). Asn46 and Asp46 contribute equally to the ΔG_{TS-GS}, the difference in free energy between the transition and ground states, while their counterpart in the N46A mutant does not ($[k_{cat}/K_M]^{N46A} < [k_{cat}/K_M]^{wild\text{-}type} = [k_{cat}/K_M]^{N46D}$). This model differs from the conclusions of Inoue et al. (1992) with regard to the roles of the wild-type Asn46 residue in substrate association and catalysis, but is limited by the reasonable assumption that there is little specific destabilization of the transition state attributable to the Ala46 side chain.

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REFERENCES

Banerjee, S. K., Kregar, J., Turk, V., & Rupley, J. A. (1973) *J. Biol. Chem. 248*, 4786−4792.
Banerjee, S. K., Holler, E., Hess, G. P., & Rupley, J. A. (1975) *J. Biol. Chem. 250*, 4355−4367.
Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967a) *Proc. R. Soc. Ser. B 167*, 365−377.
Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967b) *Proc. R. Soc. Ser. B 167*, 378−388.
Capon, B. (1969) *Chem. Rev. 69*, 407−498.
Corey, M. J. (1990) Ph.D. Dissertation, University of California, Berkeley, CA.
Harata, K., Muraki, M., Hayashi, Y., & Jigami, Y. (1992) *Protein Sci. 1*, 1447−1453.
Holler, E., Rupley, J. A., & Hess, G. P. (1975a) *Biochemistry 14*, 1088−1094.
Holler, E., Rupley, J. A., & Hess, G. P. (1975b) *Biochemistry 14*, 2377−2385.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) in *The Enzymes* (Boyer, P., Ed.) 3rd ed., Vol. 7, pp 665−868, Academic Press, New York.
Inoue, M., Yamada, H., Yasukochi, T., Miki, T., Horiuchi, T., & Imoto, T. (1992) *Biochemistry 31*, 10322−10330.
Kundrot, C. E., & Richards, F. M. (1987) *J. Mol. Biol. 193*, 157−170.
Laemmli, U. K. (1970) *Nature 227*, 680−684.
Malcolm, B. A., Rosenberg, S., Corey, M. J., Allen, J. S., De Baetselier, A., & Kirsch, J. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 133−137.
Matsumura, I., & Kirsch, J. F. (1996) *Biochemistry 35*, 1881−1889.
Maurel, P., & Douzou, P. (1976) *J. Mol. Biol. 102*, 253−264.
Muraki, M., Jigami, Y., Morikawa, M., & Tanaka, H. (1987) *Biochim. Biophys. Acta 911*, 376−380.
Nakatani, H., & Hiromi, K. (1974) *J. Biochem. (Tokyo) 76*, 1343−1346.
Phillips, D. C. (1966) *Sci. Am. 215*, 78−90.
Schinder, M., Assaf, Y., Sharon, N., & Chipman, D. M. (1977) *Biochemistry 16*, 423−431.
Sinnott, M. L. (1990) *Chem. Rev. N7*, 1171−1202.
Strynadka, N. C. J., & James, M. N. G. (1991) *J. Mol. Biol. 220*, 401−424.
Vernon, C. A. (1967) *Proc. R. Soc. Ser. B 167*, 389−401.

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