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Reexamination of the Role of Asp^{20} in Catalysis by Bacteriophage T4 Lysozyme†

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ABSTRACT: Replacement of Asp^{20} in T4 lysozyme by Cys produces a variant with (1) nearly wild-type specific activity, (2) a newly acquired sensitivity to thiol-modifying reagents, and (3) a pH–activity profile that is very similar to that of the wild-type enzyme. These results indicate that the residue at position 20 has a significant nucleophilic function rather than merely an electrostatic role. The intermediate in catalysis by lysozyme is probably a covalent glycosyl-enzyme instead of the ion pair originally proposed.

Lysozyme provides an essential function in the life cycle of the phage—the destruction of the bacterial cell wall necessary for the release of progeny phage particles. The enzyme catalyzes the hydrolysis of the β-1,4 glycosidic linkages of the alternating copolymer of *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial peptidoglycan. Little has been published on the mechanism of catalysis by T4 lysozyme, in contrast to the abundant literature on the catalytic mechanism of hen egg white (HEW) lysozyme. HEW lysozyme is the first enzyme whose atomic structure was visualized by X-ray diffraction analysis (Blake et al., 1965). The three-dimensional structure of T4 lysozyme is also known (Remington et al., 1978; Weaver & Matthews, 1987). The remarkable similarity of the geometries of the active sites of HEW and T4 lysozymes (Anderson et al., 1981; Matthews et al., 1981) strongly suggests that the two enzymes catalyze hydrolysis of glycosides via a similar or common mechanism. Inspection of the structure of HEW lysozyme in complex with substrate analogues led directly to a detailed proposal for its mechanism (Blake et al., 1967; Vernon, 1967), depicted in Figure 1A, which is presented in many textbooks of biochemistry to exemplify principles of enzymatic catalysis. The first step of that mechanism is protonation by a Glu residue (Glu^{35} in HEW or Glu^{11} in T4) of the oxygen (O_4'), which in the substrate links C_1 of the incipient reducing sugar and C_4' of the departing glycosyl moiety. The next step, bond cleavage between the protonated O_4' and C_1, is assisted by participation of the ring oxygen (O_5), yielding an endocyclic oxocarbonium ion intermediate. The glycosyl carbonium ion is thought to be stabilized by electrostatic interaction with the carboxylate side group of an Asp residue (Asp^{52} in HEW or Asp^{20} in T4). Finally, the proposed carbonium ion intermediate reacts with water (or some other acceptor) to complete the reaction. Covalent catalysis by the carboxylate of the active-site Asp (Figure 1B) was considered but regarded as unlikely in the original formulation of the mechanism (Blake et al., 1967; Vernon, 1967). Many subsequent assessments have concurred with this view, but no available evidence conclusively demonstrated or ruled out the existence of either of the intermediates shown in Figure 1 (Kirby, 1987).

While carrying out systematic studies of the effects of single amino acid substitutions on the function of T4 lysozyme, we made the initially surprising observation that substitution of Asp^{20} with Cys produced a functional enzyme. Comparison of the properties of the purified mutant T4 lysozyme (hereafter designated D20C) with those of the wild-type protein, described in this paper, provides strong evidence against a merely electrostatic role for the residue at position 20 in this enzyme and instead implies that catalysis occurs with substantial covalent bond formation between the carboxylate of the active-site Asp and C_1 of the glycosyl-enzyme intermediate.

Experimental Procedures

Phage Methods. Hybrid P22 phages bearing mutant and wild-type alleles of the T4 lysozyme gene (*e*) were constructed by crosses of P22 *Kn321 sieA44 m44* with plasmids, as previously described (Rennell & Poteete, 1989; see Figure 2). Those regions of DNA which were single-stranded in the gapped duplex DNA (gdDNA)[1] were sequenced in the hybrid phages as described (Rennell & Poteete, 1989) to ensure that only the desired mutations were present. *Salmonella typhimurium* and *Escherichia coli* strains, phage, media, and culture methods were described by Rennell and Poteete (1989).

Plasmid Construction. Plasmid pTP400 contains (in order) the following sequences: the segment of pZ152 (Zagursky & Berman, 1983) bearing the plasmid replication origin, filamentous phage IG sequence, and *bla* gene, bounded by *Pvu*II (converted to *Bam*HI in pTP400) and *Eco*RI (converted to *Sal*I) sites; P22 DNA from the *Rsa*I site in gene *13* to the proximal *Hin*fI site in gene *19* (244 bp; Rennell & Poteete, 1985); a synthetic linker, formed by two oligomers (5′-AATCTAAGC-3′ and 5′-TAAGCTTAG-3′); T4 DNA from

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[1] Abbreviations: dNTP, 2′-deoxynucleoside triphosphates; EDTA, disodium ethylenediaminetetraacetate; gdDNA, gapped duplex DNA; Tris, tris(hydroxymethyl)aminomethane.

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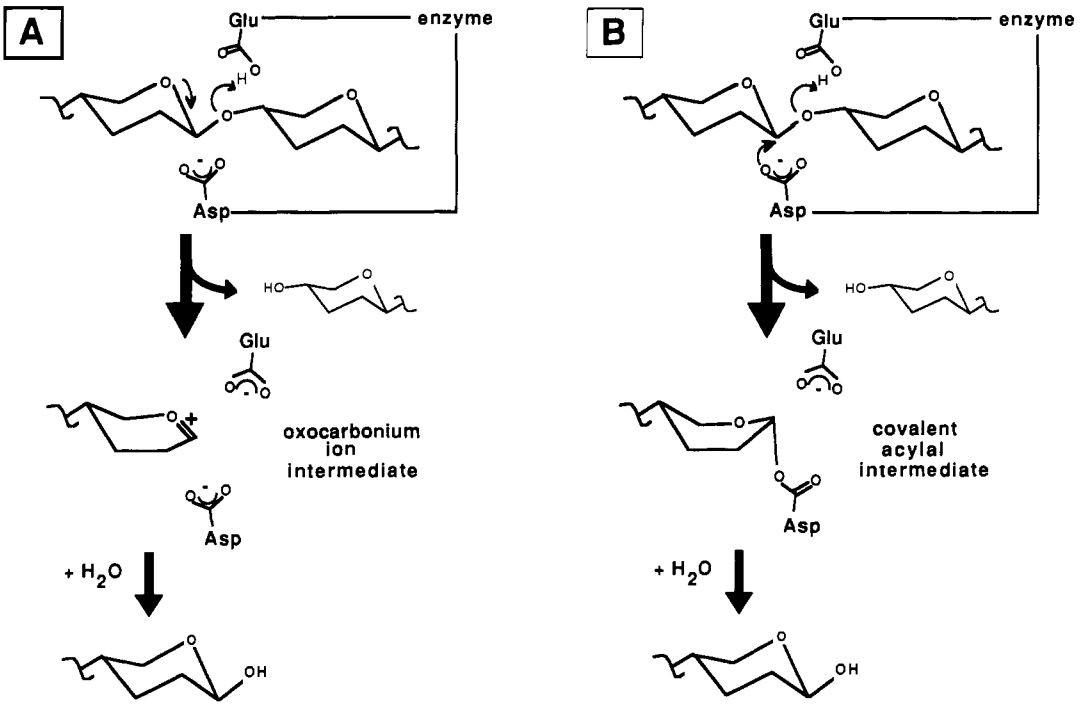

FIGURE 1: (Panel A) Mechanism of lysozyme-catalyzed hydrolysis of glycosides proposed by Blake et al. (1967) and Vernon (1967), involving stabilization of an oxocarbonium ion by electrostatic interaction with the carboxylate of Asp at the active site. The structure of the substrate is simplified for clarity. (Panel B) Mechanism of lysozyme catalysis involving the formation of a covalent intermediate by nucleophilic attack of the Asp carboxylate on C_1 of the incipient reducing sugar.

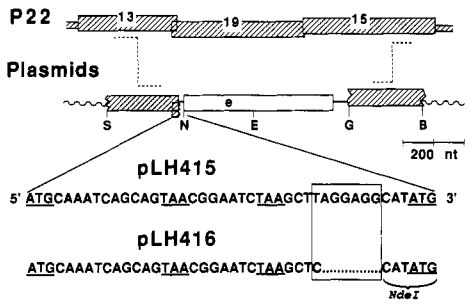

FIGURE 2: Partial structures of phage P22 genome in the vicinity of the P22 lysozyme gene *19* and of plasmids pLH415 and pLH416. The plasmids contain the coding sequences of the T4 lysozyme gene *e* (open box), flanked by the P22 sequences (filled boxes) on either side of *19* directly above the plasmid structure. Each phage hybrid is generated by crossing a plasmid bearing the requisite *e* allele, flanked by P22 sequences, with prophage P22 Kn321 (Rennell & Poteete, 1989). This prophage is too large to be effectively packaged after induction, due to a 5.5-kb insert in the middle of gene *19*, and must recombine with the plasmid in order to lose the insert and acquire a functional lysozyme gene. The recombination is equivalent to the double crossover event represented here by the broken lines. The underlined triplets in the sequences shown at the junction between the 3′ end of *13* and the 5′ end of *e* are (from left to right) the start codon for *19*, the termination codon for *13*, an artificial termination codon for *19* created to prevent translation into the *e* sequence, and the start codon for *e*. Sequence differences between pLH415 and pLH416 are boxed. The *Nde*I site at the start codon for *e* allows facile interchange of expression control elements. Unique restriction endonuclease cleavage sites in the plasmids are represented as follows: S, *Sal*I; N, *Nde*I; E, *Eco*RI; G, *Bgl*II; and B, *Bam*HI.

the *Dde*I site immediately upstream of gene *e*, including gene *e* and 42 bp downstream from its termination codon (Owen et al., 1983); a *Bgl*II linker; P22 sequences from the Val^{54} codon in gene *15* to the *Hpa*I in gene *15* (237 bp; Casjens et al., 1989); and a *Bam*HI linker. The linker sequence joining the P22 *Hin*fI site to the T4 *Dde*I site introduces a TAA codon that terminates P22 lysozyme translation after 8 sense codons, as well as a *Hin*dIII site. Plasmid pLH415 was constructed from pTP400 by two consecutive steps: (1) elimination of the unique *Nde*I site in the pBR322-derived sequences of pTP400 by cleavage with *Nde*I, filling in the ends with *E. coli* DNA polymerase large fragment, and religation and (2) introduction, by mismatched oligonucleotide-directed mutagenesis, of a new *Nde*I site overlapping the initiation codon of gene *e* (see Figure 2). Plasmid pLH416 was derived from pLH415 by introduction of a synthetic *Nde*I linker into the filled-in *Hin*dIII site upstream of the *e* gene Shine–Dalgarno sequence, with subsequent deletion of the Shine–Dalgarno sequence by cleavage with *Nde*I and religation. Plasmid pLH475 is a derivative of pLH415 with a ca. 250-bp P_{tac}-containing fragment inserted into the *Hin*dIII site, so oriented as to transcribe gene *e*. A version of pLH475 bearing the D20C mutant allele[2] was constructed by replacing the 539-bp *Nde*I–*Bgl*II gene *e* containing segment with the corresponding sequences from pLH416eD20C (described below).

Mutagenesis. Mutations were introduced into the T4 lysozyme gene (*e*) by mismatched oligonucleotide primer-directed DNA synthesis (Zoller & Smith, 1983), using a gdDNA as a target. The primers were synthetic 19-mers with two- or three-base mismatches in the center designed to introduce amber mutations in codon 11 or codon 20 or to convert codon 20 to a Cys (TGT) codon. Single-stranded plasmid pLH416 (unmethylated at *dam*-recognition sites) was prepared by infecting *E. coli* strain GM1675 (*Δlac–pro thi supE dam* F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15) bearing the double-stranded plasmid with phage f1 IR1 as described by Zagursky and Berman (1984). The strand of plasmid DNA packaged by the filamentous phage is the template strand for transcription of gene *e*. The restriction fragment used to prepare gdDNA was from plasmid grown in the methylation-proficient (*dam*$^+$) *E. coli* strain W3110 *lacI*^{q}L8. This fragment provides the strand that ultimately is covalently linked to the mismatched

[2] The notation D2OC uses the one-letter code for amino acids to indicate the substitution of the Asp residue at position 20 by Cys.

primer in the covalently closed heteroduplex circle produced after in vitro DNA synthesis and ligation. Hence any repair of the heteroduplex after its introduction into *E. coli* by the *dam*-directed mismatch repair system should discriminate against the wild-type sequence contained in the nonmethylated strand, thereby preserving the sequence changes we wish to introduce. In a typical preparation of gdDNA, single-stranded pLH416 (ca. 0.5 pmol) was annealed with a purified 3561-bp *Hin*dIII–*Eco*RI fragment from pLH415 (1.3 pmol) in a volume of 40 μL with 25 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 150 mM sodium chloride, and 50% deionized formamide by heating at 70 °C for 15 min followed by 37 °C for 30 min. The DNA was precipitated with ethanol and redissolved in 10 μL of buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 5 mM sodium chloride). Gapped duplex was separated from single-stranded phage DNA and excess fragment by electrophoresis through a 0.7% gel of low-melting agarose (SeaPlaque, FMC Corp.) in Tris–borate–EDTA buffer containing 0.5 μg/mL ethidium bromide for 10 h at 50 V. The gdDNA, which migrates in such gels more slowly than either the restriction fragment or the single-stranded circles, was recovered from the agarose by extraction with a butanol solution of hexadecyltrimethylammonium bromide (Langridge et al., 1987). A mixture of 5–20 pmol of mismatched primer and ca. 0.2 pmol of gdDNA, in a volume of 15–22 μL, was heated to 65 °C for 15 min and annealed at room temperature for 15 min, and 0.6 mM dNTPs, 1 mM ATP, *E. coli* DNA polymerase large fragment (1 unit, New England Biolabs), and T4 DNA ligase (80 units, New England Biolabs) were added, followed by a 1-h incubation at 15 °C. Competent *E. coli* W3110 was transformed with the entire reaction mixture, transformants were selected on LB plates containing ampicillin, and clones bearing plasmids with presumptive amber mutations in gene *e* were identified by loss of ability to complement λ *Ram5* cI⁻, as described previously (Rennell & Poteete, 1989). Given the behavior of P22 *e416 am20*, we could not necessarily expect the D20C mutation to destroy lysozyme function, so a different screen was needed to identify this mutation. The Cys codon that we chose to use created a new *Rsa*I endonuclease cleavage site in pLH416, approximately equidistant from the two *Rsa*I sites already present in the plasmid. One of the twelve transformants (pLH416eD20C) contained plasmid DNA with a new *Rsa*I site at the correct location.

Purification and Characterization of D20C Lysozyme. Lysozymes were expressed in *S. typhimurium* strain MS1868 from P22 *e475* phages containing *c1-7* mutation and either the wild-type or D20C mutant alleles of gene *e*. For most of the experiments described here, the proteins were purified, from lysates of the phage-infected cells obtained from 1-L cultures, by the method of Perry and Wetzel (1986) with an additional step, as follows. The pooled lysozyme-containing fractions from the Sephadex G75 column were dialyzed into 50 mM sodium acetate buffer, pH 4.9, 1 mM EDTA, and 3 mM β-mercaptoethanol (buffer A). The dialyzed enzyme was loaded onto a column (11 cm^3 in a 12-mL syringe) of sulfopropyl- (SP-) Sephadex G25 previously equilibrated with buffer A and eluted with a linear gradient of 40 mL each of buffer A and 0.4 M sodium chloride in buffer A. This yielded 2–4 mg of homogeneous T4 lysozyme.

The lysozymes used in the experiments involving the reducing sugar assays were prepared in a slightly different way. This alternate method used cultures of *S. typhimurium* strain MS1868 that were transformed with a plasmid (pTP478), which is engineered to produce an antisense RNA to block expression of the bacteriophage P22 gene *13* (A. R. Poteete, unpublished results) and thereby suppress lysis of P22-infected cells. Two-liter cultures of *S. typhimurium* strain MS1868 harboring pTP478 were infected with P22::*e475 c1-7* containing either the wild-type or D20C mutant allele of gene *e*. Two hours after infection, the cells were harvested by centrifugation, washed, resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 20 mM β-mercaptoethanol, and frozen. The cells lysed upon thawing, and the lysozyme in these lysates were purified by the method of Perry and Wetzel (1986) with the elimination of the Sephadex G75 chromatographic step. This procedure yielded 60–70 mg of homogeneous T4 lysozyme.

Protein assays and assays of lysozyme activity by monitoring the lysis of chloroform-treated *E. coli* were performed as previously described (Knight et al., 1987). One unit of activity in the cell lysis assay is the amount of enzyme needed to decrease the absorbance at 600 nm of a suspension of chloroform-treated *E. coli* by 1 unit/min.

Lysozyme activity was also assayed by measuring the rate of production of reducing sugar from a crude preparation of *E. coli* cell walls. The cell walls were prepared as follows [adapted from the procedure of Wheat (1966)]. A cell suspension (50 g of cells in 250 mL of 0.9% aqueous sodium chloride) was passed through a French pressure cell at 16000–18000 psi and centrifuged at 16000*g* for 90 min. The pelleted cell walls were resuspended in 200 mL of 1 M sodium chloride, heated to 100 °C for 15 min, washed with 1 M sodium chloride three times, and resuspended in 40 mL of water. The resulting suspension of cell walls gave an absorbance of 0.53 at 600 nm when diluted 100-fold. The basic assay for reducing sugar was a micro modification of the Park and Johnson (1949) ferricyanide method (Ghuysen et al., 1966). Reaction mixtures contained buffer, enzyme, 0.1 mg of bovine serum albumin/mL, and sufficient cell walls to give an absorbance of 0.73 at 600 nm and were stirred at 30 °C. Reactions were initiated by adding lysozyme, and timed 250-μL samples were removed and quenched by addition to 125 μL of 0.01 M potassium cyanide and 0.05 M sodium carbonate. Each quenched sample was spun for 5–10 min in a microcentrifuge to remove residual cell walls, 300 μL of the supernatant was mixed with 100 μL of 0.05% (w/v) potassium ferricyanide, and the mixture was heated at 100 °C for 15 min. The boiled samples were allowed to cool briefly and mixed with 0.5 mL of 0.15% (w/v) ferric ammonium sulfate, 0.1% (w/v) sodium dodecyl sulfate, and 0.15 M sulfuric acid. After the samples stood for 15 min to allow development of the blue color, the absorbance at 690 nm was measured. The assay was calibrated by reference to a standard curve for the color developed with *N*-acetylglucosamine and was linear up to at least 8 nmol of *N*-acetylglucosmaine added, with a slope of 0.077 absorbance unit at 690 nm/nmol.

Thiol titration with 5,5′-dithiobis(nitrobenzoic acid) was done by the method of Habeeb (1972) on samples of the proteins dialyzed into 8 M guanidine hydrochloride. For inhibition studies, samples of enzyme (4–8 μg/mL) were reacted for 2 min at room temperature in 0.2 M potassium phosphate, pH 7.0, with 10 mM iodoacetamide, 20 mM iodoacetate, or 1.2 mM *p*-hydroxymercuribenzoate and diluted 10–500-fold for assay by the cell lysis assay.

Results

New Hybrid Phage System for Studies of T4 Lysozyme Mutants in Vivo. In our previous studies (Knight et al., 1987), mutations were introduced into the T4 lysozyme gene (*e*) borne by a plasmid, pTP352. The mutant alleles were subsequently

Table I: Production of Lysozyme Activity by Hybrid Phages

genotype[a]	promoter	ribosome binding site	lysozyme activity[b] (units/mg of protein)
P22(wild type)	P22 P_{late}	near consensus[c]	6
P22 *e352*	P_{tac}+ P22 P_{late}	consensus	0.2
P22 *e415*	P22 P_{late}	consensus	50
P22 *e416*	P22 P_{late}	poor	11
P22 *e475*	P_{tac}+ P22 P_{late}	consensus	350

[a] Each phage strain bears the *c1-7* mutation to ensure entry into lytic growth after infection. [b] Average of duplicate determinations with the cell lysis assay, which gave values within 5% of each other. [c] The sequence just upstream from the P22 gene *19* is 5′-GCCGGAGTCGATGATG-3′, where the underlined triplet is the *19* start codon (Rennell & Poteete, 1985).

transferred into phage P22 by homologous recombination in vivo, which resulted in the production of a hybrid phage with the T4 lysozyme gene inserted into the P22 lysozyme gene; the hybrid phages (designated P22 *e352*) depend on their T4 lysozyme gene for plaque formation. The *e352* insertion includes sequences that negatively regulate translation of the T4 lysozyme mRNA and consequently produces a very low amount of lysozyme (Table I).

To generate hybrid phages more suitable for systematic genetic studies of T4 lysozyme, we constructed a series of plasmids that recombine with P22 as diagrammed in Figure 2. In the resulting hybrid phages, the P22 lysozyme gene (*19*) is interrupted by synthetic sequences that stop its translation after the eighth codon. [It is not possible to eliminate all gene *19* sequences from the hybrids, because *19* overlaps the essential P22 gene *13* (Rennell & Poteete, 1985).] In P22 *e415*, P22 sequences comprising the rest of gene *19*, as well as the amino-terminal two-thirds of gene *15*, are replaced by T4 gene *e* and its Shine–Dalgarno sequence. To decrease lysozyme production, the Shine–Dalgarno sequence was deleted in P22 *e416*; to increase production, it was supplemented with a P_{tac} promoter in P22 *e475*. The hybrid phages depend on T4 lysozyme function for their ability to form plaques; the absence of functional gene *15* in the hybrid phages has little effect on plaque-forming ability unless the plating medium is supplemented with divalent cations (Casjens et al., 1989).

The hybrid phages vary greatly in the level of expression of lysozyme, as shown in Table I, covering a greater than 1000-fold range, with wild-type P22 and the hybrid P22 *e416* close to each other near the middle of the range. As the most nearly normal of the substitutions, *e416* was chosen for systematic genetic studies; we have introduced a large number of lysozyme mutations into it (Rennell et al., 1991).

Properties of D20C Lysozyme. The activity of the D20C lysozyme was initially indicated by the successful suppression of a T4 gene *e* amber 20 mutant by a synthetic Cys amber suppressor [derived from that of Normanly et al. (1986)], as indicated in Table II. The ability of a P22 *e416* amber phage to form a plaque on, for instance, a *supE* strain of *Salmonella* indicates that replacement of the wild-type residue with Gln yields a lysozyme variant with at least some activity. An assessment of just how inactive a lysozyme variant must be to render the phage unable to form a plaque of normal size is provided by the data in Tables I and II. Wild-type P22 *e416* produces at least 50 times as much lysozyme activity as does P22 *e352* (Table I), yet both phages produce the same size plaques as wild-type P22 (Table II). Hence any lysozyme variant of P22 *e416* must produce less than 2% of the wild-type level of lysozyme activity before this is manifested in a reduced plaque size. The suppression pattern of P22 *e416 am11* (Table II) suggests that replacement of Glu^{11} with either Leu, Gln, Ser, Tyr, Phe, or Cys results in a variant with no more than 2% of the specific activity of the wild-type enzyme (an upper limit, assuming that suppression is 100% efficient). This result supports the idea that Glu^{11} is either catalytically or structurally essential. In contrast, P22 *e416 am20* forms large plaques efficiently on the Cys suppressor strain (Table II), suggesting either that Asp^{20} is neither structurally nor catalytically essential or that the role of this Asp residue can be fulfilled by Cys. The latter idea is more reasonable since other substitutions result in more severe phenotypes.

We converted codon 20 in gene *e* to a Cys codon, to eliminate potential ambiguities from the possible nonspecific insertion of other residues at the position of the amber codon, overproduced and purified the wild-type and D20C proteins, and compared their properties (Table III). The D20C mutant protein has three titrable thiols, whereas the wild type has two, as predicted by their DNA sequences. The specific activities of the two lysozymes were surprisingly similar (Table III).

We measured the activities of the enzymes using two assay systems. Measured by the cell lysis assay, the activity of D20C lysozyme at pH 7.0 is 0.77 that of the wild-type enzyme. This relative activity is nearly identical with the value of 0.81 obtained from assays of the two enzymes by monitoring the production of reducing sugar from isolated cell walls. Although a defined synthetic substrate would have been preferable to intact cells or cell walls, phage T4 lysozyme has stringent requirements for its substrate's structure (Mirelman et al., 1975; Jensen, et al., 1976). The phage enzyme will not cleave the synthetic substrates, such as *N*-acetylglucosamine oligosaccharides, which are commonly employed in studies of the catalytically more promiscuous HEW lysozyme. However, the production of reducing sugar from the isolated cell walls indicates that the D20C variant of phage T4 lysozyme, like the wild-type enzyme, catalyzes the hydrolysis of glycosidic linkages in the peptidoglycan.

The ultimate degree of cell lysis, or of reducing sugar produced from cell walls, observed with the D20C variant of T4 lysozyme is identical with that observed for the wild-type enzyme (Figure 3). We see no evidence for a subset of cell wall linkages that are especially labile to cleavage by D20C

Table II: Suppression Patterns of Amber Mutant Phages[a]

genotype	wild type[b]	amber suppressor[c]						
		none	Gln	Tyr	Leu	Ser	Phe	Cys
P22 (wild type)		++	++	++	++	++	++	++
P22 *e352*		++	++	++	++	++	++	++
P22 *e416*		++	++	++	++	++	++	++
P22 *e416 am11*	Glu	−	−	−	−	−	−	−
P22 *e416 am20*	Asp	−	±	±	−	±	−	++

[a] Entries denote the ability of the specified phage to form plaques on an amber suppressor strain that inserts the indicated amino acid residue. ++, large plaques; ±, very small plaques at slightly reduced efficiency of plating; −, pinpoint plaques at greatly reduced efficiency of plating or no plaques above the apparent frequency of reversion. [b] Amino acid specified by the wild-type codon at the position of the amber mutation. [c] *S. typhimurium* strains described previously (Rennell & Poteete, 1989).

Table III: Properties of Wild-Type and D20C Mutant T4 Lysozymes

	wild type	D20C mutant
equiv of thiol/mol of protein	1.9	2.8
specific activity		
cell lysis assay[a] (units μg^{-1})	62	48
reducing sugar assay[b] (μmol min^{-1} mg^{-1})	26	21
residual activity following inhibition (%)		
by iodoacetamide	>95	<1
by iodoacetate	>95	<1
by *p*-hydroxymercuribenzoate	>95	<1

[a] Performed in 0.2 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA at 25 °C. [b] Average values from pH 7.3 to 7.8, in 50 mM potassium phosphate buffer at 30 °C.

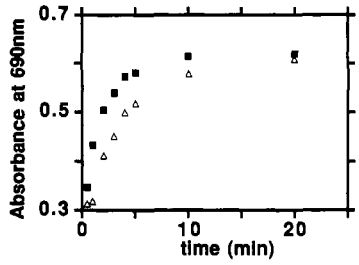

FIGURE 3: Typical time course for release of reducing sugar from cell walls by T4 lysozyme, as described under Experimental Procedures. The values of the absorbance are not corrected for the constant amount of color due to the presence of bovine serum albumin in the assay. These reactions were done at pH 7.8 and contained 0.146 μg of wild-type T4 lysozyme/mL (filled squares) or 0.153 μg of the D20C variant/mL (open triangles).

lysozyme, as has been suggested by Malcolm et al. (1989) for the HEW lysozyme variant in which Asp[52], analogous to Asp[20] in T4 lysozyme, has been altered to Asn.

The wild-type enzyme is resistant to inactivation by a variety of chemical reagents that react with thiols (Table III); this resistance is consistent with the fact that either or both Cys residues in the wild-type sequence can be replaced without affecting the specific activity of the enzyme (Perry & Wetzel, 1987). However, the D20C enzyme is nearly completely inactivated by thiol-modifying reagents, consistent with an essential role for the thiol of Cys[20].

The activities of wild-type T4 lysozyme and the D20C variant, measured by either the cell lysis assay or the reducing sugar assay, show nearly identical dependencies on pH (Figures 4 and 5). The pH dependency of the activity of wild-type T4 lysozyme reported by Tsugita (1971), using a cell lysis assay, is very similar to that reported here with the cell lysis assay. However, the pH dependencies observed with the cell lysis assay (Figure 4) differ somewhat from those observed with the reducing sugar assay (Figure 5). Note that the observed variations with pH with either assay system cannot be assigned to changes in k_{cat} or K_M since these parameters were not quantitated with the crude substrates. The differences in the pH dependencies observed with the two assays has precedence in a previous study of HEW lysozyme. Davies et al. (1969) reported discrepancies between the pH dependencies of the rate of cell lysis and the rate of hydrolysis of the tetramer of *N*-acetylglucosamine by HEW lysozyme.

Discussion

The fact that most of the T4 lysozyme variants having substitutions for Glu[11] or Asp[20] lack activity was not surprising. Chemical or mutagenic alterations of these residues, or of the equivalent residues in HEW lysozyme, were previously reported by other investigators to greatly decrease or abolish the

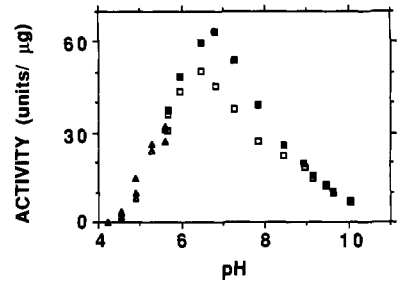

FIGURE 4: Variation of the activities (measured with the cell lysis assay) of wild-type (filled symbols) and D20C (open symbols) T4 lysozymes with pH in 0.2 M acetate buffers (triangles), prepared by mixing 0.2 M sodium acetate with 0.2 M sodium acetate and 0.2 M sulfuric acid; or 0.2 M phosphate buffers (squares), prepared by mixing 0.2 M monobasic potassium phosphate with 0.2 M tetrapotassium pyrophosphate. All buffers also contained 1 mM EDTA. Each point represents an individual assay.

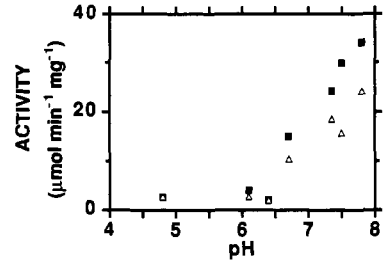

FIGURE 5: Variation of the activities (measured with the reducing sugar assay) for wild-type (filled squares) and D20C (open triangles) T4 lysozymes with pH in 0.05 M sodium acetate (pH 4.8) or 0.05 M potassium phosphate buffers. Each point represents the average of duplicate or triplicate assays, except the values at pH 7.5, which are for single determinations. Note that the pH range covered is less than that in Figure 4.

activity of the altered enzymes (Kuroki et al., 1986; Anand et al., 1988; Malcolm et al., 1989). These observations are consistent with major catalytic roles for these two residues but do not constitute *proof* for such roles nor intrinsically reveal what those roles are.

The result that ran counter to our expectations was the retention of nearly wild-type level of activity by the D20C variant of T4 lysozyme. The fact that Asp[20] can be replaced by Cys with retention of activity suggests that the residue at position 20 does something besides provide an anion to balance the positive charge of an oxocarbonium ion and that its nucleophilicity is significant. A Cys thiolate could act either as an anion or a nucleophile. However, if the residue at position 20 must be anionic for successful catalysis, a marked difference between acidic limbs of the pH versus activity profiles of the wild-type and D20C variant lysozymes should be evident, due to the four logarithmic units separating the pK_a values of aliphatic carboxylates and thiols. In fact, the activities of the two enzymes show nearly identical dependencies on pH (Figures 4 and 5). In the crystal structure of wild-type T4 lysozyme (Remington et al., 1978; Weaver & Matthews, 1987), Asp[20] is completely exposed to solvent water, so that the pK_a of either Asp or Cys at this position is probably not perturbed. [The corresponding Asp in HEW lysozyme, Asp[52], is also solvent exposed and has been reported to have a pK_a of 4.5 (Parsons & Raftery, 1972).] Hence the active form of the D20C lysozyme contains the (neutral) sulfhydryl form of Cys[20], which cannot stabilize a cation electrostatically but which is potentially an even more powerful carbon nucleophile than the oxygen of a carboxylate.

In the absence of detailed mechanistic studies of the phage enzyme, our expectations were based upon the mechanism of HEW lysozyme proposed by Phillips and co-workers (Blake

et al., 1967; Vernon, 1967). This seems reasonable, since the two enzymes catalyze the same reaction and have strikingly similar active-site geometries. (We discuss below the limitations of this assumption.) Our results are more consistent with a nucleophilic role for Asp^{20} than with the electrostatic role proposed by Phillips for the equivalent Asp^{52} in HEW lysozyme. This discrepancy led us to reexamine the evidence in the extensive studies of HEW lysozyme regarding the mechanistic role of Asp^{52}.

Koshland (1953) first pointed out that nucleophilic catalysis could explain how some glycosidases, like lysozyme, yield products with configuration identical with that of the substrate. A double displacement (two inversions) leads to net retention of configuration. Retention is always observed with HEW lysozyme–catalyzed transglycosylations, even with as small an acceptor as methanol (Dahlquist et al., 1969a). The latter acceptor might be expected to produce at least some inversion with a carbonium ion intermediate unless one side of the ion is completely occluded. Jencks (1969) has estimated that the lifetime of a (nonstabilized) glycosyl oxocarbonium ion is comparable in duration to a molecular vibration (ca. 10^{-15} s). Recent work by Bennett and Sinnott (1986) suggests that such ions, when solvated by water, may have a considerably longer lifetime, perhaps as long as 10^{-10} s, and indicates that the acid–catalyzed (nonenzymatic) hydrolyses of methyl glucopyranosides in pure water is unimolecular. The glycosyl intermediate in lysozyme-catalyzed reactions must survive long enough to participate in the transglycosylation reactions catalyzed by HEW lysozyme (Chipman et al., 1968). The stabilization that this requirement demands of the enzyme, based on the estimated 10^{-15}-s lifetime for the intermediate, is *at least* 5–7 kcal/mol (Young & Jencks, 1977). Warshel and Levitt (1976) have calculated that, in the case of HEW lysozyme, coulombic interaction between the glycosylic oxocarbonium ion and the carboxylate of Asp^{52} would provide ca. 9 kcal/mol of stabilization, so the stabilization required by the oxocarbonium ion mechanism is theoretically feasible. Indeed, these authors and Vernon (1967) have argued that this stabilization is the most significant factor in catalysis, although there is no experimental support for this view from model reactions (Dunn & Bruice, 1970; Anderson & Capon, 1972; Anderson & Fife, 1973; Loudon & Ryono, 1976).[3]

Many arguments *in favor* of the primacy of the oxocarbonium ion intermediate have actually relied upon the evidence *against* either direct displacement or the collapse of the ion pair into an acylal intermediate. Vernon (1967) and Blake et al. (1967) discounted the possibility of a covalent intermediate after inspecting the structure of the active site, citing steric difficulty in forming a bond between C_1 and the Asp carboxylate oxygen. Estimates of the distance from crystallographic data collected on complexes of substrate analogues with T4 lysozyme (Anderson et al., 1981; Matthews et al., 1981) or HEW lysozyme (Kelly et al., 1979) give values of 3–3.6 Å, approximately twice the length of known carbon–heteroatom single bonds. These static measurements do not consider the conformational changes that may accompany catalytic turnover in both T4 and HEW lysozymes (Remington et al., 1978; Weaver & Matthews, 1987; Kelly et al., 1979). Strained geometry in the covalent intermediate may provide the destabilization needed for successful catalysis (Jencks, 1969). Young and Jencks (1977) have noted that an acylal formed during lysozyme catalysis might be expected to accumulate. However, the fact that this proposed intermediate is not apparent kinetically with HEW lysozyme (Holler et al., 1975) does not prove that an acylal intermediate does not exist. Moreover, the rate constants for chemical hydrolysis of acylals (Fife, 1965) indicate that an acylal could be competent intermediate in lysozyme catalysis, particularly if the geometry of the intermediate were strained.

A major problem with postulating a covalent glycosyl-lysozyme intermediate is that the thioglycoside intermediate for the D20C enzyme would be expected to be considerably more stable than the acyl-glycoside intermediate for the wild-type enzyme. Thiolates are rather sluggish leaving groups (Sinnott & Whiting, 1971); hence the nonenzymatic hydrolysis of thioacetals proceeds considerably more slowly than that of acylals. However, strain in the enzyme-bound intermediate might accelerate the departure of the thiol or thiolate leaving group. The covalent intermediate in catalysis by the D20C lysozyme would presumably be even more strained than that formed with the wild-type enzyme, since the sulfur of Cys^{20} should be ca. 1 Å further away from C_1 than the carboxylate oxygen of Asp^{20} is. (This assumes no major rearrangement of the mutant protein's structure).

The secondary α-deuterium kinetic isotope effect (k_H/k_D) of 1.11 for hydrolysis of arylglycosides catalyzed by HEW lysozyme (Dahlquist et al., 1969b), once considered to rule out nucleophilic catalysis, is no longer compelling in light of the range of values for k_H/k_D from 1.05 to 1.18 (Craze et al., 1978; Knier & Jencks, 1980) in model reactions that are known to proceed by S_N2 mechanisms. (Kinetic isotope effects reveal the structures of transition states but not necessarily those of discrete intermediates.) These model reactions are classified as borderline S_N2, in which substantial carbonium ion character is developed in the transition state (Knier & Jencks, 1980), and the observed rate enhancements are, not surprisingly, only weakly dependent upon the nucleophilicities of the catalysts (Craze et al., 1978). ^{18}O kinetic isotope results indicate a low degree of bonding between C_1 and O_4, in the transition state for the first irreversible step for lysozyme-catalyzed reactions (Rosenberg & Kirsch, 1981), consistent with this being a borderline S_N2 reaction with a dissociative ("exploded") transition state. It is therefore reasonable to suggest that the nucleophile which forms the proposed glycosyl-lysozyme intermediate can be either a carboxylate (Asp) or an unchanged thiol (Cys).

An alternative explanation for our results with the D20C variant of T4 lysozyme is that Asp^{20} is neither structurally nor catalytically essential. We consider this possibility unlikely due to the more severe effects on lysozyme activity caused by substitutions other than Cys at residue 20. The failure of Ser to successfully substitute for Asp^{20} could be due to one of several factors. The intermediate that is formed in that case may be too stable to turn over. Alternatively, the Ser hydroxyl may not have sufficient intrinsic nucleophilicity or may be too distant, due to the difference in size of sulfur and oxygen. Confirmation of covalent catalysis by T4 lysozyme awaits trapping of the hypothetical intermediate.

Our results may be relevant to the mechanism of HEW lysozyme, since the case for the existence of an oxocarbonium ion intermediate is not compelling. Precedence for a glycosyl-enzyme intermediate exists for numerous other glycosyl transferases, recently reviewed by Sinnott (1990). However,

[3] An alternative proposal for the catalytic role of the active-site Asp of lysozyme (Bakthavachalam & Czarnik, 1987), prompted by the failure of the nonenzymic models, relies on the extensive binding interactions between the enzyme and substrate that have been suggested to compel distortion of the substrate toward oxocarbonium ion geometry. The proposal involves ground-state destabilization via lone pair repulsion between the carboxylate of the Asp and the glycosyl ring oxygen of the incipient intermediate.

it is conceivable that T4 and HEW lysozymes proceed via different mechanisms, notwithstanding the structural homology of their active sites. Conclusive evidence regarding the mechanism of the mechanistic paradigm, HEW lysozyme, must come from experiments on HEW lysozyme itself.

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