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Biochemistry

Structural basis of the conversion of T4 lysozyme into a transglycosidase by reengineering the active site

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ABSTRACT **In contrast to hen egg-white lysozyme, which retains the β-configuration of the substrate in the product, T4 lysozyme (T4L) is an inverting glycosidase. The substitution Thr-26 → His, however, converts T4L from an inverting to a retaining enzyme. It is shown here that the Thr-26 → His mutant is also a transglycosidase. Indeed, the transglycosylation reaction can be more effective than hydrolysis. In contrast, wild-type T4L has no detectable transglycosidase activity. The results support the prior hypothesis that catalysis by the Thr-26 → His mutant proceeds via a covalent intermediate. Further mutations (Glu-11 → His, Asp-20 → Cys) of the T26H mutant lysozyme indicate that the catalytic mechanism of this mutant requires Glu-11 as a general acid but Asp-20 is not essential. The results help provide an overall rationalization for the activity of glycosidases, in which a highly conserved acid group (Glu-11 in T4L, Glu-35 in hen egg-white lysozyme) on the β-side of the substrate acts as a proton donor, whereas alterations in the placement and chemical identity of residues on the α-side of the substrate can lead to catalysis with or without retention of the configuration, to transglycosidase activity, or to the formation of a stable enzyme-substrate adduct.**

There are at least two types of glycosidase: those such as hen egg-white lysozyme (HEWL) that have transglycosidase activity (1–3), and those that do not, such as T4 phage lysozyme (T4L) (1). The mechanistic difference between these two types of enzymes is not well understood (4).

Early structural studies showed that three different classes of lysozymes, typified by HEWL, T4L, and goose egg-white lysozyme (GEWL) have overall similarities in three-dimensional structure, although their amino acid sequences are almost entirely different. The active sites of these enzymes have a glutamic acid that is highly conserved (Glu-35 in HEWL, Glu-11 in T4L, Glu-73 in GEWL). Otherwise, the active site residues show substantial variability (5, 6). For example, the position of Asp-20 in T4L does not correspond to the "essential" Asp-52 of HEWL, and GEWL has no apparent counterpart at all to either of these acidic residues (6).

More recent structure determinations have shown that glycosidases, in general, need not have the "lysozyme fold" at all. They can, for example, have a "TIM barrel" structure (2, 7) or a right-handed parallel β-helix (8). In common with the lysozymes they have a highly conserved glutamic acid on the β-side of the substrate (2). As a group, however, these enzymes display considerable variability on the α-side. Consistent with these observations, various studies have shown that mutation of the "invariant" glutamate is highly deleterious, whereas some substitutions are tolerated on the α-side of the saccharide (2, 3, 9–12). These findings suggest that changes on the α-side of the saccharide may be key in explaining differences in the catalytic activities of these enzymes.

In the case of T4L it was found that the mutation Thr-26 → Glu (T26E) produced an enzyme that cleaved the cell wall of *Escherichia coli* but left the product covalently bound to the enzyme (13). Structural analysis revealed that the introduced glutamate side chain, located on the α-side of the substrate, had reacted to form a covalent adduct. This result encouraged us to further modify the catalytic site by introducing other types of nucleophilic residues at site 26. It was found that the mutation Thr-26 → His was catalytically active (albeit less than wild type, WT) but that the anomeric form of the product was changed from α in WT to β in the T26H mutant (14). Here we demonstrate that T26H, in contrast to WT T4L, also has acquired transglycosylase activity. A preliminary description of some of these results has been given (15). The importance of transglycosylation and its inhibition by a modified form of vancomycin in eliminating multidrug-resistant Gram-positive bacteria has been emphasized recently (16, 17).

MATERIALS AND METHODS

Materials. Several substitutions at the position of Glu-11 (Glu-11 → Gln, Asp, Asn, or His), Asp-20 (Asp-20 → Ala, Ser, Asn, Cys, or Glu), and Thr-26 (Thr-26 → Glu, Gln, Asp, or His) were constructed in cysteine-free pseudo-WT (WT*) lysozyme and purified as reported (13, 14, 18, 19). Mutant T26E forms a covalent adduct with a cell-wall fragment, the structure of which has been described (13). By incubating for 30 min at 37°C, pH 3.0, the adduct was released and the free enzyme was purified in the standard way except that the sequence of use of the SP-sephadex and CM-Sepharose columns was reversed.

Analysis of Enzymatic Products. The substrate used to analyze the catalytic activity of the different mutant lysozymes was the peptidic tetrasaccharide (NAG-NAM-DAla-DGlu-DAPA)-(NAG-NAMr-DAla-DGlu-DAPA) shown in Fig. 1 (NAG is *N*-acetylglucosamine, NAM is *N*-acetylmuramic acid, NAMr is the reduced form of NAM, and DAPA is diaminopimelic acid). The substrate was produced by the partial hydrolysis of *Escherichia coli* cell walls as described (14). Substrate (8×10^{-5} M) was mixed with mutant lysozyme (5×10^{-7} M) in 20 mM sodium acetate buffer at pH 5.0 and 0°C for 60 min.

The reaction mixture was analyzed as a function of time by HPLC with a reversed-phase column (3.9 × 150 mm) of μ-Bondasphere (Waters C18, 300 Å). The column was eluted with a gradient from 1% CH_3CN containing 0.05% trifluoro-

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Abbreviations: HEWL, hen egg-white lysozyme; T4L, T4 phage lysozyme; GEWL, goose egg-white lysozyme; WT, wild type; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; DAPA, diaminopimelic acid.

Data deposition: The structural coordinates have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 1QTV, 1QTZ, 1QT3–1QT8).

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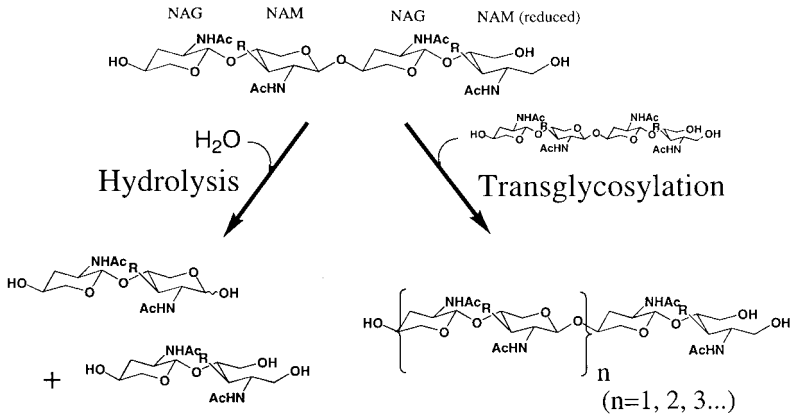

FIG. 1. Substrate and reaction paths for mutant lysozyme T26H. The substrate (*Top*) was produced by partial digest of *E. coli* cell walls with HEWL as described (14). R = DAla-DGlu-DAPA. The terminal NAM was reduced, as shown, to distinguish the respective NAG-NAM units produced by hydrolysis (*Bottom Left*). In addition, this reduction also makes it possible to determine whether the product shown bottom left is the α- or β-anomer. Transglycosylation (*Right*) can lead to a variety of products. For example, the condensation of a disaccharide of NAG-NAM (*Bottom Left*) with the original substrate (*Top*) leads to the hexasaccharide ($n = 2$) (*Bottom Right*).

acetic acid (TFA) to 15% CH_3CN containing 0.02% TFA at a flow rate of 1.0 ml/min. The molecular weight of each peak was determined by a Sciex III mass spectrometer.

Enzymatic Activity. The halo assay of Streisinger *et al.* (20) was used to estimate the activity of the mutant T4Ls. Although this assay is not quantitative, it is one of the most sensitive methods to detect enzymatic activity and was appropriate in the present context.

X-Ray Crystallography. The mutants were crystallized by using ≈2 M phosphate at pH ≈7.0, conditions similar to those used for WT (21). Diffraction data were collected by using either a San Diego Multiwire x-ray diffractometer (22) or a Rigaku R-AXIS IIc diffractometer. The model structures were refined by using the method of Tronrud *et al.* (23).

RESULTS

Enzymatic Activities of the Mutant T4Ls. As reported, a number of single mutations at the position of Thr-26 resulted in characteristic changes in the function of the enzyme. The mutation Thr-26 → Glu leads to a covalent adduct in which the cell wall peptide glycan of *E. coli* binds to the enzyme (13). Thr-26 → His was found to be active and produced the β-anomer as product (14). To investigate the mechanism of both of these mutants, further substitutions were introduced and the activity was measured (Table 1).

Replacement of Glu-11, except in the case of aspartic acid, eliminates all catalytic activity, confirming the overall importance of this residue (11, 24). Three different mutants, all containing the substitution Thr-26 → Glu, formed a covalent adduct. This finding shows that the presence of Glu at position 26 is critical, but, at the same time, the glutamate at site 11 also needs to be retained. The presence of a histidine at site 26 was essential to obtain the β-anomer but, again, a glutamate at site 11 also was required. In particular, the double mutant E11H/T26H did not show activity in the halo assay.

Product Analysis of the Mutant Lysozymes. When mutant T26H of T4L is incubated with substrate for 1 hr at 0°C at least five different products are obtained (Fig. 2). The mass of each product, as determined by MS, is given in Table 2. Peak number 4 in Fig. 2 corresponds to the starting material. Peak 1 corresponds to the reduced form of NAG-NAM-DAla-DGlu-DAPA (Fig. 1), while peaks 2 and 3, also labeled β and α, correspond, respectively, to the β- and α-anomers of the nonreduced form of the same molecule. These products have been seen before and were characterized by Kuroki *et al.* (14). The peaks numbered 5 and 6 are new. Their molecular weights exceed that of the starting material and, as shown in Table 2, they correspond, respectively, to a trimer and a tetramer of NAG-NAM-DAla-DGlu-DAPA. A peak suggesting the pentamer also can be seen in Fig. 2. The simplest interpretation of these results is that mutant T26H catalyzes transglycosylation. Furthermore, the products shown in Fig. 2 that result from transglycosylation exceed those coming from hydrolysis by about 10:1, suggesting that transglycosylation can be substantially more efficient than hydrolysis.

For comparison, the anomeric form of the products produced by both GEWL and HEWL also was determined.

Table 1. Enzymatic activity and nature of product of mutant T4L

Mutant	Active site residues* 11	20	26	Activity, halo assay	Anomeric form of product
WT*	Glu	Asp	Thr	+++	α
T26D			Asp	+	n.d.
T26E			Glu	−	Covalent adduct†
T26Q			Gln	−	n.p.
T26H			His	++	β
D20C		Cys	Thr	+++	α
D20C/T26D		Cys	Asp	+	n.d.
D20C/T26E		Cys	Glu	−	Covalent adduct†
D20C/T26H		Cys	His	++	β
D20S		Ser	Thr	+	n.d.
D20T		Thr		+	n.d.
D20A		Ala		++	α
D20E		Glu		++	α
D20N		Asn		−	n.p.
D20N/T26D		Asn	Asp	−	n.p.
D20N/T26E		Asn	Glu	−	n.p.
E11D	Asp	Asp	Thr	++	α
E11D/T26E	Asp		Glu	−	Covalent adduct†
E11Q	Gln	Asp	Thr	−	n.p.
E11Q/T26E	Gln		Glu	−	n.p.
E11N	Asn	Asp	Thr	−	n.p.
E11N/T26E	Asn		Glu	−	n.p.
E11H	His	Asp	Thr	−	n.p.
E11H/T26E	His		Glu	−	n.p.
E11H/T26H	His		His	−	n.p.
E11H/D20C	His	Cys	Thr	−	n.p.

n.d.: Could not be detmined; n.p.: no product.

*Unnamed residues are as in WT.

†Mutants T26E, D20C/T26E, and E11D/T26E give double bands when purified by SDS/PAGE. In the case of T26E this was shown by MS to be caused by a mixture of the mutant protein plus the covalent adduct. The presence of the adduct is inferred but was not verified directly for D20C/T26E or E11D/T26E.

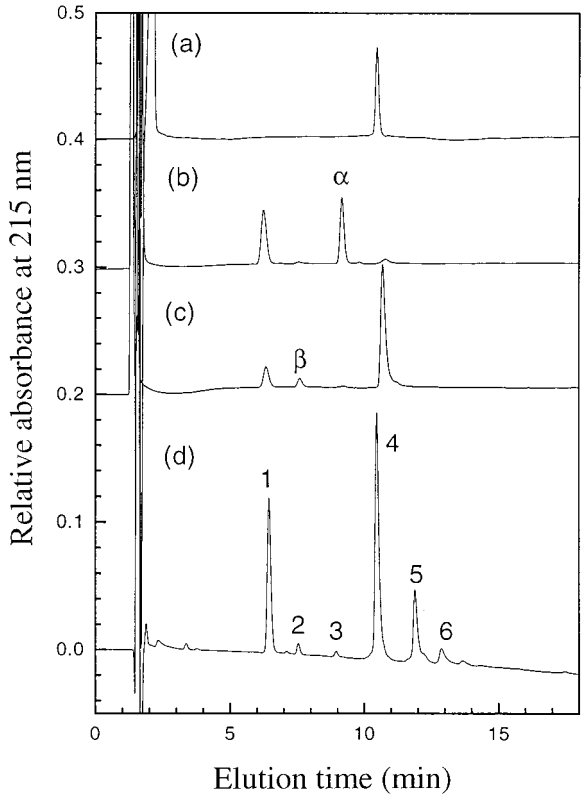

FIG. 2. Comparison of the products formed by the digestion of the substrate shown in Fig. 1 with WT lysozyme and mutant T26H. (*a*) Substrate alone. (*b*) Digestion of 2×10^{-6} M substrate by 8×10^{-8} M WT* lysozyme for 5 min at 25°C (14). (*c*) Digestion of 2×10^{-6} M substrate by 5×10^{-7} M T26H for 5 min at 25°C (14). (*d*) Digestion of 8×10^{-5} M substrate by 5×10^{-7} M T26H for 60 min at 0°C. See text for additional details and explanation of peaks.

HEWL was found to produce the β-anomer as well as the transglycosylated product, consistent with prior studies (25, 26). In contrast, GEWL was found to produce only α-anomer (data not shown).

Structure Determination of Mutant Lysozymes. To better understand the roles played by the different residues located at the active site of T4L, the three-dimensional structures of the mutant T4Ls (E11H, E11N, D20C, D20E, T26D, T26E, T26H, E11H/T26H, D20C/T26D, and D20C/T26H) were determined by x-ray crystallography (Table 3). In general, the structures of the mutants were similar to WT, confirming that these mutations do not affect the overall structure of T4L.

Fig. 3 shows a superposition of the active site residues as seen in 12 different mutant structures, including solvent. The structures were aligned so as to optimize the agreement between the α-carbon atoms of residues 11, 20, 26, and 105 (i.e., Glu-11, Asp-20, Thr-26, and Gln-105 of WT*). These four residues were chosen because they are closest (within 5.5 Å) to the C1 carbon of NAM as seen in the peptidoglycan fragment bound to T26E (13). As can be seen, the side chains at sites 11, 20, 26, and 105 maintain similar or analogous positions in each of the mutant structures. There are also two places where water molecules occupy very similar sites in a number of different structures. The first is a site where water is hydrogen-bonded to O^{γ} of Thr-26 and to $O^{\delta 1}$ of Asp-20. The second is where water is hydrogen-bonded to $O^{\varepsilon 1}$ of Glu-11. The first of these solvent-binding sites is close (0.8 Å) to the position occupied by $N^{\varepsilon 2}$ of His-26 in the T26H mutant. It is also close (0.9 Å) to the site occupied by $O^{\varepsilon 1}$ of Glu-26 in the apo-T26E mutant.

DISCUSSION

It previously was shown that the substitution Thr-26 → His in the active site of T4L causes the product to change from the α- to the β-anomer (14). It was argued that, in the mutant, catalysis proceeded via a double-displacement mechanism in which the substituted side chain, His-26, formed a covalent adduct with the substrate.

At the low concentration of substrate (*ca.* 10 μM) used by Kuroki *et al.* (14) the two primary products were the two muramyl peptides, NAG-NAM-DAla-DGlu-DAPA, one in the reduced form and the other the β-anomer (Fig. 1). The recovery of the two was, however, unequal, suggesting that some of the β-anomer might remain covalently bound to the enzyme. To investigate this result further the concentration of substrate was increased, leading, at 0.08 mM, to the result shown in Fig. 2.

The presence of the higher-order saccharides including the trimer, tetramer, and probably pentamer strongly suggests that mutant T26H of T4L has glycosidase activity. Furthermore, the apparent ratio of transglycosylation to hydrolysis, after a 60-min reaction, was about 10:1, showing that the transglycosylation can be quite effective. What is the origin of this new enzymatic activity?

It can be stated, at the outset, that transglycosylation does not come from a radical reorganization of the active site. The crystal structure of mutant T26H has been determined (14) and is very similar to WT* (21, 27), with rms discrepancy between C^{α} atoms of 0.2 Å. There is no apparent difference in the respective substrate binding sites (including both the saccharide and peptide binding regions) except for the substitution of the histidine on the α-side of the saccharide. The ε-nitrogen of His-26 is close to the site occupied by a water molecule in the native enzyme (Fig. 3) that is thought to be important for catalysis (13). It now also has been possible to determine the structure of apo-T26E (Table 3) (i.e., as distinct from the covalent adduct that it forms). In this structure oxygen $O^{\varepsilon 1}$ also occupies a site close to that occupied by the same water molecule. The overall relationship between the active site of the WT enzyme, T26H, and T26E is shown diagrammatically in Fig. 4. It appears that Glu-11, on the β-side of the bound substrate, is poised to act as a proton donor to oxygen O-1 of the NAM, whereas the group on the opposite (α) side is poised to attack the C-1 carbon. This group can, alternatively, be (*i*) a water molecule as in WT, leading to an

Table 2. Peak assignment by electrospray MS

Peak	Formula	Observed mass, daltons	Theoretical mass, daltons
1	NAG-NAM(reduced)-DAla-DGlu-DAPA ("GMP3r")	870.5	870
2	β-GMP3	868.5	868
3	α-GMP3	868.5	868
4	GMP3-GMP3r	1,721.6	1,720
6	GMP3-GMP3-GMP3r	2,571.2	2,570
7	GMP3-GMP3-GMP3-GMP3r	3,422.4	3,420

Peak numbering is as indicated in Fig. 2.

Table 3. Crystallographic data for mutant lysozymes

Mutant	E11H	E11N	D20E	D20C	T26D	T26E (Apo)	T26H	T26Q
Cell dimensions, Å								
a, *b*	61.1	61.1	61.0	61.0	60.9	61.2	61.5	61.1
c	96.8	96.8	97.0	97.1	96.9	96.3	97.7	96.3
Total reflections	40,327	33,301	53,978	28,398	39,326	30,082	105,345	33,225
Independent reflections	17,643	17,670	19,961	15,116	19,920	9,358	22,032	15,148
R_{merge}, %	6.3	6.8	7.6	4.4	3.0	7.0	6.4	4.3
Resolution used	20–1.9	20–1.8	20–1.8	20–2.0	20–1.85	19–2.3	20–1.9	20–2.1
Reflections included	16,175	18,223	20,727	13,237	14,752	9,350	17,129	11,761
Completeness, %	92	88	95	87	78	96	95	89
$\Delta_{\text{bond length}}$, Å	0.013	0.013	0.018	0.019	0.014	0.016	0.017	0.015
$\Delta_{\text{bond angle}}$, °	2.6	2.4	3.0	2.9	2.6	2.6	3.0	2.8
Δ_{planes}, Å	0.010	0.011	0.014	0.015	0.012	0.017	0.014	0.012
R, %	16.5	16.5	16.7	16.5	15.5	18.4	16.9	16.6

WT* lysozyme crystallizes in space group $P3_221$ with cell dimensions $a = b = 61.2$ Å, $c = 96.8$ Å (21). The crystals of all mutants are isomorphous. R_{merge} gives the agreement between independently measured intensities while *R* is the crystallographic residual after refinement. $\Delta_{\text{bond lengths}}$, $\Delta_{\text{bond angle}}$, and Δ_{planes} give, respectively, the average deviations of bond lengths, bond angles, and planar groups from their expected values. X-ray date also were measured for the double mutants E11H/T26H, D20C/T26D, and D20C/D26H. Preliminary analysis did not suggest anything unusual about these structures. They were not refined and are not included here.

inversion of the anomeric form, (*ii*) Glu-26 as in T26E, leading to a relatively stable covalent intermediate (13), or (*iii*) a histidine as in T26H, which in turn, can yield either hydrolysis products or to transglycosylation.

The experiments reported here provide supporting evidence for the proposed mechanism of T26H as outlined in Fig. 4*B*. As with the proposed mechanism of action of the WT enzyme (Fig. 4*A*), Glu-11 is presumed to play a critical role as a proton donor in the first step of catalysis. Consistent with this hypothesis, the replacement of Glu-11 with histidine completely abolishes activity of the T26H mutant (Table 1). Conversely, Asp-20 is assumed to play a less important role (Fig. 4*B*), and the replacement of Asp-20 with cysteine does not substantially interfere with the activity of T26H. The most compelling evidence, however, comes from the change in the product from the α- to the β-anomer (Fig. 2). This is the key difference between the attack of a water molecule on the C-1 carbon (Fig. 4*A*). and the attack of His-26 (Fig. 4*B*). The former converts from the β-anomeric substrate to the α-anomeric product in a single-displacement reaction. The latter, in a double-displacement reaction, retains the β-anomer in the final product. Presumably, the formation of this adduct retains the NAG-NAM unit within the active site (Fig. 4*B*, *Middle*), but in such a way that water cannot readily attack the C-1 carbon. This situation perhaps might be for structural reasons (e.g., none of the preferred solvent-binding sites seen in Fig. 3 are close to the requisite position). It also might be for electronic reasons (e.g., the formation of the covalent adduct with His-26 might move the C-1 carbon away from Glu-11, weakening its effect on the attacking water molecule). In any event, a preference for transglycosylation over hydrolysis can reasonably be envisaged in the context of His-26 forming a covalent adduct, but would be very hard to explain were this not the case.

The results obtained above help provide an overall rationalization for glycosidase hydrolysis. Withers and coworkers (3, 28) have suggested that the active sites of the glycosidases contain a pair of carboxylic acids. In the retaining enzymes the two acids are about 4.3–5.5 Å apart, one acting as an acid/base catalyst, the other as a nucleophile/leaving group. The inverting glycosidases also contain two carboxylic acids, but are 8–9.5 Å apart, one acting as an acid catalyst and the other as a base catalyst. The increase in separation between the carboxylates in the latter case is thought to allow the intervention of a water molecule.

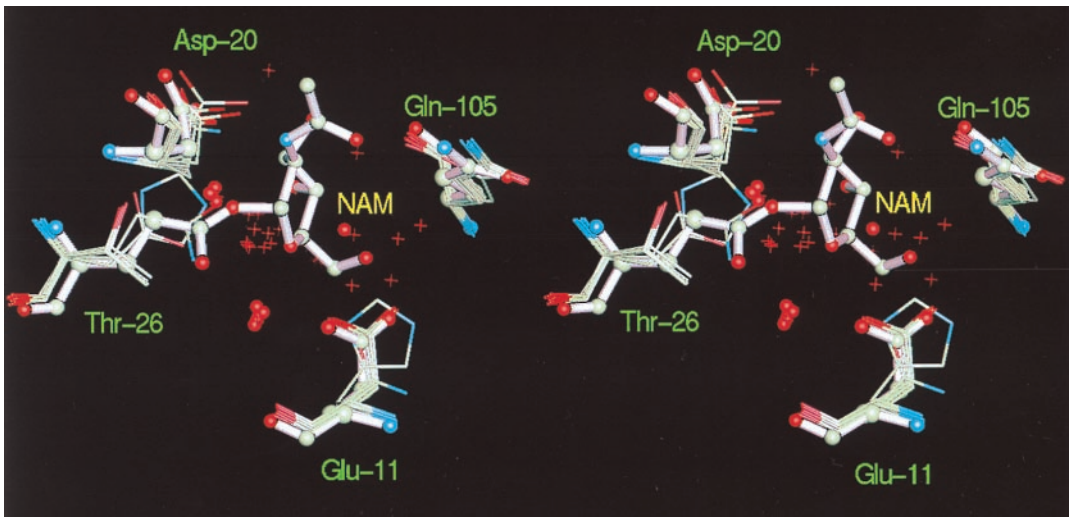

FIG. 3. Superposition of 12 structures of mutant lysozymes (see text). The figure includes WT*, E11H, E11N, D20A, D20S, D20N, D20C, D20E, T26D, T26H, T26Q, and T26E with the covalent adduct formed by this mutant (13). Water molecules clustered together at the two sites discussed in the text are shown as red spheres. The water molecule present in the covalent adduct also is shown as a red sphere. Other solvent molecules in the vicinity of the active site are shown as red crosses.

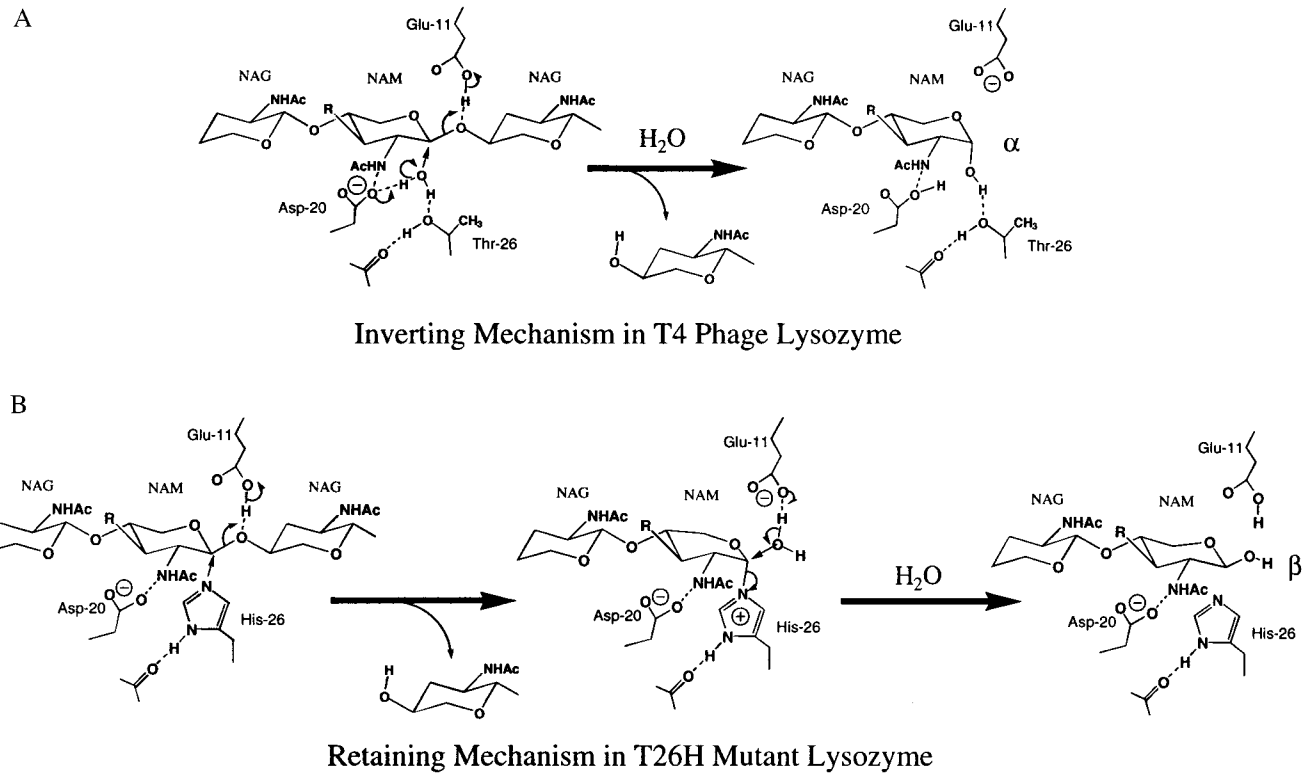

FIG. 4. Schematic diagram showing the overall relationship between the active-site structures and the presumed mechanisms of WT* T4L and mutants T26E and T26H. (*A*) In WT* lysozyme the water molecule hydrogen-bonded to Asp-20 and Thr-26 is presumed to act as the nucleophile, attacking the C-1 carbon of NAM. (*B*) In mutant T26H the nitrogen of His-26 occupies a position close to the water molecule in WT* and is presumed to act as the nucleophile, leading to a covalent adduct. This adduct can either break down by the addition of water, as shown, or can be subject to attack by another disaccharide, leading to transglycosylation. In T26E (not shown) an oxygen of Glu-26 occupies a position close to the water molecule and attacks the C-1 carbon to yield a stable enzyme-substrate adduct (13).

The properties of representative T4L mutants with altered catalytic properties are summarized in Table 4. WT T4L is an inverting enzyme and Glu-11 ($O^{\varepsilon 2}$) and Asp-20 ($O^{\delta 1}$) are 7.2 Å apart [somewhat shorter than the range given by Wang *et al.* (28)]. When Thr-26 is replaced by a histidine the separation between the introduced imidazole ($N^{\varepsilon 2}$) and Glu-11 ($O^{\varepsilon 2}$) is 5.9 Å. For the apo-T26E structure the distance between the closest carboxylate oxygens of Glu-11 and Glu-26 is 5.3 Å (in the covalent adduct the distance decreases to 4.4 Å). All of these distances are consistent with the principle of Wang *et al.* (28) that a decrease in the separation between the key catalytic residues correlates with the enzyme changing from an inverting to a retaining glycosidase. In the case of T26H there is a difference in that the role of the nucleophile/leaving group is played by a histidine rather than by a carboxylate. A carboxylate at this position is, however, provided in the mutant Thr-26 → Glu. In this case a stable adduct is formed (13), showing directly that Glu-26 acts as a nucleophile, consistent with the short distance.

We also have included in Table 4 the relevant data for GEWL. This protein, although thought to be evolutionarily related to HEWL (5, 6), has been enigmatic in not having any obvious counterpart to Asp-52. This nonconservation led to the proposal that the "second carboxylate" was not essential for lysozyme-catalyzed hydrolysis (24). A prediction of this hypothesis is that, in contrast to HEWL, which is a retaining glycosidase (25), GEWL must be an inverting enzyme. By using the same cell-wall fragment as was used for T4L (Fig. 1) (14), it has been shown that GEWL does, in fact, invert the chirality of the product (Table 4). This behavior can be considered as a formal extension of the scheme of McCarter and Withers (3) in that the second carboxylate is, in effect, removed to infinity. In this sense the present results suggest that a retaining glycosidase is characterized by a pair of

Table 4. Active site elements of inverting and retaining glycosidases

	T4L				
Lysozyme	WT	T26E	T26H	HEWL	GEWL*
(A) Acid/base catalyst	Glu-11 ($O^{\varepsilon 2}$)	Glu-11 ($O^{\varepsilon 2}$)	Glu-11 ($O^{\varepsilon 2}$)	Glu-35 ($O^{\varepsilon 1}$)	Glu-73 ($O^{\varepsilon 1}$)
(B) General acid	Asp-20 ($O^{\delta 1}$)	—	—	—	Asp-97 ($O^{\delta 2}$)
(C) Nucleophile/leaving group	(H_2O)	Glu-26 ($O^{\varepsilon 1}$)	His-26 ($N^{\varepsilon 2}$)	Asp-52 ($O^{\delta 2}$)	(H_2O)
Separation A–B, Å	7.2				9.0
Separation A–C, Å	5.7	5.3	5.9	6.2	n.d.
Configuration of products	α	Covalent adduct	β	β	α
Transglycosylase	No	n.d.	Yes	Yes	n.d.

n.d., not determined.

*In GEWL Asp-97 is thought not to participate in catalysis (24). Its distance from Glu-73 is given for reference. The location of the solvent that acts as the presumed nucleophile has not been determined.

catalytic residues comprising either two carboxylates or a carboxylate plus an imidazole, with a separation in the range of 4.3 to 5.9 Å. In contrast, an inverting glycosidase often is characterized by a pair of carboxylates 7.2–9.5 Å apart, but in some cases a single carboxylate will suffice.

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