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Chemical Conversion of Aspartic Acid 52, a Catalytic Residue in Hen Egg-White Lysozyme, to Homoserine

(enzyme mechanism/specific chemical modification of proteins/affinity labeling/active site/ester bond reduction)

YUVAL ESHDAT, ARNOLD DUNN*, AND NATHAN SHARON

Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT **Hen egg-white lysozyme (EC 3.2.1.17) was specifically esterified at aspartic acid 52 by the affinity labeling reagent 2′,3′-epoxypropyl β-glycoside of di-(*N*-acetyl-D-glucosamine) [Eshdat *et al.* (1973) *J. Biol. Chem.* 248, 5892]. The disulfide bonds of the affinity-labeled enzyme and the aspartic acid 52-ester bond were reduced with dithiothreitol and sodium borohydride, respectively, resulting in the removal of the affinity label. The reduced protein contained 0.9 mole of homoserine and 1 mole less of aspartic acid per mole of protein, as compared to the native enzyme. It was reoxidized by a mixture of reduced and oxidized glutathione to yield a modified protein that possessed one-tenth of the activity of native lysozyme (presumably due to a contamination by regenerated lysozyme formed as a result of hydrolysis of the aspartic acid 52-ester bond during the chemical treatment). The native enzyme, after reduction and reoxidation in the same manner, retained its amino-acid composition, full enzymatic activity, and fluorescence properties. The modified lysozyme, containing homoserine 52, showed the same fluorescence spectrum as the native enzyme. With both proteins, the fluorescence maximum shifted to the blue to a similar extent upon the addition of the saccharide inhibitors tri-(*N*-acetyl-D-glucosamine) and the cell-wall tetrasaccharide (GlcNAc–MurNAc)$_2$. The modified enzyme bound these two saccharides with nearly the same binding constants as those found for native lysozyme and for lysozyme that was reduced and reoxidized. Since the side chain of homoserine is similar in size to that of aspartic acid, it is concluded that the loss of enzymatic activity is the direct result of the chemical modification of the carboxyl side chain of aspartic acid 52, thus showing that this amino acid is essential for the catalytic action of the enzyme.**

The introduction of a specific chemical modification into the binding site of a biologically active protein may result in changes in its activity, the reasons for which are usually difficult to ascertain. Often, such a modification changes not only the chemical properties of the binding site, but also introduces an additional bulky group into this area. The modified protein may, however, be subjected to a second chemical reaction, resulting in a new product in which the modified residue is similar in size to the original one, but differs from the latter in its chemical properties. By this approach, serine 221, located in the active center of subtilisin, was converted to a cysteine residue after the modification of the enzyme by phenylmethanesulfonyl fluoride (1, 2) according to the "quasi-substrate" method (3). The thiosubtilisin obtained proved to be an excellent derivative for the examination of the role of serine 221 in the catalytic activity of subtilisin.

We have now combined this approach with the method of affinity labeling to convert specifically the side chain of aspartic acid 52 in the active site of hen egg-white lysozyme (EC 3.2.1.17) to a homoserine residue, the size of which is somewhat smaller than that of aspartic acid. The modified protein, though catalytically inactive, still binds specifically saccharide inhibitors of lysozyme, corroborating the suggested role of aspartic acid 52 as being essential for the activity of the enzyme. This approach has distinct advantages in that affinity labeling has been successfully used to modify specifically different residues in the binding sites of a wide variety of proteins, such as antibodies, enzymes, and receptors (4–7).

MATERIALS AND METHODS

Materials. Hen egg-white lysozyme (lysozyme), twice recrystallized, salt-free, was purchased from Worthington Biochemical Corp. "Tris" was obtained as the free base (Trizma quality) from Sigma Chemical Co., as were dithiothreitol, reduced glutathione, and oxidized glutathione. Urea (ultra pure) and DL-homoserine were obtained from Mann Res. Lab. Sodium [^{3}H]borohydride (870 mCi/mmole) was a product of the Radiochemical Centre, Amersham. Other chemicals, purchased from commercial sources, were of the highest purity available and were used without further purification.

Hen egg-white lysozyme, affinity labeled with 2′,3′-epoxypropyl β-glycoside of [^{14}C]di-(*N*-acetyl-D-glucosamine), was prepared as described (8). It contained 1.0 mole of the reagent per mole of protein and its specific radioactivity was 1.4×10^7 cpm/mmole.

The cell-wall tetrasaccharide [*N*-acetyl-D-glucosamine β(1 → 4) *N*-acetylmuramic acid]$_2$ [(GlcNAc–MurNAc)$_2$] (9) and tri-(*N*-acetyl-D-glucosamine) [(GlcNAc)$_3$] (10) were prepared as described.

Amino Acid, Glucosamine, and Homoserine Analyses. Analysis of amino acids and of glucosamine was performed as described (8). For the determination of [^{3}H]homoserine, a Packard Tri-carb flow analyzer scintillation spectrometer 3022 was connected directly to the outlet of the long column (0.9 × 50 cm) of a Beckman 120B amino-acid analyzer. Before

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid; lysozyme, hen egg-white lysozyme; [Hse^{52}]-lysozyme, lysozyme in which aspartic acid 52 was replaced by homoserine.

* Permanent address: Department of Biological Sciences, University of Southern California, Los Angeles, Calif. 90007, U.S.A.

analysis, the dried acid hydrolysate was incubated in pyridine–acetic acid buffer (pH 6.5) at 110° for 75 min, to convert the homoserine lactone formed during the acid hydrolysis to the open form of the hydroxy acid. After evaporation of the buffer, the residue was dissolved in 1 ml of 0.2 M sodium-citrate buffer, pH 2.2, and applied to the long column of the analyzer. Elution was performed at 45°. When the peak of threonine had appeared on the recorder, the temperature was raised to 55°. This procedure resulted in good separation of homoserine from threonine and from glutamic acid. The integration constant for homoserine (71% of the value for aspartic acid) was determined by subjecting homoserine in mixture with an equimolar amount of lysozyme to the standard conditions of hydrolysis (6 M HCl, 110°, 22 hr) followed by incubation at pH 6.5 and analysis on the amino-acid analyzer as described above.

Reduction of the Affinity-Labeled Lysozyme. ^{14}C-Affinity-labeled lysozyme (5 mg) was dissolved in 0.08 M Tris buffer at pH 8.5 (0.5 ml), to which 30 mg of urea and 0.5 mg of EDTA (disodium salt) were added. Reduction with 1.6 mg of dithiothreitol was for 2 hr at 23°, after which 4 mg of sodium[^{3}H]-borohydride was added, followed by the addition of 20 μl of *n*-octanol to avoid foaming. After 1 hr at 37°, the incubation mixture was acidified to pH 4 by dropwise addition of 0.1 M HCl, and was applied to a column (1.75 × 90 cm) of Sephadex G-25 (fine) pre-equilibrated with 0.1 M acetic acid. Elution was with the same solvent and 5-ml fractions were collected. The absorption of the fractions at 280 nm and their radioactivity were determined as described (8). In experiments with two radioactive labels, ^{14}C and ^{3}H were simultaneously determined with a three-channel liquid scintillation spectrometer; results obtained were corrected for background and for crossover between channels, but not for percent efficiency. The experiments were designed so that the corrections for crossover were always small (less than 1% for ^{3}H to ^{14}C and less than 12% for ^{14}C to ^{3}H). Fractions containing the radioactive protein were collected and lyophilized, to yield reduced tritium-labeled [Hse^{52}]lysozyme. All lyophilized products were stored under reduced pressure at −14°.

Reoxidation of Reduced Lysozyme. Three milligrams of reduced tritium-labeled [Hse^{52}]lysozyme was dissolved in 15 ml of 0.1 M acetic acid, which was then added to 135 ml of 0.05 M Tris buffer, pH 8.5, containing 5 mM reduced glutathione and 0.5 mM oxidized glutathione (11). After 2 hr at 37° the solution was dialyzed exhaustively against 0.08 M Tris buffer, at pH 8.5, and then against water. The dialyzed material was lyophilized, dissolved in 1 ml of 0.1 M ammonium acetate, and applied to a column (1.75 × 94 cm) of Sephadex G-25 (fine), pre-equilibrated with 0.1 M ammonium acetate. Elution was performed with the same buffer, and 10-ml fractions were collected. Absorbance and radioactivity were measured as described before. Fractions containing the radioactive protein, [Hse^{52}]lysozyme, were pooled and either lyophilized or kept frozen.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in β-alanine–acetic acid at pH 4.5 (12). The gel was divided into 1-mm sections, and each section was incubated with shaking at 37° for 16 hr in 0.2 ml of NCS tissue solubilizer (Amersham/Searle). Toluene scintillation fluid was added, and the radioactivity of the protein was measured (8).

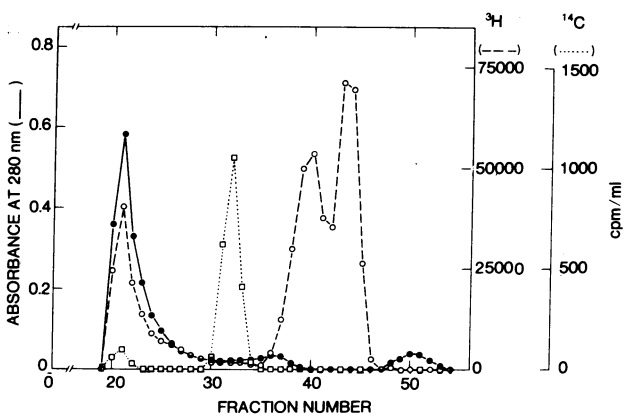

FIG. 1. Purification of reduced tritium-labeled [Hse^{52}]lysozyme (first peak on *left*) by gel filtration on a column of Sephadex G-25 (fine). For experimental details, see *text*.

Fluorescence Measurements were performed in 0.1 M ammonium acetate (pH 6.7) and at 23° as described by Chipman *et al.* (13) with an illuminating wavelength of 280 nm. For estimation of the binding constants of $(GlcNAc)_3$ and of $(GlcNAc–MurNAc)_2$, emission was measured at 340 nm and 370 nm, respectively. Association constants were calculated as before (13).

RESULTS

Purification of the Reduced Affinity-Labeled Protein. After reduction of the ^{14}C-affinity-labeled lysozyme by dithiothreitol and by sodium[^{3}H]borohydride, the product was purified by gel filtration on a Sephadex column (Fig. 1). Only one radioactive peak with an absorption at 280 nm was obtained (fractions 20–26). The specific radioactivity throughout this peak (cpm of ^{3}H per absorbance unit) was constant. A second ^{3}H radioactive peak, eluted much later than the protein, represents most probably decomposition products of the excess of NaB^3H_4. Two radioactive peaks, which contained ^{14}C-labeled materials, were also detected. The first of these, containing 5% of the total counts, coincided with the protein peak. This radioactivity represented apparently a small amount of the affinity-labeled enzyme from which the label was not removed. The second ^{14}C radioactive peak, which was well-separated from the protein peak, contained 95% of the total ^{14}C counts. After acid hydrolysis and analysis on the amino-acid analyzer, only glucosamine was found in this peak. This glucosamine is derived from the affinity label which was cleaved from the protein during the reduction by NaB^3H_4.

Fractions 20–26 containing the protein were combined and lyophilized. The product obtained, reduced [Hse^{52}]lysozyme, was enzymatically inactive, and its amino-acid analysis revealed, in addition to the expected composition, the presence of 0.9 mole of [^{3}H]homoserine and 20.0 moles of aspartic acid (19.8 and 20.2 moles were found in the analysis of two different preparations) instead of 21 moles in the native enzyme (14). Only a trace amount (less than 0.1 mole/mole of protein) of glucosamine was detected.

Reduced lysozyme was obtained from native lysozyme by the same procedure. Its amino-acid composition was identical with that of the native enzyme.

Isolation of the Reoxidation Products. Reoxidation of the eight cysteine residues present in reduced [Hse^{52}]lysozyme was

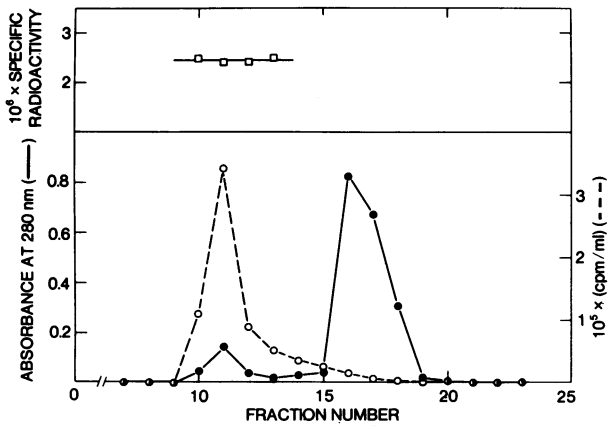

FIG. 2. Separation of tritium-labeled [Hse^{52}]lysozyme (O- - -O) from glutathione (●——●) by gel filtration on a column of Sephadex G-25 (fine). For experimental details, see *text*.

carried out by the disulfide exchange reaction (11). The soluble material obtained after dialysis and lyophilization was further purified by gel filtration (Fig. 2). A single radioactive peak was obtained with absorbance at 280 nm and a constant specific radioactivity which was lower (by 15%) than that of the reduced tritium-labeled [Hse^{52}]lysozyme. A second UV-absorbing peak, which was not radioactive, was eluted much later. Amino-acid analysis of the fractions in this peak revealed the presence of glutathione only, derived from the reoxidation reagent.

The same procedure was used for the preparation of regenerated lysozyme from the reduced native lysozyme. Upon acrylamide gel electrophoresis at pH 4.5, both proteins, as well as native lysozyme, migrated as single peaks and at identical rates. All the radioactivity associated with tritium-labeled [Hse^{52}]lysozyme was located under the protein peak.

Enzymatic Activity. The specific lytic activity of regenerated lysozyme, tested on *Micrococcus luteus* cells (pH 6.7, 26°) (8), was identical with that of the native enzyme, whereas [Hse^{52}]lysozyme was only 10% as active (Table 1). This residual activity was abolished by treatment of tritium-labeled [Hse^{52}]lysozyme (1.25 μM) with the 2′,3′-epoxypropyl β-glycoside of the $(GlcNAc)_2$ (6 mM, pH 5.5, 37°). The inactivation rate was of first order (8), with $\tau_{1/2}$ = 4.2 hr. Inactivation experiments with lysozyme and with regenerated lysozyme, carried out under identical conditions, gave $\tau_{1/2}$ = 2.8 hr and 3.2 hr, respectively.

TABLE 1. *Enzymatic activities and association constants of lysozyme and its derivatives**

Protein	Relative enzymatic activity†	Association constants (M^{-1}), pH 6.7, 23°†	
		$(GlcNAc)_3$	$(GlcNAc–MurNAc)_2$
Native lysozyme	1.00	1.1×10^5‡	4.2×10^3
Regenerated lysozyme	0.97	3.3×10^4	1.4×10^3
[Hse^{52}]lysozyme	0.10	1.2×10^4	6.3×10^3

* For experimental details, see *text*.

† The experimental error of the values given in the table is ±10%.

‡ From Chipman *et al.* (13), at pH 5.4 and 25°.

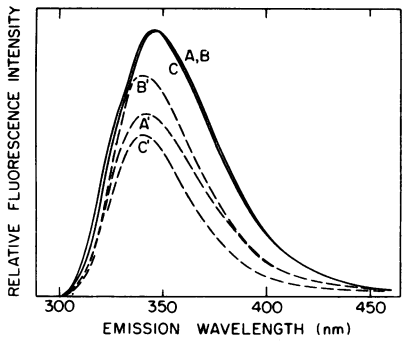

FIG. 3. Fluorescence spectra of lysozyme and of its derivatives determined at pH 6.7 and 23° with exciting wavelength 285 nm, in the absence and presence of an inhibitor. (*A*) Lysozyme; (*B*) regenerated lysozyme; (*C*) [Hse^{52}]lysozyme. (*A′*, *B′*, and *C′*) as *A*, *B*, and *C* with 1 mM $(GlcNAc–MurNAc)_2$. Protein concentration is 1.2 μM.

Fluorescence Measurements. The fluorescence emission spectra of [Hse^{52}]lysozyme and of regenerated lysozyme in the absence and presence of a large excess (saturation conditions, see ref. 13) of $(GlcNAc–MurNAc)_2$ or $(GlcNAc)_3$ are given in Figs. 3 and 4. In all cases a blue shift in the emission spectra and a change in the emission maxima intensity are observed in the presence of the above saccharides. No changes were observed upon the addition of 5 mM cellotetraose to the protein solutions.

The fluorescence intensity of regenerated [Hse^{52}]lysozyme at a given wavelength varied with the concentration of the saccharides in a manner consistent with reversible formation of an enzyme–inhibitor complex. Fig. 5 shows a typical plot for the binding of the cell-wall tetrasaccharide $(GlcNAc–MurNAc)_2$ to the modified protein. For comparison, the data for the binding of the same saccharide to lysozyme and to regenerated lysozyme are also given. In all three cases the slope is very close to unity, indicating formation of a 1:1 complex. The association constants for the three complexes, calculated by the method of Chipman *et al.* (13), are given in Table 1.

DISCUSSION

A new approach was used in the present study to establish the importance of aspartic acid 52 for the enzymatic activity of hen egg-white lysozyme. For this purpose the enzyme, fully inactivated by the affinity label 2′,3′-epoxypropyl β-glycoside of $(GlcNAc)_2$ was used (8). Attempts to remove the affinity label from the intact enzyme, using reagents such as hydroxylamine or methoxyamine, were unsuccessful unless the disulfide bonds of the protein were reduced and the resulting polypeptide chain was unfolded. In the present study the affinity-labeled enzyme was reduced with dithiothreitol in urea, and then treated with $NaBH_4$ to reduce the aspartic acid 52-ester bond between the affinity label and the protein. In this procedure, the exposure time of the protein to $NaBH_4$ was shortened to 1 hr. The resulting product, which was inactive enzymatically, contained 0.9 mole of homoserine per mole of protein and one mole of aspartic acid less than the native

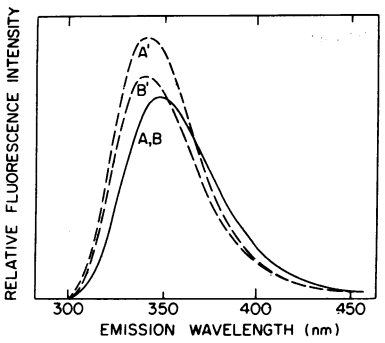

FIG. 4. Fluorescence spectra of lysozyme and of its derivatives at pH 6.7 and 23° with exciting wavelength 285 nm, in the absence and presence of an inhibitor. (*A*) Regenerated lysozyme; (*B*) [Hse[52]]lysozyme. (*A'* and *B'*) as *A* and *B* with 0.8 mM $(GlcNAc)_3$. Protein concentration is 1 μM.

enzyme. This value is in agreement with the finding that more than 95% of the affinity label was released from the inhibited enzyme by reduction with $NaBH_4$. Since the affinity label was originally attached to aspartic acid 52 in the inactivated enzyme (8), we may safely deduce that the homoserine residue has replaced aspartic acid 52 in the primary structure of hen egg-white lysozyme.

Upon reoxidation of the cysteine residues of the doubly reduced product, a modified lysozyme, tritium-labeled [Hse[52]]lysozyme, was obtained with only a small loss (about 15%) in its specific radioactivity. This loss is probably due to an incomplete reduction of the disulfide bonds in the affinity-labeled enzyme by dithiothreitol, which may have subsequently been reduced by sodium[^{3}H]borohydride; most likely this extra radioactivity was removed from the protein upon reoxidation of the thiol groups. The modified lysozyme migrated on acrylamide gel electrophoresis at pH 4.5 at the same rate as the native enzyme. It exhibited about 10% of the specific enzymatic activity of lysozyme or of lysozyme that was reduced and reoxidized under exactly the same conditions. This residual activity may be ascribed to either the catalytic power of [Hse[52]]lysozyme or to the contamination of the modified lysozyme by a small amount of regenerated lysozyme; this regenerated lysozyme may have resulted from partial hydrolysis of the aspartic acid 52-ester bond in the affinity-labeled enzyme under the alkaline conditions of the reduction with $NaBH_4$. Our experiments show that the [Hse[52]]-lysozyme preparation reacted with the affinity label, 2',3'-epoxypropyl β-glycoside of $(GlcNAc)_2$, at approximately the same first-order rate as found for the inactivation of lysozyme or regenerated lysozyme, resulting, within 24 hr, in the complete loss of the residual enzymatic activity. Since it is extremely unlikely that the affinity label will react with the alcoholic side chain of homoserine 52, or with any other neighboring group at such a rate, it is suggested that the reaction occurs between the reagent and aspartic acid 52 of the regenerated lysozyme present as a contaminant in the modified lysozyme preparation. This, together with the finding that the modified enzyme contained, per mole, 0.9 mole of homoserine (instead of the expected 1.0 mole), implies that the residual activity of the [Hse[52]]lysozyme preparation arises from a contamination of regenerated lysozyme-aspartic acid 52, and is not an intrinsic property of the modified enzyme.

The fluorescence spectrum of [Hse[52]]lysozyme was identical with that of lysozyme (Fig. 3). Of particular significance is

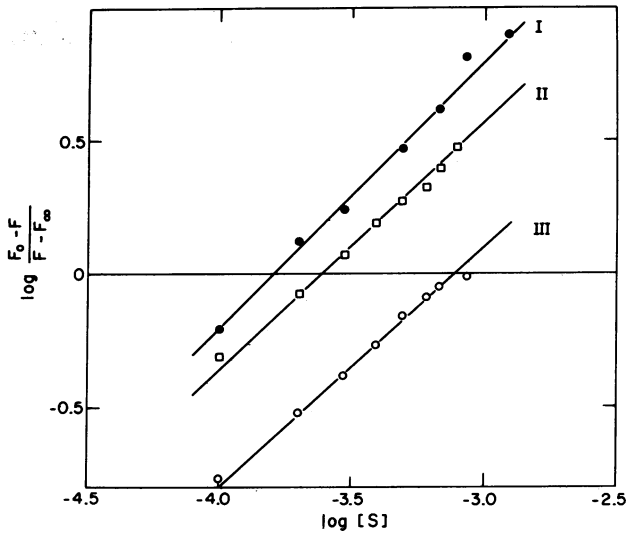

FIG. 5. Determination of the association constants of $(GlcNAc\text{–}MurNAc)_2$ to [Hse[52]]lysozyme (*I*), lysozyme (*II*), and regenerated lysozyme (*III*), at pH 6.7 and 23°, according to the method of Chipman *et al.* (13). F_0, F, and F_∞ are the relative fluorescence emission intensities at 370 nm of the protein alone, the protein in the presence of a concentration [S] of saccharide, and the protein saturated with saccharide, respectively.

the characteristic blue shift in the spectrum caused by the addition of saccharide inhibitors (but not by the noninhibitory saccharide cellotetraose), which is identical both qualitatively and quantitatively for lysozyme and [Hse[52]]lysozyme (Figs. 3 and 4). The differences in the binding constants found for the native and the modified enzyme (Table 1) are probably the result of minor changes in the architecture of subsites *A–D* (15, 16) caused by the reduction and reoxidation steps. This is supported by the finding that the association constants with saccharides of the reduced and reoxidized lysozyme also differ somewhat from those of the native one (Table 1).

Another important finding is that [Hse[52]]lysozyme still binds the tetrasaccharide $(GlcNAc\text{–}MurNAc)_2$. Since this saccharide binds to lysozyme at subsites *A–D* (13, 16), we conclude that the conversion of aspartic acid 52, which forms part of subsite *D*, to homoserine 52, did not result in any marked changes in the architecture of this subsite. Therefore, the loss of enzymatic activity must be ascribed to the conversion of aspartic acid 52 into homoserine 52.

Lin and Koshland (17) prepared an active lysozyme derivative in which all the carboxyl groups except aspartic acid 52 and glutamic acid 35 were modified. Modification of aspartic acid 52 in this derivative led to complete inactivation of the enzyme. Parsons and Raftery (18) prepared an inactive lysozyme derivative in which the β-carboxyl group of aspartic acid 52 was ethylated, and which bound $(GlcNAc)_3$. However, the above studies did not provide any evidence for the binding of a saccharide residue at subsite *D*, where the modified side chain is located. The introduction of a bulky group, as was done in these studies, might have blocked either binding or catalysis, or both. Therefore the results do not prove that aspartic acid 52 is essential for the catalytic activity of lysozyme. In [Hse[52]]lysozyme binding of a MurNAc moiety most likely takes place at subsite *D*, similarly to the mode of binding of this saccharide to lysozyme (13, 16). Moreover, homoserine is less bulky than aspartic acid, which may per-

haps account for the stronger binding of $(GlcNAc\text{-}MurNAc)_2$ to [Hse^{52}]lysozyme as compared with its binding to native lysozyme.

The approach described in this work has marked advantages over other methods for specific chemical modification of active-site residues. As shown, the method was used to prove the requirement of aspartic acid 52 for the catalytic action of lysozyme. The availability of the specifically modified lysozyme opens the way for a variety of experiments on the structural and physiochemical properties of the active site of the enzyme in which advantage is taken of its ability to bind saccharides without cleaving them.

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