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A study of D52S hen lysozyme-GlcNAc oligosaccharide complexes by NMR spectroscopy and electrospray mass spectrometry

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The production of a mutant hen lysozyme is described in which Asp-52, one of the catalytically important residues, is replaced by Ser. The mutant enzyme has very low catalytic activity but NMR studies show that its structure is closely similar to that of the wild-type protein. NMR experiments also show that well defined complexes are formed with $GlcNAc_4$ and $GlcNAc_6$ bound in the active site of the mutant enzyme. These complexes have been examined using electrospray mass spectrometry (ESMS). The most intense peaks arise from the uncomplexed protein indicating that dissociation takes place in the mass spectrometer under the conditions used here. Peaks from minor species corresponding to complexes between the protein and the oligosaccharides are, however, also observed. The possibility that the latter arise from novel covalent enzyme–saccharide complexes is discussed.

Hen lysozyme; Active site mutant; ^1H NMR; Electrospray mass spectrometry

1. INTRODUCTION

Lysozyme catalyses the hydrolysis of $\beta(1\rightarrow4)$-glycosidic bonds in certain bacterial cell wall oligosaccharides and in chitin [1] and was the first enzyme for which a crystal structure was determined [2]. Subsequent studies of the structure of the enzyme bound to the inhibitor $GlcNAc_3$ led to the first detailed proposal for the mechanism of an enzyme [3]. A key feature of this mechanism involved catalytic enhancement via steric strain. Recent evidence supporting this model has come from a crystal structure of the enzyme complexed with MurNAc-GlcNAc-MurNAc [4]. Detailed verification of the model, however, is limited by difficulties in obtaining access in the crystalline state to the lower portion of the active site cleft, and by the high turnover number of the enzyme–$GlcNAc_6$ complex in solution [1]. Theoretical calculations have suggested that the role of strain in the hen lysozyme mechanism could be negligible [5] and has not been included in a recent proposal for the lysozyme mechanism [6]; there is as yet, however, no direct experimental evidence in support of this latter model. In this paper we describe the preparation of a mutant lysozyme in which one of the catalytically important residues in the active site, Asp-52, is replaced by Ser, and preliminary studies by NMR and ESMS of complexes of the mutant with $GlcNAc_4$ and $GlcNAc_6$ are presented. Previous studies have shown that mutation or modification of Asp-52 leads to a dramatic loss in catalytic activity of the resulting enzyme [7,8]. Our results suggest that the strategy of using recombinant DNA technology to replace catalytically important functional groups to reduce or abolish enzymatic activity can be an important approach to the study of the structures of productive enzyme–substrate complexes.

Abbreviations: D52S lysozyme, the site-directed mutant of hen lysozyme in which Asp-52 is replaced by Ser; $GlcNAc_n$, $\beta(1\rightarrow4)$-linked *N*-acetyl glucosamine of order n; MurNAc, *N*-acetylmuramic acid; ESMS, electrospray mass spectrometry; DQF COSY, double quantum filtered correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; NMR, nuclear magnetic resonance spectroscopy.

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2. MATERIALS AND METHODS

$GlcNAc_6$ and $GlcNAc_4$ were prepared by partial acid hydrolysis of chitin [9] followed by fractionation on Biogel P4 [10]. D52S hen lysozyme was expressed in *Aspergillus niger* (12 mg/l) and isolated using protocols established for the production of the wild-type enzyme [11]. ^1H NMR experiments were performed at 600 MHz on a Bruker AM600 spectrometer at 35°C. Protein samples were unbuffered in 90% H_2O:10% D_2O at pH 3.8. Phase-sensitive DQF COSY [12–14] and NOESY [15,16] data sets comprised 512 t_1 increments of 64 transients and were acquired using time-proportional phase incrementation [17]. Spectra were resolution-enhanced using a 6°-shifted sine bell and after zero filling the digital resolution in both dimensions was 3.4 Hz/Pt. Samples for study by CD contained 0.2 or 1.0 mg/ml enzyme in

unbuffered solution at pH 3.8. Spectra were recorded from 260–190 nm and from 340 to 250 nm on a Jasco 720 spectropolarimeter in cells with 1 mm path length. The catalytic activity of lysozyme was measured against *Micrococcus lysodeikticus* cells at pH 6.2 and 25°C as described in [11]. ESMS spectra were measured on a VG BIO Q triple quadrupole atmospheric pressure mass spectrometer equipped with a VG electrospray interface. Samples (10 μl) were injected into the electrospray source via a loop injector (Rheodyne 5717) as a solution, typically 25–50 pmol/μl, in water/methanol (1:1), at a flow rate of 2 μl per min (Applied Biosystems model 140A dual syringe pump). The mass spectrometer was scanned over the mass range 900–1900 Da. The instrument was calibrated with wild-type hen lysozyme.

3. RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of D52S lysozyme (Fig. 1a) is closely similar to that of the wild-type enzyme which has been described in detail previously [18]. Analysis of DQF COSY and NOESY spectra show that for the majority of the resonances changes in chemical shift arising from the mutation are very small; for only 32 of the more than 500 resonances assigned in the spectra are the differences in chemical shift greater than 0.05 ppm (for only 11 of these does the difference exceed 0.1 ppm). The largest changes (up to 0.34 ppm) are limited to the β-sheet region of the protein, which contains the site of the mutation, and to the active site residues Asn-59, Trp-62 and Trp-63. No significant changes in NOE intensities were observed as a consequence of the mutation, indicating that any change in conformation of the mutant enzyme is likely to be limited to atomic movements of 0.5 Å or less.

Addition of $GlcNAc_3$ to D52S lysozyme results in changes in the NMR spectrum which are similar to those found on addition of $GlcNAc_3$ to the wild-type protein [19]. In particular, the resonances of the N(1)H protons of Trp-63 and Trp-108 are severely broadened, which is attributed to the effects of chemical exchange [19–21] and the existence of multiple bound states [19]. Addition of $GlcNAc_6$ to D52S lysozyme, however, results in a very different and well resolved NMR spectrum from that obtained with D52S lysozyme and $GlcNAc_3$. Resonances can, for example, be attributed to the indole N(1)H protons of all 6 tryptophan residues (Fig. 1b). This indicates that a well-defined and tightly bound complex has been formed, and that no free D52S lysozyme is detectable in the presence of a slight molar excess of $GlcNAc_6$. Distinct shifts in the spectrum are observed for a number of resonances, including many of those in the active site. Essentially identical 1D NMR spectra were also observed when the experiment was repeated using $GlcNAc_4$ instead of $GlcNAc_6$, showing that this species also forms a well defined complex which is structurally closely similar to that formed on addition of $GlcNAc_6$.

In order to explore further the nature of the complex we have carried out ESMS experiments on solutions prepared under the conditions used for the NMR experiments, except that immediately prior to injection the

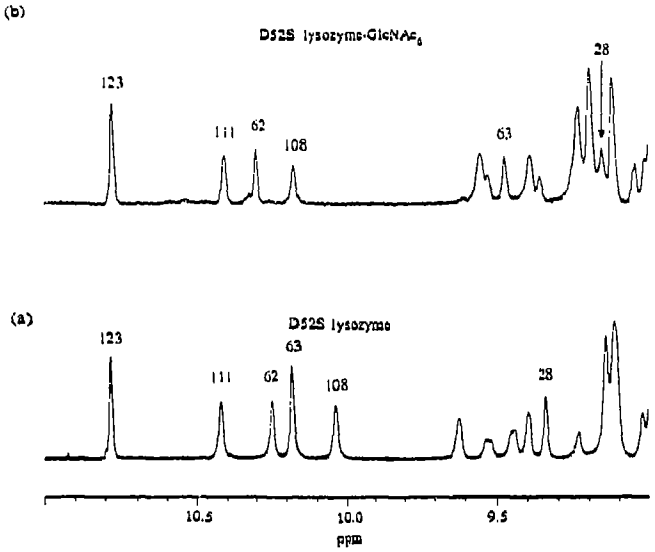

Fig. 1. 600 MHz ^{1}H NMR spectra of (a) 0.6 mM D52S lysozyme and (b) 0.6 mM D52S lysozyme and 0.75 mM $GlcNAc_6$ at 600 MHz, 35°C and pH 3.8 in 90% H_2O:10% D_2O. Tentative assignments of the indole N(1)H resonances of the tryptophan residues in (b) are indicated.

samples were diluted into 50% MeOH. The protein was shown not to be denatured in this solvent system by ^{1}H NMR and CD spectroscopy. Initially, a solution of D52S lysozyme and an equimolar solution of D52S lysozyme and $GlcNAc_3$ were investigated (Fig. 2). The ESMS spectra indicated an identical mass (14279±2 Da) in both cases, confirming the substitution of Asp for Ser (calculated M_r 14277) and suggesting that the complex had dissociated in the mass spectrometer. The spectra differed, however, in the intensities of the 10^+, 11^+ and 12^+ ions, which had markedly increased in abundance relative to the 9^+ and 8^+ ions, in the sample containing the oligosaccharide. This observation was found to be highly reproducible, and suggests that it is possible to discriminate, using ESMS, between free and bound lysozyme under these conditons.

The ESMS spectrum of an equimolar solution of D52S lysozyme and $GlcNAc_6$, recorded 3 min after mixing, again shows peaks corresponding to the mass of the uncomplexed enzyme; the intensities of the 10^+, 11^+ and 12^+ ions being similar to those in the presence of $GlcNAc_3$ (Fig. 3). Closer examination of the spectrum, however, reveals the presence of an additional set of peaks of much lower intensity, corresponding closely in mass to that expected for a complex of D52S lysozyme with $GlcNAc_6$ (experimental M_r 15516±3; calculated M_r 15513). The same relative intensities of the 2 sets of peaks were also obtained in the presence of a 9-fold molar excess of $GlcNAc_6$. A similar experiment with $GlcNAc_4$ also resulted in a set of low intensity peaks, this time corresponding to the mass expected for a complex of D52S with $GlcNAc_4$ (experimental M_r 15101±1; calculated M_r 15110). When ESMS spectra were recorded several hours after mixing the

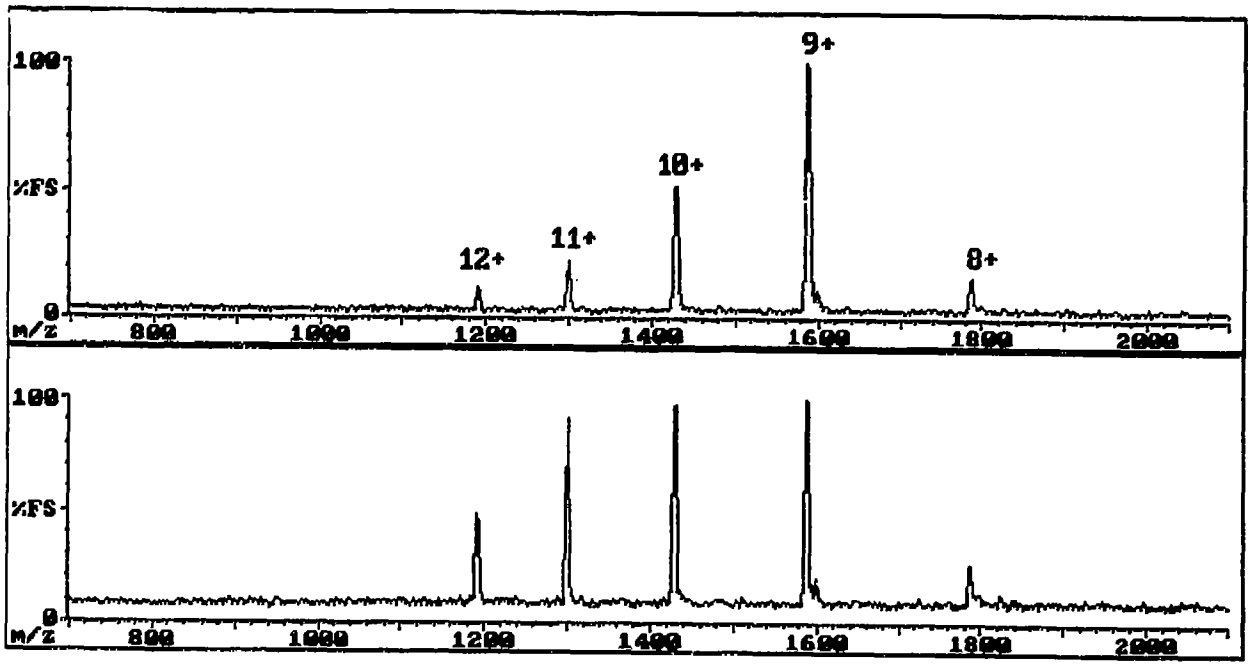

Fig. 2. ESMS spectra of (a) D52S lysozyme and (b) an equimolar solution of D52S lysozyme and $GlcNAc_3$ in 1:1 (v/v) methanol: water containing 1% acetic acid. Each spectrum is the result of 60 scans. The spectra were completely reproducible for 3 samples. The peaks are marked in (a) with the charge state. The peaks in (b) have the same charge and mass but are in different proportions.

enzyme with $GlcNAc_6$, the minor peaks arising from the $GlcNAc_6$ complex are replaced by peaks from a $GlcNAc_4$ complex (experimental M_r 15110±1; calculated M_r 15108) (Fig. 3). By contrast, 3 min after mixing a solution of wild-type hen lysozyme and $GlcNAc_6$, the ESMS spectrum contains peaks from hen lysozyme (experimental M_r 14305±1; calculated M_r 14305) and from unbound $GlcNAc_4$ ions only. Thus, under these conditions, hydrolysis of $GlcNAc_6$ by the wild-type enzyme is complete within 3 min. Wild-type hen lysozyme initially catalyses the hydrolysis of $GlcNAc_6$ to $GlcNAc_4$ and $GlcNAc_2$ [9]. The present results are consistent with D52S lysozyme catalysing the hydrolysis of $GlcNAc_6$ in a similar manner, but at least 10^4-times more slowly. This is consistent with the activity of D52S lysozyme against *M. lysodekticus*, which is less than 1% of that of the wild-type enzyme.

The observation of these minor components in the spectrum suggests, therefore, that species corresponding to oligosaccharides bound to the protein are detectable in the ESMS experiments under these conditions. The possibility arises, therefore, that these represent that proportion of the non-covalent complex which survives passage through the mass spectrometer. Such a situation has very recently been suggested by studies of the wild-type protein using ion-spray mass spectrometry [22]. It is generally assumed, however, that only in exceptional cases would non-covalent complexes be observed under the conditions of our experiments. An example of the survival of a non-covalent complex during ion-spray MS from water as a solvent has recently been reported for the macrolides FK 506 and rapamycin to their receptor, FKBP [23]. In the case of myoglobin, however, where the heme/protein complex was found to remain intact when the sample was injected from water, the complex dissociates in methanol-water mixtures such as those used in the present study [24]. Several reports, however, show that covalent adducts formed from substrates with enzymes can be seen by ESMS [25,26]. The possibility therefore arises that the minor peaks observed in the D52S lysozyme complex are covalently linked species. Indeed, minor low intensity peaks can be detected in the NMR spectrum of the D52S–$GlcNAc_6$ complex (Fig. 1), although the nature of the species giving rise to these small peaks has not yet been determined.

Support for the existence of covalent complexes is obtained from ESMS experiments in which the D52S –$GlcNAc_4$ and D52S–$GlcNAc_6$ mixtures were diluted into 80% MeOH/water prior to injection. Under these conditions both NMR and CD measurements suggest that the protein is substantially (>95%) denatured. Minor peaks corresponding to the D52S oligosaccharide complex are, however, still observed by ESMS. It may be expected that Ser-52 could form a covalent bond to the oxocarbonium ion postulated to exist in both the Phillips [3] and the Post and Karplus [6] mechanisms. A more likely possibility, however, for the production of covalent species is that they arise by formation of a hemiacetal from the mutated residue, Ser-52, with the reducing sugar bound in site D; $GlcNAc_4$ binds in the A-D subsites of the active site of

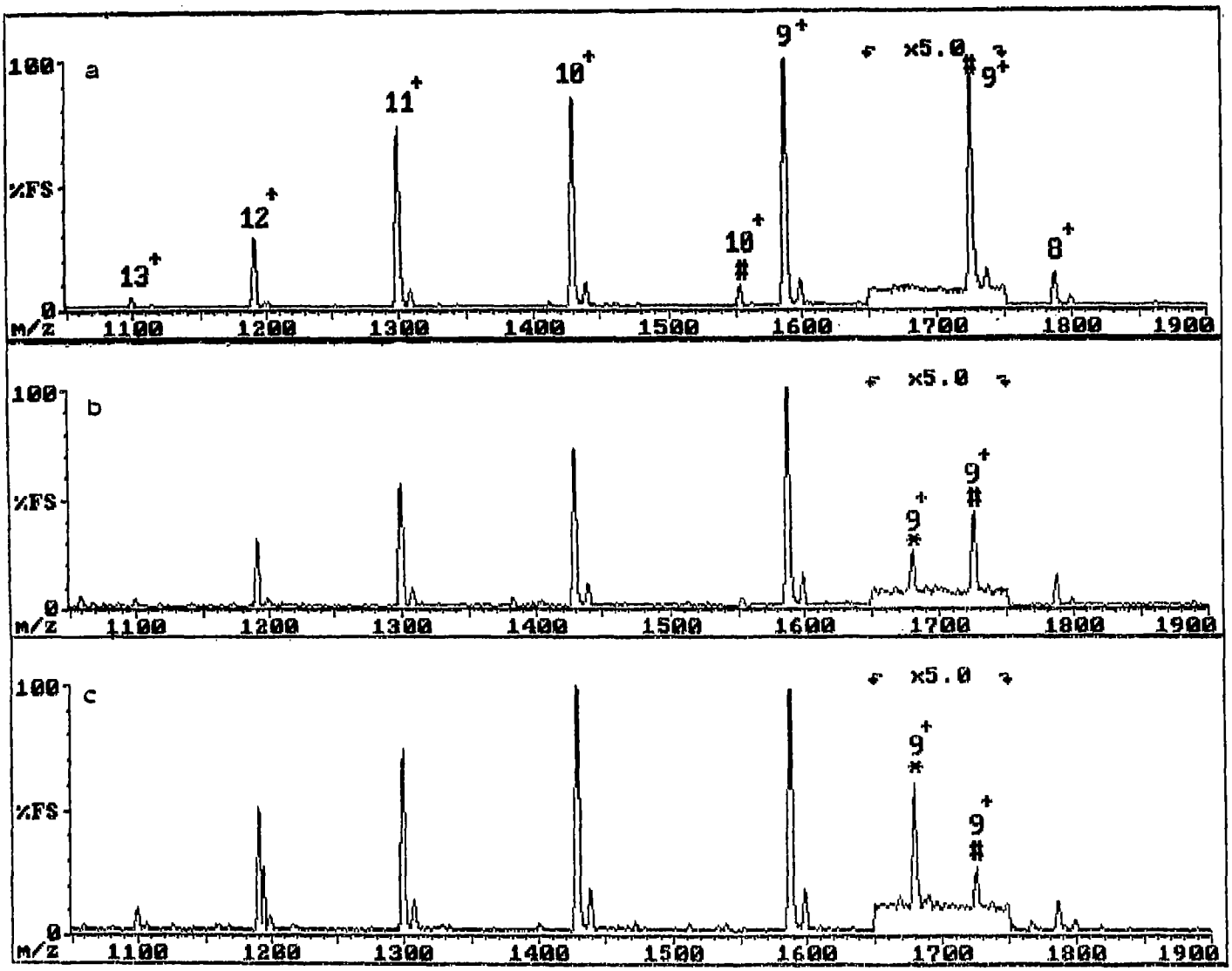

Fig. 3. Changes in the ESMS spectrum of an equimolar solution of D52S lysozyme and $GlcNAc_6$ with time. ES mass spectra were recorded (a) approximately 3 min after initial mixing at 35°C and pH 3.8. and (b), after 10 h, and (c) 32 h under these conditions. Samples were prepared for ESMS by diluting the above solutions into 1:1 (v/v) water:methanol to give a solution which was 50 μM in protein and saccharide. Peaks with the m/z of D52S lysozyme are labelled with the charge state in arabic numerals only in (a). The relative intensities of these peaks are consistent with D52S lysozyme forming a complex with the oligosaccharide in solution as shown directly by the NMR experiments. Peaks arising from the proposed covalent adduct of D52S lysozyme with $GlcNAc_6$ are labelled with the charge state and by #. Peaks arising from the proposed covalent adduct of D52S lysozyme with $GlcNAc_4$ are labelled with the charge state and by *. The peak intensities for $9^{+\#}$ and 9^{+*} are enhanced by a factor of 5. Each spectrum is the result of 60 scans. The spectra were completely reproducible for 3 samples.

wild-type hen lysozyme [27]. Similar binding to the mutant enzyme would place the reducing sugar of $GlcNAc_4$ near to Ser-52. If the sugar were then to undergo mutarotation in the active site the possibility arises that the intermediate acyclic aldehyde may be available to react with the hydroxyl group of Ser-52. Since $GlcNAc_6$ binds non-productively, as well as productively, to lysozyme [28], the covalent adduct between D52S lysozyme and $GlcNAc_6$ could also be formed from the acyclic aldehyde of $GlcNAc_6$ and Ser-52 if bound in the A-D subsites of D52S lysozyme.

The results presented here indicate that the major complex formed between D52S lysozyme and $GlcNAc_6$ appears to be a tightly bound non-covalent species. The ESMS data provide clear evidence, however, for a minor species which may be a novel covalent adduct. The $GlcNAc_6$ adduct, although highly stable relative to $GlcNAc_6$ in the presence of the wild-type enzyme, is hydrolysed slowly at 35°C and pH 3.8. ESMS indicates, however, that the non-covalent complex is stable for a period of several weeks at lower temperature (4°C, pH 3.8). This will permit detailed structural studies on the non-covalent D52S lysozyme-$GlcNAc_6$ complex and possibly also the proposed covalent adduct. This

provides an important opportunity to carry out a structural study on an enzyme–substrate complex, and thereby test mechanistic proposals for the enzyme in a novel manner.

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