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A Three Dimensional Model of the Digestion of Peptidoglycan by Lysozyme

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Abstract. The digestion of single peptidoglycan chains of the recently proposed conformation (Formanek et al., 1974) can be described with the same enzymatic mechanism as proposed by Phillips for a hexasaccharide consisting of alternating N-acetylglucosamine, N-acetylmuramic acid residues (Phillips, 1966). It is shown by model building, that in a peptidoglycan lysozyme complex the peptide chains do not exhibit any sterical hindrance.

The digestion of the peptidoglycan sacculus by lysozyme may occur at lattice defects of its paracrystalline structure. A slit of about 30 Å lenght and 10–15 Å width between peptidoglycan micells may be sufficient for the attachment of lysozyme.

Key words: Peptidoglycan – Lysozyme – Model Building.

Introduction

Murein or peptidoglycan is the rigid layer of the bacterial cell wall. It can be regarded as derivative of chitin with a D-lactyl residue forming an ether link to every second sugar residue (Fig. 1). This substituted sugar residue is called muramic acid. Peptides consisting of alternating L- and D-amino acid residues are bound through their N-terminus to the carboxylgroups of the muramic acid residues (Weidel and Pelzer, 1964; Schleifer and Kandler, 1972).

The possibility, that the carbohydrate chains of murein and chitin have the same conformation, has been discussed by several authors (Langer et al., 1969; Tipper, 1970; Higgins and Schockman, 1971; Kelemen and Rogers, 1971; Braun et al., 1973; Oldmixon et al., 1974). Similarities between the secondary and tertiary structure of murein and chitin could be shown by X-ray diffraction (Formanek et al., 1974; Giesbrecht et al., 1974) and infrared spectroscopy (Formanek et al., 1974).

Since both chitin and murein can be digested by lysozyme, they should bind to its active site without sterical hindrance. The structure of a complex of lysozyme

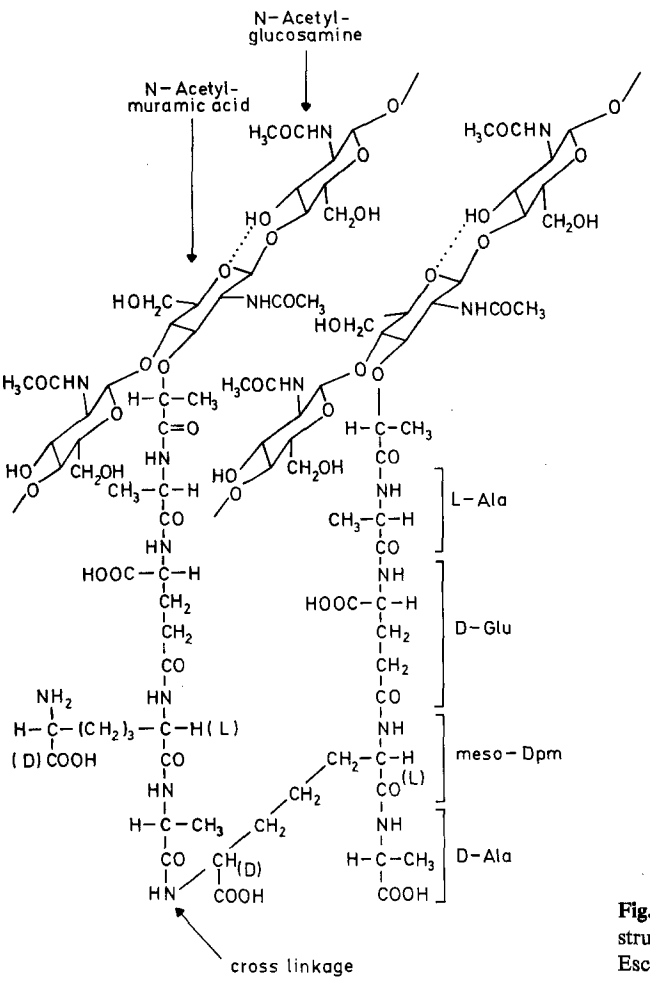

Fig. 1. Fragment of the primary structure of peptidoglycan of Escherichia coli

with a hexasaccharide consisting of alternating N-acetylglucosamine, N-acetylmuramic acid residues has already been described (Phillips, 1966). Based on experimental data and model building experiments, a possible structure of the complex of lysozyme with a peptidoglycan chain, consisting of three disaccharide tetrapeptide subunits is described in this paper.

Material and Methods

Chitin was purchased from Carl Roth, Karlsruhe. Lysozyme was purchased from Merck, Darmstadt. Spirillum serpens was obtained from Dr. Metz, Merck, Darmstadt.

A suspension of Chitin was prepared according to the method of Ch. Jeuniaux (1966). Cell walls of Spirillum serpens were isolated according to the modified method of Weidel et al. (1960), (Formanek et al., 1974).

X-ray diffraction: Foils of chitin and cell walls of Spirillum serpens were prepared by drying the suspensions at 80° C in a vacuum of 10^{-2} torr until no decrease in weight could be detected. Debye-Scherrer photographs of these foils were taken with CuKα radiation in a vacuum camera. Specimen film distance 70 mm.

Digestion of chitin and peptidoglycan by lysozyme was performed in 0.5 M ammoniumacetate buffer at pH 6.2. The course of the digestion can be followed by the decrease of the turbidity of a suspension of peptidoglycan or chitin. The turbidity was measured at 550 nm.

Model Building

All models were built with Nicholson Molecular Models purchased from Labquip, 18 Rosehill Park Estate, Caversham, Reading RG4 8XE, England. The scale of the models is 1 cm = 1 Å. Apart from hydrogen each atom is represented by a sphere whose radius is one third the van der Waal's radius. Hydrogen is not represented by a sphere but the position of the centre of a H-atom is at the end of each short arm. Hydrogen bonds were performed by connecting the units with steel wires pushed into the plastics. The steel wires were covered with plastic sleeves.

The complete set for building a model of lysozyme, together with its coordinates was purchased from Labquip.

Since the photographs of the models are too complex, schematic drawings of them are depicted.

Derivation of a Three Dimensional Model of Peptidoglycan

Till now it has not been possible to obtain single crystals of peptidoglycan. Therefore no exact three dimensional structure data are available.

In order to get a three dimensional model of peptidoglycan an experimental approach combined with theoretical consideration is necessary. For the construction of a three dimensional model of peptidoglycan the following available experimental data have been used:

1. The Primary Structure of Peptidoglycan (Weidel and Pelzer, 1964)

This includes the following information:
a) The carbohydrate moiety of peptidoglycan is similar to chitin.
b) Peptidoglycan has a network structure (Fig. 2). The dimensions of the meshes of this network can be described by two distances.

2. X-ray Diffraction (Fig. 3) (Formanek et al., 1974; Giesbrecht et al., 1974)

Two Debye Scherrer rings corresponding to distances of about 10 Å and 4.5 Å have been obtained from foils of both peptidoglycan and chitin.

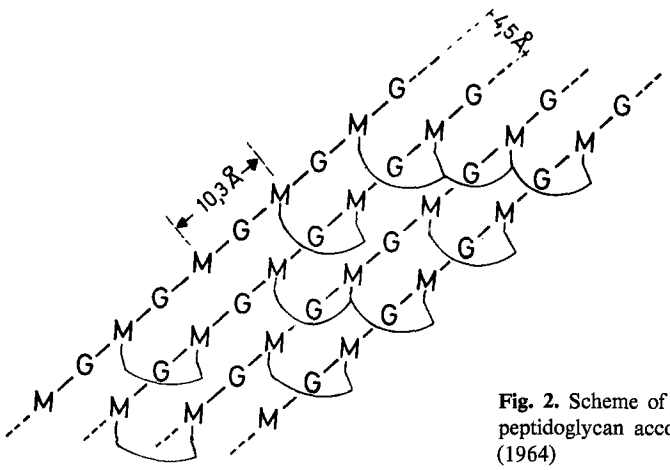

Fig. 2. Scheme of the network structure of peptidoglycan according to Weidel and Pelzer (1964)

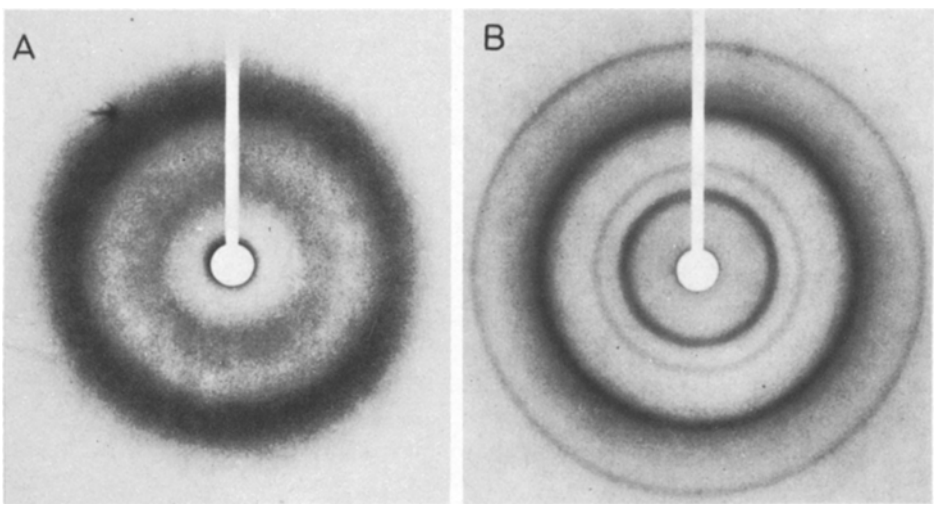

Fig. 3. Debye-Scherrer photographs of foils prepared from suspension of **A:** Peptidoglycan of Spirillum serpens, **B:** Chitin

3. Infrared Spectroscopy (Formanek et al., 1974; Formanek et al., 1976)

a) Similar infrared spectra are obtained for chitin and murein.
b) No stretching vibration of free (not hydrogen bond) NH-groups are found in both spectra.
c) Infrared spectra proved, that both peptidoglycan and its isolated peptide subunits have no α-helical- and no β-structure.

4. Density Gradient Centrifugation (Formanek et al., 1974)

The same high density of $\varrho = 1.46$ g/cm^3 was obtained for both peptidoglycan and chitin.

5. Chemical Modification of Peptidoglycan (Formanek et al., 1975; Schleifer and Kandler, 1972)

Peptidoglycans of different Gram positive bacteria show a variety of differences in the peptide moiety. In the carbohydrate moiety the C-6 OH group of the muramic acid residues may be acetylated or phosphorylated. The infrared spectra and the Debye-Scherrer photographs of different peptidoglycans are identical.

Conclusion from the Experimental Data (Formanek et al., 1974)

1. The chemically closely related structures of chitin and the carbohydrate moiety of peptidoglycan show identical hydrogen bonds (infrared spectra), identical lattice periodicities (Debye-Scherrer photographs) and identical densities. Therefore their tertiary structures should be identical.

2. The peptide chains are stabilized by hydrogen bonds and have no α-helical or pleated sheet conformation.

Conclusions Following from Sterical Requirements and Experimental Data

Densely packed (ϱ = 1.46 g/cm^3) carbohydrate stands of β-1,4-linked polyglucosides like cellulose, chitin and the carbohydrate moiety of peptidoglycan have periodicities of about 10 Å in the direction of the carbohydrate chains (Ramachandran et al., 1963). The single carbohydrate chains can be packed side by side with minimal distances of about 4.5 Å between one another (Carlström, 1957). Debye Scherrer rings corresponding to these distances of about 10 Å and 4.5 Å have been found in foils of both chitin and peptidoglycan (Formanek et al., 1974). The tertiary structure of chitin is well known (Carlström, 1957). Its N-acetylgroups at C-2 form interchain hydrogen bonds. Infrared spectroscopy shows these interchain hydrogen bonds both for chitin and peptidoglycan. Comparing the sterical requirements and the experimental data of the well known structure of chitin with the carbohydrate moiety of murein, the conclusion is justified that they have identical tertiary structures.

Necessary consequence of the identity of the tertiary structures of chitin and the carbohydrate moiety of peptidoglycan.

1. The sugar residues in the carbohydrate chain are tilted by angles of 180° against one another (Ramachandran et al., 1963; Formanek et al., 1976). Therefore the peptide chains linked to every second sugar residue of the peptidoglycan protrude all in the same direction.

2. The peptide chains of peptidoglycan have to assume the periodicities of the carbohydrate moiety.

Theoretical Considerations

1. Since the peptide chains of peptidoglycan have to fit in the periodic structure of the carbohydrate moiety of about 10 Å × 4.5 Å, they have to be very flat. Flat

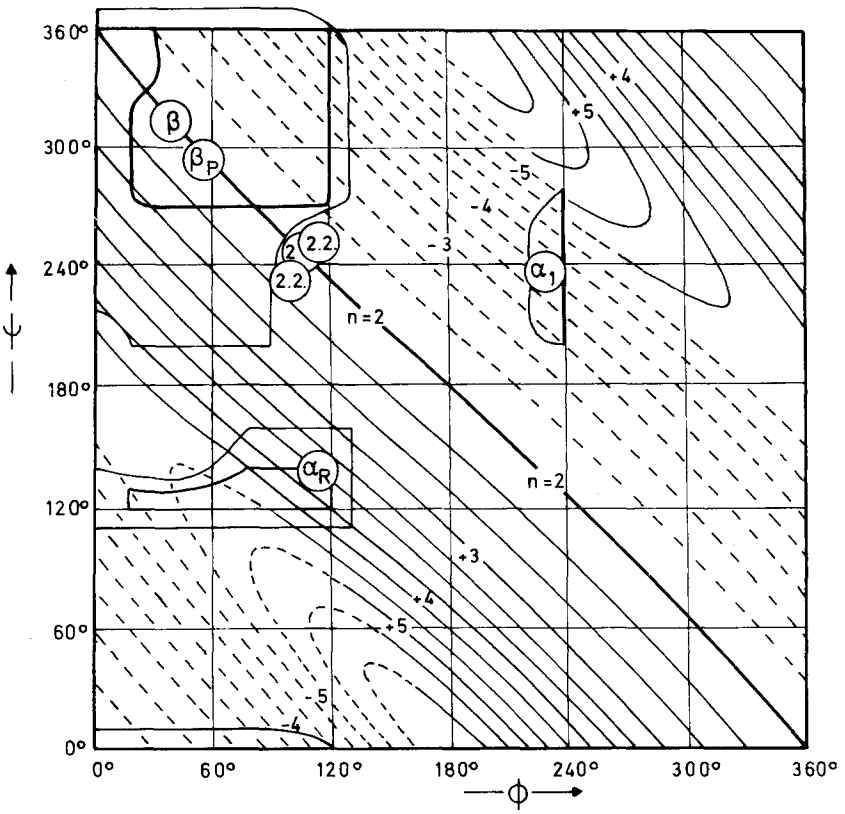

Fig. 4. Ramachandran plot with contours of equal n. n = Number of amino acids per turn of a helix, —— = right-handed helices, – – – = left-handed helices, —— = twofold ribbon structures, α_R = right handed α-helix, α_L = left handed α-helix, β = antiparallel pleated sheet, β_P = parallel pleated sheet, 2 = twofold ribbon, 2.2 = 2.2_7-helices

peptide structures are on the axis $n = 2$ of the Ramachandran plot (Fig. 4) (Ramachandran, 1963).

2. Peptides consisting of L-amino acids form mainly right handed helices. Peptides consisting of D-amino acids form mainly left handed helices. Peptides consisting of alternating L- and D-amino acids should form flat twofold ribbon structures on the axis $n = 2$, of the Ramachandran plot.

3. As found by infrared spectra (Formanek et al., 1976) the peptide should be stabilized by hydrogen bonds.

Two peptide models fullfill these conditions the pleated sheet (Kelemen and Rogers, 1971) and the 2.2_7 helix (Formanek et al., 1974). The pleated sheet structure can however be excluded by infrared measurements (Formanek et al., 1976).

From experimental data and theoretical considerations we could therefore derive a three dimensional model for peptidoglycan, whereby the glycan moiety resembles the tertiary structure of chitin and all peptide subunits in the 2.2_7 helical conformation protrude on the same direction away from the carbohydrate layer, whose repeating periodicities they have to assume. Figure 5 shows the three dimensional model of peptidoglycan.

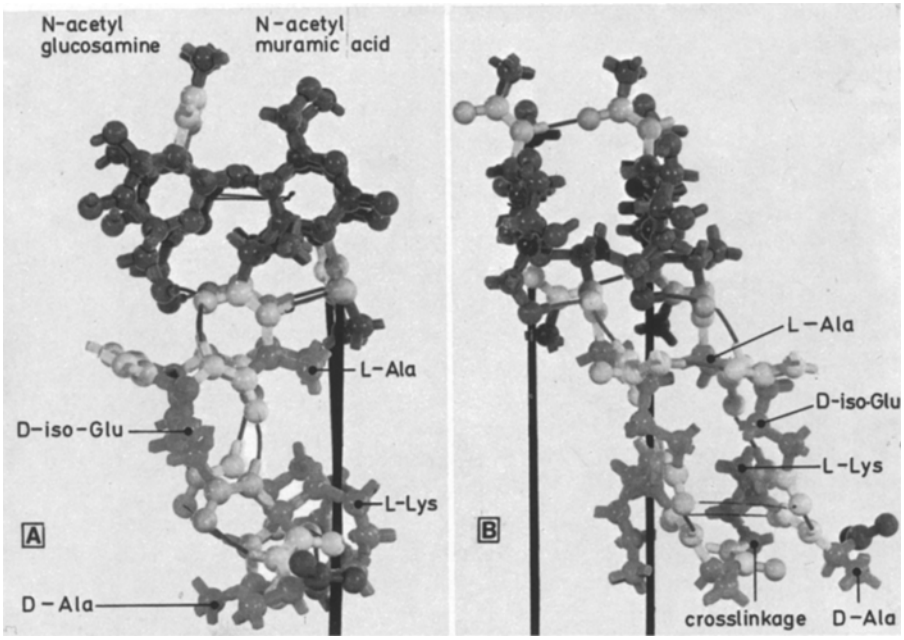

Fig. 5. Three dimensional model of peptidoglycan. **A:** Two peptidoglycan chains vertical to the plane of the paper, **B:** Two peptidoglycan chains behind one another

Comparison Between the Model of Peptidoglycan and the Crystal Structures of Chitin, N-acetylglucosamine and Muramic Acid

1. N-*acetyl-α*-D-*glucosamine* (Johnson, 1966)

The molecules are packed in the crystal with a periodicity of 4.8 Å vertical to the planes of the carbohydrate rings. The crystals have densities of $\varrho = 1.54$ g/cm^3. Weak intermolecular hydrogen bonds are performed between adjacent N-acetyl-groups.

2. Chitin (Carlström, 1957)

The polymer chains consisting of β-1,4-linked N-acetylglucosamine residues are packed side by side with lattice periodicities of about 4.7 Å. The fibres have densities of $\varrho = 1.46$ g/cm^3. Strong intermolecular hydrogen bonds are performed between adjacent N-acetylgroups.

3. N-*acetyl-α*-D-*muramic acid* (Knox and Murthy, 1974)

In this crystal structure an intramolecular hydrogen bond is found between the NH of the acetamino group and the carbonyl oxygen of the lactyl group. This is the only

possible intramolecular hydrogen bond in a neutral aqueous medium since the carboxylgroups cannot be protonated at pH values above 3. The molecules are packed in the crystal with a periodicity of 8 Å vertical to the planes of the sugar rings. Compared with N-acetylglucosamine, chitin and peptidoglycan the crystals of N-acetylmuramic acid have the relatively low density of $\varrho = 1.38$ g/cm^3.

4. Peptidoglycan (Formanek et al., 1974)

If the carboxylgroup of the lactylresidue is not free as in N-acetylmuramic acid but involved in a peptide bond as in peptidoglycan an intermolecular hydrogen bond between its NH group and the CO of the N-acetylgroup is possible (Fig. 5). The formation of this hydrogen bond enables another intrachain hydrogen bond between the C6-OH group of the N-acetylglucosamine residue and the lactyl carbonyl of the N-acetyl muramic acid residue. This hydrogen bond has already been discussed by Tipper (1970) and Formanek et al. (1974). Molecules forming these hydrogen bonds can be packed with periodicities of about 4.5 Å vertical to the plane of the carbohydrate rings, explaining the 4.5 Å Debye-Scherrer ring and the dense packing of $\varrho = 1.46$ g/cm^3 experimentally obtained for peptidoglycan. This dense packing enables the interchain hydrogen bonds between adjacent N-acetylgroups required from infrared spectroscopy of peptidoglycan and chitin.

Digestion of Peptidoglycan by Lysozyme

The three dimensional model of the digestion of peptidoglycan by lysozyme is based on the following experimental results:

1. The three dimensional structure data of the complex of lysozyme with a hexasaccharide consisting of alternating N-acetylglucosamine- and N-acetylmuramic acid residues (Phillips, 1966).

2. Acetylation or phosphorylation at C6-OH of the N-acetylmuramic acid residues of peptidoglycan inhibits its digestion by lysozyme. All other modifications of peptidoglycan, as different interpeptide chains or amidation of the α-carboxylgroups of D-Glu have no influence on the action of lysozyme.

3. Berger and Weiser (1957) found, that preparations of both chitin and peptidoglycan can be digested by lysozyme.

4. Comparison between the X-ray diffraction and digestability of our preparations of chitin and peptidoglycan by lysozyme.

a) Our suspended chitin could hardly be digested by lysozyme. Nearly no decrease of extinction at 550 nm has been observed after one day. Foils prepared from this suspension show relatively sharp Debye Scherrer rings (Fig. 3B).

b) The suspended peptidoglycan of Spirillum serpens was easily digested by lysozyme. A decrease of the extinction at 550 nm from 0.600 to 0.170 was observed within 10 min. Foils prepared from this suspension show diffuse Debye-Scherrer rings (Fig. 3A).

Based on these results the following models of the digestion of both single peptidoglycan chains and whole murein sacculi can be derived:

Digestion of Single Peptidoglycan Strands by Lysozyme

The complex of lysozyme with a hexasaccharide consisting of alternating N-acetylglucosamine, N-acetylmuramic acid residues (Phillips, 66) is stabilized by several hydrogen bonds between the enzyme and the carbohydrate chain. On sterical reasons Phillips could determine, that only the sugar binding sites B, D and F (Fig. 6) can be occupied by N-acetylmuramic acid residues.

If the C6-OH of the N-acetylmuramic acid residues are acetylated or phosphorylated this complex cannot be formed on sterical reasons. In our model building experiments we fitted a peptidoglycan strand with three disaccharide tetrapeptide subunits, in the conformation proposed (Formanek et al.; Kandler and Formanek, 1975), in the active site of lysozyme, in a way, that the N-acetyl-muramyl-tetrapeptide residues occupy positions B, D and F (Fig. 6). The carbohydrate chain of this strand can then be fixed with hydrogen bonds in the same position as the hexasaccharide described by Phillips. The peptide chains in the conformation described (Formanek et al., 1974), do not exhibit any sterical hindrance with lysozyme. In addition to the hydrogen bonds between the carbohydrate chain and lysozyme (Fig. 6), the following hydrogen bonds may be performed between the peptide chains and the enzyme (Fig. 6):

1. Between the carbonylgroup of L-Ala of the peptide subunit linked to a muramic acid residue in position D and the guanidine group of Arg 112
2. Between the carbonylgroup of D-Ala of the peptide subunit linked to a muramic acid residue in position D and the α-amino group of Lys 116
3. Between the free or amidated carboxylgroup of D-Glu of the peptide subunit linked to a muramic acid residue in position F and the carbamide group of Asn 113.

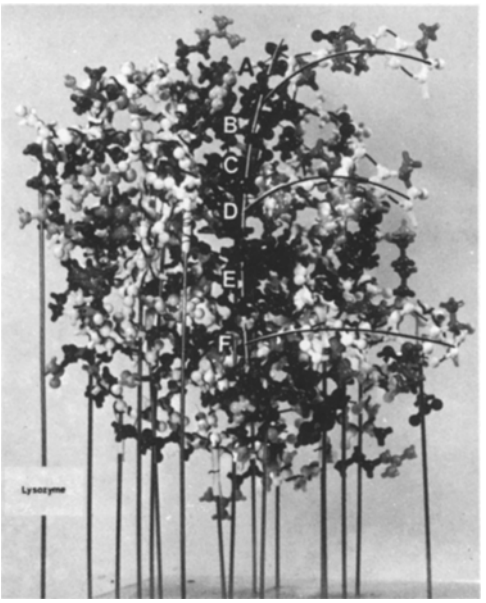

Fig. 6. A: Three dimensional model of lysozyme with a peptidoglycan chain consisting of three disaccharide tetrapeptide subunits in its active site,

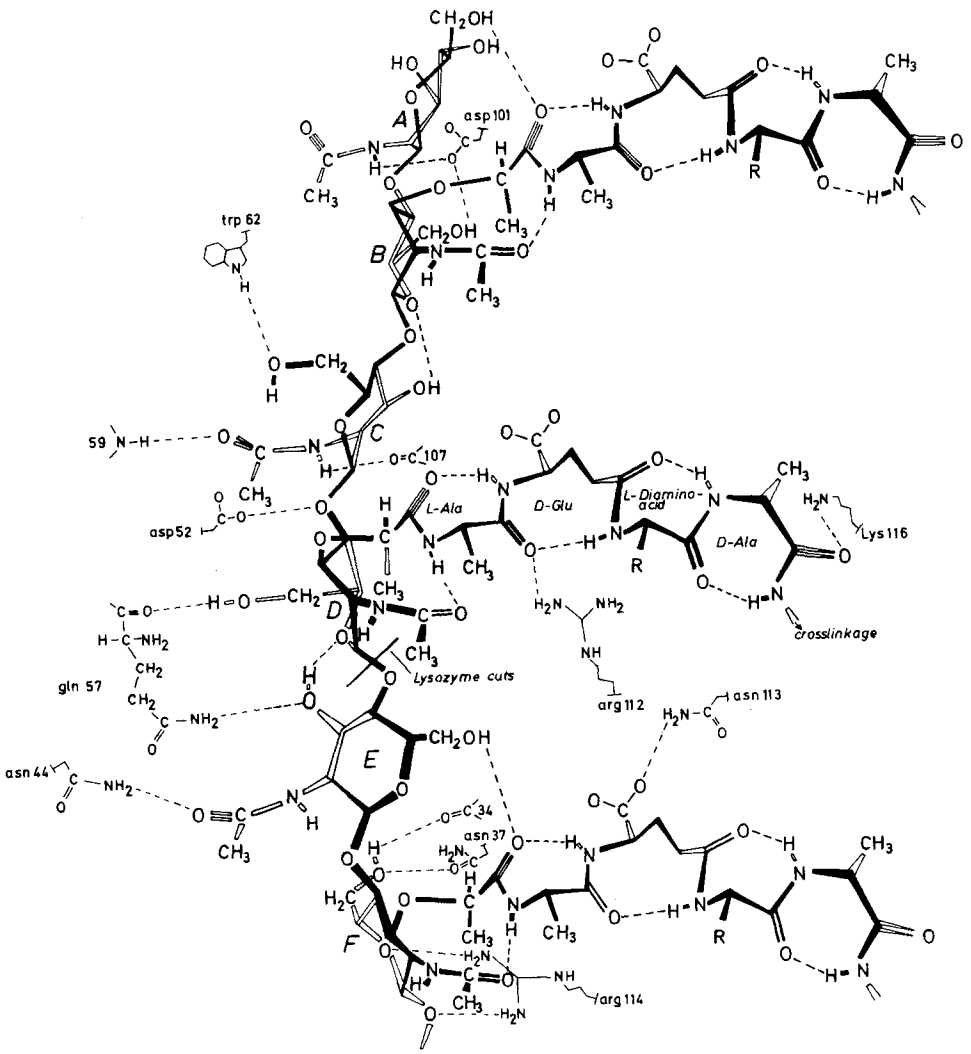

Fig. 6. B: Schematic drawing of the interaction of a single peptidoglycan chain with lysozyme. – – – Hydrogen bonds. The peptidoglycan chains consist of three disaccharide tetrapeptide subunits

The peptide subunit linked to the muramic acid residue in position B has no contact with lysozyme.

The Digestion of the Murein Sacculus

Model building experiments have shown, that the digestion of single strands of chitin and murein can be described with the same enzymatic mechanism. But model building shows also, that lysozyme cannot attach to intact lattices of both chitin or murein.

If the murein envelope of the bacterial cell wall would exhibit an ideal crystalline structure as shown in this model (Formanek et al., 1974) the cell wall would be impermeable. Ideal crystal structures are however improbable in biological and synthetic polymers. These substances often perform paracrystalline structures (Hosemann, 1973; Hosemann 1976). In contrast with the crystalline state the ordered regions (mosaic blocks) in paracrystals are very small. Unlike small molecules, natural and synthetic polymers most time do not crystallize completely, therefore more or less extended amorphous regions (lattice defects) are between the mosaic blocks, which can be tilted against one another. These irregularities of the paracrystalline structures are responsible for the combination of both stability and flexibility of fibres and foils (Zachmann, 1974).

The murein lattice can be regarded as a paracrystalline foil with amorphous regions and crystalline mosaic blocks.

The extension of these mosaic blocks is inversely proportional to the broadening of the Debye-Scherrer rings obtained from a solid (Klug and Alexander 1954). A comparison between the Debye-Scherrer photographs of chitin and peptidoglycan (Fig. 3) with the action of lysozyme on both compounds shows the following result:

The mosaic blocks of our preparation of chitin are much more extended than the mosaic blocks of peptidoglycan. Our suspension of chitin can hardly be digested by lysozyme because of its ordered structure. The suspension of peptidoglycan however can easily be digested by lysozyme. Chitin, whose lattice structure has been widely destroyed by the methods of preparation, is also digestible by lysozyme (Berger and Weiser, 1957).

The degree of ordering derived from the X-ray pictures seems to be a property of the native peptidoglycan. This is probable if one considers, that biological structures are self assembling systems which are in an energetically favoured and therefore stable equilibrium conformation (Eigen, 1971).

Based on the peptidoglycan model derived (Formanek et al., 1974) one can explain the formation of the murein layer by self association in the following way:

During biosynthesis polymer chains consisting of disaccharide peptide chains are formed with twofold serew axes possible both in the carbohydrate and in the peptide chains.

Association of these flat peptidoglycan strands can take place in a similar way as with the polysaccharide chains of chitin (Carlström, 1957) and the carbohydrate molecules of N-acetylglucosamine crystals (Johnson, 1966) by the formation of intermolecular hydrogen bonds between the N-acetylgroups of adjacent carbohydrate molecules (Fig. 5B). Experimental results obtained on the biosynthesis of peptidoglycan could be explained with these considerations (Hammes and Kandler, 1976). There is however evidence, that the ordered conformation which only can be recognized by X-ray diffraction is in a dynamic equilibrium with other slightly different structures involving localized coiling (Blundell and Johnson, 1976). This coiling can only take place within the sterical requirements (Ramachandran et al., 1963).

It is reasonable to regard the murein layer as a paracrystalline structure, where single peptidoglycan chains may extend over both crystalline and amorphous re-

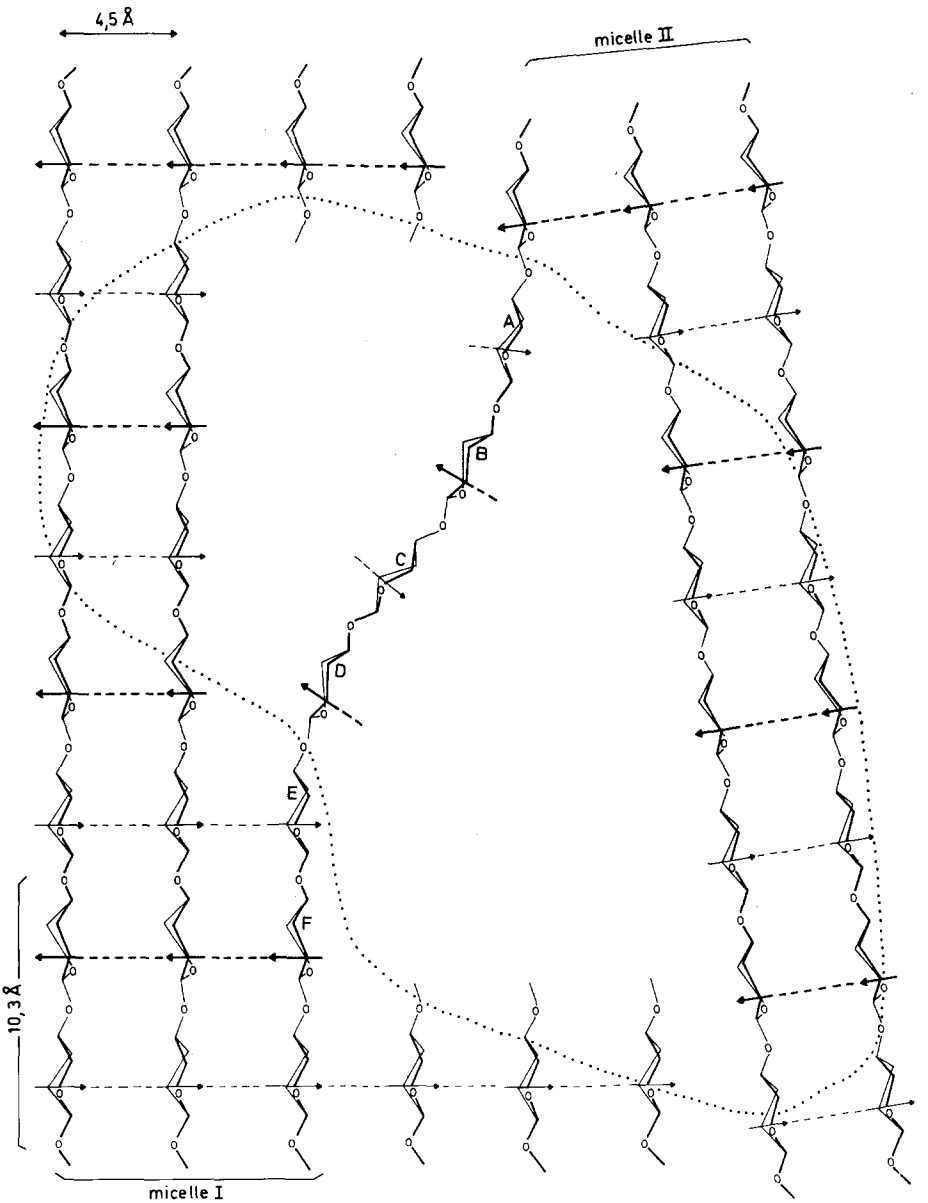

Fig. 7. Schematic drawing of lysozyme attached to a lattice defect in a murein layer. The peptidoglycan chains are symbolized by their sugar residues. → N-acetylgroups. Sugar residue with arrows pointing to the left side symbolize N-acetylmuramic acid residues, the other ones symbolize N-acetylglucosamine residues. – – – Interchain hydrogen bonds between the N-acetylgroups of adjacent carbohydrate residues. · · · · Approximate circumference of lysozyme attached to sugar residues A–F

gions like in cellulose (Meyer, 1950). At these lattice defects the murein layer may be permeable and lysozyme may start with the digestion of the cell wall.

A possible arrangement of peptidoglycan strands at a lattice defect has been obtained by model building. The result, schematically drawn in Figure 7 shows two micells of murein performing an angle of about 10° between one another. One peptidoglycan chain may connect both micells in a region where they are about 10–15 Å distant from one other (Fig. 7). The sugar residues E and F are part of the ordered structure of micell I. Therefore the sum of the two torsion angles about their connecting glycosidic oxygen atom is 180°, characteristic for the twofold screw axis in the regular lattices of cellulose (Ramachandran et al., 1963), chitin (Ramachandran et al., 1963) and murein (Formanek et al., 1974). The torsion angles between the sugar residues A till E in the amorphous region of the peptidoglycan chain may assume any combination of sterically allowed values. One set of angles may cause a conformation similar to the hexasaccharide fitting into the cleft of lysozyme and a model of lysozyme can be fixed to these sugar residues (A–F in Fig. 7) with the already described hydrogen bonds (Fig. 6).

In the areas of the lattice defect not occupied by lysozyme, two or thre peptidoglycan chains may be attached to micell I (shown in Fig. 7) or micell II by model bulding. The resulting slit of about 30 Å length and 10–15 Å width in the murein layer (Fig. 7) is smaller than the diameter of the lysozyme molecule. It is however sufficient for the attachment of the enzyme and may also allow the permeation of small molecules. The macromolecules of the cytoplasmic membrane however may only be pressed against the lattice defect by the osmotic pressure.

The difficulties to correlate the experimental data leading to an ordered structure of murein and the finding, that lysozyme digests peptidoglycan in an unordered region can be overcome with the help of the paracrystalline state which is related to the crystalline state but allows a certain degree of disorder (Hosemann, 1973; Hosemann, 1976).

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