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OLIGOMANNOSIDE UNITS OF CELL SURFACE GLYCOPROTEINS AS RECEPTORS FOR BACTERIA

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Abstract - The adherence of many strains of *Escherichia coli* and of other enterobacteria to animal cells is inhibited by low concentrations of D-mannose, methyl α-D-mannoside or mannan. This adherence is mediated by lectins present on the bacterial surface, primarily in the form of type 1 fimbriae (pili). Such fimbriae can be dissociated reversibly into subunits that retain their mannose-binding activity.

Detailed studies of the sugar specificity of the fimbrial lectins have been carried out by quantitative inhibition of the agglutination of mannan-containing yeast cells by the bacteria. The best inhibitors of the agglutination by *E. coli* 346 were the oligosaccharides Manα1→6[Manα1→3]Manα1→6[Manα1→2Manα1→3]ManαOMe, Manα1→6[Manα1→3]Manα1→6[Manα1→3]-ManαOMe and Manα1→3Manβ1→4GlcNAc. and the aromatic glycoside *p*-nitrophenyl α-D-mannoside, all of which were 20-30 times more inhibitory than methyl α-D-mannoside. The disaccharides Manα1→3Man, Manα1→2Man and Manα1→6Man, the tetrasaccharide Manα1→2Manα1→3Manβ1→4GlcNAc and the pentasaccharide Manα1→2Manα1→2Manα1→3Manβ1→4GlcNAc, were all poor inhibitors. A very good correlation was found between the relative inhibitory activity of the different sugars tested with intact *E. coli*, with the fimbriae isolated from this organism and with *Klebsiella pneumoniae*. Our findings show that the combining sites of the mannose specific lectins of *E. coli* and *K. pneumoniae* are extended corresponding to the size of a trisaccharide, that they contain a hydrophobic region, and that they are in the form of a pocket on the surface of the lectins. Since the "best fit" for the combining sites are structures found in short oligomannoside chains of N-glycosyl linked glycoproteins, this strongly suggests that such structures serve as bacterial receptors on epithelial cells.

Methyl α-D-mannoside or mannose have been shown to prevent experimental infection by mannose specific strains of *E. coli*, *K. pneumonia* or *Shigella flexneri*. Detailed knowledge of the structure of the oligosaccharide receptors for different bacteria may lead to the design of highly effective inhibitors of adherence that may prove to be useful in the prevention of bacterial infections.

INTRODUCTION

Research carried out in various laboratories during recent years has led to the conclusion that carbohydrate-mediated recognition plays a key role in many cell-cell interactions, either homotypic or heterotypic ones. One of the best characterized examples of such an interaction is the adherence or attachment of bacteria to epithelial and other types of cell.[1] This adherence, believed to be a prerequisite for bacterial infection, is in numerous cases inhibited specifically by low concentration of monosaccharides that are constituents of cell surface glycoconjugates.[2, 3] Thus, many strains of *Escherichia coli*, of *Klebsiella*, *Salmonella* and *Shigella* are inhibited by D-mannose or methyl α-D-mannoside; such bacteria are designated as mannose-specific, or MS. Certain strains of *E. coli* are classified as mannose-resistant (MR) since their adherence to cells is not inhibited by D-mannan. Several of these strains are also sugar specific, their adherence to epithelial cells being effectively inhibited by oligosaccharides

and glycolipids containing the structure Galα(1→4)Galβ.[4a, 4b] Microorganisms specific for other sugars have also been identified, e.g., *Actinomyces viscosus* for D-galactose, certain *Chlamydia* for N-acetyl-D-glucosamine, and *Mycoplasma* for N-acetylneuraminic acid.

Ofek et al.[4c] who in 1977 reported on the interaction of *E. coli* with buccal epithelial cells were the first to propose that the sugar specific adherence is mediated by lectins on the bacterial surface which bind to D-mannose residues on the animal cells. This proposal was based primarily on the finding that no attachment of the bacteria to the epithelial cells was observed following treatment of the latter with sodium metaperiodate (a reagent known to oxidize sugar residues on cell surfaces) or with concanavalin A (a lectin that binds specifically to D-mannose or D-glucose residues on cells). It was also in accord with earlier observations that MS bacteria agglutinate cells of yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* that are covered by mannans.[2, 3] Subsequent work by Ofek and Beachey[5] has shown that, as expected, oxidation of yeast cells with periodate abolished their ability to bind MS *E. coli*. It has also been demonstrated that MS *E. coli* bind yeast mannan,[4c] and that there is a high correlation between the mannan binding capacity of the bacteria and their attachment to animal cells.[6]

PHYSICOCHEMICAL PROPERTIES OF THE BACTERIAL SURFACE LECTINS

The MS lectins of *E. coli* may occur on the surface of the bacteria in different forms, most commonly as pili (or fimbriae), elongated protein appendages, of a diameter of ~7 nm. The participation of pili in the MS adherence of *E. coli* to cell surfaces was first pointed out by Duguid and Gillies as early as 1957.[2] They found a striking correlation between the occurrence of pili and the MS hemagglutination exhibited by the bacteria. The pili responsible for this hemagglutination were designated as "type 1 pili". They consist of identical protein subunits, known as pilin[7] with molecular weight (MW) of 17,000 dalton. Ofek and Beachey[5] fractionated cultures of *E. coli* into non-adherent and adherent populations by adsorption of the latter on buccal epithelial cells followed by elution with methyl α-D-mannoside. The bacteria thus eluted exhibited a high degree of mannose binding activity and were heavily piliated, whereas the non-adherent bacteria lacked such activity, and were devoid of type 1 pili. There is also direct evidence that the MS lectin of *E. coli* may indeed be in the form of type 1 pili. Isolated and purified type 1 pili agglutinated guinea pig erythrocytes and adhered to monkey kidney cells grown *in vitro*.[8] All these reactions were specifically inhibited by low concentrations of mannose or methyl α-D-mannoside. It was also shown that purified type 1 pili from *E. coli* or *Salmonella typhimurium* strongly agglutinated yeast cells.[9, 10]

In the past, studies of type 1 pili have been greatly hampered by difficulties encountered to dissociate them into biologically functional subunits. For example, type 1 pili are not readily denatured by sodium dodecylsulfate (SDS) even with boiling, and the bulk of the protein does not penetrate SDS-polyacrylamide gels. The dissociation of the pili into their subunits could only be achieved under conditions such as boiling at low pH or chemical modification, which usually lead to irreversible protein denaturation.

We have found[11] that incubation of pili in saturated guanidine-HCl (8.3 M) at 37°C leads to their complete dissociation, as evidenced by nephelometry and electron microscopy. Gel chromatography of the dissociated pili on a Sepharose CL-6B column in the presence of saturated guanidine-HCl yielded a single protein peak corresponding to a MW of about 17,000 dalton, which is attributed to pilin. Dialysis of this "peak" against 5 mM Tris-HCl (pH 8.0) and rechromatography in the same buffer afforded a major protein peak, probably consisting of pilin dimers. The pilin dimers gave a single protein band (MW 16,600) upon polyacrylamide gel electrophoresis in the presence of 0.1% SDS or 10 M urea, and penetrated completely into 7% gels in the absence of denaturants.

Exposure of pili to 8.6 M guanidine-HCl was sufficiently mild so that the subunits could reassemble in low yield (30%) into structures resembling short fragments of intact pili, as observed in the electron microscope. Of special significance is the findings that the subunits formed by dissociation with 8.6 M guanidine-HCl retained their ability to bind to D-mannose. Thus, about 25% of the subunits after removal of the guanidine-HCl by dialysis were bound to immobilized mannan and could be eluted by methyl α-D-mannoside. We cannot adequately explain why only part of the subunits were bound specifically to the mannan column. Perhaps the majority of the subunits were not renatured after removal of guanidine-HCl to molecules capable of binding D-mannosyl residues. On the other hand, it is possible that only a portion of the pilin monomers in each intact pilus had D-mannose-binding activity, implying biological heterogeneity of the individual pili subunits. Whatever the significance of the low proportion of binding activity, the fact that there was binding at all is additional and perhaps definitive proof that type 1 pili mediate the D-mannose-binding activity of *E. coli*.

Studies in our laboratory have demonstrated D-mannose-specific lectins may be present on the bacterial surface in forms other than pili. We have isolated preparations of flagella that agglutinate yeast cells from *E. coli* 7343[12, 13] and *Serratia marcescens* 8347.[14] In preliminary experiments (T. Pistole and N. Sharon, unpublished) it has been found that in certain bacteria (e.g., *S. typhemurium*) the MS lectin(s) may be tightly attached to the outer membrane.

Eshdat et al.[10] have isolated outer membrane vesicles from *E. coli* 2699, a mannose-specific strain which agglutinated yeast cells whether the bacteria were piliated or not. The outer membrane preparations exhibited stronger agglutinating activity than the pili isolated from the same organism. Examination by electron microscopy did not reveal any intact or fragmented pili in the outer membrane preparations. These and other findings led Eshdat et al.[10] to suggest that the membrane bound lectin may be either in the form of pilin monomers inserted into the outer membrane without polymerization, or that it consists of protein subunits different from pilin.

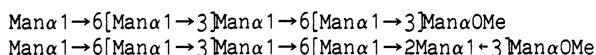
CHARACTERIZATION OF THE SUGAR-BINDING SITE OF THE LECTINS

Until recently, the sugar specificity of MS bacteria had been examined only with a limited variety of sugars, mostly monosaccharides. Perhaps the only exception is the study of Old[15] who tested the effect of various monosaccharides, as well as several simple oligosaccharides, on the mannose-specific agglutination of guinea pig or horse erythrocytes by certain strains of *S. typhimurium* or *Shigella flexneri*. Modification of the hydroxyl groups at the C-2, C-3, C-4 or C-6 position of the D-mannopyranosyl molecule resulted in loss of inhibitory activity, showing that these groups are required for binding to the sugar combining site on the bacteria. The α-configuration of the D-mannose was also found to be important, since carbohydrates containing β-linked D-mannose were much poorer inhibitors than those containing the α-linked monosaccharide.

Detailed characterization of the combining site of the bacterial lectins is important for gaining a better insight into the nature of the interaction between the bacteria and epithelial cell surfaces. It may also provide information for the design of more effective inhibitors for the prevention of adhesion, of colonization of the mucosal surfaces and of infection as well.

We have examined the inhibitory effect of a large number of mannose-containing oligosaccharides on the agglutination of yeast cells by *E. coli* 346 and the type 1 pili isolated from this organism,[16] as well as by *Klebsiella pneumoniae*. The validity of such an approach for the determination of the combining sites of plant and animal lectins is well established.[17] However, no studies of this type had been reported for the bacterial lectins.

A quantitative evaluation of the inhibitory activity of various mannose derivatives on the agglutination of yeast cells by the bacteria was obtained using an aggregometer[5]. In all cases, a linear correlation was observed between the percent inhibition and the logarithm of sugar concentration, within the range tested, although the slopes of the lines were not always identical. For each sugar, the concentration causing 50% inhibition was derived from its inhibition line and the relative inhibitory activity in comparison to that of methyl α-D-mannoside was calculated. The results showed that two of the oligosaccharides tested:



were strong inhibitors of yeast agglutination by *E. coli* 346. Their inhibitory activity was 30 times higher than that of methyl α-D-mannoside. The trisaccharide Manα(1→3)Manβ(1→4)GlcNAc, as well as the aromatic glycoside of p-nitrophenyl α-D-mannoside, were also strong inhibitors (21 and 30 times more than methyl α-D-mannoside, respectively).

On the other hand, the disaccharides Manα(1→2)Man, Manα(1→3)Man and Manα(1→6)Man were considerably weaker inhibitors, similar in activity to methyl α-D-mannoside. We assumed therefore that the combining site of this *E. coli* lectin is an extended one. The finding that the trisaccharide Manα(1→3)Manβ(1→4)GlcNAc was a much better inhibitor than the disaccharide Manα(1→3)Man, the tetrasaccharide Manα(1→2)Manα(1→3)Man-β(1→4)GlcNAc and the pentasaccharide Manα(1→2)Manα(1→2)Man α(1→3)Manβ(1→4)GlcNAc, strongly suggests that the combining site corresponds to the size of a trisaccharide, and that it is in the form of a depression or pocket on the surface of the lectin. Extended carbohydrate-binding sites have been described for enzymes (e.g., lysozyme), several lectins and antibodies. In the case of the *E. coli* lectin, there are probably three adjacent subsites, each of which fits a monosaccharide residue. The fact that there was only little difference between the inhibitory activity of methyl α-D-mannoside and the D-mannose disaccharides tested is an indication that the binding to one of the subsites is weak. The strong inhibition by p-nitrophenyl α-D-mannoside may be due to the existence of a hydrophobic region, adjacent to one of the subsites. Such hydrophobic regions have been found in other lectins as well (e.g., concanavalin A and soybean agglutinin).

Our data demonstrate that the combining site of the *E. coli* 346 lectin best fits structures found in short oligomannoside units of N-glycosyl linked glycoproteins. The proposed site is, however, quite different from that of concanavalin A, a well characterized lectin with closely related sugar specificity. Firstly, while the latter is inhibited by both D-mannose and D-glucose, the *E. coli* lectin is not inhibited by glucose. Secondly, Manα 1→2 Man is a considerably better inhibitor of concanavalin A than is methyl α-D-mannoside, whereas there is almost no difference between the inhibitory activity of this disaccharide and methyl α-D-mannoside on yeast agglutination by *E. coli*. Studies with additional oligosaccharides, as well as detailed examination of molecular models of the various inhibitory compounds, are necessary in order to obtain further insight into the structure of the combining site of the *E. coli* lectin.

The very good correlation found between the relative inhibitory power of the different sugars tested with isolated pili and intact bacteria shows that the pili are indeed responsible for the MS adherence of the bacteria to cells. The variation in the slopes for the different sugars tested may be accounted for by assuming that the bacterial pili or their subunits exist as a family of lectins (or isolectins) with closely related specificities.

EFFECT OF INHIBITORY SUGARS ON EXPERIMENTAL INFECTION

To evaluate directly the role of the MS bacterial lectins in initiating infection, Aronson et al.[18] have tested the possibility of preventing infection by inhibition of adherence. In these experiments, diuretic mice which are highly susceptible to urinary tract infection were used. Mannose specific *E. coli* isolated from cases of pyelonephritis in humans were injected into the bladders of mice. The results showed that when injection was in methyl α-D-mannoside (10%) there was a significant decrease (by a factor of 3) in the number of bacterium/mice in comparison with the control group. As expected, methyl α-D-glucoside (which is not an inhibitor of MS adherence) did not affect the number of infected animals. Inspection under the microscope of stained bladders of mice that were injected with *E. coli* in methyl α-D-mannoside, revealed a considerably lower number of adherent bacteria than control mice injected with bacteria in saline. It should be noted that exposure of the *E. coli* strains used in this study to a 20% solution of methyl α-D-mannoside at 37°C for 24 hours did not decrease their viabilty. When *Proteus mirabilis* was injected into the mice, instead of *E. coli*, no effect on the rate of infection was observed for either of the sugars tested, in accordance with the inability of these sugars to inhibit the adherence of *P. mirabilis* to epithelial cells.

In a subsequent study by Fader and Davis[19] MS piliated and nonpiliated organisms derived from a mannose-specific strain of *K. pneumoniae*, were injected into rat bladders. Nonpiliated bacteria, which were not MS, caused little infection. When the bacteria were injected in the presence of methyl α-D-mannoside (5%) a significant decrease in the number of infected rats was observed, whereas in the control group or when injection was in the presence of D-glucose, the majority of the animals became infected.

The role of MS adherence in the pathogenicity of infection was also assessed in a different system, by examining the effect of D-mannose on the ability of a virulent and piliated strain of *Shigella flexneri* to cause experimental keratoconjunctivitis in guinea pigs.[20] For this purpose albino guinea pigs were innoculated in the right eye with the bacteria suspended in D-mannose (2%) and in the left eye with the same number of bacteria in D-glucose (also 2%). Daily observations were then made for one week. A 3-4 fold decrease in the infectivity of the bacteria occurred in the presence of D-mannose, as compared to that of D-glucose.

These independent studies clearly demonstrate that MS lectins are a key factor in the ability of various MS bacteria to cause experimental infection, by mediating the attachment of the bacteria to the surfaces of the target tissues. It remains however to be seen whether inhibitors of lectin mediated adherence may also be useful in preventing natural infection.

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