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Investigation of the selectivity of maltoporin channels using mutant LamB proteins: mutations changing the maltodextrin binding site

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Wild-type and seven mutant maltoporins were purified and their channel-forming activities studied after reconstitution into black lipid membranes. The proteins were assayed for alterations at the maltodextrin binding site by measuring the sugar-dependent blockage of ion flux through these channels. Some substitutions (R8H, W74R) caused reduced channel affinity for all maltodextrins without changing single channel conductivities. The channel with a GlySer insertion after residue 9 was also poorly blocked by sugars but unique to this protein, the channel showed a striking, almost exponential increase of affinity with increasing maltodextrin chain length. In mutants with AspPro insertions after residues 79 and 183, there was an increase in affinity for glucose and maltose but not longer maltodextrins. The additional negative charge in the AspPro insertion mutants increased the cation selectivity of maltoporin channels, as did the decrease in positive charge resulting from the R8H substitution. A mutant with a W126C substitution also showed an increased affinity for glucose and maltose but reduced affinity for longer maltosaccharides. In contrast, a Y118F substitution resulted in an 8-fold increase in maltotriose affinity, but lesser improvements for other sugars. These results are interpreted to reflect changes in subsites contributing to an extended binding site within the channel, which in turn determines the overall sugar affinity of maltoporin.

Introduction

Maltoporin (or LamB protein) functions as a maltosaccharide facilitator in the outer membrane of *Escherichia coli* [1–6]. The protein contains a maltooligosaccharide binding site with increasing affinity for longer maltodextrins, up to and including starch polysaccharides [7]. Cell-sorting techniques capable of separating mutants with alterations in starch binding [8–10] have permitted the selection of maltoporin variants with sugar affinity changes and the identification of residues of the protein involved in affinity functions [11]. The residues identified as being important in maltodextrin binding are also completely conserved in two other recently identified sugar-specific porins with sequence relatedness to the LamB protein [12,13].

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A great deal of information is available on the topology of maltoporin of *E. coli*, from work using phages, proteases and antibodies as probes of structure [14–19]. Based on the topological data as well as sequence-based predictions, a detailed model maltoporin folding across the outer membrane has been derived [20]. This model has been tested and supported by more recent immunological and genetic evidence [12,18]. The binding sites for starch and Lambda have been further mapped by cysteine mutagenesis, and these studies also support the structural models and confirm the involvement of residues in sugar-specific functions [21,22].

Maltoporin reconstituted into vesicles or black lipid membranes is a malto-saccharide-selective pore protein [1,3,5]. To investigate the role of particular residues in transport specificity, pore selectivity in the variant maltoporins needs to be studied. A liposome swelling study with maltoporin mutants indicated some sugar-selective flux changes [23] but this earlier study was

only capable of measuring fluxes of sugars at high concentrations and limited data on sugar affinity and no data on ionic selectivity of mutant pores was obtained. The only mutants properly characterized were in the study by Dargent et al. [3], who studied mutants probably altered at the mouth of the channel rather than genuinely at the binding-site [11].

This paper examines the channel properties of four maltoporins altered by single amino acid substitution and three proteins with two amino acid insertions. The sites of substitution/insertion are shown in Fig. 1, superimposed on the model of folding of maltoporin across the outer membrane [20]. Three of the mutations were in proposed transmembrane segments of the protein. All but one of these mutations were shown to qualitatively affect maltodextrin binding in vivo (using an assay involving the maltoporin-dependent binding of bacteria to starch [8,21,24,25]. The precise affinity changes resulting from these mutations could not be quantitated previously. Using the sugar-specific blocking of maltoporin channels [1], this study permitted the determination of the association constants for malto- and non-maltosaccharides and hence a more precise analysis of the role of the residues at the maltodextin binding site in determining the sugar selectivity of maltoporin.

Materials and Methods

Protein purification

The source strains used for maltoporin purifications and their derivation have been previously described [10,21,23]. The protein from BW1042 (with an Arg8 → His, R8H substitution in maltoporin), BW1333 (Trp-74 → Arg or W74R) and BW1402 (Tyr-118 → Phe or Y118F) was extracted exactly as described in Nakae et al. [23]. The wild-type protein from BW1022 [8] as well as proteins from BW2646 (GlySer insertion after residue 9, or +GS(9)), BW2645 (AspPro insertion after residue 79, or +DP(79)), BW2655 (Trp-120 → Cys, or W120C) and BW2642 (AspPro insertion after residue 183, or +DP(183)) were purified to homogeneity by

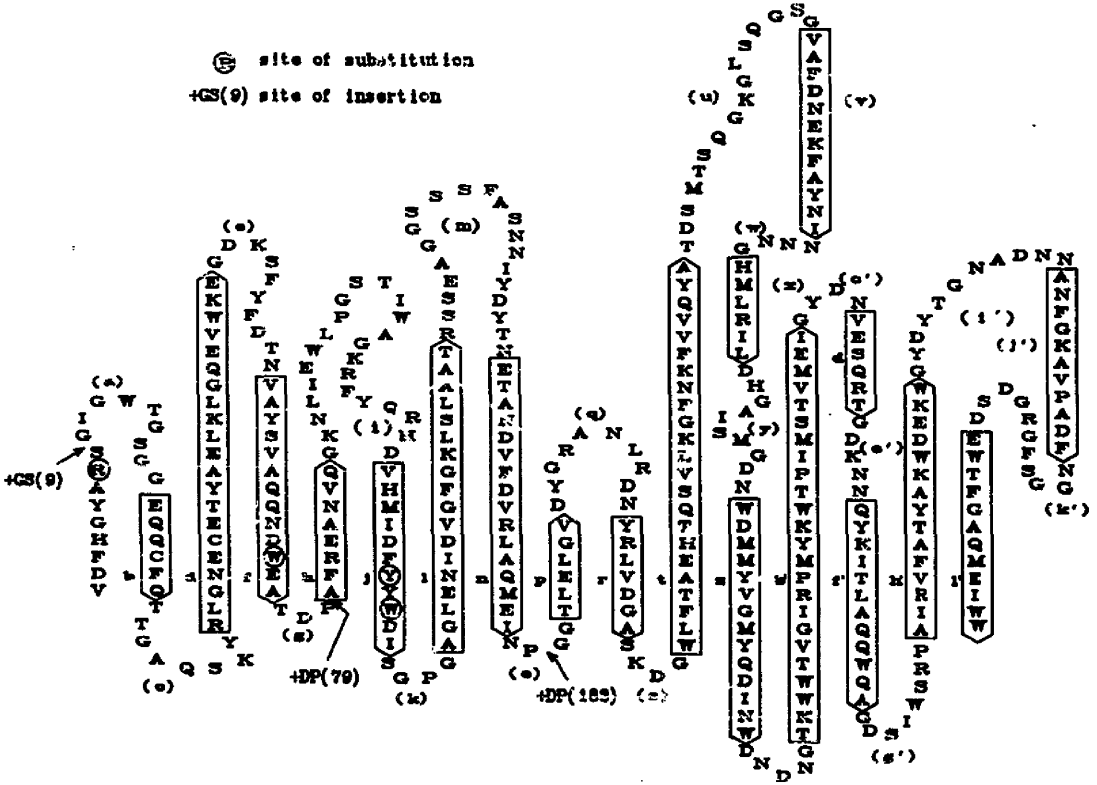

Fig. 1. A model of maltoporin folding across the outer membrane. The model derived in Ref. 20 is shown with the predicted structurally ordered segments boxed in the figure. The sites of substitution and insertion are shown by circled and arrowed residues, respectively. The segments with alternating unstructured and ordered sequences (a)–(l') are shown.

affinity chromatography on starch-Sepharose exactly as described by Francis et al. [22]. The maltose-eluted proteins were precipitated with isopropanol and redissolved and stored in a buffer containing 0.5% Triton X-100, 10 mM Tris-HCl, 5 mM EDTA and 5 mM PMSF (pH 7.2).

Black lipid membrane experiments

The method using black lipid membranes for the reconstitution of membrane proteins has been previously described [1,2]. The apparatus consisted of a Teflon cell with two compartments filled with salt solution; the wall between the two compartments had small circular holes with an area of either 2 mm^2 (for macroscopic conductance experiments) or 0.1 mm^2 (for single-channel experiments). A 1% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) in *n*-decane was painted over the holes to make the membrane. The incorporation of maltoporin into these membranes was as previously described [2], and was started after the lipid membrane had turned black as seen with incident illumination, indicating bilayer formation. The aqueous KCl solutions (Merck, Darmstadt, FRG) were used unbuffered and had a pH around 6. The temperature was kept at 25°C throughout. The different sugars used were from Sigma, St. Louis, MO, and added from concentrated stock solutions stored at 4°C prior to use.

The membrane current was measured with a pair of calomel electrodes switched in series with a voltage source and an electrometer (Keithley 602). In the case of single-channel recordings, the electrometer was replaced by a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded with a tape or a strip chart recorder. Zero-current membrane potential experiments were performed by establishing a salt gradient across membranes containing 100 to 1000 maltoporin channels as has been described earlier [2].

Results

Purification of mutant proteins

The mutant proteins tested in this study belong to two classes, those which still show considerable maltodextrin binding activity (i.e., the insertion mutants +DP(79) and +DP(183) and the Y118F and W120C mutants) and those with greatly reduced maltodextrin binding activity (the R8H and W74R mutants as well as the insertion +GS(9)). Wild-type protein and the first of these classes of mutant protein could be isolated to high purity by the affinity-chromatographic method recently described [21] since these mutants still bound the starch used as the affinity matrix. The R8H and

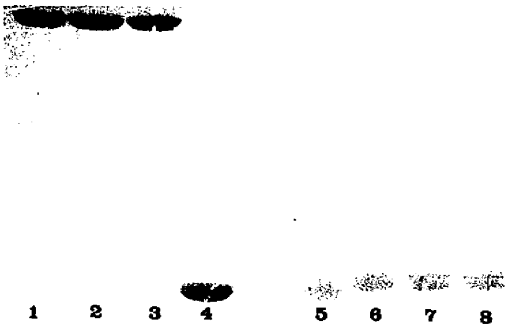

Fig. 2. Stability of maltoporins used in channel reconstitutions. Purified wild-type protein (tracks 1–4) and +GS(9) protein (tracks 5–8) were diluted in SDS-containing sample buffer [30] and electrophoresed in the acrylamide gel system of Laemmli et al. [30]. The proteins were applied to the gel directly at room temperature (tracks 1 and 5) or after heating for 5 min at 55° (tracks 2 and 6), 75° (tracks 3 and 7) or 100° (tracks 4 and 8).

W74R mutants could not be purified using this method due to low retention in these affinity columns; these proteins were obtained exactly as previously described in Ref. 23. Somewhat surprisingly, the protein with the +GS(9) insertion was retained by starch-Sepharose, even though bacteria with this protein show reduced affinities for maltose and maltohexaose [10]. An explanation of this retention is suggested below; this protein has an exponentially increasing affinity with increasing size of maltodextrin, and this presumably permitted the affinity for starch during purification.

The properties of the +GS(9) protein also differed from other maltoporins in that the stability of the trimeric form of the protein was reduced. As shown in Fig. 2, wild-type maltoporin (as well as all the other mutant protein proteins except from BW2646, not shown) was stable as the oligomer (trimer) in SDS at temperatures up to 75°C, but the protein with the GlySer insertion was dissociated into subunits even at room temperature in SDS. This is the most unstable maltoporin trimer yet reported. The reduced stability properties of this mutant were suspected from earlier studies which showed the temperature-sensitive expression of the protein in the outer membrane [10]; this is ascribable to thermolability at the higher temperatures. The reduced stability may also be responsible for the two closely migrating bands of the +GS(9) protein eluted from starch-Sepharose columns, one of which may be a proteolytic product of the unstable protein. The variable nature of the channels formed by this protein (see below) may also be due to trimer instability or the presence of the modified maltoporin in the mutant protein preparation.

Reconstitution of channels and single channel conductance experiments

The channel properties of each of the mutant proteins was tested in single channel experiments. These were performed by adding small amounts of the mutant proteins to 1 M KCl solutions bathing the black lipid membranes (final concentration of protein was 10 ng/ml for all mutants except +GS(9), which was added at 50 ng/ml). Step increases in conductance were observed shortly after addition of protein to one or both sides of the lipid bilayer membrane. The single-channel recording shown in Fig. 3A shows the conductance steps observed with the W120C protein. The average single-channel conductance of all mutants (except +GS(9)) was 140 to 160 pS under these conditions (1 M KCl). The +GS(9) mutant BW2646 formed irregular channels with a large variation (30–120 pS) of the single channel conductance (see Fig. 3B).

A histogram of the conductance steps obtained with protein from W120C mutant BW2655 (see Fig. 4) shows that there is a narrow range of step sizes in the LamB preparation. Hence the protein purified by affinity chromatography was homogeneous with respect to channel size and was not contaminated with the general diffusion porins OmpF and OmpC, confirming the purity of these preparations demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 1 and Ref. 22).

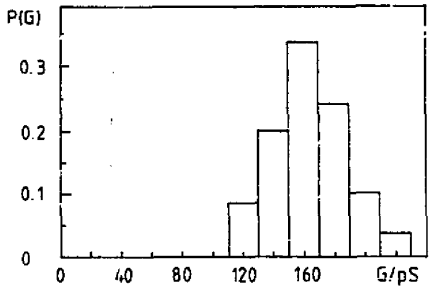

Fig. 4. Histogram of the conductance steps observed with diphytanoylphosphatidylcholine/*n*-decane membranes in the presence of 10 ng/ml LamB mutant W120C (BW2655). The average single-channel conductance was about 160 pS for 148 steps. The aqueous phase contained 1 M KCl, the temperature was 25°C: V_m = 50 mV.

The addition of 1 mM maltohexaose reduced ion flux through all mutant channels (see Fig. 1C), as it does LamB channels from wild-type protein from both *E. coli* [1,2] and *S. typhimurium* [26]. Also as demonstrated with lamB from *S. typhimurium* [26], the addition of sugar to the mutant proteins increased the current noise of the single-channel recordings (see Fig. 3C). However, in the proteins purified without affinity chromatography, there was a low level of residual channel activity after addition of maltodextrin due to

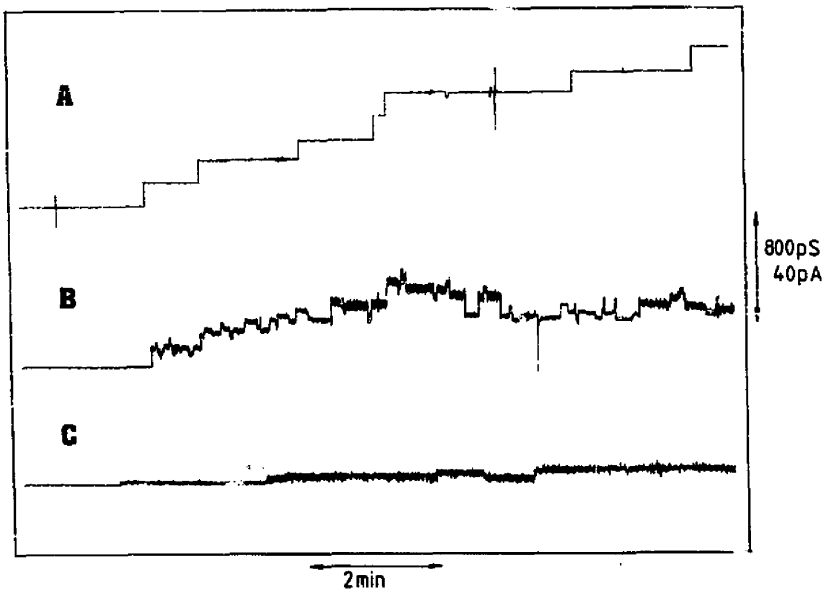

Fig. 3. Single-channel recordings of diphytanoylphosphatidylcholine/*n*-decane membranes after the addition of various LamB mutant proteins to the aqueous phase containing 1 M KCl (pH 6.0). The temperature was 25°C and the applied membrane potential, V_m, was 50 mV. The traces show conductivities of membranes with: (A) 10 ng/ml W120C protein (BW2655); (B) 50 ng/ml+GS(9) protein (BW2646), with irregular channels; (C) as (A), but in the presence of 1 mM maltohexaose. Note the absence of conductance steps in (C) and the increased current noise because of the association-dissociation reaction between binding site and sugar.

the non-specific large channels previously demonstrated in this kind of LamB preparation [1]. There was no difference in the properties of wild-type protein whether purified by affinity chromatrography or the older method (results not shown). Overall, these results suggested that some affinity for large maltosaccharide was retained in mutant proteins, and the affinities for a range of sugars was further investigated in the macroscopic conductance experiments below.

Ion selectivity measurements

The wild-type maltoporin channel is cation selective. To study the influence of the mutations on ion selectivity, we measured the zero-current membrane potential in the presence of salt gradients. After incorporation of 100–1000 channels into the membrane, the KCl concentration on one side of the membrane was raised in small steps from 10 to 100 mM. For each gradient, the zero current potential was measured, and the permeability ratio P_{cation}/P_{anion} was calculated according to the Goldman-Hodgkin-Katz equation [1,2]. The results are summarized in Table I for those mutants that did not have contamination with large, non-specific channels. The values are the means of at least three membranes at each experimental condition. For all the mutants shown, the more dilute side was positive which indicated a preferential movement of cations through the channel. The wild-type, +GS(9) and W120C proteins showed little significant difference, but the increased cation selectivity of the +DP(79) and +DP(183) proteins was presumably due to the additional negatively charged amino acid in these latter two mutants. The R8H substitution also increased cation selectivity, hence removal of a strongly positively-charged residue at pH 6 was equivalent to the introduction of a negative charge in the DP insertion mutants.

TABLE I

Zero-current membrane potentials of mutant maltoporins

Mutant	V_m (mV) [a]	P_c/P_a [b]
W.T.	44	12
BW1042(R8H)	48	19
BW2646 (+GS, 9)	45	13
BW2645 (+DP, 79)	47	17
BW2655 (W120C)	44	12
BW2642 (+DP, 183)	49	20

[a] The V_m, or zero-membrane potential, was measured in diphytanoyl-phosphatidylcholine/*n*-decane membranes in the presence of different LamB mutants, measured for a 10-fold gradient of KCl.

[b] The P_c/P_a is the permeability ratio for cations/anions and was calculated from the Goldman-Hodgkin-Katz equation as previously described [1].

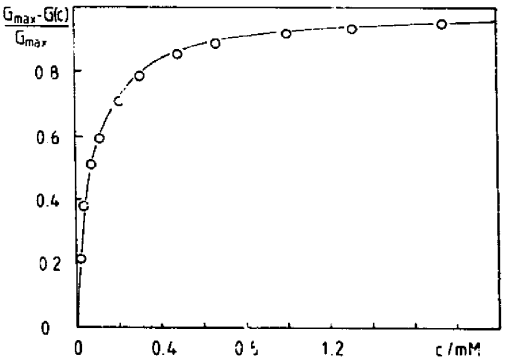

Fig. 5. Plot of the relative conductance inhibition $(G_{max}-G(c))/G_{max}$ as a function of the maltohexaose concentration in the aqueous phase. The data were derived from a titration experiment in which the membrane conductance ($G(c)$) mediated by the LamB mutant W120C (BW2655) was measured as a function of increasing concentrations of maltohexaose. The membrane was formed from diphytanoylphosphatidylcholine/*n*-decane in an aqueous solution containing 1 M KCl and 100 ng/ml mutant protein. The solid line was drawn with Eqn. 7 of Ref. 2, corresponding to a stability constant of sugar binding of 110000 M^{-1} or a half-saturation constant of 90 μM.

Binding affinity of sugars to maltoporin variants

The single-channel data suggested the binding of maltooligosaccharides to the LamB mutants. Macroscopic conductance experiments were performed to determine the stability constants of sugar binding. The experiments were performed in the following way. The mutant proteins were added to the black lipid membrane in concentrations between 100 ng/ml (wild-type and mutants other than +GS(9)) and 500 ng/ml (for +GS(9)). The macroscopic conductance was allowed to reach its maximum after addition of the proteins (within 30 to 40 min). At this point, increasing concentrations of sugar was added to the 1 M KCl solutions on both sides of the membrane. There was an increasing inhibition of macroscopic conductance with increasing concentrations of sugar. Fig. 5 shows an experiment of this type with BW2655 (W120C) and maltohexaose. The addition of maltohexaose up to 1.75 mM resulted in an almost complete inhibition of the membrane conductance (see Fig. 5). Assuming that the two-barrier, one-site model of maltoporin channels is valid [2], then the sugar-induced blockage of ion current through the mutant proteins can be used for the evaluation of the stability constant K as previously described [2]. The half-saturation constant $K_S(=1/K)$ can be obtained from a plot of sugar binding as a function of sugar concentration as given in Fig. 5 or from a Lineweaver-Burk plot of the same data. Using this formalism, a half-saturation constant (K_S) of 90 μM can be calculated from the inhibition curve for the W120C protein permeability by maltohexaose.

TABLE II

Stability constant, K, for the binding of sugar to wild-type and mutant maltoporins

Sugar	Mutant proteins [a]							
	W.T.	BW1042 R8H	BW2646 +GS(9) [b]	BW1333 W74R	BW2645 +DP(79)	BW1402 Y118F	BW2655 W120C	BW2642 +DP(183)
Glucose	8	8	< 1	5	31	20	17	21
Maltose	110	40	10	52	220	660	240	230
Maltotriose	2800	470	900	440	4700	23000	1900	3600
Maltotetraose	9000	2100	1500	–	9600	–	6000	7500
Maltopentaose	14000	2900	2800	3300	20000	19000	7700	13000
Maltohexaose	16000	2700	4000	3600	19000	23000	10000	22000
Maltoheptaose	17000	2700	7000	5000	25000	27000	13000	20000
Lactose	22	5	< 5	< 5	16	10	19	38
Sucrose	60	13	< 5	6	110	36	170	170

[a] The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The unbuffered aqueous solutions (pH around 6) contained 1 M KCl and 100 ng/ml purified maltoporin except for BW2646 protein, which was added at 500 ng/ml; $T = 25°C$; $V_m = 20$ mV. The stability constant K was calculated from the mean of at least three experiments from data similar to that shown in Fig. 5.

[b] The substitutions or insertions are indicated using the one-letter amino acid code. The residue numbers in brackets show the position of amino acid insertions.

Similarly, half-saturation constants and stability constants were calculated for maltosaccharides between maltose and maltoheptaose and three non-maltosaccharides. These constants are listed in Table II for wild-type and all purified mutant proteins. As was expected from the properties of these proteins in vivo, the affinity of maltoporin was modified in the mutant channels; both increases as well as reductions in affinity were observed for some ligands, in line with qualitative data indicating binding changes in starch-Sepharose binding assays [8,10,21,24].

Saturation of the maltodextrin binding site with larger oligosaccharide

The changes in the association constants of each mutant for maltodextrins was further analyzed in two

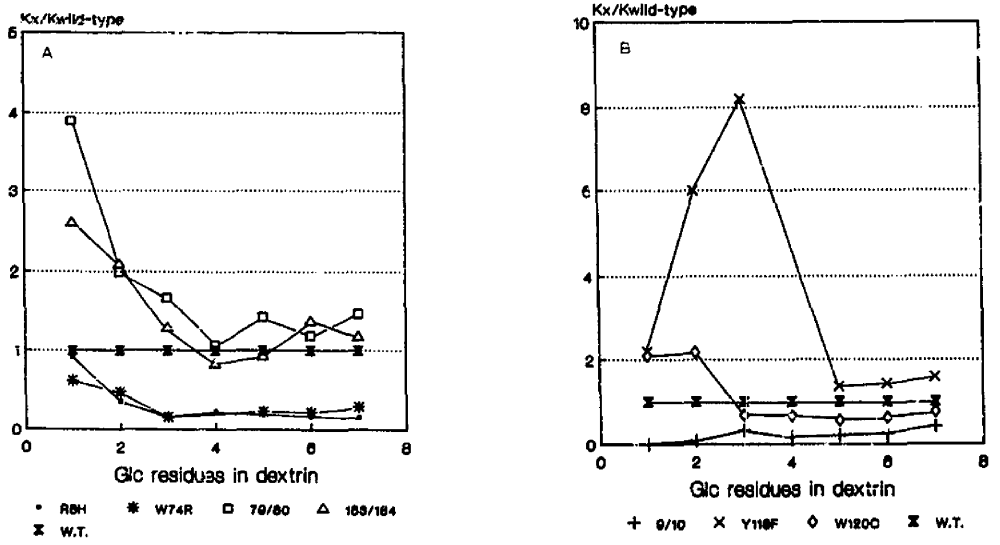

Fig. 6. Plots of the affinities of mutant proteins relative to the that of the wild-type for different maltosaccharide substrates. Using the data in Table II, the stability constant of a mutant (K_x) was plotted relative to that of the wild type for sugars between glucose and maltoheptaose, with the wild-type affinity for each sugar taken as unity in this plot. In Fig. 6(A), the proteins from BW1042 (R8H, ■), BW1333 (W74R, •), BW2645 (+DP(79), □) and BW2642 (+DP(183), △) are compared and in Fig. 6(B), the mutants BW1402 (Y118F, ×), BW2655 (W120C, ◇) and BW2646 (+GS(9), +) are shown.

ways. Firstly, Fig. 6 shows the changes in binding affinity for each ligand relative to wild-type for the same sugar (in plots of $K_x/K_{w.t.}$ versus maltosaccharide chain length). From this analysis, it is clear that the sequence changes in the mutant proteins can differentially affect affinity for particular substrates. Compared to wild-type, the mutants R8H and W74R as well as the insertion mutant +GS(9) showed reduced affinities for maltosaccharides of all sizes tested, as well as for non-maltosaccharides lactose and sucrose (Table II). Yet the changes to the binding sites were not identical in these mutants, as the defect in binding was greatest for large sugars in R8H but for small sugars in BW2646 protein (the +GS(9) insertion mutant). In all of these mutants, there were definite trends in changes of affinity with chain length of ligand, as shown in Fig. 6. This also holds true in mutants that showed an increase in affinity selectively for some maltodextrins; in mutants with AspPro insertions after residues 79 and 183, there was a significant increase in affinity for glucose and maltose but not longer maltodextrins. The progressive nature of this change with chain length is clear from Fig. 6A. Fig. 6B also illustrates the chain-length-dependent increase in affinity in the Y118F mutant. The major difference in the Y118F binding site compared to wild-type was a particularly large increase in affinity for dextrins close to maltotriose in size.

The stability constant of the maltodextrin binding site in wild-type channels saturates with increasing chain length. Hence there is normally only a marginal increase in affinity for maltoheptaose as against maltohexaose, even though there is more than a 150-fold increase in affinity between maltose and maltopentaose [2,7]. This point is illustrated in Fig. 7, in which the stability constant of a protein for a particular maltodextrin is compared to the stability constant of the same protein for maltose. This approach, of plotting $K_X/K_{maltose}$ versus chain length, highlights the relative changes in the saturation of the mutant maltodextrin binding sites with increasing chain length.

As with wild-type protein, in most mutants the increase in affinity with chain length begins to saturate with maltohexaose and maltoheptaose (Fig. 7A). In the Y118F mutant, this process already occurs with maltotriose reinforcing that the largest increase in affinity occurs for shorter dextrins in this mutant. Consequently, there is only a 40-fold increase in affinity between maltose and maltoheptaose. The channel with the R8H substitution is another example of a protein that is already saturated with maltopentaose, although in this case with a much lower overall affinity than wild-type. In extreme contrast, the BW2646 (+GS(9)) protein shows a large, near-exponential increase in affinity with increasing chain length and there was a 700-fold increase in binding constants between maltose and maltoheptaose. This is clearly evident from Fig. 7B, despite the result that the overall affinity of the protein is down compared to wild-type, even for maltoheptaose, as shown in Fig. 6.

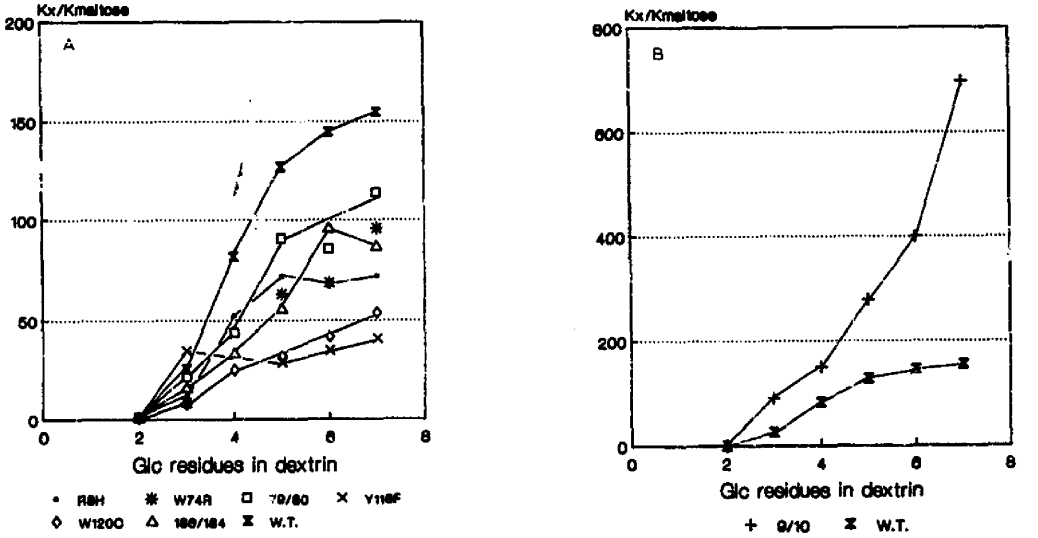

Fig. 7. Plots of the affinities of mutant proteins for longer maltosaccharides relative to that for maltose. As a measure of the increasing affinity of maltoporin with increasing chain length, the stability constant of mutants for maltosaccharide (K_x) was compared relative to the stability constant for maltose in the same mutant. In part (A), proteins derived from wild-type strain BW1022 and the mutants BW1042 (R8H, ■), BW1333 (W74R, *), BW2645 (+DP(79), □), BW1402 (Y118F, ×), BW2642 (+DP(183), △) and BW2655 (W120C, ◇) are compared and in Fig. 7(B), the mutant BW2646 (+GS(9), +) and wild-type protein (×) are shown.

Discussion

The residual sugar affinity of the channels discussed in this paper permitted quantitation of the binding changes inside the channel. Adoption of the sugar-dependent blockage of channels as the sugar binding assay made it feasible to quantitate the stability constants of the maltodextrin binding site even in mutants that had a large reduction in affinity in FITC-amylopectin binding assays; these latter assays were too insensitive to measure stability constants when the affinity for amylopectin or longer maltosaccharides dropped by a factor of 5–10 [8].

Residue changes in the 8–10 region of maltoporin significantly reduce the sugar affinity of channels. The amino acid substitution R8H reduced affinities for both malto- and non-maltosaccharides without changing the single channel conductivity of channels, hence it is unlikely that the affinity changes arise from a change in pore size. The mutation, which also did not affect other properties of maltoporin (antigenic or phage binding), is therefore highly specific for the sugar binding site.

The insertion of two residues near residue 8 (between residues 9 and 10) also drastically affected channel function. In addition, the insertion strongly affected the trimer stability of the protein and resulted in variable single-channel conductivities, so the mutation was more non-specific. It may be that as in the *Rhodobacter* porin structure [27], this N-terminal portion is significant in completing the β-barrel structure of maltoporin. But the most interesting characteristic of this mutation is the change in saturability of the binding site with increasing chain length of dextrin; this protein is unique in not being 'saturated' even with maltoheptaose. The simplest explanation of this property is that an extended binding site is so distorted that residues contributing to the binding sites are moved apart by the insertion and only reached by long dextrins.

In the four mutants with increased affinity for smaller sugars (with W120C, Y118F substitutions and DP insertions at 79 and 183) the possible explanation is a displacement in the binding site bringing subsites closer, better fitting shorter maltosaccharides. Alternatively, a subsite fitting a monosaccharide unit in the substrates is improved, and this notion is supported by the increase in affinity for glucose in all four mutants.

The ion selectivity of maltoporin channels was shifted even further towards a preference for cations in the three proteins, but not in mutants with amino acid changes involving neutral substitutions or insertions. The R8H substitution as well as two negatively charged AspPro insertions near residues 80 and 183 increased cation selectivity. The sites at 80 and 183 are postulated to be in tight turns on the periplasmic side of the membrane in Fig. 1. It is not necessarily the case that ion selectivity was shifted because these sites are directly in the channel, as the additional negative charge may be delocalized and influence ion conductivity at a distance [29].

Overall, these results are consistent with current views of models of the role of the maltodextrin binding site in channel function. A clearer view of the changes caused by these mutants will emerge once the crystal structure is solved for maltoporin [28]; perhaps the mutations investigated in this paper influence an 'eyelet' type structure in the channel found in the *Rhodobacter* porin [27].

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