Vol. 121, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1984 Pages 34-40

AFFINITY ENGINEERING OF MALTOPORIN: VARIANTS WITH ENHANCED AFFINITY FOR PARTICULAR LIGANDS

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Received April 13, 1984

SUMMARY Affinity-chromatographic selection on immobilized starch was used to selectively enhance the affinity of the maltodextrin-specific pore protein (maltoporin, LamB protein, or λ receptor protein) in the outer membrane of E. coli. Selection strategies were established for rare bacteria in large populations producing maltoporin variants with enhanced affinities for both starch and maltose, for starch but not maltose and for maltose but not starch. Three classes of lamB mutants with up to eight-fold increase in affinity for particular ligands were isolated. These mutants provide a unique range of modifications in the specificity of a transport protein.

Maltoporin (or λ receptor protein; LamB protein) is involved in the transport of maltodextrins across the outer membrane of E. coli (1-4) and has a binding specificity and transport selectivity for ligands containing linear α, 1-4 linked glucose residues (5,6). The binding affinity of the protein increases with increasing size of ligand, with a K_D of 15 mM for maltose but approximately a hundred-fold higher affinity for starch polysaccharides (5). Genetic modification of the affinity of maltoporin should provide a useful system to probe the molecular basis of transport specificity. Towards this end, this study describes the selection of unique mutants with increased affinities for maltodextrin.

The basis for these selections was the finding that E. coli cells exhibit binding to covalently immobilized starch which is maltoporin dependent and which is optimal at defined concentration of immobilized starch (7) and at neutral pH (8). Wild-type bacteria bound to starch-Sepharose can be eluted by competing ligands wit an efficiency corresponding to their respective affinities: this means in practice that high concentrations of maltose are required to elute bacteria bound to starch

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0006-291X/84 $1.50

under optimal conditions (7). These properties have permitted the establishment of an affinity chromatographic system whereby separation of bacteria is on the basis of maltoporin function (9). In this study, we used affinity-chromatographic selections in the isolation of unique lamB mutants with (a) enhanced affinities for all ligands, (b) enhanced affinity for starch relative to maltose and (c) enhanced affinity for maltose relative to starch.

MATERIALS AND METHODS

Strains and Growth Conditions The Escherichia coli K12 strains and the bacterial culture conditions were previously described (7).

Affinity Chromatography of Bacterial Populations The affinity matrix, starch-Sepharose, was synthesized as previously described (7). The standard starch-Sepharose used in these studies contained 6.4 mg immobilized starch in 1 ml packed volume matrix. Low-starch Sepharose was synthesized as in (7), with the modification that the starch concentration in the coupling step was reduced to 20 mg/ml, resulting in material containing 0.46 mg immobilized starch in 1 ml matrix. The preparation and chromatography of bacteria was under the general conditions previously described (7) and the specific selective conditions were as defined in the text.

Estimation of the Affinity of Maltoporin for Ligands The affinity of maltoporin for maltose (Merck, biochemical grade) maltohexaose (Boehringer) and amylopectin (Sigma) was estimated in binding competition experiments with fluorescein-labelled amylopectin (5). The concentration of fluorescent ligand was 4 µg/ml in the standard assay (5).

RESULTS AND DISCUSSION

To isolate LamB variants with increased affinity for all ligands, rare spontaneous mutants in a population were selected that bind to starch-Sepharose better than wild-type under suboptimal conditions and are eluted by subsaturating concentrations of maltose. As shown in Fig.1a wild-type E. coli populations with fully induced levels of maltoporin were incapable of binding to starch-Sepharose columns containing 0.46 mg/ml immobilized starch when the bacteria were applied and eluted with buffer at pH 10.2. This is in contrast to binding under optimal conditions (6.4 mg/ml immobilized starch, pH 6.8, Fig.1e) when wild-type bacteria were chromatographically retained. Less than 0.5% of an applied wild-type population containing 2×10^9 bacteria was eluted by buffer containing low or high concentrations of competing maltose at the high pH (Fig.1a). Spontaneous mutants producing LamB variants with high affinity for both starch and maltose would be expected to be both better retained under these conditions and to be eluted by 5 mM maltose. The few bacteria (<0.5%) present in the pH 10.2, 5 mM maltose fraction

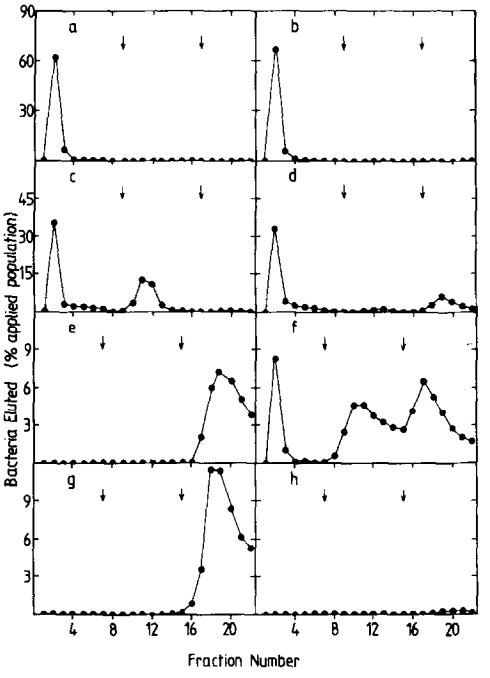

Figure 1: Elution properties of Escherichia coli populations subjected to affinity selections.

The preparation of bacteria for chromatography on starch-Sepharose was under the conditions previously described (7). In experiments (a)-(d), chromatography of bacteria was under conditions non-optimal for maltoporin-dependent binding of the affinity matrix. The starch content of the 1 ml columns was 0.46 mg and the columns were equilibrated with 25 mM bicarbonate buffer, pH 10.2. Columns (e)-(h) were run under optimal conditions for binding and contained 6.4 mg starch in a 1 ml column and the equilibration buffer was a phosphate-based salts medium without carbon source, minimal medium A (14), pH 6.8. Each column was loaded with 2 x 10^9 bacteria and eluted with the equilibration buffer. Bacteria in 0.3 ml fractions were determined from absorbance (A_{580}) measurements. At the points indicated, elution was changed to the same medium containing 5 mM and 100 mM maltose respectively.

Columns (a) and (e) were loaded with maltose-grown E. coli wild-type for lamB, strain BW1022 (7); (b) and (f) maltose-grown BW1330, selected for high affinity for maltose but not starch; (c) and (g) maltose-grown BW1400, selected for high affinity for both starch and maltose; (d) and (h) maltose-grown BW1500, selected for high affinity for starch but not maltose.

were therefore isolated, grown up on maltose and enriched by further rounds of chromatography under the same conditions. After passage of 2 x 10^9 bacteria through each of 23 consecutive rounds of chromatography, the 5 mM maltose fraction increased to 20-25% of the applied population. A single colony isolate from this population, BW1400, exhibited the elution patterns shown in Fig.1c and 1g, with a clear low-maltose peak at pH 10.2. The enhanced retention in this isolate was due to a mutation in lamB, since transfer of lamB by P1 transduction into a Mal^- recipient (pop 1730) with a partial deletion of lamB (MBΔ17) (10) resulted in all Mal^+

Table 1

Affinity of isolates for ligands of maltoporin

	Amylopectin (µg/ml)		Maltohexaose (mM)	Maltose (mM)	
	pH 6.8	pH 10.2	pH 6.8	pH 6.8	pH 10.2
BW 1022 (wild-type)	400	310	1.6	19	16
BW 1400	65	75	0.20	7.8	2.2
BW 1500	50	55	0.45	40	19

The values quoted are the concentrations of ligands giving a 50% inhibition of LamB-dependent binding of fluorescein-isothiocyanate-coupled amylopectin (5). The maltohexaose affinity of this wild-type strain WB 1022 was reproducibly lower than the affinity measured previously for strain pop6432 (2).

transductants with chromatographic properties of BW1400 and not the wild-type binding behaviour. The ligand affinities of this LamB variant were tested by the fluorescent ligand binding assay (5) and are shown in Table 1. In comparison to wild-type, an increased affinity was found for all ligands at pH 6.8, and the enhanced binding at high pH was not due to a shift in the pH optimum of the mutant. Clearly, the strategy yielded a lamB-variant with the properties specified by the selection. Interestingly, as shown in Table 1, the low binding of wild-type LamB to starch-Sepharose at pH 10.2 (8) and (Fig.1a) was not due to a lower affinity of the protein than at pH 6.8, but due to a lower number of available sites per bacterium (unpublished observations). The nature of this reversible inactivation of maltoporin binding at high pH is not known.

The second strategy, used to isolate variants with high affinity for large ligands but not maltose, was essentially as above except the maltose concentration used to elute bacteria from low-starch Sepharose columns was increased to 0.1M. At this higher concentration, the competing smaller ligand would be expected to elute bacteria with the low, wild-type affinity for maltose (Table 1). Again subjecting 2×10^9 bacteria to consecutive rounds of enrichment, it took only 5 cycles of chromatography to obtain a population from which approximately 20% of the bacteria could bind low-starch Sepharose at pH 10 and could be eluted by 0.1 M (but not 5 mM) maltose. An isolate from this population, BW1500, was also altered in lamB, as

analyzed by transductional mapping as described above. The elution pattern of this isolate (Fig.1d and 1h) and its binding affinities (Table 1) were also investigated. Both results are consistent with an increase in affinity for starch and amylopectin, as well as for the intermediate ligand maltohexaose. In contrast, the affinity of this mutant for maltose at pH 6.8 was actually reduced but was also consistent with the reduced elution by maltose from starch columns at neutral pH (Fig.1h). The properties of BW1500 also closely correspond to the type of mutant specified by the selection strategy.

To modify the affinity of maltoporin for small but not large ligands, the affinity-chromatographic selection was carried out at a saturating column concentration of immobilized starch and at neutral pH. Since wild-type bacteria are efficiently retained under these conditions (Fig.1e), there was no preselection for enhanced starch affinity. However enhanced affinity for maltose was selected through elution of bacteria bound under these optimal conditions by low 5 mM maltose which was insufficient to elute wild-type bacteria at pH 6.8 (Fig.1e). Less than 1% of applied wild-type bacteria were eluted by 5 mM maltose in such a selection and these bacteria were also collected and subjected to 27 consecutive cycles of chromatographic enrichment, each time collecting bacteria eluted by 5 mM maltose. After these cycles of selection, 15% of the population could be eluted by low maltose. The elution properties of an isolate from this population is shown in Fig.1b and 1f. This strain, BW1330, was actually less well retained by starch-Sepharose than wild-type E. coli at neutral pH suggesting that the mutant had a partial reduction in affinity for starch. This was confirmed in binding assays, in which BW1330 bound less than 15% of the fluorescently labelled amylopectin ligand bound by wild-type bacteria in the standard binding assay (5). Since quantitation of affinities depends on the competition for amylopectin binding, this made it impossible to analyze accurately the affinities of this isolate. However, the elution of BW1330 by low maltose was not due solely to the partial reduction in starch affinity. Other mutants previously isolated with comparable reductions in starch affinity, such as BM101, were not eluted by 5 mM but were eluted by 0.1 M maltose(7) (and unpublished observations). Secondly, BW1330 carried at least a double mutation in

lamB, since transfer of the lamB region into pop1730 resulted in two classes of recombinant. The large majority of transductants had the same properties as BW1330, but 2 out of 24 tested were not eluted by 5 mM maltose but were still partially impaired in starch-binding activity. Hence the two alterations in affinity could be genetically separated and suggests that a second mutation was necessary for the enhanced response to low maltose concentrations. It may be speculated that the enrichment of BW1330 was a result of the conditions in the selection being too optimal in favour of starch to permit isolation of a mutant with enhanced maltose affinity but without a reduction in starch affinity.

The three isolates from these selections produced normal amounts of maltoporin, as checked by quantitative electrophoresis of outer membrane proteins (11). Each of the mutants also retained the surface interaction site for phage lambda, since all three strains were still sensitive to λ_{vir} (12). Hence the lamB alterations selected specifically affected the maltodextrin binding site of the protein.

The isolation of these mutants illustrates the exquisite specificity of affinity selection as a protein engineering technique and the responsiveness of the enrichments to specified changes in chromatographic conditions. The strategies successful in modifying the LamB protein should be readily applicable to the engineering of other bacterial surface proteins as considered elsewhere (9). The flexibility of selection strategies is confirmed not only by the isolation of the lamB mutants with enhanced affinity but also by the previous isolation of mutants with partial or total reductions in maltodextrin affinity (7) and the positive selection of isolates with enhanced LamB expression (11). The mutants now isolated provide a unique range of binding site alterations of the LamB protein in further studies aimed at determining the specificity and pore structure of this transport protein as well as in identification of the residues in the lamB sequence (13) involved in maltodextrin binding.

ACKNOWLEDGEMENT

This work was supported by the Australian Research Grants Scheme.

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