Characterization of TreR, the Major Regulator of the Escherichia coli Trehalose System*

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The pathway of trehalose utilization in Escherichia coli is different at low and high osmolarity. The low osmolarity system takes up trehalose as trehalose 6-phosphate which is hydrolyzed to glucose and glucose 6-phosphate. treB and treC, the genes for the enzymes involved, form an operon that is controlled by TreR (encoded by treR), the repressor of the system, for which trehalose 6-phosphate is the inducer. We have cloned and sequenced treR. The protein contains 315 amino acids with a molecular weight of 34,508. TreR was purified and shown to bind as a dimer trehalose 6-phosphate and trehalose with a $K_d$ of 10 and 280 $\mu M$, respectively. The conformations of the protein differ from each other with either one or the other substrate-bound. Protease treatment removed the DNA-binding domain from the intact protein leaving the dimerization domain (a 29-kDa carboxy-terminal fragment) intact. Nuclease protection experiments revealed a palindromic sequence located directly upstream of the −35 promoter sequence of treB that functions as the operator of the system.

The nonreducing disaccharide trehalose (α-D-glucosyl-1→1-α-D-glucoside) is known to maintain the fluidity of membranes under conditions of dryness and desiccation (1) as well as to stabilize enzymes, foods, pharmaceuticals, and cosmetics even at high temperatures (2). Escherichia coli synthesizes trehalose when exposed to high osmolarity (3–5). The synthesis of trehalose requires the enzymes trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, encoded by otsA and otsB, respectively (6, 7). otsB and otsA form an operon (8) that is induced at high osmolarity and during entry into stationary phase. The induction is dependent on RpoS, which is the alternative $\sigma$ factor for stationary phase (9). Trehalose itself is not involved in the regulation of the operon. The accumulated trehalose is broken down to glucose by the cytoplasmic trehalase (TreF) (10), ensuring a continuous turnover of the osmoprotectant.

E. coli is also able to use trehalose as the sole carbon source at low and high osmolarity. The problem of the simultaneous degradation and synthesis of trehalose at high osmolarity is solved by separating the two metabolic pathways. The synthesizing enzymes are located in the cytoplasm, and the degradative enzymes encoded by treA are located in the periplasm. TreA (11, 12) hydrolyzes trehalose to glucose, which is subsequently taken up by the enzyme II of the phosphotransferase system specific for glucose (13). Mutants lacking treA can no longer grow on trehalose as the sole carbon source at high osmolarity, but they still grow at low osmolarity (11). This is due to a second system for trehalose degradation that is only expressed at low osmolarity. This system is composed of a phosphotransferase system enzyme II specific for trehalose (EiICB) encoded by treB), which together with EIHA transports trehalose as trehalose 6-phosphate to the cytoplasm (14–15). The cytoplasmic trehalose-6-phosphate hydrolase (encoded by treC) splits the accumulating trehalose 6-phosphate to glucose and glucose 6-phosphate (16). treB and treC form an operon at 96.5 min on the chromosome with treB as the promoter-proximal gene (15). The treB/C operon is induced by trehalose 6-phosphate and dependent on the cAMP catabolite gene activation protein (CAP), therefore it is subject to glucose-mediated catabolite repression (13). Trehalose 6-phosphate is thus a central metabolite being the crossing point between the synthesizing and degradative pathways for trehalose. To prevent the cells from futile cycles a proper regulation of both pathways is necessary.

Recently, we have described an open reading frame directly upstream of treB (with the same direction of transcription as the treB/C operon). This open reading frame, called treR, encodes the regulator of the system (15). Here we present the molecular characterization of treR and its encoded protein TreR (the trehalose repressor). We show that TreR is able to bind DNA at a palindromic sequence located upstream of the −35 region of the treB promoter. We show that TreR binds trehalose 6-phosphate as well as trehalose but that only the binding of trehalose 6-phosphate results in a reduction of the operator binding affinity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—Bacterial strains used in this study are described in Table I. Pl transductions (17) were used for strain construction. Cultures were grown in Luria broth (LB). Growth was monitored by measuring the optical density at 578 nm ($A_{578}$).

Construction of Plasmids—Standard DNA methods were used (18). Cloning of treR and construction of pHo500 was described previously (15). Digestion of pHo500 with SspI/PstI and cloning of the 1162-bp treR-carrying fragment into pBR322 that was opened with the same restriction enzymes yielded plasmid pHo501. To obtain a fragment containing the promoter of treR, plasmid pHo500 was digested with SspI/SmaI. The resulting 198-bp fragment was ligated into promoter vector pTAC3575 (lacZ, phaA) (19) opened with SmaI, yielding plasmid pTACpcreR. The orientation of the insert was confirmed by restriction analysis to be the promoter of treR, which was controlling the transcription of lacZ. To overexpress TreR, the coding sequence of treR was amplified by polymerase chain reaction from plasmid pHo500 using two primers: treR NoEI, carrying a NoEI site at the ATG translation start (Fig. 1, Table II), and treR EcoRI, downstream of the

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1 The abbreviations used are: CAP, cAMP catabolite gene activation protein; PAG, polyanhydride gel electrophoresis; LB, Luria broth; bp, base pair(s).
open reading frame (Fig. 1, Table I). The amplified DNA was digested with NdeI and EcoRI and ligated into pCYTEXP1 digested with the same enzymes, yielding plasmid pHoTreR. The cloned sequence was verified by sequencing.

**Sequence of treR and Sequencing Strategy**—The sequence of *treR* was determined from plasmid pHo500 by the dideoxy chain termination method of Sanger et al. (20) with the modification of Biggin et al. (21) using customized sequencing primers 209/1–5 and 209/k from MWG (Gesellschaft für Angewandte Biotechnologie GmbH, Ebersberg, Germany) and the Sequenase kit version 2.0 (Amersham). The primer sequences are shown in Table II, and their positions are indicated in Fig. 1. [32P]dATP was purchased from Amersham International. Sequence reaction products were separated on a 4% sequencing gel containing urea according to the manufacturer’s manual (Pharmacia Biotech Inc.). The DNA sequence and the deduced amino acid sequence were identical to another sequence submitted to GenBank™ later (accession number U14003) (22). Sequence homologies were found by querying the latest releases of all available data bases using the BLAST server at the National Center for Biotechnology, Bethesda, MD (23). Protein homology comparisons were done with MegAlign (DNA Star Inc.).

**Determination of Transcriptional Start Point**—The mRNA start point was mapped by the reverse transcriptase method of primer extension according to Ref. 24 using 20–50 µg of total cellular RNA and 5 pmol of primer 209/3 (Table II). The sequence reaction was done with the same primer.

**Purification of TreR—**Strain SF120 (25) was transformed with plasmid pHoTreR and grown at 28 °C in 1 liter of LB containing ampicillin (100 µg/ml). The nucleic acid remaining in the supernatant was precipitated with 60% saturated ammonium sulfate and stored in precipitated form at −20 °C. During purification the different fractions were analyzed by SDS-PAGE using 15% polyacrylamide (26).

**Gel Filtration of Purified TreR—**100 µl of TreR (0.5 mg/ml) in buffer A was applied on a Superdex 200 column (Pharmacia). Trehalose 6-phosphate or trehalose, when present, had a concentration of 10 mM. Elution was with the same buffer and done at a flow rate of 0.3 ml/h. The marker proteins chymotrypsin (25 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), and dimer of bovine serum albumin (132 kDa) were analyzed in separate runs using identical conditions. The molecular weights were plotted half-logarithmically against their elution volume forming a straight line from which the molecular weight of TreR was interpolated.

**Proteolysis of TreR—**5 µg of TreR at 2 mg/ml was incubated at 37 °C with different concentrations of thermolysin in buffer A supplemented with 4 mM CaCl₂. After 2 h an excess of EDTA (6 mM) was added, and the products were analyzed on a 15% SDS-PAGE gel (26).

**Gel Retardation Assay—**4 µg of plasmid pHo400 was digested with AvoI and NeoI. After heat inactivation of the enzymes, the restricted sites were filled up with 4 units of Klenow enzyme using 10 µCi of [32P]dATP and an excess of the other unlabeled nucleotides (2 mM each). After heat denaturation, the labeled DNA was diluted (1/100) and incubated with different concentrations of purified TreR in buffer A containing bovine serum albumin (0.1 mg/ml). As indicated, the sugars trehalose and trehalose 6-phosphate were added to a final concentration of 3 µM. The mixture was separated on a 4% acrylamide gel (18) and subjected to autoradiography.

**DNase I Footprinting—**4 pmol of the primer treB FP2 (Table II) were labeled with 10 units of T4 polynucleotide kinase (New England Bio-labs) using 5 µCi of [γ-32P]dATP (Amersham). After labeling, the complete mixture was used to perform a polymerase chain reaction together with unlabeled primer Foot1 (Table II) and plasmid DNA as template. After precipitation with ethanol, the pellet was resuspended with water to a value of 50,000 cpm/µl. 2 µl of the labeled DNA was incubated with varying amounts of TreR in a total volume of 50 µl using buffer A containing bovine serum albumin (0.1 mg/ml) and 100 µg/ml poly(dI-dC) (Pharmacia). Trehalose and trehalose 6-phosphate were added to a final concentration of 1 mM. After an incubation of 10 min at 37 °C, 3 µl of DNase I (Boehringer Mannheim, 0.007 units/µl) was added, and the reaction was performed at 37 °C for 3 min. It was stopped by the addition of 50 µl of stop solution (600 mM sodium acetate, pH 4.8, 20 mM EDTA). The DNA was precipitated by the addition of 2 volumes of ethanol. The resulting pellet was resuspended in 1 × sequencing stop solution (Amersham) and analyzed on a sequencing gel. A sequencing reaction with the corresponding primer was used to localize the resulting binding site.

**RESULTS**

**Sequence of treR—**The DNA region upstream of *treB* located on plasmid pHo500 was sequenced on double-stranded DNA with the dideoxy chain termination technique using the customized primers indicated. The result is shown in Fig. 1. An
The open reading frame was found starting with ATG at position 155 and ending with the TGA stop codon at position 1100 of the sequence shown in Fig. 1. This open reading frame contains 945 bp encoding 315 amino acids (molecular mass of 34,508 Da) that represent TreR. Upstream of the ATG start codon we recognized sequences that are likely to function as regions of a s-dependent promoter. Primer extension analysis was used to determine the transcriptional start point (Fig. 2). Four closely spaced start points were identified, and they are indicated in Fig. 1 together with their corresponding promoter region. Also shown in Fig. 1 downstream of the TGA stop codon of treR, a sequence was found that could form a palindrome able to act as a potential transcription termination signal (stem and loop structure).

Comparison of the deduced amino acid sequence of TreR with that of the LasI (27), PurI (28), and other members of the LacI-GalR subfamily shows a high degree of homology at the NH2-terminal part of the protein sequence. This part is known to contain the helix-turn-helix DNA-binding motif (Fig. 3). In the case of TreR the recognition helix is KSTVSRVLN.

By cloning a 198-bp fragment containing the identified promoter of treR into a promoter probe vector with promoterless lacZ and phoA genes we obtained plasmid clones where lacZ was fused to a promoter within treR but oriented opposite to treR transcription (data not shown). Therefore, a second even stronger promoter than the identified treR promoter exists on this very short DNA fragment but with the opposite orientation. This finding is consistent with a report of an active lacZ fusion to this hypothetical promoter (29).

**Table 1.** Sequence of treR. Shown is the DNA sequence containing the open reading frame of treR (capital letters). The deduced amino acid sequence is also shown. The strongest identified promoter of treR is given as black boxed sequences. The transcriptional start points are indicated by an asterisk, and the large asterisk is pointing to the strongest transcription. The potential Shine-Dalgarno sequence (SD) and the potential termination structure (stem loop) are indicated below the sequence. The hypothetical −10 and −35 region of the “anti” promoter is indicated by a gray boxed sequence. The positions of the primers are shown as underlined numbers above the DNA sequence (the dashed extension points to the introduction of restriction sites).
TreR is a dimer of identical subunits. The dimeric state of TreR was not changed in the presence of 10 mM trehalose or 10 mM trehalose 6-phosphate, the inducer of the system (data not shown).

**TreR Binds Trehalose 6-Phosphate and Trehalose**—Previously, we had identified trehalose 6-phosphate as the inducer of the treBtreC operon (30). Therefore, TreR should bind trehalose 6-phosphate. Using the intrinsic fluorescence of TreR we found that the emission (excitation at 280 nm) changed in the presence of trehalose 6-phosphate (Fig. 5A). The reduction in the emission at 340 nm was used to measure binding of trehalose 6-phosphate (Fig. 5B). Half-maximal saturation identical with the K_d of binding occurred at 10 μM trehalose 6-phosphate. To our surprise, the addition of trehalose also caused a change in the fluorescence emission of the protein (Fig. 5A) with the half-maximal concentration being 280 μM (Fig. 5B). Other sugars at 10 mM concentration such as glucose, maltose, and sucrose did not elicit changes in the emission spectrum (data not shown).

**TreR Consists of at Least Two Domains**—During purification of TreR, particularly when purified from extracts of a strain not carrying mutations in different proteases, the rapid formation of a proteolytic breakdown product of 29 kDa was observed. This proteolytic fragment was still able to dimerize (data not shown). During elution from heparin-Sepharose, a heterodimer of a proteolytic breakdown product of 29 kDa was observed. The stability of this fragment allowed the determination of its amino-terminal sequence. The sequence obtained indicated the existence of three different proteolytic fragments differing only in the last 3 or 6 amino-terminal amino acids (Fig. 7). Therefore, this region is likely to represent a linker between the carboxyl-terminal dimerization domain and the amino-terminal DNA-binding domain of the intact protein.

**TreR Binds DNA Containing a Specific Palindromic Sequence**—By use of DNase I footprinting analysis we determined the sequence of the DNA recognized and bound by TreR. Increasing amounts of TreR were incubated with linear DNA carrying the promoter fragment of treB/C prior to incubation with nuclease. Depending on the concentration of TreR, a DNA segment with the centered perfect palindromic sequence 5'-CGGGAAAC→GTTCCCG-3' was protected from DNase I (Fig. 8, lanes 1–8). At concentrations of 1 nM TreR or lower no protection of the DNA was visible and at 5 nM TreR protection was complete. At the highest concentration used (250 nM), no unspecific binding of DNA was detectable. When trehalose 6-phosphate (1 mM) was added to the assay, the DNA-protecting ability of TreR changed. No binding to DNA was obtained at low TreR concentrations (5 nM). At higher concentrations (>50 nM), the protein was able to bind to the same DNA sequence as in the absence of the inducer (Fig. 8, lanes 9–15). The addition of trehalose (1 mM) did not change the DNA binding ability of TreR (Fig. 8, lanes 16–22). The TreR binding site is located near the hypothetical cAMP/CAP binding site and partially overlaps the −35 region of the major promoter of treB/C (Fig. 9). The topology of the DNA had no influence on the results obtained since identical binding patterns were visible with supercoiled DNA (data not shown). Similar results concerning the binding of TreR to its operator were obtained by gel retardation assays (data not shown).

**Discussion**

In this paper, we describe the molecular and biochemical characterization of TreR, the regulatory protein of the treB/C operon of E. coli. treR had previously been identified by the phenotype of an insertion in this gene. treR mutants are constitutive in the expression of the treB/C operon and become repressed when treR is overexpressed on a multicopy plasmid (15). Therefore, TreR acts as the repressor of treB/C, the genes encoding the trehalose-utilizing enzymes. The detailed analysis of treR and its encoded protein TreR reported here firmly establishes the regulation of the metabolic pathway of trehalose utilization at low and high osmolality.

TreR of E. coli and the very closely related bacterium Salmonella typhimurium (31) is homologous to other repressor proteins of the LacI-GalR family, but significant homology only exists in the amino-terminal part of the protein known to contain the helix-turn-helix motif for DNA binding (32).

By the use of substrate-dependent fluorescence quenching, we established that trehalose 6-phosphate is bound by TreR. Binding of this sugar phosphate not only causes a fluorescence quench but also a shift in the emission maximum, which is indicative of a conformational change of the protein upon binding substrate. By titration we calculated a K_d of 10 μM. This is significantly lower than the K_m of TreC (6 mM) (16), which is the trehalose 6-phosphate-hydrolyzing enzyme. From the maximal rate of treB-mediated transport in a fully induced strain and the capacity of TreC, we could estimate that the equilibrium concentration of internal trehalose 6-phosphate at saturating external trehalose concentration is about 0.6 mM (16). This is far above the limiting concentration for saturation of TreR thus ensuring full induction.
In the absence of trehalose 6-phosphate, TreR binds to DNA harboring the control region of the treB/C operon. This can be seen by gel shift as well as nuclease protection assays. The analysis of the latter reveals the palindromic sequence 5'-CGGGAA-C-3' as the only TreR binding site of the operator of the treB/C operon. Significantly, this sequence contains a central CG which has been found in all operators of the LacI family (33).

Mueller-Hill and co-workers (34, 35) have proposed rules for the interaction of the recognition helix of repressors with their cognate operators. In particular, bases 4 and 5 of the symmetrical operator sequence (counted from the center of symmetry toward the 5' direction) only allow a narrow range of amino acid side chains in positions 1 and 2 of the recognition helix for effective repression. When the lac operator sequence was mutated to carry GG at positions 4 and 5 the only amino acids tolerated for effective repression at positions 1 and 2 of the equally mutated recognition helix were Lys for position 1 and Ser for position 2 (34). Exactly this combination occurs in the tre system for the operator (Fig. 8) and recognition helix (Fig. 3). This finding lends further support to the validity of rules in repressor-operator recognition.

The operator sequence partially overlaps the putative binding site for cAMP/CAP. One could therefore argue that the effective transcription of the operon is solely due to the activation by the cAMP-CAP complex, and the function of TreR is to prevent this activation. The TreR concentration needed to protect the operator-DNA from nuclease attack is at least 50 nM in the presence of 1 mM trehalose 6-phosphate versus 5 nM in its absence (Fig. 9). The presence of 1 mM trehalose neither affects the ability of TreR to protect the DNA from nuclease action nor
its ability to elicit a gel retardation effect. As seen by footprint analysis, the protected DNA region is somewhat larger when TreR has bound trehalose 6-phosphate. This indicates a conformational change of the dimeric repressor protein resulting in an increased distance of its two DNA-binding motifs.

To our surprise, we found that TreR is also able to bind trehalose, although with a much higher $K_d$ than it binds trehalose 6-phosphate ($280 \text{ M}$ versus $10 \text{ M}$). The $K_d$ for binding trehalose is far lower than the measured concentration of this sugar when synthesized at high osmolarity (about $400 \text{ mM}$) (5) and therefore should be of physiological significance. The conformational change deduced from the fluorescence change was not the same as the one that is caused by binding trehalose 6-phosphate. Nevertheless, the addition of excess trehalose 6-phosphate to TreR that already contained bound trehalose lead to the typical spectrum seen in the presence of trehalose 6-phosphate only. This was also true for the addition of a large excess of trehalose to TreR that contained bound trehalose 6-phosphate. This indicates that both sugars bind competitively to the same site but their binding causes different conformations of the protein.

Because trehalose is synthesized internally at high osmolarity and $treB/C$ (the genes encoding the degradative enzymes under TreR control) become uninducible under these conditions, one may conclude that binding of trehalose could enhance the affinity of TreR for the operator. However, this is not the case. Yet, the simple competition by the high trehalose concentration for the binding of trehalose 6-phosphate to TreR will also slow down or even prevent induction. Trehalose thus behaves as an anti-inducer of the $tre$ system. Similar observations have been made in the case of the Lac repressor where it was found that sugars other than the inducer isopropyl-1-thio-
β-o-galactopyranoside, such as o-nitrophenyl-β-o-fucoside or even glucose, in high concentrations could compete with the binding of inducer but did not elicit repression by themselves (36). Thus, the synthesis of trehalose can cause the physiologically important undecouability of the treB/C operon independently of the enzymatic activity of the trehalose 6-phosphate-degrading enzyme (OtsB) as we have considered previously (30). Therefore, TreR is the major control element to ensure the proper separation of the two crossing pathways of synthesis and degradation of trehalose in E. coli.

The strong evidence of a promoter element divergent to the treR promoter opens the possibility of a treR specific antisense RNA pointing to an additional form of regulation. More studies are necessary to unravel this aspect.

Recently, the repressor for the genes encoding the trehalose-utilizing enzymes in Bacillus subtilis was described (37). These enzymes, enzyme IIB (21) and trehalose-6-phosphate hydrolase (38, 39), are homologous to the corresponding enzymes from E. coli. Surprisingly, this is not true for the repressor.

The biochemical analysis of TreR showed that the protein forms a dimer of two identical subunits irrespective of its bound ligand. Like in other repressors of this family, the dimerization domain is homologous to the substrate binding cleft of the LacI repressor, the TreR repressor only forms a dimer. This is due to the lack of the four-helix bundle oligomerization domain (33) has fully confirmed this concept. The determination of the crystal structure of the TreR repressor is due to the four-helix bundle oligomerization domain in TreR that is present at the extreme carboxyl terminus of the Lac repressor (41, 42).

Early on it had been proposed that the inducer-binding domain of repressors is homologous to the substrate binding cleft in the well studied sugar-binding proteins involved in transport and chemotaxis (43, 44). Indeed, the elucidation of the three-dimensional structure of the Lac repressor (42, 45) and the PurR repressor (33) has fully confirmed this concept. The determination of the crystal structure of the TreR repressor that is under way will add to our knowledge of the molecular properties of these interesting proteins.

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