Determinants of Translocation and Folding of TreF, a Trehalase of Escherichia coli*

Received for publication, April 3, 2000, and in revised form, May 10, 2000
Published, JBC Papers in Press, May 17, 2000, DOI 10.1074/jbc.M002793200

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One isoform of trehalase, TreF, is present in the cytoplasm and a second, TreA, in the periplasm. To study the questions of why one enzyme is exported efficiently and the other is not and whether these proteins can fold in their nonnative cellular compartment, we fused the signal sequence of periplasmic TreA to cytoplasmic TreF. Even though this TreF construct was exported efficiently to the periplasm, it was not active. It was insoluble and degraded by the periplasmic serine protease DegP. To determine why TreF was misfolded in the periplasm, we isolated and characterized Tre+ revertants of periplasmic TreF. To further characterize periplasmic TreF, we used a genetic selection to isolate functional TreA-TreF hybrids, which were analyzed with respect to solubility and function. These data suggested that a domain located between residues 255 and 350 of TreF is sufficient to cause folding problems in the periplasm. In contrast to TreF, periplasmic TreA could fold into the active conformation in its nonnative cellular compartment, the cytoplasm, after removal of its signal sequence.

Secretary and cytoplasmic proteins differ not only by the signal sequence but also in their folding properties. It is thought that the export competence of secretory proteins is the result of slow folding prior to export. Cytoplasmic proteins are believed to fold more rapidly and thus are not substrates of the cellular secretion apparatus. For a better understanding of the mechanism of translocation, the folding of cytoplasmic and secretory proteins needs to be characterized. Folding of polypeptides is determined by the primary amino acid sequence but also could be influenced by the particular properties of a cellular compartment. However, little is known about whether and how the cytoplasm and the periplasm specifically influence protein folding, i.e. whether there are other elements besides redox state and the presence/absence of ATP. To study these aspects, we used TreA and TreF, the two trehalases of Escherichia coli, as model proteins.

Periplasmic TreA is synthesized as a precursor of 565 amino acids. The signal sequence of 30 amino acids is rather long. Mature TreA has a molecular mass of 58 kDa (1, 2). The $K_m$ of the purified enzyme is 0.8 mM, the $V_{max}$ is 66 µmol of trehalose hydrolyzed/min/mg of protein, and the pH optimum is 5.5 (3). The expression of treA is independent of the presence of trehalose in the growth medium but is stimulated 10-fold at high osmolarity (1, 2). Also, treA is regulated by RpoS, the stationary phase sigma factor (4).

Cytoplasmic TreF has 549 amino acids and a molecular mass of 64 kDa. The $K_m$ of the purified enzyme is 1.9 mM, the $V_{max}$ is 54 µmol of trehalose hydrolyzed/min/mg of protein, and the pH optimum is 6.0. Like treA, the expression of treF is independent of trehalose, is dependent on RpoS, and is induced at high osmolarity. Both TreA and TreF are monomeric enzymes, and they share an amino acid sequence identity of 47%. TreA has an extended C terminus of about 30 residues, which is not present in TreF, and TreF has a 61-residue extension at its N terminus, which is not present in TreA (5).

Trehalose metabolism in E. coli can occur either in the periplasm via TreA or after transport into the cytoplasm via a trehalose-specific enzyme II encoded by treB (6). Transport results in the formation of trehalose 6-phosphate. Trehalose 6-phosphate is cleaved by TreC into glucose 6-phosphate and glucose (7). When trehalose is synthesized in the cytoplasm in response to high osmolarity of the growth medium, it can be degraded by cytoplasmic TreF. Cells expressing treF from the chromosome exhibit very low trehalase activity (5).

We are interested in the phenomenon that, despite their high similarity, TreA and TreF are localized in different cellular compartments, and we wished to determine whether these compartments have an effect on folding and specific activity. To study these questions we expressed both enzymes in their nonnative cellular compartment, i.e. treA in the cytoplasm and treF in the periplasm, and investigated whether they would fold into the active conformation. Whereas treA was actively expressed in the cytoplasm, periplasmic TreF was inactive. To determine why TreF is misfolded in the periplasm, we isolated and characterized Tre+ revertants of periplasmic TreF. To identify regions in TreF responsible for misfolding in the periplasm, we selected and characterized TreA-TreF hybrid proteins.

**EXPERIMENTAL PROCEDURES**

Bacteria and Plasmids—E. coli strains used were derivatives of DHB3 (araD139 Δtara-leu 7697 ΔlacX74 ΔphoA (PvuII) phoR ΔmalF3 galE galK thi rpsL) (8) or of MC4100 which is F− ΔlacU169 araD136 rbsR relA rplA (9). KU92 is DHB3, treA:Spec$^R$ ΔtreB ΔtreC ΔtreA ΔmalF3 ΔaraD74 leu::Tn10 Tet$^R$; KU93 is KU92 pRA4 Kan$^R$; KU95 is MC4100 treA::Spec$^R$ treC ΔtreC ΔtreA ΔmalF3 ΔaraD74 leu::Tn10 Tet$^R$; KU101 is KU92 ΔaraD74 leu"; KU104 is KU101 degr::Tet$^R$; KU105 is KU104 pRA4 Kan$^R$. Plasmids used were pBAD22, a pBR22-derived plasmid that has the arabinose promoter followed by a linker containing multiple restriction sites (10); pBADtreF has the coding region of wild-type treF in pBAD22 (5); ptre11 has wild-type treA under its own promoter in pBR322 (2).

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* This work was supported by a grant from the Deutsche Forschungsgemeinschaft (to M. E.) and by fellowships from the Studienstiftung des deutschen Volkes (to M. M. and C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Construction of Plasmids—pSestreA was generated by cleaving ptre11 with BglI followed by T4 DNA polymerase treatment and PstI digestion. The 2.7-kilobase DNA fragment containing ΔstreA was ligated into pBAD22, which was cleaved with Asp718I, followed by treatment with Klenow enzyme and PstI digestion. Because of these manipulations, the ptre11 fragment was expressed without its signal sequence and the first five amino acids of mature TreA (Glu, Glu, Thr, Pro, and Val) were replaced by Met, Val, and Leu.

ptreA′-treF was constructed by subcloning a 2.27-kilobase EaqI fragment from ptre11 into pBADtreF which was cleaved with EaqI. This plasmid contains the wild-type treA promoter and the 404 codons of the 5′-end of the selection site and the 3′-end of ptre11 cleaved with KpnI and EcoRI. Primers used were 5′-GGGGTACCagctactgaagaattcaaac-3′ and 5′-CGGATCTcttggttcgccgtaca aacc-3′. The 2.3-kilobase EaqI fragment from pBADtreF was cloned with EaqI and SacI.

ptreA′-treF expresses a gene fusion of the first 265 codons of treA and all of treF. It was constructed by cloning a polymerase chain reaction product of treF flanked by a KpnI restriction site at the 5′-end and an EcoRI restriction site at the 3′-end into ptre11 cleaved with KpnI and EcoRI. Primers used were 5′-GGGGTACCagctactgaagaattcaaac-3′ and 5′-aagggctagccagggaggatg-3′. This construct contained a treA sequence including the first two codons of mature treA, followed by codons for Gly and Thr and the entire treF gene. The additional Gly and Thr residues were a consequence of the introduced KpnI site immediately upstream of the translational initiation codon of treF.

Trehalase Assay—To determine the $K_m$ for trehalase of the various trehalase constructs, treA treC mutant strains KU92 or KU95 expressing the trehalase constructs from plasmids were grown overnight in rich medium. Cultures were washed twice in minimal medium and were broken in a French pressure cell at 9000 p.s.i. The remaining intact cells were removed by centrifugation (15,000 rpm, 30 min, 4°C, SS34 rotor). The DNA of the cell extract was precipitated with 2% streptomycin sulfate and pelleted by centrifugation (15,000 rpm, 30 min, 4°C, SS34 rotor). Subsequently, the protein concentration of the cell extract was adjusted to 0.2–0.4 mg/ml. Alternatively, to assay periplasmic trehalase activity of whole cells, cells were grown overnight in rich medium. Cultures were washed twice in minimal medium and were resuspended in trehalase assay buffer.

Trehalase assays were performed in 100 μl of 20 mM potassium phosphate buffer, pH 6.0. The reaction was started by the addition of 0.25 to 10 μl trehalase and incubated for 5–15 min at room temperature. The reaction was stopped by boiling the samples for 5 min. Cleavage of trehalase was assayed by determining glucose concentration by the glucose test kit from Merck.

Labeling of Cells, Cell Fractionation, and Antibodies against TreF—Protein was labeled in cultures of strain KU101 expressing either treA or sstreF from plasmids ptre11 and pstreF, respectively, growing exponentially (Δ600 0.4) in minimal medium 9 (11), 0.2% glucose, 1 μg/ml thiamine, 50 μg/ml ampicillin, supplemented with each common amino acid except Cys and Met. 1 ml of cell cultures was exposed to [35S]methionine (>$1000 $Ci$/mmol$) at 50 $μCi$/ml for 1 min and subsequently cooled on ice for immunoprecipitation or the labeling period was followed by a chase with excess cold methionine (50 mM final concentration) for the time indicated. To assay the effect of the temperature-sensitive lepB9 mutation on secretion of ΔstreF, strain IT41 (12) was grown at 28°C and shifted to 42°C for 30 min before exposure to [35S]methionine. Immunoprecipitation and gel electrophoresis were done as described by Ito et al. (13) and Laemmli (14). To precipitate TreA, TreF, and DegP, polyclonal antibodies against TreA, TreF, and DegP were used, respectively.

Rabbit polyclonal antiserum against TreF was obtained by immunization with purified TreF. The antiserum against TreF cross-reacts only weakly with TreA. Rabbit polyclonal antiserum against TreA, MBP, DegP, or SecA were kind gifts of W. Boos, J. Beckwith, and P. C. Tai. The cold osmotic shock procedure was carried out according to Neu and Heppel (15).

Selection of TreF′ revertants of sstreF—Strain KU101 containing pstreF was grown in LB in the presence of dianinopurine (600 μg/ml). pstreF was isolated and reintegrated into KU101. TreF′ revertants were selected on minimal medium A containing 0.2% trehalose (11). After purification on the selection medium, candidates were tested for elevated trehalase activity on MacConkey agar plates containing 1% trehalose. Candidates exhibiting red color on MacConkey plates were further tested by trehalase assays. The mutants of the TreF′ revertants were determined by nucleotide sequencing of the entire treF gene.

Selection of TreA′treF and of ΔstreA/TreF hybrids—Strain KU92 containing ptreA′-treF was grown overnight in rich medium. A 25-ml culture was washed twice in minimal medium, and aliquots corresponding to 2.5 ml of cell culture were plated onto one minimal medium agar plate containing 0.2% trehalose. After incubation overnight, TreF′ revertants were identified by their clear zone of growth on the plate. To show that complementation of the tre defect was linked to ptreA′-treF, plasmid DNA of such candidates was reintroduced into strain KU92. In all cases growth on minimal trehalose media was detected. This indicated that plasmid-derived mutations were sufficient to generate growth on selection plates. These cells were used for further characterization. The fusion joints were determined by nucleotide sequencing. Selection of ΔstreA/TreF hybrids was carried out as described above for selection of TreA′TreF hybrids except that strains KU93 and KU95 containing pSstreA′-treF′ were used.

Electron Microscopy—For electron microscopy, strains were grown overnight at 28°C in LB medium. Cells were harvested by centrifugation (4000 × g, 10 min, 4°C) and fixed for 1.5 h at room temperature with 2.5% glutaraldehyde in buffer A (0.1 M sodium phosphate buffer, pH 7.1). The pellet was washed three times with buffer A and embedded in 2% low melting agarose. These samples were fixed in buffer A containing 2% OsO4 (1 h, room temperature), washed five times with buffer A, dehydrated in 50 and 70% acetone (10 min, room temperature), contrasted with 2% uranylacetate in 70% acetone (1 h, room temperature), and subsequently further dehydrated with 90 and 100% acetone. Embedding was done according to Spurr (16). After polymerization ultrathin sections were prepared and contrasted with 2% aqueous uranylacetate and lead citrate (17). The specimen were examined in a EM10 C2 (Zeiss, Oberkochen, Germany) under 80 kV at a primary magnification of ×17,600.

RESULTS

The two trehalases of E. coli are highly homologous and have similar enzymatic properties. The main apparent difference is their cellular localization. Therefore, these enzymes must contain signals allowing either efficient translocation and folding in the periplasm or exclusion from export and proper folding in the cytoplasm. To obtain information on how these isoenzymes have adapted to their environment we expressed both in their nonnative cellular compartment, periplasmic treA in the cytoplasm, and cytoplasmic treF in the periplasm.

Export and Folding of Cytoplasmic Trehalase—To test whether periplasmic TreA can fold in the cytoplasm, we constructed a signal sequenceless TreA derivative. This plasmid was termed pΔstreA. ΔstreA was expressed in ΔtreA strain KU101. Signal sequenceless treA was actively expressed in the cytoplasm (Table I). The kinetic parameters of cytoplasmic TreA were determined in whole cell extracts.

| Plasmids | Whole cells KU101 | Extract KU101 | Whole cells KU104 (degP) |
|----------|------------------|--------------|-------------------------|
| treF′     | 3.91 ± 1.33      | 2.46 ± 0.19  | 3.96 ± 1.09              |
| pΔstreA  | 0.62 ± 0.11      | 2.98 ± 0.05  | 0.60 ± 0.12              |
| pstreF   | 0.06 ± 0.03      | 0.07 ± 0.01  | 0.12 ± 0.05              |
| pΔstreA  | 0.37 ± 0.08      | n.d.         | 0.71 ± 0.16              |
| pstreF/T2 | 0.33 ± 0.10     | n.d.         | 0.88 ± 0.21              |

a Trehalase activity was determined by assaying the release of free glucose from trehalose. Assays were done in 20 mM potassium phosphate buffer (pH 6.0) in the presence of 100 mM trehalose. Trehalase activity determined in whole cells corresponds to periplasmic trehalase activity whereas both cytoplasmic and periplasmic trehalase activities contribute to the activity determined in whole cell extracts.

b no plasmid.
c n.d., not determined.
sequence, we expected comparable levels of expression. Be-
under

ing either wild-type prlA because it could be exported in
addition, signal sequenceless TreA remained export competent,
export of signal sequenceless secretory proteins (18).

efficient processing of ssTreF could be excluded.

activity in whole cell extracts (see below), this explanation for
mature form of ssTreF, TreF would fold into the active confor-
matic activity. There was no difference when comparing tre-

FIG. 1. Translocation of ssTreF. A, cells of strain KU101 express-
either wild-type treA or ssTreF from plasmids pre11 or pstreeF,
respectively, were labeled with [35S]methionine for 60 s (0) or 60 s
followed by chase periods of 0.5, 1, or 5 min with excess cold methionine.
Cells were lysed and immunoprecipitated with antibodies to TreA or
TreF. The positions of TreA, of mature TreF, and of DnaK are indicated by
arrows. B, cells of strain IT49 (lepB9) expressing ssTreF from pstreeF
were grown at 28 °C and shifted to 42 °C for 30 min before exposure to
[35S]methionine. Cells were labeled for 60 s (0) or 60 s followed by chase
periods of 1, 5, or 10 min with excess cold methionine. Cells were lysed
and immunoprecipitated with antibodies to TreF and DegP. The posi-
tions of DnaK, which was recognized by the antiseraum against TreF,
and of precursor and mature forms of TreF and DegP are indicated. C,
Western blot of strains KU101 (degP::Tn10) and KU104 (degP::Tn10)
expressing ssTreF after overnight growth in LB. These cultures were
concentrated 10-fold. To detect TreF, polyclonal antibodies against
TreF were used. The positions of TreF and DnaK are indicated.

extracts of strain KU101. Signal sequenceless TreA had a K_m of
0.16 mM, whereas periplasmic TreA had a K_m of 0.31 mM
trehalase. Cytoplasmic wild-type TreF had a K_m of 1.5 mM.
In addition, signal sequenceless TreA remained export competent,
because it could be exported in prlA4 background (data not
shown). prlA mutants are derivatives of secY allowing the
export of signal sequenceless secretory proteins (18).

To test whether treF could be actively expressed in the
periplasm, we fused the signal sequence of TreA to the N
terminus of TreF. This plasmid was termed pstreeF. TreF was
efficiently exported to the periplasm. Pulse-chase experiments
indicated that more than 95% of the signal sequences of the
TreF precursor population were processed within 1 min (Fig.
1A). Only under nonpermissive conditions in the temperature-
sensitive lepB9 strain IT41 (12) could the ssTreF precursor be
detected (Fig. 1B). We concluded that processing of the signal
sequence occurred as a consequence of translocation. Also, if a
cytoplasmic protease would be responsible for generating the
mature form of ssTreF, TreF would fold into the active confor-
mation in the cytoplasm. Because we did not detect trehalase
activity in whole cell extracts (see below), this explanation for
efficient processing of ssTreF could be excluded.

sstreF was expressed at lower levels compared with treA, i.e.
37 ± 6% of wild-type treA (Fig. 1). Because treF was expressed
under treA promoter control and was fused to the TreA signal
sequence, we expected comparable levels of expression. Be-
cause the periplasmic DegP protease is known to degrade a
large variety of misfolded proteins (19), we tested whether
DegP is involved in degradation of periplasmic TreF. We de-
tected an increase in treF expression in the degP null mutant
strain KU104. This effect of the degP mutation was stronger in
overnight than in log phase cultures (Fig. 1C). It should be
noted that 60 times more cells were used for Western blotting
compared with pulse-chase experiments. Other mutants of cell
envelope proteases such as ompT, ptr, hhoA, and hhoB had no
pronounced effects on TreF expression.

Enzymatic Activity of Periplasmic TreF—Periplasmic TreF
had only background enzymatic activity regardless of whether
TreF was expressed in degP* or degP mutant strains (Table I).
This finding corresponded to the weak growth of strain KU101
expressing ssTreF on minimal trehalose agar plates. When
growth was dependent on the presence of periplasmic TreA,
single colonies were detected after overnight incubation at
28 °C. When growth was dependent on periplasmic TreF,
strains needed 3 days to form single colonies.

Trehalase activity was determined in either whole cells
or whole cell extracts of treA::spec ΔtreBC strains, lacking
periplasmic trehalase TreA and the trehalase transporter
TreB. Trehalase activity of whole cells represented only
periplasmic trehalase activity because in ΔtreB mutants tre-
halase is not transported across the cytoplasmic membrane.
When trehalase activity was determined in whole cell extracts,
periplasmic and cytoplasmic trehalase contributed to enzy-
matic activity. There was no difference when comparing

FIG. 2. ssTreF and TreA'-TreF are not released by cold osmotic
shock. A, Western blot of periplasm and pellet fractions of strain
KU104 (degP::Tn10) expressing ssTreF and treA'-treF after overnight
growth in LB. To detect TreF, TreA'-TreF, SecA, or MBP polyclonal
antibodies against TreF, SecA, and MBP were used. SecA and MBP were
used as controls for cytoplasmic and periplasmic proteins, respectively.
The positions of TreF, TreA'-TreF, SecA, and MBP are indicated. S is
cold osmotic shock fluid, and P is the pellet fraction of osmotically
shocked cells. B, electron microscopy of degP* and degP* strains ex-
pressing ssTreF. The images show representative details of the cell
envelope of degP* strain KU101 (a and b) and degP* strain KU104 (c
d). Ultrathin sections were contrasted with uranylacetate and lead
citrate. Aggregated forms of improperly folded ssTreF were detected in
the periplasm of the degP mutant (arrowheads). Magnification was
equal, and the bar represents 100 nm.
were obtained with MBP mutants (20). From these data we conclude that TreF can be exported to the periplasm where it is present in a misfolded and inactive form and is a substrate for DegP protease.

**Genetic Selection of Periplasmic TreF with Increased Trehalase Activity**—To determine why TreF is misfolded in the periplasm, we isolated and characterized TreF revertants of periplasmic TreF. To obtain mutants of periplasmic TreF with increased enzymatic activity, pssTreF was mutagenized using 2-aminopurine. Mutagenized plasmids were transformed into treA::specDtreBC strain KU101 and plated on minimal trehalose agar plates (Fig. 3). Colonies showing improved growth were screened for increased trehalase activity on MacConkey trehalose agar plates. Subsequently, trehalase activity of 25 candidates was assayed in whole cells. Two candidates showed more than a 5-fold increase in trehalase activity. Retransformation verified that the isolated mutations were linked to pssTreF. Both mutants, termed treF82 and treF172, exhibited about a 6-fold increase in trehalase activity (Table I). Nucleotide sequencing indicated that they were independent mutants, because treF82 had an A82T and treF172 had a T172I exchange (Fig. 4). Interestingly, the T172I exchange detected in treF172 is located in the N-terminal trehalase signature. An amino acid sequence alignment shows that most trehalases have hydrophobic residues at position 172, whereas wild-type TreF has a Tyr residue (Fig. 5). This may explain why the T172I exchange leads to elevated trehalase activity.

The expression of treF82 and treF172 was found to be identical to periplasmic TreF. It is therefore likely that the mutations did not change the overall structure of periplasmic TreF but might have an effect only on folding of the catalytic site.

**Characterization of a TreA′-TreF Hybrid**—We reasoned that a hybrid protein composed of a fragment of TreA and TreF might mediate contact to potential periplasmic chaperones or folding catalysts and may thus influence folding of the TreF moiety. Fusing the N-terminal 263 amino acids of the TreA precursor to TreF (Fig. 6) led to translocation of the hybrid protein but did not increase trehalase activity (Table II) or solubility of the protein compared with periplasmic TreF (Fig. 2).

To test whether the presence of the 233 amino acids of mature TreA would interfere with folding of TreF we constructed a signal sequenceless derivative. It was expressed in the cytoplasm and exhibited high trehalase activity (Table II). Thus, the N-terminal 233 amino acids of TreA did not interfere with the folding of TreF in the cytoplasm and did not stimulate folding of periplasmic TreF.

**Genetic Selection for Functional TreA′-TreF Hybrids**—To identify regions responsible for misfolding of periplasmic TreF, we wanted to test whether functional hybrids can be constructed composed of N-terminal fragments of TreA and C-terminal fragments of TreF. The genetic selection for active
Trehalase-signature  PGGRFx2EXXWDXY
Tref  E. co  TVPGRFPELYWDXY
Treff172  E. co  TVPGRFPELYWDXY
Tre  E. co  WVPGRFPELYWDXY
Tre  S. ce  AVPGRRNLGYWDXY
TreB  S. ce  AVPGRRNLGYWDXY
TreA  C. al  WVPGRFPELYWDXY
TreA  K. la  AVPGRRNLGYWDXY
TreB  N. cr  AVPGRRNLGYWDXY
TreA  B. mo  TVPGRFKETYWDXY
TreA  T. mo  TVPGRFKETYWDXY
TreA  O. cu  TVPGRFKETYWDXY
TreA  H. sa  TVPGRFKETYWDXY

Fig. 5. Consensus sequence of the N-terminal trehalase signa-
ture. Aminoacyl residues 166–180 of TreF and of ssTreF172 are
compared with the relevant sequences of other trehalases. The sequences
were extracted from the latest release of SwissProt data base. Accession
numbers are given in parenthesis. TreA and TreB of S. ce are from
Saccharomyces cerevisiae (P52356 and P53172). E. co is Candida abil-
cans (P52949). K. la is Kluyveromycetes lactis (P49381). N. cr is Neuro-
spora crassa (O42783). B. mo is Bombyx mori (P32358). T. mo Tenebrio
moltitor (P32359). O. cu is Oryctolagus cuniculus (P19813), and H. sa is
Homo sapiens (O43280).

trehalase hybrids was identical to that used for selection of
Tre r revertants of streF. It was carried out in strain KU92
(treA::spec streBC), which requires periplasmic trehalase ac-
tivity for growth on minimal trehalose medium (Fig. 3). To
select for functional TreA-TreF hybrids, plasmids were con-
structed containing the 5′-end of treA until codon 373 fused to
the 3′-end of treF starting at codon 166. The region of over-
lapping homology was about 750 base pairs. In this construct,
termed treA′·treF, the reading frames of treA and treF were
nonidentical. This construct did not confer trehalase activity,
and no growth on minimal trehalose medium was observed
after expression in strain KU92.

When plating cultures of KU92 containing ptreA′·treF spon-
taneous Tre r revertants were isolated at high frequency. Plas-
mids of 12 candidates were retransformed into strain KU92
and checked for trehalase activity. Three candidates were
chosen for further analysis. Nucleotide sequencing indicated that
treA-F1 encodes a hybrid protein precursor of 535 amino acids
composed of the N-terminal 335 residues of TreA precursor and
the C-terminal 200 residues of TreF. treA-F8 encodes a hybrid
protein precursor of 534 amino acids composed of the N-termi-
nal 239 residues of TreA precursor and the C-terminal 295
residues of TreF. treA-F10 encodes a hybrid protein precursor
of 535 amino acids composed of the N-terminal 342 residues of
TreA precursor and the C-terminal 193 residues of TreF (Figs.
4 and 6). Processing of each precursor protein removes the
N-terminal signal peptide of 30 residues. Because the fusion
joints of treA-F1 and treA-F10 were nearly identical, we did not
further investigate treA-F10.

Enzymatic Activity of Periplasmic TreA′·TreF Hybrids—The
hybrid proteins differed in their enzymatic activity. Whereas
TreA-F1 exhibited high trehalase activity, TreA-F8 had low
trehalase activity. Like all other constructs described above,
the hybrids were substrates of DegP protease. In degP null
mutant derivatives of strain KU92, protein expression (data
not shown) and enzymatic activity increased (Table II). Also,
we detected a difference in solubility. Whereas about 50% of
TreA-F1 could be released by cold osmotic shock, only about
10% of TreA-F8 was released, indicating that a larger popula-
tion of TreA-F8 was misfolded (Fig. 7). Because TreA-F8 had
similar properties to periplasmic TreF but had only about 50% of
TreF sequence, we suspect that the C-terminal half of TreF
contains a major determinant for misfolding of periplasmic
TreF. Because TreA-F1 exhibited enzymatic properties similar
to TreA (see below) even though it contains the 200 C-terminal
residues of TreF, the region causing folding problems could be
further restricted to residues 255–350 of TreF.

Because wild-type TreA and TreF differ in their Km values
for trehalose, i.e. 0.3 mM for TreA and 1.5 mM for TreF, we
asked whether the trehalase hybrid TreA-F1 would resemble
more closely TreA or TreF. The Km value for trehalose was
determined as 0.3 mM, which was identical to that of periplas-
mic TreA. Thus, the high affinity binding site should be located
in the TreA part of TreA-F1. A possible candidate for the high
affinity binding site is the N-terminal trehalase signature (Figs.
4 and 5).

Genetic Selection and Characterization of Functional
ΔssTreA′·TreF Hybrids—We repeated the selection described
above using signal sequenceless versions of ptreA′·treF. These
were expressed in prlA4 strain KU93 allowing export of signal
sequenceless secretory proteins. As above, we selected for
periplasmic trehalase activity. Tre r revertants were obtained
at a 1000-fold lower frequency compared with the original
selection using ptreA′·treF encoding the TreA signal sequence.
Fortuitously, one isolate had exactly the same fusion joint as
TreA-F1 and was thus termed ΔssTreA-F1. The fusion joint
was such that a new trehalase gene was generated, lacking the
extended N terminus of TreF and the extended C terminus of
TreA. This trehalase was composed of 503 residues, whereas
wild-type TreA has 535 residues and wild-type TreF has 549
residues. ΔssTreA-F1 contained three additional residues (Met,
Val, and Leu) at its N terminus, which are a consequence of
subcloning, 300 residues of TreA (37–334), and the 200 C-
terminal residues of TreF (350–549). The enzymatic activity
of ΔssTreA-F1 was about 12-fold higher as detected for the signal
sequence-containing construct TreA-F1 and was mostly local-
ized in the cytoplasm (Table II). After expression in prlA degP
mutant strain KU105, the activity of ΔssTreA-F1 was 0.89 ±
0.02 units, which was only about 40% higher than detected in
prlA degP strain KU101 (Table II) indicating that ΔssTreA-F1
was only a poor substrate of the prlA secretion apparatus.
Therefore, we conclude that the C-terminal 200 residues of
TreF were sufficient to block export by the PrlA secretion
apparatus. The Km of ΔssTreA-F1 was determined in whole cell
extracts as 0.16 mM for trehalose, which was identical to that of
ΔssTreA. This result and the finding that the activity of
ΔssTreA-F1 was the same as that of ΔssTreA (Tables I and II)
indicated that the exclusion from export was not the conse-
quence of improper folding of ΔssTreA-F1.

DISCUSSION

We used genetic approaches to study the intramolecular
signals and the effects of the cellular compartments on trans-
location and folding of the two trehalases of E. coli. Periplasmic
trehalase TreA folded properly in the periplasm and in the
cytoplasm. Also, TreA-TreF hybrid proteins containing 334
amino acids of TreA and 200 residues of TreF had enzymatic
properties comparable to those of wild-type TreA, no matter
whether these constructs were expressed in the cytoplasm or in
the periplasm. The experimental data obtained from these
hybrid proteins indicated that the main determinant of sub-
strate affinity must be localized in the N-terminal 334 residues
of TreA. Trehalases have two conserved signatures typical for
glycosyl hydrolases. One is localized at the N terminus and the
other at the C terminus (Fig. 4). Thus, we speculate that the
N-terminal trehalase signature is responsible for the lower Km
values of TreA.
Export and Folding of Cytoplasmic Trehalase

In contrast to TreA, cytoplasmic TreF could not fold in its nonnative cellular compartment. Periplasmic TreF was misfolded and enzymatically inactive. One TreF mutation, T172I, located in the N-terminal trehalase signature, led to 6-fold higher periplasmic trehalase activity. This finding supported the model that the N-terminal Tre box is important for enzymatic activity. However, this mutation did not abolish the problems of solubility and protease sensitivity of periplasmic TreF.

It is difficult to secrete native cytoplasmic proteins of *E. coli*, which is consistent with the idea that cytoplasmic proteins fold too rapidly to be substrates of the secretion apparatus. Therefore, successful translocation of native cytoplasmic *E. coli* proteins has been reported only for a few cases. Thioredoxin 1 can be translocated to the periplasm when fused to the signal sequence of alkaline phosphatase or of DsbA (21, 22), and β-galactosidase was exported as tripartite protein, LamB-LacZ-PhoA (23), or when fused to the signal sequence of OmpA (24). Compared with thioredoxin and β-galactosidase, secretion of TreF was more efficient, which may be because of the exceptionally long and hydrophobic signal sequence of TreA. We are currently investigating whether a more hydrophobic signal sequence is leading to a more efficient export of native cytoplasmic proteins.

A model predicting that the cytoplasm and the periplasm have different properties influencing the folding of polypeptides may explain why TreF did not fold properly in the periplasm. Periplasmic *E. coli* amylase MalS is another example because signal sequenceless MalS cannot fold into the active conformation in the cytoplasm (25). The cellular factors responsible for these effects are unknown. Possible candidates are different sets of molecular chaperones, for example ATP-dependent chaperones are present in the cytoplasm but not in the periplasm. Also, because TreF contains three Cys residues, which tend to form intermolecular disulfide bonds in purified cytoplasmic TreF, these Cys residues could be responsible for misfolding of periplasmic TreF. When testing whether the inability of periplasmic TreF to fold was dependent on DsbA, a catalyst for disulfide bond formation, no increase in TreF activity or solubility was observed in *dsbA* knock out strains.1 Also, there are no Cys residues in the TreF part of the hybrid TreA-F8, which was as insoluble as full-length ssTreF. However, one region comprising residues 255–350 of TreF was

1 K. Uhland, unpublished results.
identified as sufficient to cause folding problems in the periplasm. These 95 residues, of which only 24 are nonconserved with respect to TreA, are located between the fusion joints of treA-F1 and treA-F8. We are expecting that further work on TreF will allow us to study the determinants of solubility, folding of the active site, and protease sensitivity by using genetic methods.

Acknowledgments—We thank Eberhard Spiess for help with the EM work done in his lab. We thank Jon Beckwith, P. C. Tui, and Winfried Boos for antibodies; Ross Dalbey for bacterial strains; and Ann Flower for comments on the manuscript.

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