Mutations That Affect the Folding of Ribose-binding Protein Selected as Suppressors of a Defect in Export in *Escherichia coli*

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It has been proposed (Randall, L. L., and Hardy, S. J. S. (1986) Cell 46, 921–928) that export of protein involves a kinetic partitioning between the pathway that leads to productive export and the pathway that leads to the folding of polypeptides into a stable conformation that is incompatible with export. As predicted from this model, a decrease in the rate of export of maltose-binding protein to the periplasmic space in *Escherichia coli* resulting from a defect in the leader sequence was able to be partially overcome by a mutation that slowed the folding of the precursor, thereby increasing the time in which the polypeptide was competent for export. [Liu, G., Topping, T. B., Cover, W. H., and Randall, L. L. (1988) *J. Biol. Chem.* 263, 14790–14793]. Here we describe mutations of the gene encoding ribose-binding protein that were selected as suppressors of a defect in export of that protein and that alter the folding pathway. We propose that selection of such suppressors may provide a general method to obtain mutations that affect the folding properties of any protein that can be expressed and exported in *E. coli.*

Subsequently the site of the mutational change and the effect, if any, on folding can be determined. If enough aminoacyl substitutions are studied it may be possible to identify the patterns that are crucial in determination of folding pathways. There are several variations of this genetic approach currently in use. Mutational substitutions that alter the functional activity of proteins have been shown to alter the folding and stability of proteins (Alber *et al.*, 1987; Beasty *et al.*, 1986; Pakula *et al.*, 1986; Shortle and Meeker, 1986). King and his co-workers (King *et al.*, 1987; Sturtevant *et al.*, 1989) have developed a system that selects directly for effects on folding. By isolating temperature-sensitive mutations in the gene that encodes the tail spike of the *Salmonella* phage P22 they have identified aminoacyl substitutions that alter the kinetics of folding but not the stability of the polypeptide. Here we describe another method for direct selection of changes that alter folding. This method may be of general application to studies of proteins that can be exported by *Escherichia coli.*

Proteins are marked for export by the presence of an aminoterminal extension of aminoacyl residues, called a leader sequence or signal sequence, that is proteolytically removed during export to generate the mature protein. It has been proposed that export involves a kinetic partitioning between the productive translocation of proteins to the periplasmic compartment and the folding of precursor forms within the cytoplasm. Once the precursor has attained the conformation characteristic of the mature protein it is not competent for export (Randall and Hardy, 1986). Normally, the export process is efficient and little precursor remains inside the cell. However, if an alteration in the leader sequence of the precursor decreases the efficiency of export, a significant proportion of the precursor accumulates within the cell in a folded, export-incompetent form. The model of kinetic partitioning predicts that the defect in export could be partially overcome by retarding the folding of the polypeptide and allowing more time for the precursor to interact with components of the export apparatus. A mutation in the gene encoding maltose-binding protein originally isolated as a suppressor of a defect in export resulting from an altered leader sequence has been shown to drastically retard folding. The mutational alteration was the change of a tyrosinyl residue to an aspartyl residue at position 283 in the mature sequence (Cover *et al.*, 1987; Liu *et al.*, 1988). In order to test the idea that selection for suppression of defective export provides a general means of obtaining mutations that affect folding, we undertook a study of the periplasmic ribose-binding protein. Here we describe two mutations that were isolated because they enhance export of a precursor carrying a defective leader sequence. Both mutational changes were located in the mature portion of the polypeptide and each affects the kinetics of folding and the
stability of mature ribose-binding protein. Thus selection for restoration of export may afford a means to isolate mutations affecting folding.

### EXPERIMENTAL PROCEDURES

**Materials**—Guanidinium chloride (ultrapure) was purchased from SIGMA-Aldrich and Trizma Base was purchased from Sigma. Q-Sepharose Fast Flow anion exchange resin, Polybuffer 94 and Polybuffer 74 were purchased from Pharmacia LKB Biotechnology Inc. "[5S]Methionine (1000 Ci/mmol) was purchased from Du Pont-New England Nuclear.

**Bacterial Strains and Growth Media**—All bacterial strains used in this study are derivatives of E. coli K-12. Strains and plasmids are given in Table I. Luria broth, T broth, and M9 minimal salts are as described above. Plasmid DNA was isolated from each revertant and used to transform UH889 (rbsB::Tn10) harboring a plasmid that carries rbsB103, which encodes a precursor of ribose-binding protein mediated by the wild-type leader peptide. The fragment of the plasmid pAI12 containing the rest of the coding sequence was placed under the control of the promoter of the rbsB gene, which encodes proteins with ribose-binding ability. The fragment of the plasmid pAI12, which carries the vector DNA and the portion of the rbsB gene which includes the leader sequence was ligated to the 2.8-kb BstXI-Hind111 fragment of pSP108 or pSP109 which carries the vector DNA and the portion of the rbsB gene which includes the leader sequence was ligated to the 2.8-kb BstXI-Hind111 fragment of pSP108 or pSP109, which carries the vector DNA and the portion of the rbsB gene which includes the leader sequence.

**Construction of Recombinant Plasmids**—For analysis of the effect of the suppressor mutational alterations on export of ribose-binding protein mediated by the wild-type leader peptide, the portion of the gene carrying the mutated leader sequence in plasmids pH07 and pH07W was replaced by the wild-type leader sequence. Two fragments are produced by double digestion with BamHI and BamHI of each of the plasmids pAI12 (harboring the wild-type rbsB gene), pH07, and pH07W. The large fragment (5.0 kb) carries all the vector and the first 30 codons of the rbsB gene which encode the leader sequence and the amino terminus of ribose-binding protein. The small (3.1 kb) fragment contains the rest of the coding sequence. The fragments were purified by agarose gel electrophoresis followed by electroelution. The 3.1-kb fragments from pH07 and pH07W were separately ligated to the 5.0-kb fragment from pAI12 to produce recombinant plasmids (pH07W and pTS6W) carrying genes which encode proteins with the suppressor changes in the mature portion and the wild-type leader sequence. To facilitate purification of the various species of ribose-binding protein, the genes were placed under the control of the λ P1 promoter. The alleles rbsB103 and rbsB103, rbsB106, an allele with the original mutation causing a defect in export as well as a suppressor mutation located in the leader sequence, have been cloned previously under the λ P1 promoter in plPC2833 (Park et al., 1988) to give the resultant plasmids pSP108 (rbsB103) and pSP109 (rbsB103, rbsB106). The 3.0-kb BstXI-HindIII fragment of pSP108 or pSP109 which carries the vector DNA and the portion of the rbsB gene which includes the leader sequence was ligated to the 2.8-kb BstXI-HindIII fragment of pH07 or pH07W. These constructions produced the recombinant plasmids pH101, pH102, pH103, and pH104.

**Determination of the Nucleotide Sequence**—Plasmids harboring rbsB103 or rbsB102 were purified by precipitation with polyethylene glycol (Maniatis et al., 1989) and the nucleotide sequence was determined using the T7 polymerase kit from Pharmacia LKB Biotechnology Inc. DNA primers used, designed to cover the entire rbsB gene in ~500-base pairs intervals, were synthesized with an oligonucleotide synthesizer.
Pulse-labeling—Cells (strains used: CP627, CP628, CP630, CP631, CP649, CP650) were grown to a density of 2 × 10^8 cells/ml in M9 minimal salts medium supplemented with 0.0004% vitamin B_12 and 0.4% glycerol and radiolabeled for 15 s by the addition of 60 μCi/ml [35S]methionine and nonradioactive methionine to give a final concentration of 90 nM methionine. At 15 s nonradioactive methionine was added to give a final concentration of 22 μM and at the times indicated samples of 10^6 cells were pipetted directly into trichloroacetic acid to give a final concentration of 5% trichloroacetic acid. The samples were processed for immunoprecipitation with antisera to ribose-binding protein and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (Randall and Hardy, 1986). The gels were dried and overlaid with x-ray film, and the bands of ribose-binding protein quantified by densitometry.

Purification of Various Species of Ribose-Binding Protein—Using the strains (CP610, CP611, CP632, CP633, HB786, HB787, HB1151, and HB1152) harboring plasmids that were constructed to express the rbsB genes under the control of the λ Pr promoter the ribose-binding proteins were purified by a combination of ammonium sulfate precipitation, anion exchange chromatography, and chromatofocusing as described in detail elsewhere (Teschke, 1990) (Fig. 1). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. To facilitate discussion, the positions of aminoacyl residues in the leader peptide are designated −1 to −25 with −25 referring to the extreme amino-terminal residue of the precursor. The amino-terminal residue of the mature form is designated +1. The mutant proteins will be named according to the mutational change as follows: the gene name, the wild-type amino acid, the position of the change, and then the changed amino acid. Thus the precursor protein encoded by the mutated allele rbsB103 is named RbsB L(−17)P, meaning that the leucyl residue at position −17 of the leader has been altered to a prolyl residue.

The amino-terminal amino acid sequence of purified ribose-binding proteins was found to be intact except for the first methionine residue. The precursor RbsB L(−17)P, S(−15)P was missing the first 5 aminoacyl residues and the preparation of precursor RbsB L(−17)P contained a mixture of both species.

Fluorescence Spectroscopy—The fluorescence of tyrosine is sensitive to the environment of the aminoacyl side chain. When tyrosyl residues that are buried in the hydrophobic interior of a folded protein are exposed to a more polar environment by the unfolding of the polypeptide chain, the amplitude of the fluorescence signal decreases (Lakowicz, 1983). Thus the intrinsic fluorescence of tyrosine can be used to monitor folding and unfolding reactions. Fluorescence measurements were made with a Shimadzu RF-540 fluorescence spectrophotometer with an excitation wavelength of 280 nm (slit width, 2 nm) and an emission wavelength of 303 nm (slit width, 5 nm). All fluorescence measurements were performed at 25°C.

Equilibrium Studies of Unfolding Transitions—Native proteins (final concentration of 18.5 μg/ml) were added to varying concentrations of guanidinium chloride in 10 mM Hepes, pH 7.7, and after equilibrium had been achieved the fluorescence intensity of each sample was measured. The fraction of the protein that was unfolded was taken as (F_e−F_u)/(F_e−F_u), where F_e refers to the observed fluorescence, and F_e and F_u refer to the fluorescence of the native and the unfolded protein at the particular concentration of guanidinium chloride. The values for F_e and F_u were obtained by linear extrapolation into the region of the unfolding transition (Creighton, 1987). The concentration of the guanidinium chloride was confirmed by measuring the conductance of the solution.

Kinetic Studies of Protein Folding Reactions—Ribose-binding protein in either the native or unfolded state was subjected to a rapid change in conditions and the approach to the new equilibrium was followed by monitoring the change in the fluorescence intensity of the sample. The relaxation time was extracted from a plot of the log of the change in fluorescence versus time. Unfolding reactions were initiated by addition of the purified protein in the native state to a solution of guanidinium chloride buffered with 10 mM Hepes, pH 7.7, held in a cuvette in the chamber of the spectrophotometer, to give a final protein concentration of 18.5 μg/ml and a concentration of guanidinium chloride as indicated for each experiment. For refolding studies, protein was unfolded by incubation in guanidinium chloride buffered with 30 mM Hepes, pH 7.7. Folding was initiated by addition of the denatured protein to a predetermined value of solution of buffered guanidinium chloride such that the final protein concentration would be 18.5 μg/ml and the final concentration of denaturant would be as indicated for each experiment.

RESULTS

Isolation of Intragenic Suppressors—Ribose-binding protein is a periplasmic protein in E. coli that functions in transport of ribose into the cell and in chemotaxis to ribose. The protein is synthesized in the cytosol as a precursor that carries a leader sequence comprising 25 amino-terminal residues. The gene encoding the ribose-binding protein, rbsB, is a member of the Rbs operon which contains the genes for a high-affinity (K_F ~ 1 μM) transport system as well as the rbsK gene which encodes ribokinase (Iida et al., 1984; Lopilato et al., 1984). Strains that are defective in the Rbs transport system but that have an active ribokinase are able to grow on ribose if it is present at high concentration indicating that the cells must have at least one other transport system which has a low affinity for ribose; however, they grow very poorly on minimal medium supplemented with 0.05% ribose. The allele rbsB103 encodes a precursor that has a defective leader sequence (leucine at position −17 changed to proline) and is not exported to the periplasm, but accumulates within the cell in an unprocessed form. Cells harboring this gene cannot grow on 0.05% ribose, thus providing a means to isolate mutations which restore export simply by selecting for growth on ribose at a low concentration.

Selection in liquid medium yielded revertants from 13 of 20 independent cultures, whereas selection on plates yielded 140 revertants (see “Experimental Procedures” for details). To eliminate revertants that had gained the ability to grow on ribose by a mechanism other than restoration of export of ribose-binding protein, chemotaxis toward ribose was as-

![Fig. 1. SDS-polyacrylamide gel electrophoresis of purified ribose-binding proteins.](image-url)
The bacteria at the site of inoculation metabolize the ribose creating a steep gradient of the sugar at the periphery of the extragenic suppressors, and 1 suppressor, rbsB201, had a mutation in the mature portion of ribose-binding protein. The 6 strains carrying either true reversions or extragenic suppressors will be described elsewhere. Of the 140 revertants isolated by the plate selection only 8 showed taxis toward ribose suggesting that most of these strains do not export ribose-binding protein to the periplasm. The chemotactic responses to ribose of strains harboring rbsRI03 in addition to either rbsB201, the one suppressor isolated from liquid culture, or rbsB202, one of the 8 suppressors isolated by the plate technique, are compared in Fig. 2 with the normal tactic response exhibited by a strain producing wild-type ribose-binding protein and with the defective response of a strain producing the export-defective precursor RbsB L(-17)P, the product of rbsB103.

Mutational Changes in the Primary Structure—Comparison of the entire nucleotide sequence of rbsB201 with the published sequence of wild-type rbsB (Groarke et al., 1983) revealed seven differences in bases. All but one of the differences occur in wobble positions and it is likely that they are due to the fact that the origin of the rbsB gene used in this study differs from that of the gene sequenced by Groarke et al. (1983). The codon that corresponds to the 50th aminoacyl residue of the mature protein is GTG in the wild-type and GAG in the mutated gene. This difference results in the replacement of the valine of the wild-type ribose-binding protein by a glutamate. In the case of rbsB202 the only difference in the nucleotide sequence that alters the aminoacyl sequence is that the replacement of G by A in the 27th codon of the wild-type sequence (GCG to ACG) causes alanine to be replaced by threonine in the suppressor protein. The mature ribose-binding proteins encoded by rbsB201 and rbsB202 are thus designated RbsB V50E and RbsB A27T, respectively.

Kinetics of Processing of the Ribose-binding Proteins—Investigations of export in vivo show that precursor ribose-binding protein carrying a wild-type leader sequence was proteolytically processed to the mature, periplasmic form within a minute after synthesis (Fig. 3A). In contrast, at 10 min after synthesis only 5% of the precursor RbsB L(-17)P that has an altered leader sequence was matured (Fig. 3A). A revertant of rbsB103, described previously (Iida et al., 1985), that exhibits near normal kinetics of processing has a second mutational change in the leader peptide (allele, rbsB103, rbsB106; protein product RbsB L(-17)P, S(-15)F). Presumably, this mutational change restores the function of the leader directly. The suppressors isolated in this study that cause changes in the mature portion of the protein are not as effective as the revertant with the altered leader sequence, but they improve the rate of export of the defective precursor substantially (Fig. 3, B and C).

Fig. 2. Chemotactic phenotype of the suppressors. The ability of the suppressors to form a chemotactic ring on a semisolid agar swarm plate containing ribose was tested as described in the text. The strain CP626 which does not produce ribose-binding protein from the chromosome (rbsB102::Tn10) was carrying the following plasmids: 1, pAI12 encoding wild-type ribose-binding protein; 2, pHOT encoding the suppressor RbsB L(-17)P, V50E; 3, pHO7 encoding RbsB V50E with a wild-type leader; 4, pTS6 encoding the suppressor RbsB L(-17)P, A27T; 5, pTS6W encoding RbsB A27T with a wild-type leader; 6, pAI27 encoding the defective precursor, RbsB L(-17)P.

Fig. 3. Kinetics of processing or ribose-binding proteins. Cultures growing exponentially were labeled as described under "Experimental Procedures." A, the kinetics of processing of wild-type ribose-binding protein (Δ, V represent duplicate experiments), defective precursor RbsB L(-17)P (C), and the suppressor with a second change in the leader sequence RbsB L(-17)P, S(-15)F (O). B, the kinetics of processing of suppressor RbsB L(-17)P, V50E (C) and RbsB V50E with a wild-type leader (O). C, the kinetics of processing of suppressor RbsB L(-17)P, A27T (Δ, V represent duplicate experiments) and RbsB A27T with a wild-type leader (O). In B and C, the dashed line represents the kinetics of processing of the defective precursor RbsB L(-17)P and the dotted line the processing of wild-type ribose-binding protein taken from A.
At steady state, as judged by the level of Coomassie Blue staining of the immunoprecipitated proteins, RbsB L(−17)P produced no mature ribose-binding protein, whereas the mature forms of RbsB V50E and RbsB A27T were present in variable amounts corresponding to approximately 10–50% of the amount of mature wild-type ribose-binding protein. The total amount of ribose-binding protein present at steady state in the strains harboring the suppressors was similar to that present in the strain carrying the wild-type gene; however, whereas all wild-type precursor ribose-binding protein is processed to mature, substantial amounts of the precursors carrying the defective leader and the suppressor mutations accumulated within the cell in the unprocessed form thus accounting for the decreased amount of matured proteins in the periplasm.

When the mutational alterations in the mature portion were combined with a wild-type leader peptide, the kinetics of export were normal (Fig. 3, B and C). Thus, it is unlikely that the aminoacyl substitution in the mature suppressors by restoring a crucial interaction between the altered leader and the mature portion of the protein. If this were the case, the mutational change in the mature portion would be expected to have an adverse effect on interaction with a wild-type leader. An alternative explanation of the suppression is provided by a model of the export process that includes a kinetic partitioning between the productive translocation of the precursor across the membrane and the folding of the precursor inside the cell to a state incompatible with translocation. Leader peptides have multiple roles in export. They function during the initial interactions with the export apparatus, the entry phase, and also during translocation (Randall and Hardy, 1989). An alteration in the leader might decrease the efficiency of any of several steps. Indirect suppression of these various defects could occur if the folding of the precursor into the incompetent state were slowed, thus allowing more time for the defective precursor to be exported. We, therefore, examined these proteins to determine whether the aminoacyl substitutions had altered the folding properties.

Equilibrium Studies of the Unfolding Transitions of Ribose-binding Proteins—Ribose-binding protein contains tyrosinyl residues at positions 32, 115, and 261 of the mature protein, but has no tryptophanyl residues (Groarke et al., 1983), thus the intrinsic fluorescence of tyrosine could be used to investigate the stabilities and folding properties of the protein. The denaturant-induced, reversible, unfolding transitions of the various species of mature and precursor ribose-binding proteins were compared (Figs. 4–7). All of the transitions are well described by a two-state model involving a nature and unfolded state:

\[ \text{native} \leftrightarrow \text{unfolded} \]

The midpoint of the transitions for the wild-type mature, the wild-type precursor, and the precursors RbsB L(−17)P and RbsB L(−17)P, S(−15)F all occurred at a guanidinium chloride concentration of approximately 475 mM indicating that these proteins have similar stabilities (Figs. 4 and 5). Relative to those proteins, which have the wild-type sequence in the mature portion, all forms that contain the mutational substitutions in the mature portion showed a decreased stability. The midpoint of the equilibrium transition for the mature form, RbsB A27T and for the precursor form containing both

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**Equilibrium transition curves for unfolding of wild-type precursor and mature ribose-binding proteins.** The unfolding of ribose-binding proteins induced by the addition of guanidinium chloride was monitored by the intrinsic fluorescence of tyrosine could be used to investigate the stabilities and folding properties of the protein. The denaturant-induced, reversible, unfolding transitions of the various species of mature and precursor ribose-binding proteins were compared (Figs. 4–7). All of the transitions are well described by a two-state model involving a nature and unfolded state:

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The stability of the native state as compared to the unfolded state was calculated from the relationship \( \Delta G_s = -RT \ln K_N \) where \( K_N \) is \( [N/U] \). The value of \( \Delta G_s \) in the presence of denaturant was derived from the equilibrium data. The value for \( \Delta G_s \) at 0 M guanidinium chloride was obtained by extrapolation. In this analysis \( \Delta G_s \) was taken to be a linear function of the concentration of denaturant (Pace, 1975; Schellman, 1978). Energies of stabilization are expressed in kcal mol\(^{-1}\). ND, not determined.

<table>
<thead>
<tr>
<th>Leader</th>
<th>Mature</th>
<th>Wild type L(-17)P</th>
<th>L(-17)P, S(-15)F</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>-4.8</td>
<td>-4.9</td>
<td>-4.1</td>
<td>-5.1</td>
</tr>
<tr>
<td>V50E</td>
<td>ND</td>
<td>-1.1</td>
<td>ND</td>
<td>-1.6</td>
</tr>
<tr>
<td>A27T</td>
<td>ND</td>
<td>-2.4</td>
<td>ND</td>
<td>-1.8</td>
</tr>
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Fig. 8. The GuHCl dependence of the relaxation times for folding and unfolding of wild-type mature and wild-type precursor ribose-binding proteins. The experiments are described under “Experimental Procedures.” The open symbols denote the relaxation times of folding and the closed symbols the relaxation times of unfolding. The squares are the relaxation times for wild-type mature ribose-binding protein and the circles for wild-type precursor ribose-binding protein. To unfold wild-type mature or wild-type precursor ribose-binding protein, precursors RbsB L(-17)P or RbsB L(-17)P, S(-15)F, the proteins were incubated at room temperature for 2 h in a solution of 1.25 M GuHCl.

the change in the mature portion as well as the alteration, L(-17)P, in the leader occurred at 225 mM guanidinium chloride and the midpoint of the transition for the corresponding polypeptides carrying the substitution V50E in the mature was at 55 mM guanidinium chloride (Figs. 6 and 7). Quantitative analysis of the equilibrium data indicates that the mutational changes destabilize the proteins relative to the wild-type by approximately 2.5-3.8 kcal/mol (Table II). Since the equilibrium between the native and unfolded state of a protein reflects the ratio of the rate constants \( k_u/k_f \), the mutational changes could destabilize the protein by affecting the rate of folding, the rate of unfolding or both rates unequally. Studies of the kinetics of folding of the proteins were carried out to determine which reactions were affected.

Kinetic Studies of the Folding Reactions of Ribose-binding Proteins—Purified protein, either in the native or in the unfolded state, was subjected to a rapid change in conditions that required the protein to reach a new equilibrium mixture of the native and unfolded states. The relaxation time to reach the new equilibrium was determined by monitoring the change in fluorescence with time. A series of experiments was carried out for each form of ribose-binding protein and the results are presented as a plot of the relaxation time as a function of the final concentration of guanidinium chloride.

Fig. 9. The GuHCl dependence of the relaxation times for folding and unfolding of precursors RbsB L(-17)P and RbsB L(-17)P, S(-15)F. The experiments are described under “Experimental Procedures.” The open symbols represent the relaxation times of folding and the closed symbols the relaxation times for unfolding. The squares are the relaxation times for precursor RbsB L(-17)P and the circles the relaxation times for precursor RbsB L(-17)P, S(-15)F. The proteins were unfolded as described in Fig. 8. The dotted line indicates the relaxation times for the wild-type ribose-binding protein taken from Fig. 8 and is added for comparison.

Fig. 10. The GuHCl dependence of the relaxation times for folding and unfolding of precursor RbsB L(-17)P, V50E and mature RbsB V50E. The experiments are described under “Experimental Procedures.” The relaxation times for folding are denoted by open symbols and for unfolding by closed symbols. The squares are the relaxation times for mature RbsB V50E and the circles the relaxations for precursor RbsB L(-17)P, S(-15)F. To unfold mature RbsB V50E or precursor RbsB L(-17)P, V50E, the proteins were incubated at room temperature for 2 h in 0.2 M GuHCl.
The GuHCl dependence of the relaxation times for folding and unfolding of precursor RbsB L(-17)P, A27T and mature RbsB A27T. The experiments are described under "Experimental Procedures." The folding relaxation times are shown in the open symbols and the unfolding in the closed symbols. The squares are the relaxation times for mature RbsB A27T and the circles the relaxation times for precursor RbsB L(-17)P, A27T. To unfold mature RbsB A27T or precursor RbsB L(-17)P, A27T, the proteins were incubated at room temperature for 2 h in 1 M GuHCl.

FIG. 12. Structure of ribose-binding protein. The stars indicate the positions of the mutational changes V50E (right) and A27T (left). The amino (N) and carboxyl (C) termini are labeled.

(Figs. 8-11). For all proteins studied the unfolding and refolding reactions are described by a single kinetic phase that accounts for greater than 90% of the expected change in fluorescence intensity. In the unfolding reaction we are monitoring the rate-limiting step of the native protein unfolding to whatever species follows it. The refolding reaction observed is clearly the reverse of the rate-limiting step in unfolding since the relaxation times for unfolding and refolding connect smoothly at the inflexion point (Figs. 8-11). The relaxation time (r) is related to the rate constants as follows: r^-1 = k_r + k_i. Under conditions in which the final concentration of guanidinium chloride is high, k_r >> k_i and r^-1 \approx k_i. When the concentration of denaturant is low k_r \gg k_i and r^-1 \approx k_i.

The presence of a leader on ribose-binding protein, whether it be the wild-type leader (Fig. 8), the defective leader or the leader which is functional because it carries a second mutational change (Fig. 9), decreases the rate of folding relative to that of the mature species. Extrapolation of the relaxation times to 0 M guanidinium chloride indicates that the relaxation time is slowed from 7 to 12 s. The substitution in RbsB V50E slows the folding of the precursor 13-fold (relaxation time, 156 s at 0 M guanidinium chloride) and mature species 15-fold (relaxation time, 107 s) relative to the wild type. The substitution of threonine for alanine in RbsB A27T also slowed the folding reactions. The relaxation time (extrapolated to 0 M denaturant) for folding of the precursor was 236 s and 117 s for folding of the mature, changes, respectively, of 19- and 16-fold over wild type.

The most dramatic effect on unfolding was observed for RbsB V50E; both the precursor and mature forms unfolded more rapidly than did the corresponding wild-type species (Fig. 10). In contrast the substitution A27T slowed unfolding but the magnitude of the effect was small compared to that of V50E.

DISCUSSION

The leader sequence mutation rbsB103 leads to a defect in the export of ribose-binding protein to the periplasm. As a result the cells grow poorly on low concentrations of ribose and are defective in chemotactic response to ribose. Suppressors were selected for the ability to grow on low concentrations of ribose and subsequently screened for normal chemotactic response. In all cases examined a mutational event that simultaneously restored high-affinity transport and chemotaxis also restored export of the altered precursor ribose-binding protein. Two of the suppressors that were shown to be intragenic were studied in detail. Both strains exported an increased amount of ribose-binding protein relative to the amount exported by the strain expressing the precursor with the altered leader sequence. The suppressor mutations were both localized to the region of the gene encoding the mature portion of the polypeptide. In one, valine at the 50th residue was changed to glutamate (V50E) and in the other alanine at position 27 was changed to threonine (A27T). Both protein species bound ribose though the affinity for the sugar was lower than that of the wild-type protein: for RbsB V50E the affinity was 20-fold lower and for RbsB A27T, 2-fold lower. Both mutational changes drastically decreased the rate of unfolding. A suppressor mutation, selected by the ability to restore export, that also altered folding was first described for maltose-binding protein (Cover et al., 1987; Liu et al., 1988). A change in the mature protein from tyrosine at position 283 to aspartate drastically reduced the folding rate with very little effect on the unfolding rate.

Maltose-binding protein and ribose-binding protein belong to a family of periplasmic proteins that function as receptors for many small molecules during transport and chemotaxis in Gram-negative bacteria. The periplasmic binding proteins are characterized by two structurally similar domains each comprising a central \( \beta \) sheet structure positioned between two layers of \( \alpha \)-helices. Each domain is made up of noncontiguous segments of the polypeptide chain with the chain passing between the two globular domains three times. Both of the mutational substitutions which change the kinetics of folding of ribose-binding protein lie in \( \alpha \)-helices in the globular domain that contains the aminoterminus of the polypeptide (domain 1, Fig. 12). The nucleotide sequences of an additional seven intragenic suppressors isolated as described above have been determined and the mutational changes in all but one are localized in domain 1. Interestingly, the maltose-binding

2 C. Park, unpublished results.
protein that was identified by selection for suppression of defective export carries a change in domain 1 (Y283D, Spurli, 1988) of that protein. Since mutational changes that were generated randomly in two different binding proteins all lie in domain 1, it seems likely that the acquisition of structure in this domain is the rate-limiting step in the folding of the binding proteins. The determination of the folding properties of the remaining suppressor species should firmly establish this point.

The selection procedure described here can be easily extended to other members of the binding protein family and may be applicable to any protein that can be expressed in E. coli and that undergoes a kinetic partitioning between export and folding.

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Folding Mutants Selected as Suppressors of Export Defects

REFERENCES

Adler, J. (1973) J. Gen. Microbiol. 74, 77-91
Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Schellman, J. (1978) Biopolymers 17, 1305-1322