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Modulation of Folding Pathways of Exported Proteins by the Leader Sequence

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Leader peptides that function to direct export of proteins through membranes have some common features but exhibit a remarkable sequence diversity. Thus there is some question whether leader peptides exert their function through conventional stereospecific protein-protein interaction. Here it is shown that the leader peptides retarded the folding of precursor maltose-binding protein and ribose-binding protein from *Escherichia coli*. This kinetic effect may be crucial in allowing precursors to enter the export pathway.

EFFICIENT TRANSFER OF PROTEINS through membranes during the processes of secretion and mitochondrial assembly in eukaryotes and of export in prokaryotes requires that the polypeptide has not adopted the stably folded structure of the mature species (*1, 2*). The current models for the export process incorporate the effects of protein conformation in different ways. One hypothesis prevalent among workers studying eukaryotic systems is that cells contain factors that actively unfold structured precursors by using the hydrolysis of nucleotide triphosphates as a source of energy (*3, 4*). A different notion, proposed for bacterial export, is that components within the cells bind to precursors before they fold into the final mature conformation and thereby maintain (or create) the export-competent state without having to disrupt established tertiary structure (*2, 5*). A defining and essential feature of a protein destined for export to the periplasm of *Escherichia coli* is that at its amino terminus it contains a leader sequence that is proteolytically removed to generate the mature species upon translocation across the membrane. Although functional leader peptides have some common features, there is no sequence similarity among such peptides. In bacteria the leader peptide is likely to be involved in several phases of export, initially in mediating entry into the export pathway, and subsequently in establishing interaction with the membrane at export sites (*6–8*). The precise role of the leader sequence in these separate steps may differ. Gierasch and co-workers (*9*) have suggested that during the encounter of the precursor with the membrane, the hydrophobicity and conformation of the leader are crucial for its proper insertion into the bilayer. We propose that, in addition, at an earlier step leader sequences allow the initial interaction with components of the export apparatus by modulating the folding pathways of precursor polypeptides, and we present evidence that the leader sequences of two periplasmic proteins from *Escherichia coli,* those of maltose-binding protein and ribose-binding protein, decrease the rates of folding of these proteins into their mature conformations.

Since we were interested in investigating the effect of leader sequences on the pathway of protein folding in the absence of interaction with any other cellular components, we purified the precursor and mature forms of the proteins (*10, 11*) and compared the kinetics of their folding in vitro. Both maltose-binding protein (molecular weight 38,500) and ribose-binding protein (molecular weight 29,000) are monomeric and contain no disulfide bonds. Maltose-binding protein contains eight tryptophanyl residues and thus the reversible unfolding-folding transition could be monitored by changes in the intrinsic fluorescence of tryptophan (*12*). In contrast, fluorescence spectroscopy could not be used to monitor the folding of ribose-binding protein, because that protein contains no tryptophan, and no change in the fluorescence of tyrosine was observed when the protein was denatured. Studies of the folding of ribose-binding protein were thus performed by measuring the resistance of the mature conformation to proteolytic degradation as the assay for folding.

Using fluorescence spectroscopy we have investigated the equilibrium, unfolding

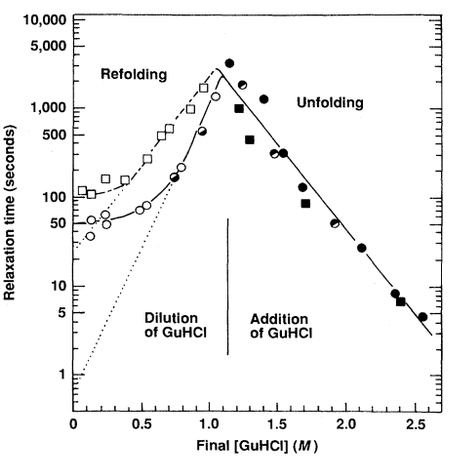

Fig. 1. Comparison of relaxation times for folding transitions of mature and precursor maltose-binding proteins. The relaxation times for folding (open symbols) and unfolding (closed symbols) transitions were obtained by monitoring the change in fluorescence of tryptophan. For unfolding transitions the protein was initially in 10 m*M* Hepes, *p*H 7.8, and guanidinium hydrochloride (GuHCl) was added to the final concentration shown. For refolding transitions, the protein was dissolved in 2*M* GuHCl, which was diluted to the final concentration shown. Fluorescence measurements were made with a Perkin-Elmer MPF-3L. The excitation and emission wavelengths used were 295 nm and 344 nm, respectively; mature maltose-binding protein (○); precursor maltose-binding protein (□). The precursor species was purified by a procedure that includes one cycle of unfolding-refolding (*10*). As a control, the mature species was subjected to a cycle of unfolding-refolding. Relaxation times determined with this material are represented by the half-closed circles; unfolding transition (◐); refolding (◑).

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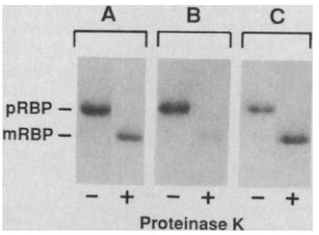

Fig. 2. Assay of the folded state of ribose-binding protein by measuring resistance to proteolytic degradation. Purified precursor ribose-binding protein (molecular weight 31,000) was subjected to the following treatments before assessing the resistance to proteolytic degradation. (**A**) Native protein, no treatment, held on ice. (**B**) Unfolded by incubation with 6*M* urea, 10 m*M* Hepes, *p*H 7.1, for 30 minutes at 30°C. (**C**) Unfolded as described for (B) and subsequently refolded by dilution of the urea to 0.1*M* with 10 m*M* Hepes, *p*H 7.1, and incubation at 30°C for 30 minutes. Following treatment, the samples were placed on ice and incubated without (−) or with (+) proteinase K (1.5 μg/ml) for 20 minutes. Proteolysis was terminated by addition of sample buffer for SDS–polyacrylamide gel electrophoresis (*2*) containing phenylmethylsulfonyl fluoride (0.2 m*M*). After incubation at 100°C for 5 minutes, the samples were analyzed by SDS–13% polyacrylamide gel electrophoresis. Only the relevant portion of the Coomassie blue–stained gel is shown. Proteolytic digestion of the precursor when it is in a folded state results in removal of the leader; the mature portion, which is resistant to degradation, is recovered as a species that migrates during electrophoresis at the position of the mature protein.

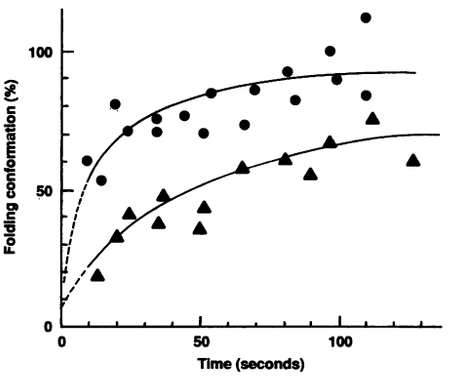

Fig. 3. Kinetics of folding of mature and precursor forms of ribose-binding protein. Purified protein was unfolded by incubation at 30°C for 30 minutes in 6*M* urea, 10 m*M* Hepes, *p*H 7.1. Folding was initiated at 12.5°C by dilution of the urea to a final concentration of 0.1*M* in 10 m*M* Hepes, *p*H 7.1. Samples were withdrawn at the times indicated and rapidly chilled by transfer into tubes held on ice. The resistance of ribose-binding protein to proteolytic degradation was assessed as described in the legend to Fig. 2. Quantification of the percent of protein that had folded was obtained from densitometric tracings of the Coomassie blue–stained gels. Samples withdrawn at 2 and 5 minutes indicated that the plateau levels of folding were 95 and 70% for the mature and precursor proteins, respectively. To determine the relative effect of the leader on folding, the times taken for the species to obtain half of the maximal folding observed were compared. Mature ribose-binding protein (●); precursor, ribose-binding protein (▲).

transition of maltose-binding protein induced by guanidinium hydrochloride (GuHCl) (*13*) and have shown that the data can be approximated by a simple two-state model. We can represent the reaction as follows:

$$\text{Native} \underset{k_f}{\overset{k_u}{\rightleftharpoons}} \text{Unfolded}$$

The experimental approach that we have taken is based on the elegant work of Matthews and co-workers (*14*). Purified maltose-binding protein either in a folded (no denaturant present) or unfolded (denaturant present) state was subjected to a rapid change in conditions that required the protein to achieve a new equilibrium mixture of the native and unfolded states. The relaxation time to reach the new equilibrium position was determined by monitoring the change in fluorescence with time. Experiments were carried out to determine the relaxation time as a function of the final concentration of GuHCl (Fig. 1).

Comparison of the data for the mature protein with those for the precursor indicates that the presence of the leader sequence does not significantly alter the relaxation time for the unfolding transition but increases the relaxation time, τ, for the folding transition, which can be related to the rate constants as $\tau^{-1} = k_u + k_f$. For experiments in which the final concentration of denaturant is high, $k_u >> k_f$ and $\tau^{-1} \cong k_u$. Where the concentration of denaturant is low, $\tau^{-1} \cong k_f$. The data show that the presence of the leader decreases the rate of folding but has little effect on the rate of unfolding. The magnitude of the effect that might be physiologically significant is difficult to define precisely since we do not know what limits the rate of folding in vivo. The in vitro rate-limiting step, which is monitored by the fluorescence, changes at approximately 0.5*M* GuHCl. Below that concentration the relaxation time is independent of the concentration of denaturant, whereas above 0.5*M* the relaxation time shows dependence on the final concentration of GuHCl. The denaturant-independent phase may reflect cis-trans isomerization of proline residues about X-Pro peptide bonds (*15*). The presence of the leader sequence increases the relaxation time for this phase by a factor of ~3. If the rate-limiting step in vivo were that reflected in the transitions above 0.5*M* GuHCl, the presence of the leader would have an even greater effect: extrapolation to 0*M* GuHCl shows that the leader increases the relaxation time of this step by about 40-fold, from 0.7 to 28 seconds (Fig. 1).

The presence of a functional leader peptide on ribose-binding protein (*11*) decreases the rate of folding of that precursor by a factor of 3 relative to the rate of folding of the corresponding mature protein (Figs. 2 and 3). Since analysis of folding of ribose-binding protein used the resistance to proteolytic degradation as the assay for the folded state (Fig. 2), refolding had to be monitored under conditions in which the folding was complete at equilibrium (that is, a rapid dilution of the urea from 6 to 0.1*M*). Thus our analysis was in this case restricted to effects on the step that is rate limiting under these conditions. The folding pathway may include another step (as was the case for the folding of maltose-binding protein) that might also be influenced by the leader. Unfortunately, we could not investigate this possibility.

The final folded state achieved by each precursor polypeptide resembles that of the corresponding mature species because treatment with proteinase K removed only the leader sequence from each precursor and left the mature protein intact [see Fig. 2 and (*2*)]. In addition, precursor maltose-binding protein was purified by its ability to bind ligand, which suggests the achievement of the native structure.

The export apparatus may not be capable of interacting with or translocating a precursor polypeptide after it has attained the conformation characteristic of the mature species. The slowing of the folding rate by leader sequences might reflect a function critical to export. Modulation of the folding pathway by the presence of the leader peptide could either expose or create an element of structure that is not accessible or not present in the mature species, but that is essential to allow functional interaction with the export apparatus as was originally proposed in the trigger hypothesis (*16*). Such a role for leader peptides could explain the immense variation that exists among sequences that function to initiate export. In addition to the wide range of naturally occurring leader sequences, a surprisingly high frequency of randomly cloned sequences function in export in yeast [20%, (*8*)] and in bacteria [between 4 and 15%, (*17*)]. It may be that many sequences, normally sequestered by the rapid folding of polypeptides, when moved out of their proper context and inserted at the beginning of an exported protein, can kinetically interfere with folding to the mature species and

allow the protein to enter the export pathway.

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    To facilitate purification of mature ribose-binding protein and the precursor form, the appropriate alleles of *rbsB* were cloned under the lambda P_L promoter on the plasmid vector pPLC2833. The procedures for cloning and purification are described in detail elsewhere [S. Park, thesis, Washington State University, Pullman (1987)]. The mature protein was purified from the periplasmic fraction obtained by osmotic shock of cells and the precursor was obtained from lysed spheroplasts. The purification procedure involved two sequential steps of ion-exchange column chromatography (DEAE-Sephadex and CM-Sephadex) followed by chromatofocusing (Pharmacia, Mono P). We were unable to obtain quantities of wild-type precursor sufficient for these studies. Therefore we used a precursor with a mutated but functional leader sequence [see (*11*)].
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Donor-Derived Cells in the Central Nervous System of Twitcher Mice After Bone Marrow Transplantation

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The twitcher mouse is an animal model of galactosylceramidase deficiency, comparable to Krabbe's disease, a lysosomal storage disease in humans. As in most lysosomal storage diseases, neurological deterioration is a prominent feature of the disease in these mice. Transplantation of enzymatically normal congenic bone marrow was earlier found to result in prolonged survival and increased levels of galactosylceramidase in the visceral organs of twitcher mice. It is now reported that bone marrow transplantation results in increased galactosylceramidase levels in the central nervous system (CNS). Concomitantly, the levels of psychosine, a highly toxic lipid that progressively accumulates in the CNS of untreated twitcher mice, stabilized at much lower levels in the CNS of treated twitcher mice. Histologically, a gradual disappearance of globoid cells, the histological hallmark of Krabbe's disease, and the appearance of foamy macrophages capable of metabolizing the storage product were seen in the CNS. By immunohistochemical labeling it was demonstrated that these foamy macrophages were of donor origin. The infiltration of enzymatically competent, donor-derived macrophages was accompanied by extensive remyelination in the CNS. It is concluded that after bone marrow transplantation, donor-derived macrophages infiltrate the affected brain tissue and are capable of inducing a partial reversal of the enzyme deficiency.

SINCE THE FIRST BONE MARROW transplantation (BMT) for lysosomal storage disease by Hobbs *et al.* (*1*), many studies on the effect of BMT in patients and animal models with lysosomal storage diseases have been reported (*2–7*). After BMT, the enzymatically normal donor-derived blood cells and tissue macrophages served as a continuous source of enzyme and led to increased enzyme levels in leukocytes, plasma, and various visceral organs (*2–7*). The effects of BMT on the neurological symptoms, which are very prominent in many lysosomal storage diseases, vary with the type of disease. For instance, in patients with metachromatic leukodystrophy, BMT led to improved psychomotor development in comparison with siblings receiving no treatment (*2, 8*), whereas in patients with Sanfilippo's disease, BMT did not lead to beneficial effects (*9*). Increased enzyme levels after BMT were reported in the central nervous system (CNS) of a dog suffering from fucosidosis, but without alleviation of the neurological symptoms (*5*). In β-glucuronidase–deficient mice and in a feline model of arylsulfatase-B

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