Ricin A Chain Fused to a Chloroplast-targeting Signal Is Unfolded on the Chloroplast Surface Prior to Import across the Envelope Membranes*

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The initial stages of chloroplast protein import involve the binding of precursor proteins to surface-bound receptors prior to translocation across the envelope membranes in a partially folded conformation. We have analyzed the unfolding process by examining the conformation of a construct, comprising the presequence of a chloroplast protein linked to ricin A chain, before and after binding to the chloroplast surface. We show that the presequence is highly susceptible to proteolysis in solution, probably reflecting a lack of tertiary structure, whereas the A chain passenger protein is resistant to extremely high concentrations of protease, unless deliberately unfolded using denaturant. The A chain moiety is furthermore active, indicating that the presence of the presequence does not prevent formation of a tightly folded, native state. In contrast, receptor-bound p33KRA (fusion protein comprising the 33-kDa presequence plus 22 residues of mature protein, linked to the A chain of ricin) is quantitatively digested by protease concentrations that have little effect on the A chain in solution. We conclude that protein unfolding can take place on the chloroplast surface in the absence of translocation and without the aid of soluble factors.

The translocation of proteins across the chloroplast envelope is a complex process requiring the activities of a number of proteins in both the outer and inner envelope membranes (reviewed in Ref. 1). Imported proteins are initially synthesized with amino-terminal presequences which contain specific targeting signals that ensure recognition by receptors on the chloroplast surface and subsequent translocation into the stroma, probably at contact sites between the two membranes (2). The molecular nature of the chloroplast-targeting signals have remained elusive, but considerable progress has been made in identifying and characterizing components of the import apparatus in the envelope membranes (3–6).

Some aspects of the overall import mechanism have been largely elucidated, for example requirements for ATP hydrolysis in both the binding and translocation processes (7, 8), but other aspects remain unclear. Of particular interest are the import characteristics of a different fusion protein (p33KRA)1 imported proteins in both the outer and inner envelope membranes (reviewed in Ref. 1). Import intermediates have been identified which span both membranes, demonstrating that precursor proteins traverse the envelope in an unfolded or partially folded conformation (2, 9). Because chloroplast protein import can proceed entirely posttranslationally, the key question arises as to how and when this unfolding is accomplished and in particular whether it is achieved before or after the precursor interacts with the chloroplast. Perhaps surprisingly, rather few studies have examined the conformations of precursor proteins in solution, possibly because most studies have employed in vitro translation systems in which proteins are synthesized in minute quantities, precluding detailed structural studies on the precursor proteins at any stage of the import process. There is some evidence that chloroplast protein presequences are substantially, if not completely unfolded (10, 11), but there is no evidence for the presence of a factor in cell-free translation systems which maintains chloroplast precursor proteins in an unfolded conformation. On the contrary, the precursor of 5-enolpyruvylshikimate-3-phosphate 1-carboxyvinyltransferase was found to be catalytically active, and thus presumably correctly folded, after synthesis in a reticulocyte lysate (12), and protease sensitivity studies on the precursor of a 23-kDa thylakoid lumen protein suggested that this protein comprised an unfolded presequence together with a folded mature moiety (13). However, the only precursor protein to be studied in detail has been that of ferredoxin (10), and this study concluded that the entire precursor protein was relatively unfolded. This may be because folding of this particular protein requires formation of the FeS groups, but it nevertheless remains the case that the structures of chloroplast protein precursors in general are poorly understood.

The driving force for the unfolding process is equally poorly defined and only one study has addressed the conformation of a protein following binding to the chloroplast. In this study (14) it was found that a presequence-dihydrofolate reductase construct was highly sensitive to proteolysis once bound to the chloroplast, whereas proteases converted the precursor protein primarily to mature size dihydrofolate reductase in solution. The interpretation was that the protein was relatively unfolded once bound to receptors. Furthermore, additional studies suggested that the unfolding activity may be quite powerful: dihydrofolate reductase becomes even more tightly folded once a substrate analog, methotrexate, binds in the active site, to the extent that translation of chimeras into mitochondria is blocked (15). However, this treatment did not prevent import into chloroplasts (16), raising the possibility that a particularly potent unfolding activity is associated with the surface-exposed import machinery. In this report we have investigated the import characteristics of a different fusion protein (p33KRA)1

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1 The abbreviations used are: p33KRA, fusion protein comprising the
in which the presequence of a 33-kDa thylakoid lumen protein (33K) is linked to ricin A chain. Previous work (17) has shown this protein to be efficiently imported into intact chloroplasts, and assays are available to assess whether this protein is active even as an in vitro translation product. We show that the fusion protein is highly active in solution and that the A chain is remarkably resistant to proteolysis as a result of its tightly folded native conformation. We also show that the protein becomes susceptible to digestion once bound to the chloroplast import machinery, providing evidence that the chloroplast is able to partially unfold tightly folded proteins without the assistance of soluble import factors.

**EXPERIMENTAL PROCEDURES**

**Synthesis of p33KRA and Binding/Import Assays—**p33KRA was synthesized in vitro by transcription of a cDNA construct followed by translation in a wheat germ lysate and incubated with protease K at the concentrations indicated above the lanes for 40 min at 0°C. After incubation, samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. RA denotes mobility of authentic mature size A chain marker. Right-hand panel, p33KRA was incubated in the absence of protease or the presence of 50 μg/ml protease K as indicated; the sample denoted imp represents the results of importing p33KRA into intact pea chloroplasts. 33KRA represents imported p33KRA which has been proteolytically processed in the chloroplasts to yield A chain containing 22 residues of mature 33K at the amino terminus.

likely that A chain could be initially targeted into the thylakoid lumen and subsequently “reverse-translocated” back to the stromal phase. However, analysis of the x-ray crystal structure reveals an alternative explanation: A chain appears to be a protein that folds extremely tightly (23), raising the possibility that the thylakoid protein import apparatus is incapable of unfolding A chain sufficiently for translocation to proceed to completion. In general, tightly folded proteins are more resistant to proteolysis, and we therefore tested the resistance of p33KRA to increasing concentrations of protease K. The results (Fig. 1) show that even low concentrations (5 μg/ml) are sufficient to quantitatively convert the fusion protein to a polypeptide that comigrates precisely with mature ricin A chain. Remarkably, this polypeptide is then largely resistant to digestion by even 1 mg/ml protease K (a massive concentration, which in our hands has totally degraded every other protein tested, including dihydrofolate reductase complexed with methotrexate). The logical interpretation of this result is that p33KRA consists of a 33K presequence/mature section that is relatively, if not completely, unfolded, together with an A chain moiety that is extremely tightly folded. The right-hand panel in Fig. 1 shows that this polypeptide is smaller than the major imported species, as noted by Roberts et al. (17) in their initial import experiments. This is because removal of the 33K presequence during import leaves 22 residues of mature 33K attached to the NH2 terminus of A chain.

Further indications of the folding characteristics of A chain are shown in Fig. 2. In this experiment we tested whether A chain becomes more susceptible to proteolysis when deliberately unfolded in urea (to exclude the unlikely possibility that the A chain sequence does not contain protease K cleavage sites). p33KRA was incubated with 8 M urea to induce unfolding, and the mixture was then diluted 4-fold with buffer containing protease K. The data show that this treatment renders the A chain far more susceptible to digestion when compared with the minus-urea control, with the majority of p33KRA now being cleaved to low molecular weight forms (defined here as polypeptides smaller than mature size A chain) by concentrations of protease K of 50 μg/ml or greater. Quantitation of the data (shown as part of Fig. 4) indicates that a small proportion of p33KRA continues to be converted to mature size A chain, even at very high protease concentrations; possibly, these are molecules that were able to quickly refold into their correct tertiary structures when diluted from 8 to 2 M urea, whereas the majority were too slow and were degraded. Overall, however, the data provide compelling evidence that
Protein Unfolding during Import into Chloroplasts

**Fig. 2.** 33KRA is protease-sensitive when deliberately unfolded in urea. p33KRA was synthesized as detailed in the legend to Fig. 1 and then diluted 4-fold into 50 mM Hepes, pH 8.0, 330 mM sorbitol-containing protease K (PK) at the indicated concentrations in μg/ml (left-hand panel) or incubated with 8 mM urea for 30 min on ice, then diluted 4-fold with the same buffer containing protease K (right-hand panel). After incubation for 40 min, samples were analyzed as in Fig. 1.

ricin A chain is highly resistant to proteolysis as a consequence of its tightly folded three-dimensional structure.

In Vitro Synthesized p33KRA Is Active on Mammalian Ribosomes—Perhaps the best test of correct folding under these circumstances is to determine whether the protein is enzymatically active, and this has been a major limitation with most proteins synthesized in vitro, because the commonly used translation systems produce extremely low quantities of protein. In the case of ricin A chain, however, this is not a limitation because, although the protein is only weakly active on wheat germ ribosomes, A chain has such high activity toward mammalian ribosomes that translational capacity in reticulocyte lysates is rapidly destroyed even by an A chain in vitro translation product during its synthesis (18). Fig. 3 shows an assay for A chain activity in which both p33KRA and mature size A chain were synthesized in a rabbit reticulocyte lysate, and the ribosomes were subsequently examined for the diagnostic, specific depurination of an adenine in 28 S RNA. This was achieved by brief treatment of the ribosomes with aniline, which has the effect of preferentially cleaving the phosphodiester backbone at the depuration site and releasing a 390-base fragment. This fragment, diagnostic of A chain-catalyzed depurination, was detected by Northern blotting with a labeled primer as detailed under "Experimental Procedures." Fig. 3 shows that both authentic A chain and p33KRA depurinate the 28 S RNA to a similar degree, confirming that the presence of the presequence does not affect folding of p33KRA to the extent that activity is blocked. The remaining panels of Fig. 3 are controls, which show that depurination does not occur in the absence of additions or when the transcription vector minus p33KRA insert is transcribed and translated.

Receptor-bound p33KRA Is Highly Sensitive to Proteolysis and Hence Apparently Unfolded—The data shown in Figs. 1–3 provide convincing evidence that the A chain moiety in p33KRA is extremely tightly folded in solution and that the presence of the amino-terminal presequence has little or no effect on the folding of this particular passenger protein. Having shown previously that p33KRA can be efficiently imported into chloroplasts, we used protease sensitivity as an indicator of folding to address the question: what is the conformation of p33KRA following binding to the chloroplast? The binding of precursor proteins to the chloroplast import apparatus requires lower concentrations of ATP than does translocation into the stroma (8), and it is possible to accumulate receptor-bound precursor by incubation with intact chloroplasts in the presence of 10 μM MgATP. Under these conditions, analysis of the chloroplasts reveals bound precursor (Fig. 4A, lane 0) but little or no imported 33KRA protein (the major imported species consisting of 22 residues of mature 33K linked to A chain). This precursor is productively bound, because increasing the ATP concentration to 1 mM leads to efficient chasing into the stromal 33KRA form (lane Ch). Significantly, incubation of the receptor-bound p33KRA with protease K at concentrations above 50 μg/ml results in the almost quantitative degradation of p33KRA to low molecular weight polypeptides (defined as smaller than mature size A chain) in the experiment outlined above (bound graph) or when soluble p33KRA is incubated with protease K in the absence or presence of urea as shown in Fig. 2 (graphs – / + urea).
tions of proteinase K (250–1000 μM), and we speculate that these molecules may in fact be nonspecifically bound to the chloroplasts rather than bound to receptors, in which case they would probably be fully folded.

In summary, we have provided further evidence that proteins can be targeted into chloroplasts without the benefit of a cytosolic antifolding factor and that the unfolding process instead takes place once bound to the chloroplasts, but before translocation across the envelope membranes. The identity of the “unfoldase” remains to be established. The precursor proteins may be passed on to an unfoldase activity after interaction with the initial import receptor, or the import receptor may fulfill both roles. It is also possible that envelope lipids play a role in the unfolding process, although it seems unlikely that lipids could achieve this level of unfolding without the input of dedicated proteins. Finally, it is interesting to speculate that the ATP hydrolysis required for stable binding of precursors may well be used to drive the unfolding process, possibly by Hsp70 molecules, which are known to be present in the import complex (9). This could be achieved either by an “active” unfoldase activity in which the protein involved physically unfolds the precursor protein or a “passive” unfolding in which the precursor protein “breathes” and partially folded forms are progressively stabilized by the unfoldase. Clearly, this topic merits further attention in order to unravel in detail the early events in chloroplast import and in understanding the factors responsible for the presumed unfolding of ricin A chain within the endoplasmic reticulum of mammalian cells.

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