The Cost of Exposing a Hydrophobic Loop and Implications for the Functional Role of 4.5 S RNA in the Escherichia coli Signal Recognition Particle*

Received for publication, December 11, 2000, and in revised form, February 13, 2001
Published, JBC Papers in Press, February 21, 2001, DOI 10.1074/jbc.M011130200

Robert M. Cleverley‡, Ning Zheng§, and Lila M. Gierasch‡§

From the ‡Department of Biochemistry and Molecular Biology and the §Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

The signal recognition particle (SRP) is an RNA-protein complex that directs ribosomes to the rough endoplasmic reticulum membrane by binding to targeting signals found on the nascent chain of proteins destined for export to the endoplasmic reticulum. We found evidence from studies with fragments of the protein component of the Escherichia coli SRP that a long hydrophobic loop (the so-called "finger loop") is detrimental to the stability of its signal peptide-binding domain, the M domain. This hydrophobic loop is highly conserved and thus may have a critical role in the function of the SRP. Given our previously reported evidence that 4.5 S RNA stabilizes the tertiary fold of the M domain (Zheng, N., and Gierasch, L. M. (1997) Mol. Cell 1, 79–87), we now propose that the functional requirement for 4.5 S RNA resides in its ability to counteract the destabilizing influence of the finger loop.

In eukaryotic cells the signal recognition particle (SRP) targets ribosomes synthesizing secretory and membrane proteins to the rough endoplasmic reticulum membrane. The Escherichia coli SRP is composed of a single protein subunit, termed Ffh, which binds to a 114-nucleotide RNA molecule, 4.5 S RNA. The E. coli SRP has been shown to direct nascent membrane proteins into the inner membrane and therefore bears a strong functional resemblance to its counterparts in eukaryotic organisms (1–4). Limited proteolysis experiments define two domains in Ffh termed the "NG" and "M" domains (5). The C-terminal M domain contains the RNA-binding site and can be cross-linked to signal sequences (6). The NG domain contains a Ras-like GTPase domain and a novel small helical N-domain (7). The crystal structure of Thermus aquaticus Ffh shows the M domain to consist of four α-helices with a long, flexible loop, denoted the "finger loop," between the first and second α-helices (Fig. 1A) (8). This loop contains a number of conserved hydrophobic residues, which are illustrated in red. A groove on the surface of the M domain formed by the finger loop and the first, second, and fourth α-helices has been proposed to represent the signal sequence-binding site (8).

As yet there is no clear explanation why RNA is necessary for SRP function (9). Based on the recent crystal structure of the M domain-4.5 S RNA complex, it has been proposed that RNA interacts directly with the signal peptide (10). On the other hand, our previous studies with E. coli Ffh point to an important structural role for RNA (11). Our circular dichroism and proteolysis experiments showed that 4.5 S RNA stabilizes the tertiary fold of the M domain, which is highly flexible in the absence of 4.5 S RNA. Indeed the recent observation that RNA catalyzes the association of Ffh with its receptor has been attributed to possible RNA-induced conformational changes in the protein (12). We now report that the metastability of the E. coli Ffh M domain in the absence of RNA can be attributed to the presence of the long, hydrophobic finger loop. This loop is conserved in all homologues of SRP and thus may have a critical role in the function of the SRP. In turn, we propose that RNA is functionally necessary to counter the destabilizing effect associated with this loop.

EXPERIMENTAL PROCEDURES

Protein and RNA Preparation—Ffh and 4.5 S RNA were prepared as described previously (11). The M domain (residues Gly326–Met435), amino deletion mutant (residues Lys365–Met435) and finger loop deletion mutant (from Gly326–Met355 with Gln340, Asp355 replaced by a GAGG linker sequence) were overexpressed from pET20b vectors (Novagen) in BL21DE3pLysS (Stratagene) cells and purified under denaturing conditions by cation exchange chromatography using CM-Sepharose CL-6B (Sigma) resin. Removal of denaturant by dialysis against water was used to refold all of these proteins. To prepare the Lys324 Ffh truncation mutant, a stop codon was introduced into the Ffh expression plasmid at the Lys325 codon (via the 3′-primer) and to introduce a second unique NdeI site downstream of the Arg339 codon (via the 5′-primer) and to introduce a unique NarI restriction site and two Gly codons into the M domain expression plasmid upstream of the Lys325 codon (via the 5′-primer) and to introduce a second NarI site downstream of the Arg339 codon (via the 3′-primer). The linear polymerase chain reaction product was then cut with NarI, purified, and circularized with DNA ligase. The identities of the M domain, amino deletion mutant, and finger loop deletion mutant were verified by matrix-assisted laser desorption ionization mass spectrometry (see Fig. 3A for a schematic description of these constructs).

Limited Proteolysis—Ffh (1 mg ml−1) was digested in the presence or absence of ~0.7 mg ml−1 4.5 S RNA at 30 °C with either 0.1 mg ml−1 (Fig. 2) or 5 μg ml−1 (Figs. 4, A and B) porcine pancreatic elastase (Roche Molecular Biochemicals) in buffer A, 50 mM triethanolamine acetate, pH 7.5, 25 mM potassium acetate, 2.5 mM magnesium acetate, 10 mM β-mercaptoethanol, and 0.1% octaethylene glycol monododecyl ether (Fluka). To purify the digests by Ni-NTA chromatography, KCl and imidazole were added to them in the amounts of 300 and 5 mM, respectively, before loading onto a Ni-NTA column; the column was
washed with at least 30 column volumes of buffer A supplemented with 20 mM imidazole and 300 mM KCl and eluted with buffer A supplemented with 500 mM imidazole and 300 mM KCl. The eluate was concentrated by precipitation with 15% trichloroacetic acid before analysis by SDS-PAGE. SDS-PAGE gels were stained with Sypro-Orange dye (Molecular Probes) and analyzed on a Molecular Dynamics PhosphorImager using ImageQuant software. To check the consistency of the observed increase in the amount of fragment A in the digest when RNA is present, we analyzed four pairs of proteolysis reactions set up under identical conditions in the presence and absence of 4.5 S RNA. To identify N-terminal protease cleavage products by Western blotting, protein digestives were electroeluted onto nitrocellulose membranes after SDS-PAGE. After a 1-h incubation in PBS (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl pH 7.5) containing 10% (v/v) nonfat dry milk and 0.05% (v/v) Tween 20, the membranes were incubated for 2 h in the same buffer containing a 1:1000 dilution of mouse antihistidine tag antibody (Amersham Pharmacia Biotech). After several washes in PBS containing 10% nonfat dry milk and 0.05% Tween 20, the membrane was incubated for 2 h in the same buffer containing a 1:1000 dilution of an anti-mouse antibody horseradish peroxidase conjugate (Amersham Pharmacia Biotech). The bands on the membrane were visualized using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Circular Dichroism—All CD data were acquired in 10 mM potassium phosphate buffer (pH 6.5) in a 2-mm path length cell with protein concentrations of 5–10 μM. For thermal unfolding experiments, the sample was heated at a rate of 20 °C h–1; ellipticity measurements at 222 nm were taken at 0.1 °C intervals with a response time of 8 s per measurement. Gel filtration analysis of the amino deletion mutant at 200 μM protein concentration confirmed that the protein is monomeric at concentrations more than 10 times those used for the CD analysis (data not shown).

Gel Shift Assay—0.2 μg 4.5 S RNA was mixed with various concentrations of Ffh and M domain in buffer A supplemented with 10% glycerol. 10-μl binding reactions were supplemented with 3 μg of baker’s yeast tRNA (Sigma) per binding reaction to reduce nonspecific binding. For competition binding reactions, all components were thoroughly pre-mixed before adding the 4.5 S RNA. The binding reactions were separated on 7% polyacrylamide gels as described by Lentzen et al. (13).

RESULTS

Destabilizing Influence of the Finger Loop—Crystal structures of the E. coli Ffh M domain-4.5 S RNA complex (10) and of the homologous M domain from the mammalian SRP (14) both revealed lengthy loops equivalent to the T. aquaticus Ffh finger loop. Moreover, sequence comparisons among homologues showed the size and character of this loop to be largely conserved (Fig. 1B). Using limited proteolysis, we found evidence that this disordered, exposed loop is retained as a feature of the E. coli Ffh M domain in solution. Elastase digestion of Ffh in the presence of 4.5 S RNA generates an 8-kDa truncated fragment of the M domain which starts, by N-terminal sequence analysis, at Lys365 (Fig. 2). Based on its approximate size on the SDS-PAGE and on analysis of the digest by matrix-assisted laser desorption ionization mass spectrometry, we concluded that this elastase-resistant fragment corresponds with Lys365–Met135. Thus, the protease cleaves within the finger loop and leaves the region that encompasses helices 2–4 intact in the presence of 4.5 S RNA. This protease-stable core of the M domain is marked in magenta on the structure in Fig. 1A.

In our subsequent analysis of a recombinant version of the elastase fragment (Lys365–Met135), termed here the “amino deletion mutant”; see Fig. 3A) and the M domain (residues Gly526–Met135; see Fig. 3A), we found that the finger loop has a markedly destabilizing effect on the fold of the M domain. CD spectra of the amino deletion mutant and the M domain both reveal a substantial amount of α-helical secondary structure (Fig. 3B). As expected from our previous studies with an M domain construct purified from a proteolytic digest of Ffh (11), thermal unfolding of the recombinant M domain construct studied here was highly noncooperative (Fig. 3C). The near-linear decrease in ellipticity at 222 nm between 4 and 80 °C shows that the M domain does not adopt a stable tertiary structure in solution. Despite this structural flexibility, the M domain construct used here retained the same binding affinity for 4.5 S RNA as Ffh (Fig. 4), arguing that it is a valid representation of the M domain within the native protein.

Remarkably, the smaller amino deletion mutant had a considerably more cooperative thermal unfolding profile than the fully intact M domain. Its 222 nm ellipticity signal changed in a sigmoidal manner between 4 and 80 °C, with the transition fully reversible. These combined structural data therefore revealed, quite unexpectedly, that the truncated amino deletion mutant has a higher propensity to form a tertiary structure than the fully intact M domain.

The unexpected gain in structural stability upon removing helix 1 and the finger loop to form the amino deletion mutant suggests that the unusually long hydrophobic finger loop (amino acids Leu318–Asp370) destabilizes the folding of the M domain. A contribution from helix 1 cannot be excluded, however. To analyze the effect of the finger loop more directly, we prepared an additional construct in which finger loop residues Gln340–Asp370 were replaced with a flexible GAGG linker sequence. Thirty-one residues in the loop, including the conserved hydrophobic residues, were thus removed, creating the finger loop deletion mutant (Fig. 3A). Strikingly, the protein remained highly α-helical by CD and had a significantly more cooperative thermal unfolding profile than did the M domain (Figs. 3, B and C). The properties of this protein were thus entirely consistent with a destabilizing influence of the finger loop on the M domain. Solvent Accessibility of the Finger Loop within the Context of Intact Ffh—We surmised that the destabilizing effect associated with the loop might be due to the exposure of its many hydrophobic residues on the protein surface. However, whether the loop is surface-exposed when the protein is in solution is left uncertain by the way it is involved in extensive intermolecular packing interactions in the crystal structures of both T. aquaticus Ffh (8) and Homo sapiens Ffh M domain (14). The facile elastase cleavage we observe within the finger loop region (Fig. 2) suggested that this loop is exposed on the surface of the M domain in solution. Milder conditions biased toward only one protease cut per molecule were used in further experiments to probe whether the loop is surface-exposed within intact Ffh, prior to cleavage of the NG-M domain linker. Ffh bearing an N-terminal dodecahistidine tag was digested with a low concentration of elastase, and N-terminal fragments were identified by monitoring for fragments that bound to Ni-NTA resin.

Two N-terminal fragments were identified that can be assigned as the products of cleavage after Lys325, the starting point of the M domain, by virtue of their slower electrophoretic mobility in comparison with that of a mutant truncated at Lys324 (Fig. 5A, fragments labeled A and B), and by the fact that they bind both to Ni-NTA resin and to an anti-His tag antibody in Western blots (data not shown). The cleavage region can be further narrowed down to between Lys325 and Lys365 because proteolysis under harsher conditions (Fig. 2) revealed that the Lys365–Met135 region is highly stable to proteolytic cleavage in the presence of RNA. Given that the bulk of the Lys325–Lys365 region encompasses the finger loop and that limited proteolysis generally cleaves flexible loops with much higher propensity than regular secondary structure elements (15), we conclude that the cleavages occur within the finger loop. Furthermore, because fragments A and B retained the linker between the NG and M domains, it follows that cleavage of the NG-M domain linker is not a prerequisite for the finger
loop to become protease-accessible. These results therefore strongly argue that the finger loop is solvent-exposed within the context of intact Ffh.

Intriguingly, analysis of the band intensities revealed an increase in the amount of fragment A when the digestion of Ffh is conducted under identical conditions in the presence of 4.5 S RNA (Fig. 5B, lanes 2 and 3). Complicating the interpretation of these data is the difficulty of quantitating the amount of fragment A in a way that accounts for any differences in gel loading or proteolysis efficiency between lanes. However, this apparent increase was observed consistently after conducting several parallel proteolysis reactions under identical conditions in the presence and absence of 4.5 S RNA. Any increase in the exposure of the finger loop to the protease must be induced in the intact protein by the RNA prior to cleavage. The alternative possibility, that the RNA protects fragment A from further cleavages, is ruled out because the product lacks the bulk of the RNA-binding region and thus should not bind RNA. It is thus possible that RNA binding drives the unfavorable exposure of the loop on the protein surface. In addition, clear changes in the pattern of 27–37 kDa-sized fragments in the presence of RNA further indicate significant RNA-induced structural changes in Ffh (Fig. 5B).

DISCUSSION

Previously we found that the Ffh M domain lacks a stable tertiary structure as an isolated fragment in the absence of 4.5 S RNA (11). We can now reconcile this finding with the pres-
ence of the finger loop, which is shown here to destabilize the fold of the M domain. This result may be explained by the nonpolar character of the loop, which contains a number of highly conserved hydrophobic residues (Fig. 1, A and B). Inspection of the crystal structure of T. aquaticus Ffh (8) revealed that these conserved hydrophobic residues neither pack into the hydrophobic core of the M domain nor interact with the NG domain but are instead buried by packing interactions between molecules in the crystal lattice. Similar packing interactions are observed in the crystal structure of the homologous M domain from H. sapiens SRP (14). In both cases the finger loop

Fig. 2. The N-terminal third of the M domain sequence contains a highly protease-susceptible region. An SDS-PAGE of the products of elastase digestion of the Ffh-4.5 S RNA complex reveals an 8-kDa fragment assignable to residues Lys<sup>365</sup>-Met<sup>435</sup> by N-terminal sequencing and mass spectroscopy (data not shown). The protease-stable core of the M domain, encompassing helices 2–4, is marked in magenta on the structure in Fig. 1A.

Fig. 3. The hydrophobic finger loop destabilizes the fold of the M domain. A, schematic illustration of truncation mutants of Ffh M domain. The amino deletion mutant (residues Lys<sup>365</sup>–Met<sup>435</sup>) corresponds to the magenta region on the M domain structure in Fig. 1A. This construct lacks helix 1 and the finger loop. In the finger loop deletion mutant, the finger loop residues Gln<sup>340</sup> through Asp<sup>370</sup> of the M domain have been replaced with a GAGG linker sequence. B, CD spectra of the amino deletion mutant, finger loop deletion mutant, and the M domain reveal highly α-helical proteins with double minima close to 208 and 222 nm. Protein concentrations are ~5 μM in all cases in 10 mM potassium phosphate buffer at 4 °C. C, CD at 222 nm as a function of temperature for the amino deletion mutant (ADM), the finger loop deletion mutant (FDM), and the M domain (MD). Thermal unfolding of both the amino deletion mutant and the mutant lacking the finger loop is more cooperative than the unfolding of the M domain.
on one molecule interacts with the proposed signal sequence binding groove on a neighboring molecule, thus satisfying the need of both molecules to sequester hydrophobic residues. These two crystal structures therefore provide evidence that the finger loop has a preference for hydrophobic environments and cannot easily accommodate its hydrophobic residues intramolecularly within the hydrophobic core of the M domain.

Notably, however, neither these crystal structures nor the recent structure of E. coli M domain bound to a fragment of 4.5 S RNA (10) provided a picture of the finger loop interactions when the M domain is in the context of soluble, intact Ffh. Our proteolysis data have provided evidence that the finger loop, despite its hydrophobicity, is exposed on the surface of the Ffh protein. Two protease-susceptible regions were identified that can be mapped to a portion of the M domain sequence dominated by the finger loop. Therefore, any destabilizing effect produced by the exposure of hydrophobic amino acids in the finger loop should be significant within intact Ffh. However, proteolytic susceptibility does not necessarily imply that the finger loop is exposed at all times. The loop could be in a dynamic equilibrium between exposed and buried states. Further work will be required to verify whether the loop is permanently exposed or partially buried, for example, at the interface between the NG and M domains.

In addition to its high content of hydrophobic amino acids, the length of the finger loop is expected to destabilize the fold of the M domain. Statistical analyses have shown that loop regions of more than 10 amino acids in length are rare in protein structures (16). Experimental studies have verified that there is an inverse correlation between loop length and the stability of proteins (17–20). Sequence analyses in fact reveal that there is an inverse correlation between loop length and the protein structures (16). Experimental studies have verified that regions of more than 10 amino acids in length are rare in protein structures (16).

Destabilizing Influence of the SRP Finger Loop

The length of the finger loop is expected to destabilize the fold of the M domain. Statistical analyses have shown that loop regions of more than 10 amino acids in length are rare in protein structures (16). Experimental studies have verified that there is an inverse correlation between loop length and the stability of proteins (17–20). Sequence analyses in fact reveal that there is an inverse correlation between loop length and the protein structures (16). Experimental studies have verified that regions of more than 10 amino acids in length are rare in protein structures (16).
The Cost of Exposing a Hydrophobic Loop and Implications for the Functional Role of 4.5 S RNA in the *Escherichia coli* Signal Recognition Particle

Robert M. Cleverley, Ning Zheng and Lila M. Gierasch

*J. Biol. Chem. 2001, 276:19327-19331.*

doi: 10.1074/jbc.M011130200 originally published online February 21, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011130200

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 22 references, 4 of which can be accessed free at http://www.jbc.org/content/276/22/19327.full.html#ref-list-1