Renaturation of Rhodanese by Translational Elongation Factor (EF) Tu

PROTEIN REFOLDING BY EF-Tu FLEXING*

(Received for publication, June 12, 1997, and in revised form, August 4, 1997)

Wieslaw Kudlicki, Ashley Coffman, Gisela Kramer, and Boyd Hardesty‡

From the Molecular Biology Institute and the Department of Chemistry & Biochemistry, The University of Texas, Austin, Texas 78712

The translation elongation factor (EF) Tu has chaperone-like capacity to promote renaturation of denatured rhodanese. This renaturation activity is greatly increased under conditions in which the factor can oscillate between the open and closed conformations that are induced by GDP and GTP, respectively. Oscillation occurs during GTP hydrolysis and subsequent replacement of GDP by EF-Ts which is then displaced by GTP. Renaturation of rhodanese and GTP hydrolysis by EF-Tu are greatly enhanced by the guanine nucleotide exchange factor EF-Ts. However, renaturation is reduced under conditions that stabilize EF-Tu in either the open or closed conformation. Both GDP and the nonhydrolyzable analog of GTP, GMP-PCP, inhibit renaturation. Kirromycin and pulvomycin, antibiotics that specifically bind to EF-Tu and inhibit its activity in peptide elongation, also strongly inhibit EF-Tu-mediated renaturation of denatured rhodanese to levels near those observed for spontaneous, unassisted refolding. Kirromycin locks EF-Tu in the open conformation in the presence of either GTP or GDP, whereas pulvomycin locks the factor in the closed conformation. The results lead to the conclusion that flexing of EF-Tu, especially as occurs between its open and closed conformations, is a major factor in its chaperone-like refolding activity.

Bacterial translation elongation factor (EF) Tu has been intensely studied for many years in relation to its role in the reaction cycle by which peptides are elongated on ribosomes. In this series of reactions, EF-Tu promotes codon-directed binding of aminoacyl-tRNA into the ribosomal A site from an EF-Tu-GTP-aminoacyl-tRNA ternary complex with concomitant hydrolysis of GTP. GDP is subsequently displaced from the resulting EF-Tu-GDP complex by the guanine nucleotide exchange factor EF-Ts. The EF-Tu complex and elongation factor G, EF-G, appear to interact sequentially with a ribosome to induce its oscillation between two conformations as part of the reaction cycle by which mRNA and tRNA are moved through the ribosome during protein synthesis (1). The early understanding of these reactions of EF-Tu allows it to serve as the archetypal guanine nucleotide-binding protein (G protein).

EF-Tu is one of the most abundant proteins in rapidly growing *Escherichia coli* cells, with approximately 5–6 copies per ribosome (2). This abundance has promoted searches for other roles for EF-Tu. Recent reports indicate that EF-1α, the eukaryotic counterpart of EF-Tu, or EF-Tu itself may participate in the degradation of N-terminally blocked proteins by the 26 S protease complex (3, 4). These results are particularly intriguing in that they suggest that EF-Tu or EF-1α may interact with a partially unfolded protein and that the peptide elongation factor may serve as a chaperone which binds to ubiquitin-conjugated proteins.

The relative molecular weight of *E. coli* EF-Tu is 43.2 (5). The crystal structure of the trypsin-modified EF-Tu complex shows a rather flat triangular molecule consisting of three domains and an unusually large hole between the domains (6). EF-Tu forms distinct complexes with at least four different components during its participation in the reactions of peptide elongation on ribosomes. These complexes involve GDP, GTP, EF-Ts, and aminoacyl-tRNA. A striking feature of EF-Tu is the large conformational differences between the form of the protein when it is complexed with GDP or GTP. Abel and Jurnak (7) reviewed these conformational differences and considered how the chemical energy of GTP hydrolysis might be converted into mechanical energy associated with a change in the conformation. EF-Tu-GTP has a relatively closed conformation, which opens upon hydrolysis of GTP or binding of GDP to give the EF-Tu-GDP complex. We speculate that this opening and closing of EF-Tu may be a critical component of the refolding of denatured rhodanese that we present here.

EF-Tu is the specific target of the antibiotics kirromycin and pulvomycin, which both bind to the factor in a 1:1 molar ratio but at different sites (8, 9). Upon complex formation with kirromycin, the elongation factor becomes locked in the closed conformation similar to that induced by GTP and in which GTP hydrolysis can take place. Aminoacyl-tRNA can bind to EF-Tu in this conformation regardless of whether GTP or GDP is present. As a consequence, EF-Tu-GDP/kirromycin remains associated with the ribosome, inhibiting peptide bond formation and leaving the ribosomes stalled on the mRNA. In contrast, pulvomycin freezes EF-Tu in the open conformation of the EF-Tu-GDP complex even in the presence of GTP and thus inhibits GTP hydrolysis. Aminoacyl-tRNA cannot bind to this EF-Tu-pulvomycin complex.

Refolding of denatured rhodanese has been used extensively to study protein folding over the last 20 years. Unassisted refolding is a relatively slow process that is critically dependent on protein concentration, temperature, and other factors (10). This refolding can be accelerated by agents such as micelles of lauryl maltoside (11) or cardiolipin liposomes (12) and certain proteins such as serum albumin (13) and α-crystallin (14). However, the molecular chaperones GroEL and GroES in an

*This work was supported by a grant from the Foundation for Research and by National Institutes of Health Grant GM 55152. 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.

‡ To whom correspondence should be addressed. Tel.: 512-471-5874; Fax: 512-471-8696.

1 The abbreviations used are: EF, translation elongation factor; GMP-PCP, β,γ-methyleneguanosine 5′-triphosphate.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
ATP-dependent reaction promote a relatively high rate and extent of rhodanese refolding (reviewed in Ref. 15). We have demonstrated that components of the protein synthesizing machinery, namely the 50 S ribosomal subunit and its 23 S RNA, promote efficient refolding of denatured rhodanese in the absence of ATP (16). Here we extend these studies and show that EF-Tu promotes refolding of denatured rhodanese. This refolding is strongly stimulated by GTP in the presence of EF-Ts but inhibited by the nonhydrolyzable analog of GTP, GDP-PCP, by GDP and by both kirromycin and pulvomycin.

EXPERIMENTAL PROCEDURES

Materials

Nucleoside triphosphates and E. coli tRNA were purchased from Boehringer-Mannheim; urea, rifampicin, amino acids, and kirromycin were from Sigma. [3H]Leucine was purchased from NEN Life Science Products and [γ-32P]GTP was from Amersham Corp. The plasmid used was pSP65, containing the rhodanese coding sequence under the SP6 promoter (17). The antibiotic pulvomycin was a generous gift from Dr. Andrea Parmeggiani (Paris, France). Preparation of electrophoretically homogeneous EF-Tu from Thermus thermophilus was carried out as described previously (18). Highly purified translational factors EF-Ts and EF-G from T. thermophilus were kindly provided by Dr. Paul Sigler and co-workers and Dr. Peter Moore and co-workers, respectively (Yale University, New Haven, CT).

Methods

Synthesis of Rhodanese, Its Denaturation, and Refolding—Rhodanese was synthesized in vitro by coupled transcription/translation in an E. coli cell-free extract as described previously (17). After synthesis, the reaction mixture was centrifuged for 45 min at 150,000 × g to separate ribosomes from the soluble fraction. The synthesized enzyme was purified from the supernatant fraction by precipitation with (NH₄)₂SO₄ and Affi-Gel blue chromatography (19). Denaturation and renaturation of in vitro synthesized and purified rhodanese was performed according to Mendoza and Horowitz (20). Rhodanese (5.2 μM) was denatured in 200 mM sodium phosphate buffer (pH 7.4) containing 1 mM β-mercaptoethanol and 8 mM urea for 45 min at 25 °C. Spontaneous and EF-Tu or EF-Tu/EF-Ts-assisted refolding was initiated by diluting 2 μl of denatured enzyme in 8 μl urea to a final volume of 130 μl of a solution containing 50 mM Tris-HCl (pH 7.8), 200 mM β-mercaptoethanol, 50 mM sodium thiocyanate, 10 mM MgCl₂, and 10 mM KCl. In addition, 1 mM GTP or GDP was added where indicated. The final concentration of rhodanese in the refolding reaction was 80 nM. The refolding reaction was carried out for 30 min at 25 °C unless indicated otherwise. Enzyme activity was used as a measure of successful refolding of rhodanese into its native conformation. Enzyme activity was determined as described (21).

Formation and Purification of EF-Tu-GDP Complex—The reaction mixture (total volume 250 μl) contained 20 μM EF-Tu and 100 μM GDP in a solution containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 50 mM NH₄Cl, and 1 mM diithiothreitol. The incubation was carried out at 37 °C for 5 min. The EF-Tu-GDP complex was isolated from the reaction mixture by gel filtration on a Sephadex G-50 column (0.5 × 10 cm) equilibrated with the same solution. Fractions of 100 μl were collected and used for interaction with kirromycin as described in the legend to Fig. 4.

Assay for GTPase Activity of EF-Tu—The GTPase activity of EF-Tu was measured as described by Conway and Lipmann (22) with modifications. For other experimental details, see legends to Tables and Figs.

RESULTS

EF-Tu-dependent Refolding of Denatured Rhodanese—One of the reasons rhodanese has been used as a test protein is because of a simple and sensitive assay for its enzymatic activity (21). The activity is taken as a quantitative measure of refolding of the denatured protein into its native conformation. Table I indicates that only a small portion (13%) of denatured rhodanese refolds spontaneously when diluted 65-fold from an 8 mM urea solution under the conditions used (refolding solution, 30 min incubation at 25 °C). This activity is expressed as a percentage of the activity of native rhodanese carried through the same procedure. The extent of spontaneous refolding is sensitive to the conditions used, particularly the final concentration of rhodanese and temperature. Inclusion of EF-Tu in the refolding reaction mixture in an approximately equal molar amount to denatured rhodanese promoted refolding up to almost half of the activity of enzyme that had not been denatured. Renaturation was found to be dependent upon the concentration of EF-Tu up to the point at which a small molar excess of EF-Tu to rhodanese was reached, yielding about 45% refolding (data not shown). Doubling the amount of EF-Tu added above this point gave only a slight increase in the percent refolding under all of the conditions tested in the experiments represented by Table I. GTP had little or no effect on renaturation mediated by highly purified EF-Tu. Similarly, addition of ATP or ADP had little or no effect (data not shown).

The extent of renaturation increased to 90% when EF-Tu was added with EF-Tu and GTP (Table I). EF-Tu had no effect by itself or when added in the presence or absence of GTP or GDP. The enzymatic activity of native rhodanese that was not subjected to denaturation was constant under all the conditions represented by Table I. These results are interpreted to indicate that all of the denatured rhodanese, except possibly for a small amount that may refold spontaneously, initially binds to EF-Tu probably in a 1:1 molar ratio, and then about half of this protein refolds into the active, native conformation and is released. Nearly all of the bound rhodanese is renatured and released from EF-Tu, if EF-Ts and GTP are present under conditions in which multiple rounds of GTP hydrolysis can occur.

A surprising result shown in Table I is that either the nonhydrolyzable analog of GTP, GDP-PCP, or GDP inhibit EF-Tu-mediated refolding from about 50% down to near the level of spontaneous refolding. The data appear to indicate that these compounds block the renaturation activity of EF-Tu that occurs in the absence of nucleotide, the increase in renaturation from about 13% for unassisted refolding to about 47% in the presence of EF-Tu only.

EF-Tu-mediated renaturation of rhodanese in the presence of EF-Tu/GTP was analyzed kinetically. In the presence of EF-Tu alone, about 47% of the denatured rhodanese was renatured in 3–5 min. (Fig. 1). When EF-Ts and GTP were added to a similar reaction mixture after 4 min rhodanese activity remained 83% in the next 60 s and 92% in 120 s. When GTP and a 1.5-fold molar excess of EF-Tu/EF-Ts over rhodanese were present initially, 5 min were required to reach 81% refolding of the denatured rhodanese (Fig. 2). If the EF-Tu/EF-Ts concentration was reduced to 16 nM each to give a molar ratio of EF-Tu/EF-Ts to denatured rhodanese of 0.2, 30 min were required to reach about 80% renaturation (Fig. 2). These results, considered with those of Fig. 1, indicate that, in the presence of...
Denaturation in 8M urea was omitted. The amount of native enzyme carried through the same incubations except danese activity is expressed as percentage of that of an equivalent at the indicated times, and rhodanese activity was determined. Reaction mixture after 4 min incubation (limiting concentration of either EF-Tu only (16 nM) or with a limiting concentration of either EF-Tu only (16 nM) (A) or EF-Tu/EF-Ts (16 nM each) (B). Aliquots were withdrawn at the indicated times, and rhodanese activity was determined. Rhodanese will refold spontaneously and that an additional portion under the conditions used about 14% of the denatured rhodanese on a molar basis, whereas this is not the case in the absence of EF-Ts and GTP. With limiting EF-Tu (molar ratio of 0.2 EF-Tu to denatured rhodanese in Fig. 2), there is only a small increase in renaturation (10% in Fig. 2) in addition to spontaneous refolding, about 14%.

Considered with the results of Fig. 1, these data indicate that under the conditions used about 14% of the denatured rhodanese will fold spontaneously and that an additional portion of the denatured enzyme, about 30%, can fold with an optimal concentration of EF-Tu alone. The results also indicate that there is a portion of the denatured rhodanese that cannot be renatured under these conditions, but can be refolded under conditions in which GTP is hydrolyzed by EF-Tu in the presence of EF-Ts. These conditions give multiple rounds of GTP hydrolysis and are assumed to cause an oscillation of EF-Tu between the closed conformation that is induced by GTP and the open conformation that is promoted by GDP (cf. Ref. 7).

**DISCUSSION**

The results presented above demonstrate that EF-Tu has chaperone-like capacity to promote refolding of denatured rhodanase. Although the results might prompt provocative hypotheses about a possible physiological role for EF-Tu in fold-
EF-Tu by itself, without nucleotides or other factors bound to it, is able to increase refolding of rhodanese from about 14% (spontaneous refolding) to about 50% of the activity of native enzyme carried through the same procedure. The level of refolding is increased to more than 90% by the addition of GTP and EF-Ts to the folding reaction mixture. Both components are required, suggesting that multiple rounds of GTP hydrolysis are involved in this second level of activity. Under these conditions, a molecule of EF-Tu can promote refolding of more than one molecule of rhodanese. However, GTP hydrolysis in itself is not adequate to promote EF-Tu-mediated refolding. GTP hydrolysis by EF-Tu is markedly increased by kirromycin, whereas refolding is inhibited to near the level of unassisted refolding. Bound kirromycin holds EF-Tu in the closed conformation that is induced by binding of GTP. GDP resulting from GTP hydrolysis is released from EF-Tu in this conformation so that successive rounds of GTP binding, hydrolysis, and release of GDP can take place in the absence of EF-Ts. Both GMP-PCP and kirromycin inhibit refolding. Denatured rhodanese appears to bind to EF-Tu in the presence of either of these compounds, but this binding results in inhibition rather than refolding of the protein into its native conformation. Inhibition of refolding was also observed when similar experiments were carried out with GDP or pulvomycin, both of which hold EF-Tu in the open conformation.

Considered together, these results lead to the conclusion that, for successful refolding of the denatured protein, GTP hydrolysis is required under conditions in which EF-Tu can cycle between the open and closed conformation. We propose the following as the mechanism by which the mechanical energy related to this change in the conformation of EF-Tu is used to promote refolding of some improperly folded molecules of rhodanese that have taken an unproductive folding path. These molecules are trapped in a semi-stable, misfolded conformation in which relatively hydrophobic regions remain exposed on their surface. Presumably, these hydrophobic regions interact with regions of EF-Tu. In terms of a computer-generated folding landscape (25), these misfolded molecules are trapped in a low energy depression or valley. By binding, probably through hydrophobic interactions across a region of EF-Tu in which a conformational change takes place, the misfolded protein may be repeatedly pulled and stretched or compressed locally by the
relative movement within the structure of EF-Tu. By this mechanism, the protein is lifted out of the low energy folding depression so that it can fold in a different way. That is, the misfolded protein is activated to a point at which it can follow another folding pathway that may lead to the native conformation. In this conformation, most of its hydrophobic regions are buried within the protein that is then released from its association with EF-Tu.

The mechanism proposed above is applicable to EF-Tu-mediated folding that is dependent on GTP hydrolysis and presumably the related change of conformation in the factor. But how could such a mechanism account for the EF-Tu-mediated refolding that does not involve GTP hydrolysis? This is the increase in refolding from 14 to about 50% that is promoted by EF-Tu in the absence of added nucleotide and EF-Ts, as shown in Table I. This refolding activity of EF-Tu is inhibited by either GMP-PCP or GDP from about 50 to 29 and 23%, respectively. It is inhibited even further to about the level of unassisted refolding by either kirromycin or pulvomycin. A common effect of each of these compounds appears to be to stiffen EF-Tu in either its open or closed conformation. E. coli EF-Tu alone appears to have considerable flexibility in solution (7). Binding of one of the antibiotics or a guanine nucleotide tends to hold the factor in a specific conformation. We suggest that it is this decrease in flexibility that causes the reduction in rhodanese refolding. It follows that flexing of the EF-Tu, probably as a result of thermal motion, is able to activate some but not all of the misfolded rhodanese molecules in a manner that is essentially similar to that described above.

Acknowledgments—We are grateful to Dr. Andrea Parmeggiani (Paris, France) for providing pulvomycin. We thank Drs. John Czworkowski and Peter Moore (Yale University, New Haven, CT) as well as Drs. Youxing Jiang and Paul Sigler (Yale University) for the generous gifts of purified EF-G and EF-Ts, respectively.

REFERENCES
1. Mesters, J. R., Potapov, A. P., de Graaf, J. M., and Kraal, B. (1994) J. Mol. Biol. 242, 644–654
2. Neidhardt, F. (1996) *Escherichia coli and Salmonella*, p. 1562, ASM Press, Washington, D. C.
3. Gonen, H., Smith, C. E., Siegel, N. R., Kahana, C., Merrick, W. C., Chakraburtty, K., Schwartz, A. L., and Ciechanover, A. (1994) Proc. Natl. Acad. Sci. 91, 7648–7652
4. Gonen, H., Dickman, D., Schwartz, A. L., and Ciechanover, A. (1996) Adv. Exp. Med. Biol. 389, 209–219
5. An, G., Bendlak, D. S., Mamela, L. A., and Friesen, J. D. (1981) *Nucleic Acids Res.* 9, 4163–4172
6. Kjeldgaard, M., and Nyborg, J. (1992) J. Mol. Biol. 223, 721–742
7. Abel, K., and Jurnak, F. (1996) *Structure (Lond.*)* 4, 229–238
8. Parmeggiani, A., and Swart, G. W. M. (1985) *Annu. Rev. Microbiol.* 39, 557–577
9. Kraal, B., Zeev, L. A. H., Mesters, J. R., Boon, K., Vorstenbosch, E. L. H., Bosch, L., Anborgh, P. H., Parmeggiani, A., and Hilgenfeld, R. (1995) *Biochem. Cell Biol.* 73, 1167–1177
10. Mendoza, J. A., Rogers, R., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem.* 266, 13587–13591
11. Tandon, S., and Horowitz, P. (1988) *Biochim. Biophys. Acta* 955, 19–25
12. Zardeneta, G., and Horowitz, P. (1992) *Eur. J. Biochem.* 210, 831–837
13. Jarabak, R., Westley, J., Dungan, J. M., and Horowitz, P. (1993) *J. Biochem. Toxicol.* 8, 41–48
14. Das, R. P., Pethash, J. M., and Surewicz, W. K. (1996) *J. Biol. Chem.* 271, 10449–10452
15. Horowitz, P. (1997) *Structure and Function of Molecular Chaperones: The Role of Chaperotins in the Life Cycle of Proteins* (Fink, A. L., and Goto, Y., eds) pp. 278–300, Marcel Dekker, New York
16. Kudlicki, W., Coffman, A., Kramer, G., and Hardesty, B. (1997) *Fold. Des.* 2, 101–108
17. Kudlicki, W., Odom, O. W., Kramer, G., and Hardesty, B. (1994) *J. Biol. Chem.* 269, 16549–16553
18. Leberman, R. B., Antonsson, R., Giovannelli, R., Guariguata, R., Schumann, R., and Wittinghofer, A. (1990) *Anal. Biochem.* 184, 29–36
19. Horowitz, P. M. (1978) *Anal. Biochem.* 86, 751–753
20. Mendoza, J. A., and Horowitz, P. M. (1994) *J. Protein Chem.* 13, 15–22
21. Sorbo, B. H. (1953) *Acta. Chem. Scand.* 7, 1129–1136
22. Conway, T., and Lipman, F. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 54, 1462–1469
23. Lefevre, Y., Varlot, G., De Vendittis, E., Sander, G., and Parmeggiani, A. (1990) *Biochim. Biophys. Acta* 1035, 53–60
24. Wolf, H., Asmann, D., and Fischer, R. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 5324–5328
25. Dill, K., and Chan, H. S. (1997) *Nat. Struct. Biol.* 4, 10–19