Folding of an Enzyme into an Active Conformation While Bound as Peptidyl-tRNA to the Ribosome†

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ABSTRACT: Rhodanese bound to bacterial ribosomes as peptidyl-tRNA can be folded into an enzymatically active conformation by generating C-terminal extensions of the wild-type enzyme. Rhodanese was synthesized by coupled transcription/translation in a cell-free *Escherichia coli* system from plasmids containing the coding sequences for the wild-type enzyme or its C-terminally extended mutants. Two proteins with extensions of 23 amino acids or longer were enzymatically active while bound to the ribosomes whereas wild-type protein and a 13-amino acid extension were not. All forms of the enzyme were active after termination and release of the full-length protein from the ribosomes. All five of the bacterial chaperones were required to substantially increase the specific enzymatic activity of the extended rhodanese while the nascent protein was bound to ribosomes. The results provide direct support for the hypothesis that proteins acquire tertiary structure as they are formed in ribosomes.

Several recent reports provide evidence that nascent proteins acquire three-dimensional structure while they are being synthesized in ribosomes (Picking et al., 1992; Kudlicki et al., 1994a; Kolb et al., 1994; Fedorov et al., 1995) and that chaperones are involved in this process (Frydman et al., 1994; Kudlicki et al., 1994b, 1995a; Craig et al., 1994; Hansen et al., 1994). These and other reports reopen the old discussion of whether proteins fold into their native conformation while they are synthesized vectorially in ribosomes, the cotranslational folding hypothesis. The evidence so far leads to the conclusion that most, probably nearly all, nascent proteins acquire three-dimensional structure before they are released from ribosomes. However, the relation of this structure to the native conformation of the proteins is not known in that all enzymes that we have studied to date were enzymatically inactive before they were terminated and released from the ribosomes. Here, we provide direct evidence that modified forms of rhodanese, altered by addition of a sequence of amino acids at the C-terminus of the wild-type enzyme, are enzymatically active while they are bound as peptidyl-tRNA to the ribosome in which they were formed.

EXPERIMENTAL PROCEDURES

Materials

Nucleoside triphosphates and *Escherichia coli* tRNA were purchased from Boehringer Mannheim. Rifampicin, puromycin, and other biochemicals were from Sigma. The chaperones DnaJ, DnaK, GrpE, GroES, and GroEL were bought from Epicentre Technologies. The following coding sequences inserted in the indicated plasmids were used: bovine rhodanese in pSP65 (Kudlicki et al., 1994a); hamster rhodanese coding sequence minus its five terminal amino acids, plus 28 unrelated amino acids in pET11d (Trevino et al., 1995); bovine rhodanese minus its stop codon fused to mouse DHFR[1] in pET11d (J.C., unpublished results).

Methods

Propagation of the plasmids, isolation of SP6 RNA polymerase and preparation of the *E. coli* cell-free extract (S30) as well as the isolation of the ribosome fraction from the S30 were carried out as described previously (Kudlicki et al., 1992). T7 RNA polymerase was isolated by a procedure identical to the one used for preparation of SP6 RNA polymerase. *E. coli* BL21 (pAR 1219) cells were used as a source for T7 RNA polymerase.

The *in vitro* system for coupled transcription/translation (usually a 30−60 μL reaction mixture) has been detailed (Kudlicki et al., 1992, 1994a). [^{14}C]Leucine was the radioactive precursor. After incubation for 30 min at 37 °C, the reaction mixtures were centrifuged in an airfuge for 40 min at 150000*g*. The resulting supernatants and the ribosomal fraction (after resuspension in a volume equal to the original reaction mixture) were analyzed separately (a) for the amount of protein synthesized by precipitation with trichloroacetic acid and determining the radioactivity in the precipitate, (b) for size of the synthesized protein by SDS−PAGE (Laemmli, 1970) and autoradiography, and (c) for enzymatic activity according to Sörbo (1953) as described in detail in Kudlicki et al. (1994a). Specific enzymatic activity is expressed as units/mg of full-length rhodanese

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[1] Abbreviations: DHFR, dihydrofolate reductase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RHO, rhodanese.

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Table 1: Enzymatic Activity of C-Terminally Extended Rhodanese[a]

rhodanese analyzed	total reaction mixture		supernatant fraction				ribosome fraction			
	[^{14}C]leucine incorporated (pmol)	enzymatic activity (units × 10^{-3})	[^{14}C]leucine incorporated (pmol)	protein (pmol)	enzymatic activity (units × 10^{-3})	enzymatic activity (units/mg)	[^{14}C]leucine incorporated (pmol)	% as full length	enzymatic activity (units × 10^{-3})	enzymatic activity (units/mg)
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
wild-type extended rho	142	58.7	64	2.7	59.2	683	70	48	0	0
+13 aa	128	44.2	55	2.3	39.1	518	68	42	0	0
+23 aa	117	23.1	42	1.3	17.5	381	67	33	5.2	217
+160 aa	82	7.5	21	0.4	5.2	170	52	31	2.9	126

[a] Wild-type rhodanese and its C-terminally extended sequences were synthesized *in vitro* by coupled transcription/translation (see Experimental Procedures). Extention by 13 amino acids was achieved by translating wild-type rhodanese mRNA in a 30 μL reaction mixture also containing 150 pmol of synthetic tRNA^{Ala} which recognizes the UGA stop codon (cf. Ma et al., 1993). Rhodanese plus 23 amino acids and plus 160 amino acids, respectively, was synthesized by using the mutant plasmids listed under Experimental Procedures. The +160-amino acid C-terminal extension was generated by fusion of the coding sequence of bacterial dihydrofolate reductase with the coding sequence lacking the stop codon for rhodanese. After 30 min of incubation at 37 °C, the reaction mixtures were centrifuged and the resulting supernatant and ribosomal fractions were analyzed separately for the amount of protein synthesized and for enzymatic activity. The results are given for a 30 μL reaction mixture.

synthesized. This latter parameter was determined after SDS−PAGE and autoradiography by cutting out and solubilizing the band from the gel that represents full-length rhodanese. Radioactivity in this band was measured in the presence of EcoLite (ICN).

RESULTS AND DISCUSSION

Rhodanese, a 33 kDa sulfur transferase, was synthesized from plasmid DNA in a cell-free transcription/translation system derived from *E. coli* (Kudlicki et al., 1992). C-terminal extensions of the cloned wild-type gene were produced in three different ways: (a) A synthetic tRNA^{Ala} which functions as a suppressor tRNA (Ma et al., 1993) was used to insert alanine at the UGA stop codon of the wild-type protein so that a naturally occurring in-frame UAG stop codon would be used after translation of an additional 13 amino acids. (b) A coding sequence had been engineered that lacked the codons for the five C-terminal amino acids of native hamster rhodanese but coded for an extension of this rhodanese species by 28 other amino acids (Trevino et al., 1995). (c) A +160-amino acid fusion protein was generated containing bovine rhodanese linked at its C-terminus to mouse dihydrofolate reductase (DHFR). The data presented in Table 1 indicate that only those plasmids with extended coding sequences to give C-terminal extensions of 23 amino acids or longer (b and c above) produced polypeptides which were able to fold into an enzymatically active conformation while still bound to ribosomes. All of the plasmids gave enzymatically active protein in the total reaction mixture (column 2 of Table 1). However, the amount of the different rhodanese species that were produced by coupled transcription/translation declined with the length of the C-terminal extension (column 1) as did the enzymatic activity (column 2). An aliquot of the reaction mixture was centrifuged to yield a ribosome and a soluble fraction. SDS−PAGE of the latter and autoradiography indicate only a single band corresponding in size to the anticipated full-length rhodanese species (Figure 1). Since the number of leucine residues in the rhodanese species are known, the molar amount of the protein that is present in the supernatant fraction can be calculated directly from its molecular weight and the [^{14}C]leucine that is incorporated into the protein. These results are given in column 4 of Table 1. Specific enzymatic activity was calculated (column 6) from the amount of protein and the enzymatic activity measured in

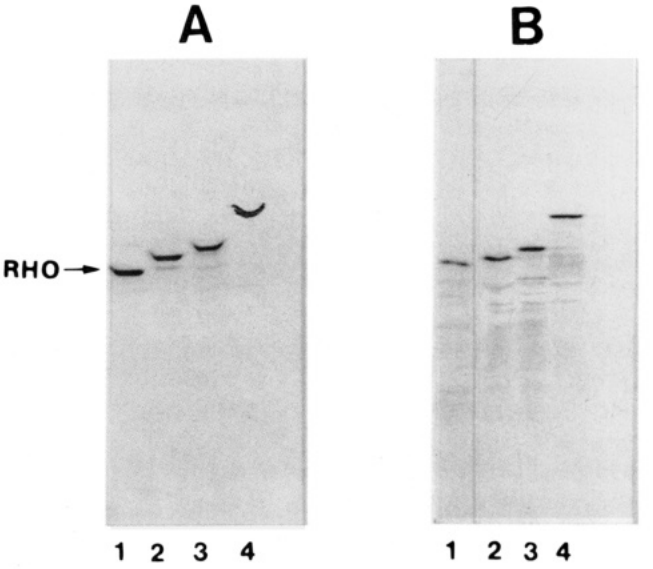

FIGURE 1: Rhodanese polypeptides in the supernatant and ribosome fractions. Wild-type rhodanese and modified forms with C-terminal extensions were synthesized *in vitro* in the cell-free system (see Experimental Procedures). The ribosomes and the supernatant fraction were separated by centrifugation. Parts A and B represent supernatant and ribosome fraction, respectively. In each case, 20 μL of the supernatant and ribosome-bound material was analyzed. Tracks 1, wild-type rhodanese; tracks 2, rhodanese with additional 13 amino acids; tracks 3, rhodanese with additional 23 amino acids; tracks 4, fusion protein rhodanese/DHFR.

the different supernatant fractions (column 5). Previously, we demonstrated that an appreciable amount of the newly formed rhodanese peptides present in the ribosome fraction were bound to the ribosomes as full-length peptidyl-tRNA (Kudlicki et al., 1994a). For the wild-type enzyme, full-length protein accounts for about half of the total newly formed peptides that are in this fraction (column 8, Table 1) as we reported previously (Kudlicki et al., 1994a). Accumulation of this material appears to reflect failure of termination and release in the cell-free transcription/translation system. Enzymatic activity for polypeptides bound to the ribosomes is given in column 9. The specific enzymatic activity was calculated (column 10) from the percentage of the total protein in the ribosome fraction that is in the form of full-length protein. Inherent in the calculation is the assumption that peptides that are less than full-length are enzymatically inactive. The percentage of full-length polypep-

Table 2: Effect of Chaperones on Activation of Ribosome-Bound Nascent Rhodanese[a]

rhodanese analyzed	rhodanese enzymatic activity (units × 10^{-3})	specific enzymatic activity (units/mg)
wild-type	0.1	2.2
extended rhodanese		
+23 aa	9.8	408
+160 aa	4.4	229

[a] An aliquot of the ribosomal fractions with bound wild-type rhodanese or extended rhodanese species (70 pmol of [^{14}C]leucine incorporated in nascent protein) isolated after synthesis was incubated for 15 min at 37 °C in the presence of the chaperones: 3.5 μg of GroEL (0.44 μM), 1 μg of GroES (0.46 μM), 2 μg of DnaK (0.9 μM), 1 μg of DnaJ (0.8 μM), and 1 μg of GrpE (1.4 μM). All reaction mixtures contained 4 μM sparsomycin, ATP, GTP, and other low molecular weight components as described previously (Kudlicki et al., 1994a). After incubation, rhodanese activity and specific activity were determined.

tide chains was determined by excising and counting the protein band from a gel after SDS−PAGE (Figure 1). The percentage of the respective full-length product was calculated from the total labeled polypeptides that were applied to the gel (Table 1, column 8).

Enzymatic activity was detected only in the two ribosome fractions containing rhodanese that was extended by at least 23 amino acids. The specific enzymatic activity of rhodanese for the 160-amino acid extension protein was half that of the species with 23-amino acid extension. The specific enzymatic activities of these species were lower than those of the corresponding protein that had been released into the supernatant fraction. Consideration of the crystal structure (Ploegman et al., 1978) suggests that the reduced specific enzymatic activity of the 23-amino acid and the 160-amino acid extension products relatively to wild-type rhodanese in its native conformation may result from interference of these C-terminal segments of the modified protein with the native conformation.

The enzymatic activity (and thus the specific enzymatic activity) of the two ribosome-bound forms was approximately doubled by incubating the respective ribosome fraction with all five bacterial chaperones (DnaJ, DnaK, GrpE, GroEL, and GroES) simultaneously while the nascent chains were held in the ribosomes (Table 2 in comparison to Table 1). Addition of only DnaJ, DnaK, GrpE, or only GroEL and GroES did not elicit this effect (data not shown). The chaperones had no effect on the ribosome-bound wild-type enzyme or on the ribosome-bound species that had been extended by 13 amino acids. The incubation of the ribosome fractions with chaperones was carried out in the presence of sparsomycin, an antibiotic that specifically inhibits the peptidyl transferase reaction (Cundliffe, 1980) and thus codon-dependent termination and release of rhodanese from the ribosomes. Less than 10% of nascent chains were released under these conditions as determined after a second centrifugation following the incubation with the chaperones in the presence of sparsomycin (data not shown). Incubation of ribosome-bound wild-type rhodanese with all chaperones in the absence of sparsomycin resulted in release of enzymatically active protein (Kudlicki et al., 1994a). We conclude that the increase in specific enzymatic activity (Table 2) reflects chaperone-mediated folding of more of the nascent ribosome-bound protein into its native conformation. It should be emphasized that the crude ribosomes in which rhodanese is synthesized contain chaperones and release factors detectable on Western blots with the respective antibodies (unpublished results). Apparently during 30 min incubation for coupled transcription/translation, the chaperones on the ribosomes appear to become limiting for termination, release, and activation of the nascent protein.

Inhibition of release by sparsomycin indicates that the peptidyl transferase reaction is involved in release of full-length polypeptides from the ribosomes and that they are held in the ribosomes as peptidyl-tRNA. This conclusion is supported by data (Table 3) indicating that puromycin (in the absence of sparsomycin) releases a high percentage of the incorporated [^{14}C]leucine that was initially present in the ribosome fraction. Puromycin is a tRNA analog that binds into the ribosomal A site and by the ribosomal peptidyl transferase reaction forms a covalent bond with the C-terminus of a peptide bound as peptidyl-tRNA in the ribosomal P site. This reaction causes release of a nascent peptide with puromycin at its C-terminus. As shown in Table 3, mutant rhodanese with 23- or 160-amino acid C-terminal extension has enzymatic activity after the puromycin reaction. The specific enzymatic activity of the 23- and 160-amino acids extension products were essentially unchanged by puromycin (Tables 1 and 3). Wild-type rhodanese released by puromycin was enzymatically inactive as reported previously (Kudlicki et al., 1994a).

From these results we conclude that lengthening the rhodanese peptide by the 23-amino acid extension is adequate to allow the nascent rhodanese to fold into an active conformation. This folding is dependent on one or more chaperone-mediated reactions that take place while the nascent enzyme is bound to the ribosome as peptidyl-tRNA. A C-terminal extension of adequate length may allow a C-terminal segment of the nascent rhodanese that is required for folding into an enzymatically active conformation to extend beyond the restrictive confines of the tunnel into a

Table 3: Release of Ribosome-Bound Peptides by Puromycin and Analysis of Rhodanese Activity in the Supernatant[a]

	−puromycin		+puromycin		
rhodanese analyzed	[^{14}C]leucine in protein (pmol)	enzymatic activity (units × 10^{-3})	[^{14}C]leucine in protein (pmol)	enzymatic activity (units × 10^{-3})	specific enzymatic activity (units/mg)
wild-type	6	0.0	48	0.0	0
mutant rhodanese					
+23 aa	4	0.2	53	4.1	214
+160 aa	7	0.1	51	2.7	109

[a] An aliquot of the ribosome-bound rhodanese was incubated at 37 °C in the absence or presence of 2 mM puromycin. After a 20 min incubation, the ribosomes were separated from the supernatant by centrifugation. The amount of rhodanese released into the supernatant and its enzymatic and specific enzymatic activities were determined.

cavity or domain in the large ribosomal subunit where the chaperone-mediated folding reactions can take place (Eisenstein et al., 1994). The results presented provide a direct demonstration that chaperone-dependent folding into an enzymatically active conformation can take place while the protein is bound as peptidyl-tRNA to the ribosome.

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Makeyev, Kolb, and Spirin recently reported that a 26-amino acid C-terminal extension resulted in enzymatically active nascent luciferase while bound to ribosomes (Frontiers in Translation, Victoria, B.C., Canada, May 20−25, 1995). A plasmid containing the mouse DHFR coding sequence was generously provided by Dr. D. Stueber, Hoffmann-LaRoche (Basel, Switzerland). We thank Dr. Horowitz and co-workers (UT Health Science Center, San Antonio, TX) for providing the plasmid with the extended hamster rhodanese sequence. We thank Ronda Barnett for preparing the typescript.

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