Complex Environment of Nascent Polypeptide Chains*

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Nascent polypeptides enter into high molecular weight complexes with other proteins during chain elongation in vitro and in vivo. The nature of these complexes was investigated using an in vitro translation system programmed with a single mRNA lacking a translational termination codon. Complexes containing nascent polypeptides (molecular mass < 20 kDa), the molecular chaperone hsp 73 and other unidentified proteins can be released from the translationally arrested polysomes by puromycin treatment. The apparent native molecular mass of the nascent chain binding complex was determined to be >700 kDa by gel-filtration analysis. Complexes between the nascent polypeptide and at least hsp 73 appear to be sensitive to (disrupted by) ATP. The presence of ATP also dramatically alters the sensitivity of the nascent polypeptide chains to dimeric chaperone hsp 70, which comprises a nascent polypeptide elongation chain binding complex. The presence of ATP also dramatically alters the sensitivity of the nascent polypeptide chains to dimeric chaperone hsp 70, which comprises a nascent polypeptide elongation chain binding complex.

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Most current models describing how proteins might achieve their final properly folded monomeric or higher ordered structure now include the actions of protein families known as molecular chaperones (1-3). In general, molecular chaperones participate in the process of protein biogenesis by preventing premature folding and/or reducing the probability of inappropriate and nonproductive intra- and intermolecular interactions of the newly synthesized polypeptide. These conclusions have been arrived at, in part, via examining the role of molecular chaperones in facilitating the refolding of proteins that were first denatured in a chaotrope medium and then diluted back into a "refolding buffer" (4-9). In addition, the interactions between molecular chaperones and polypeptides that occur after the termination step of cell-free protein synthesis have also been investigated recently (10, 11). We now have examined the interaction of nascent polypeptide chains, still in the process emerging from the ribosome, with other proteins through use of both in vitro and in vivo experiments. We show here that during chain elongation the maturing polypeptide chains enter into a high molecular weight complex. The nascent polypeptide chain binding complexes have been partially characterized and found to contain hsp 70 (and probably other proteins) and to display nucleotide-dependent dynamics.

EXPERIMENTAL PROCEDURES

Materials—Antibodies recognizing hsp 73 or both hsp 72/73 have been described previously (14, 18). Antibodies recognizing puromycin were provided by Dr. K. Matlack and Dr. P. Walter (University of California, San Francisco). Edeine was provided by Dr. Harvey Lodish (Massachusetts Institute of Technology).

In Vitro Transcription/Translation—Diguanosine triphosphate capped (19) full-length or truncated (lacking the stop codon) CAT mRNA were synthesized in vitro by SP6 RNA polymerase (Promega) transcription (20) of pSPCAT DNA, which had been linearized at the BamHI (outside the coding region) or HindIII, EcoRI (within the coding region) restriction sites. pSPCAT contains the CAT gene, a HindIII to BamHI fragment of pSFV-CAT, inserted into the BglII site of pSP64T (19). Rabbit reticulocyte lysate translation reactions, prepared as described previously (17), were performed at 22–24 °C.

Isolation of in Vitro Assembled Polyribosomes—Polyribosomes were isolated from ATP-depleted translation reactions by sedimentation into a 10–40% sucrose gradient, containing 20 m~ Heps, pH 7.4, 100 m~ KCl, 2.1 m~ MgCl2, 2 m~ DTT, and 0.1 m~ EDTA (Buffer A), for 90 min at 38,000 rpm (Beckman SW 41 rotor, 4 °C).

Disruption of Polysomes and Separation of Nascent Polypeptides from Ribosome Subunits—Nascent polypeptide chains were dissociated from ribosomes by incubation with 2 m~ puromycin (24 °C for 20 min) and 500 m~ KCl, 5 m~ MgCl2, (37 °C/5 min) (21). Separation of the puromycin-released CAT polypeptides from the ribosomal subunits was achieved by layering the the reaction mixture between 2.3 ml of Buffer A and 2 ml of 30% sucrose in Buffer A, followed by centrifugation for 135 min at 50,000 rpm (Beckman type 70 Ti rotor, 4 °C).

Gel-filtration Chromatography—Translation reaction products (depleted of ATP) were fractionated on a 12-m (1.2 cm x 10.5 cm) Sephacryl S-400 gel-filtration column developed in 20 m~ Heps, 100 m~ KCl, 2 m~ MgCl2, 1 m~ DTT at 0.2 ml/min.

Chemical Cross-linking Experiments—Translation products were passed through SephadeX G-25 and then incubated incubated (45 min, 24 °C) without or with (0.6 or 1.8 m~) the cross-linker DTSSP (Pierce). The cross-linker was quenched (40 m~ ethanolamine, pH 8), the products were denatured (80 °C in 6.5% SDS, 0.1 m~ Tris, pH 11) and immunoprecipitated with anti-hsp 73 antibodies. The cross-linked, immunoprecipitated products were boiled in the presence of Laemmli sample buffer containing 250 m~ DTG prior to SDS-PAGE.

Immunoprecipitation of in Vitro Translation Products—ATP was depleted from the translation reactions by incubation with apyrase (Sigma) (50 units/ml, 15 min/24 °C) or was regenerated at 5 m~ by phosphorylase (50 units/ml)/creatine phosphate (12.5 m~). The native or SDS-denatured reaction products were digested with 20 m~ Heps, pH 7.4, 100 m~ KCl, 2 m~ MgCl2, 1% Triton X-100, 0.1% sodium deoxycholate prior to immunoprecipitation.

Isolation of Radiolabeled HeLa Cell Polyribosomes—HeLa cells were labeled in spinner suspension (DMEM containing 10% calf serum and 3 mCi/ml 35 m~[S]methionine (4 m~ methionine)/16 h). Emetine (0.2 m~) was added to stabilize the polysomes. Cells were washed with phosphate-buffered saline/emetine and disrupted at 4 °C by Dounce homogenization in 20 m~ Heps, pH 7.4, 100 KCl, 2 m~ MgCl2, 0.1 m~ EDTA, 1 m~ DTT, 0.1% Triton X-100, 0.2 m~ emetine, and 20 units/ml apyrase. From a post-mitochondrial supernatant fraction (20,000 × g/15 min, Beckman JS-13 rotor), crude polyribosomes were concentrated by centrifugation (50,000 rpm/90 min, Beckman type 70 Ti rotor), resuspended, and then applied to a 10–40% sucrose gradient, containing 0.5 m~ KCl and 5 m~ MgCl2.

Immunoprecipitation of Polypeptides Terminated by Puromycin in Vivo—HeLa cells were incubated with 3 m~ puromycin for 10 min.
Fig. 1. Isolation of nascent polypeptide chains interacting with hsp 73 during translation in vitro. A, full-length or truncated CAT polypeptides were synthesized in vitro in a rabbit reticulocyte lysate for 60 min at 22–24 °C. Aliquots of the individual reactions were mixed together, ATP was depleted with apyrase and native immunoprecipitation was performed using an anti-hsp 73 antibody. Shown are: [35S]methionine-labeled CAT proteins synthesized from full-length mRNA (BamHI (221 aa) and three different truncated mRNAs (HincI (190 aa), NcoI (173 aa), and EcoRI (71 aa)) (lane 1), and the CAT proteins precipitated by preimmune serum (lane 2) or anti-hsp 73 antisera (lane 3). B, translation reactions programmed with truncated CAT (NcoI) mRNA were incubated with 4 mM 7-methylguanosine monophosphate and 0.01 mM edeine after 15 min to inhibit initiation. In B, following a 15-min chase period, the reaction was divided equally, ATP was depleted from one half of the reaction (−ATP) or regenerated in the other half (+ATP) and then analyzed as described in part A. Shown are starting materials for immunoprecipitations from pulse-labeled (lanes 1 (−ATP) and 3 (+ATP)) and from pulse-labeled and chased reactions (lanes 2 (−ATP) and 4 (+ATP)). Immunoprecipitates from pulse-labeled samples (lanes 6, 8, 9, and 10) or from pulse-labeled and chased samples (lanes 7, 8, 11, and 12) were prepared with preimmune serum (lanes 5, 7, 9, and 11) or anti-hsp 73 serum (lanes 6, 8, 10, and 12). ATP was depleted prior to capture of immunoprecipitates shown in lanes 5–8 and was regenerated for lanes 9–12. In C, polypeptides terminated by incubation of the reaction products from B with puromycin were analyzed as described in B. The reaction of puromycin with the nascent chains was verified by the resulting mobility shift of the chains on SDS-PAGE and by reactivity of the chains with anti-puromycin antibody. Shown are the starting materials for immunoprecipitations of puromycin-released CAT polypeptides (lane 1 (−ATP)) and (lane 2 (+ATP)), and the immunoprecipitated products from ATP-depleted (lanes 3 and 4) or ATP-supplemented (lanes 5 and 6) reactions prepared with preimmune (lanes 3 and 5) or anti-hsp 73 serum (lanes 4 and 6).

washed with methionine-free DMEM, and pulse-labeled for 20 min in DMEM containing 0.5 mM [35S]methionine and 3 μM puromycin. The cells were washed in phosphate-buffered saline, 5 mM MgCl2, and homogenized in phosphate-buffered saline, 5 mM MgCl2, and 0.1% Triton X-100. ATP was depleted from the cell lysate with apyrase (20 units/ml, 15 min at 4 °C) and the lysates used for native immunoprecipitation using anti-puromycin or preimmune antisera. Preincubation of the anti-puromycin serum with 1 μM puromycin blocked the capture of the puromycin terminated polypeptides. Alternatively, HeLa cells were labeled for 20 h in DMEM containing 0.1 mM [35S]methionine (4 μM methionine), chased for 4 h in radiolabel-free DMEM (200 μM methionine), and then incubated for 30 min with 3 μM puromycin.

RESULTS AND DISCUSSION

The proposed interaction of a newly synthesized protein with cytosolic hsp 70 (12–14) was examined during translation in vitro. A mixture of CAT polypeptides was produced by in vitro translation of synthetic truncated (lacking a termination codon) and full-length mRNAs in a rabbit reticulocyte lysate. Native immunoprecipitation was then performed using antibodies specific for hsp 73 (14). A preferential association of the nascent (truncated) polypeptides with hsp 73 was observed (Fig. 1A, lane 3) as compared to the mature (full-length) CAT molecules present in the mixed population of polypeptides (Fig. 1A, lane 1).

We next examined the interaction of nascent polypeptides with hsp 73 during an in vitro pulse-chase experiment and the possible influence of ATP (12, 13, 23) on the chaperone-substrate interaction. Translation of the truncated mRNA was carried out for 15 min (pulse). ATP was then depleted from one half of the lysate and regenerated in the other half. In parallel, after 15 min of translation, further initiation was blocked and chain elongation allowed to proceed for 15 min (chase). In the 15-min pulse-labeled lysates (depleted of ATP), the truncated CAT polypeptide was observed to co-immunoprecipitate with hsp 73 (Fig. 1B, lane 6). Following a 15-min chase period, more of the truncated CAT polypeptide was complexed with hsp 73 (Fig. 1B, lane 8). Significantly less of the truncated translation product was observed to co-precipitate in those lysates supplemented with ATP (Fig. 1B, lanes 10 and 12). Thus, through the prevention of polypeptide chain termination, via elimination of the stop codon, relatively stable (but ATP-sensitive) intermediates of a nascent chain, complexed with at least cytosolic hsp 73, can be accumulated, thereby allowing for their further physical and biochemical characterization.

To examine in detail the nascent polypeptide-hsp 73 complexes, the nascent chains were released from the polysomes by treatment with the aminocyl-tRNA analogue puromycin and high salt (21). The puromycin-released CAT polypeptides again were found to co-precipitate with hsp 73 when the reactions were depleted of ATP (Fig. 1C, lane 4), while addition of ATP decreased the relative amount of co-precipitating nascent chains (Fig. 1C, lane 6).

Having determined that stable complexes between a defined population of nascent polypeptides (or their derived puromycin-released chains) and hsp 73 could be accumulated in vitro, we examined the apparent native size of the translation products using gel-filtration chromatography. The full-length CAT polypeptide eluted at a position well included within the column profile (Fig. 2A, profile 1), consistent with the native CAT molecule being a trimer of 25-kDa monomers (15, 16). Next, as would be expected of any ribosome-associated polypeptide, the translation products of a truncated CAT mRNA (molecular mass ~ 18 kDa by SDS-PAGE) were found to elute near the void volume of the column (Fig. 2A, profile 2) (the ribosome-bound CAT polypeptides were eluted slightly later than the excluded volume of the column due to interaction of the ribosomes with the column matrix). Interestingly, upon treatment with puromycin, the released CAT polypeptides were found to elute within the void volume of the column, with a small fraction of the material exhibiting a heterogeneous profile within the included volume (Fig. 2A, profile 3). Finally, to exclude the possibility that the puromycin-released CAT polypeptides were binding to proteins present within the reticulocyte lysate after their release from the ribosome, sucrose gradient-purified polypeptides synthesizing CAT were dissociated by incubation with puromycin/high salt. Such puromycin-released chains still eluted exclusively within the void volume of the column (Fig. 2A, profile 4). The complexes containing hsp 73 and nascent chains released from the purified polypeptides were still ATP-sensitive, as determined by immunoprecipitation using the anti-hsp 73 antibody (Fig. 2C, lanes 4 and 5). Taken together, these results indicate that the nascent 18-kDa CAT polypeptide truncation product, but not mature full-length 25-kDa CAT, exists as part of a high molecular mass complex (>700 kDa). Presumably this is due, at least in part, to an interaction of the nascent chains with hsp 73.

In order to test this hypothesis further and to obtain a more quantitative estimate of the fraction of nascent polypeptides that interact with hsp 73, we used a bifunctional and reversible cross-linking reagent, DTSSP, which would allow for the isolation of hsp 73-containing nascent chain complexes under more stringent conditions. Messenger RNAs encoding globin and two truncated CAT proteins were translated, the reactions were depleted of ATP and then incubated with the cross-linker. After
purified polysome fraction were still resistant to proteolytic digestion if ATP was depleted. Complete digestion was again observed if the polysomes were incubated with ATP (Fig. 3B).

Whether proteins other than hsp 73 may also associate with nascent polyribosomes was next examined. Polysomes synthesizing truncated CAT molecules were isolated and then were dissociated by addition of puromycin/high salt. The released nascent chains were separated from the ribosomal subunits by centrifugation. The 35S-labeled nascent chains, as determined by SDS-PAGE analysis, remained near the top of the gradient (Fig. 4A). In order to detect reticulocyte lysate proteins that might be complexed with the nascent chains, the proteins present in each gradient fraction were radioiodinated. Analysis of the iodinated proteins by SDS-PAGE revealed that prominent proteins of apparent molecular mass of 30 kDa on up to 100 kDa, including a major one of approximately 70 kDa were enriched in those fractions containing the 35S-labeled nascent chains (Fig. 4A) (the major species near 14 kDa is puromycin-released globin). Note that the majority of the 35S-labeled proteins were found at the bottom of the gradient and that these proteins, but not those that were found to sediment near the 35S-labeled nascent chains, are of apparent molecular masses less than 45 kDa, in a pattern characteristic of ribosomes.

In order to determine whether nascent chain binding protein complexes can be also identified in vivo, polysomes from steady state radiolabeled HeLa cells were isolated. The polysomes, isolated under conditions (i.e. high salt) that should remove the protein synthesis initiation and elongation factors, were disassembled by incubation with puromycin. Centrifugation of the dissociated polysomes (Fig. 4B, lane 1) resulted in a ribosomal subunit fraction (Fig. 4B, lane 4) and a soluble fraction. Similar to the results with the soluble fraction containing the puromycin-released nascent chains from the reticulocyte lysate (Fig. 4, A and B), the analogous soluble fraction from the HeLa cells contained a number of polypeptides, most notably hsp 70 (Fig. 4B, lanes 2 and 3).

An independent approach using an anti-puromycin antibody was also employed to identify proteins that interact with nascent polypeptides in vivo. The anti-puromycin antibody was effective in precipitating a "smear" of radiolabeled puromycin-terminated proteins from cells pulse-labeled in the presence of a low concentration of puromycin (Fig. 4C, lanes 1–3). Next, to identify possible proteins interacting with the nascent polypeptides, cells were steady state labeled with [35S]-methionine, the radiolabel was removed and the cells further incubated in the absence of radiolabel. Now with the cellular "machineries" involved in protein biogenesis radiolabeled (Fig. 4C, lane 4), the cells were treated with puromycin for 30 min. When cell lysates were prepared and examined by native immunoprecipi-
FIG. 4. Nascent polypeptide chain binding complexes contain proteins in addition to hsp 70. A, nascent chains were released from purified polysomes translating truncated (NcoI) mRNA by incubation with puromycin/high salt. Nascent polypeptides (and their associated proteins) and the ribosomal subunits were separated by velocity gradient sedimentation (see "Experimental Procedures"). Top panel, [35S]Smethionine-labeled nascent CAT polypeptides in samples of the gradient fractions. The starting material applied to the gradient (lane 1), gradient fractions 2–9 (top of gradient to bottom, respectively, lanes 2–9), and the pellet fraction (lane 10) are shown. Bottom panel, reticulocyte lysate proteins present within the gradient fractions were detected by radioiodination. Samples of the starting material applied to the gradient and each gradient fraction were iodinated using chloramine T (22) and Na[125I] (Amerham Corp.) and analyzed by SDS-PAGE. A shorter exposure of fraction 9 (also shown to reveal the individual polypeptides (lane 9)). B, polyribosomes isolated from steady state [35S]Smethionine-labeled HeLa cells (see "Experimental Procedures") were dissociated with puromycin and applied to a sucrose step gradient as described in A. Shown are the radiolabeled proteins present in the puromycin- and high salt-treated polyribosome starting material applied to the gradient (lane 1), gradient fractions 3 and 4 (lanes 2 and 3, respectively), and the pellet fraction of the gradient (lane 4). Molecular size standards indicated by the bars on the right are the same as in A. HeLa cells were pulse-labeled with [35S]Smethionine in the presence of puromycin (see "Experimental Procedures") for 30 min. The proteins present in the cell lysate (lane 1) were subjected to native immunoprecipitation using preimmune antiserum (lane 2) or anti-puromycin serum (lane 3). To identify proteins possibly interacting with nascent polypeptides, HeLa cell lysates were prepared and examined by native immunoprecipitation using the preimmune, anti-puromycin, or anti-hsp 70 antibodies. Shown are the starting material for the immunoprecipitations (lane 4), proteins immunoprecipitated using preimmune serum (lane 5), anti-puromycin serum (lane 6), and anti-hsp 72/73 antibodies (lane 7).

Our results indicated that the anti-puromycin antibody was found to immunoprecipitate a number of labeled polypeptides of mass greater than ~45 kDa (Fig. 4C, lane 6). One of the polypeptides co-precipitated with the anti-puromycin antibody was hsp 70 (Fig. 4C, compare lane 6 to lane 7; this was confirmed by anti-hsp 70 precipitation of the 70-kDa band in lane 6 (data not shown)). The identity of those proteins, other than hsp 70, which are apparently complexed with the nascent polypeptides is currently under investigation.

Complexes containing newly synthesized polypeptides and hsp 70, first described as transient intermediates in vitro (12, 13), are formed during the elongation phase of translation in vitro (Figs. 1 and 2). Complexes containing nascent polypeptides ~20-kDa polypeptide chain exhibit a native molecular mass in excess of 700 kDa after their release from the translational machinery. Moreover, based upon a number of criteria (e.g. Figs. 2–4), these complexes appear to contain, in addition to hsp 73, other protein components that we suggest together may represent a nascent polypeptide chain binding complex. Although not shown here, the observations we have reported upon are not specific to CAT; instead, they appear to apply to nascent polypeptides in general. Other components of such a complex may be those we have identified via radiodination (Fig. 4A) or metabolic labeling (Fig. 4, B and C). Finally, owing to the recent description of an interaction between the bacterial DNA J protein and cytosolic eukaryotic nascent chains in vitro (24), it is possible that a eukaryotic DNA J homolog may be one of the components of the nascent chain binding complex.

Our suggestion of there being a general nascent chain binding complex is reminiscent of another more specialized nascent chain binding complex, the signal sequence recognition particle (25). While the signal sequence recognition particle creates a cytosolic targeting complex (26) through its binding to signal sequences, which directs polypeptides translating signal sequence containing proteins to the endoplasmic reticulum, we suspect that the nascent chain binding complex containing at least hsp 73 might act in a somewhat more generic fashion. The role of this cytosolic complex would be to ensure that nascent chains, upon emerging from the ribosome, are protected and/or prevented from improper intra- or intermolecular interactions, ideas that are consistent with the overall theme of molecular chaperone function (1–3). Our observations regarding the ATP-dependent proteolytic susceptibility of nascent chains would appear in line with this idea. Upon ATP-induced release of at least hsp 73, the nascent chains appeared remarkably sensitive to the presence of a relatively small amount of chymotrypsin, indicative of the nascent chain, perhaps present in an extended (or partially unfolded) conformation, now being accessible to the protease. Finally, we also think it likely that via an interaction with a general nascent chain binding complex, the nascent chain may be preferentially reserved for interactions with other protein biogenesis machineries. Relevant examples, may include other cytosolic chaperones (e.g. a cytosolic chaperonin like TCP-1), proteins involved in targeting to subcellular organelles and enzymes involved in co- and post-translational protein modification (e.g. the peptidyl-prolyl isomerases, protein kinases, lipid modification enzymes). Using the approach outlined here such possibilities are now being addressed.

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