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A Newly Synthesized, Ribosome-bound Polypeptide Chain Adopts Conformations Dissimilar from Early *in Vitro* Refolding Intermediates*

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Patricia L. Clark‡ and Jonathan King

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Little is known about the conformations of newly synthesized polypeptide chains as they emerge from the large ribosomal subunit, or how these conformations compare with those populated immediately after dilution of polypeptide chains out of denaturant *in vitro*. Both *in vivo* and *in vitro*, partially folded intermediates of the tailspike protein from *Salmonella typhimurium* phage P22 can be trapped in the cold. A subset of monoclonal antibodies raised against tailspike recognize partially folded intermediates, whereas other antibodies recognize only later intermediates and/or the native state. We have used a pair of monoclonal antibodies to probe the conformational features of full-length, newly synthesized tailspike chains recovered on ribosomes from phage-infected cells. The antibody that recognizes early intermediates *in vitro* also recognizes the ribosome-bound intermediates. Surprisingly, the antibody that did not recognize early *in vitro* intermediates did recognize ribosome-bound tailspike chains translated *in vivo*. Thus, the newly synthesized, ribosome-bound tailspike chains display structured epitopes not detected upon dilution of tailspike chains from denaturant. As opposed to the random ensemble first populated when polypeptide chains are diluted out of denaturant, folding *in vivo* from the ribosome may begin with polypeptide conformations already directed toward the productive folding and assembly pathway.

Although a subset of globular proteins can refold correctly *in vitro*, many other proteins fail to fold in the test tube (1, 2). For the limited set of proteins whose folding has been investigated both *in vivo* and *in vitro*, polypeptide chains fold more efficiently in their native cellular environments (3, 4). Part of the difference between folding *in vitro* and *in vivo* is due to the action of molecular chaperones; these protein complexes increase the yield of successfully folded protein for some polypeptide sequences (5, 6). Yet there are many proteins whose folding appears to be unaided by the presence of molecular chaperones, particularly *in vivo* (7–10).

The N-terminal domain(s) of some proteins are able to fold co-translationally to native-like conformations (8, 11, 12); many other proteins are unable to fold correctly in the absence of C-terminal domains (13–15). These results suggest that additional cellular components may be required for the successful folding of these polypeptide chains *in vivo*. The recent discovery that the ribosome-associated trigger factor may cooperate with the molecular chaperone DnaK to assist in the folding of some polypeptide chains (16, 17) suggests that additional parts of the cellular folding machinery may lie very close to the ribosome (18) and may involve the ribosome itself (19, 20).

What is the conformational state of nascent polypeptide chains not bound by molecular chaperones? During polypeptide synthesis, after amino acids are added to the growing polypeptide chain at the aminoacyltransferase center within the ribosome, they pass through a tunnel, emerging at the exit site on the back side of the large ribosomal subunit (21–23). Newly synthesized polypeptide chains must be held in some conformation in order to protect the nascent chain from proteolytic digestion or unproductive associations with other nascent or unfolded chains. This is true even during co-translational folding, which still appears to require the presence of a complete folding domain (50–200 amino acids) before folding can commence (10).

The experimental difficulties involved in studying a dynamically growing nascent polypeptide chain, attached to a large (2–4 MDa) ribosome, have limited studies of the conformations of nascent chains on actively translating ribosomes. Most investigations have focused on the interactions of nascent chains with chaperones (24, 25), or co-translational folding of nascent polypeptide chains to native or native-like states, detected by immunogold labeling (26), enzymatic activity (3, 8), or protection from protease digestion (13, 27). Many of these conformational studies have used stalled ribosomes, generated either chemically (28) or with truncated mRNA messages (12) and/or synchronized cell-free translation systems (13, 27), to trap and populate discrete ribosome-nascent chain species. Investigations using physiological translation systems have been rare (8).

In this paper, we examine the conformation of ribosome-bound, nascent polypeptide chains of the tailspike protein from *Salmonella typhimurium* phage P22. Tailspike is a homotrimeric protein with 666 amino acid residues per chain, arranged in an extended structure that includes a large central parallel β-helix domain (Fig. 1) (29, 30). Near the C terminus, the three separate amino acid chains wrap around one another to form an interdigitated triangular β-prism domain (31). Past folding studies have demonstrated that trimerization, and the subsequent interdigitation of the C-terminal domain, is crucial for correct formation of the native structure (32). Tailspike is unusual in that it represents one of the few proteins whose folding and assembly has been studied extensively both *in vitro* and *in vivo* (32–37). Because the SDS-resistant native state is distinguishable from its folding and assembly intermediates by SDS or native gel electrophoresis, the folding and assembly process

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‡ Supported by Postdoctoral Fellowship GM19715 from the National Institutes of Health. To whom correspondence should be addressed: Dept. of Biology 68-340D, Massachusetts Institute of Technology, Cambridge, MA 02139. Tel.: 617-253-3545; Fax: 617-252-1843; E-mail: plclark@mit.edu.

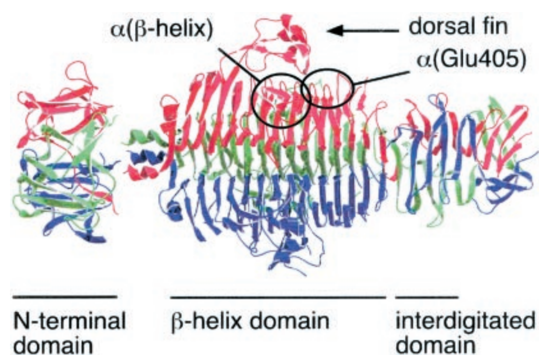

FIG. 1. **Ribbon diagram of the crystal structure of P22 tailspike (29).** The N-terminal head-binding domain was crystallized separately (30). The three polypeptide chains of the monomer are depicted in *red, blue,* and *green*. *Ovals* indicate the approximate epitope locations for the monoclonal antibodies used in this study; for clarity, the positions of the epitopes are indicated only for the *red* polypeptide chain. The dorsal fin, β-helix, and interdigitated domains are labeled.

can be monitored in crude extracts (33, 38). During folding *in vivo*, partially folded monomeric and trimeric intermediates have been identified (34). *In vitro* intermediates have been characterized by Seckler and co-workers (32, 39), using fluorescence and circular dichroism spectroscopy. Both monomeric and trimeric intermediates have been identified, corresponding to the *in vivo* intermediates (32). Fig. 2 shows a schematic diagram for the pathway of tailspike folding and assembly after release of polypeptide chains from ribosomes or dilution of chains out of denaturant.

Unlike the folding of many small globular proteins, tailspike folding exhibits Arrhenius behavior (*i.e.* linear dependence of the folding rate on temperature) (39). The temperature dependence is quite steep, resulting in an activation energy of ~165 kJ/mol (39). Although the reasons for non-Arrhenius behavior during folding are still under debate (40, 41), for the tailspike it appears that the stabilities and kinetics of the phases of folding are equally affected by temperature. A critical feature of this behavior is that tailspike intermediate populations can be trapped in the cold and remain competent for further folding for many hours (37).

The single-chain and the protrimer intermediates trapped in the cold can be separated by electrophoresis through non-denaturing acrylamide gels run in the cold (33). Unlike the microsecond and millisecond lifetimes for the folding intermediates of many small globular proteins, tailspike folding intermediates persist for several hours (32). Warming cold-trapped intermediates permits their continued folding to the native trimer (37). Cold-trapping therefore provides a method for temporarily stabilizing transient folding intermediates, permitting their investigation with more time-consuming experimental methods, without altering the tailspike folding pathway (34).

The tailspike single-chain partially folded intermediate is thermolabile, and temperature-induced destabilization of this intermediate causes tailspike folding to partition from the productive pathway to the aggregation pathway (33, 38). Although off-pathway aggregation occurs both *in vivo* and *in vitro*, the intermediates *in vivo* are less labile, requiring higher temperatures to initiate aggregation. Overexpression of GroEL/S does not alter the yield of native tailspike nor does it rescue tailspike folding mutants (42, 43). In addition, newly synthesized wild type tailspike chains do not appear to be associated with DnaK (44) or the GroE complex (43). Despite the similarities between the *in vitro* and *in vivo* folding pathways, tailspike folding *in vivo* always results in higher yields of folded protein. These inconsistencies prompted us to examine other participants in protein folding *in vivo*; specifically, whether the translation machinery itself can stabilize early intermediates *in vivo*.

A set of monoclonal antibodies isolated against native tailspike recognizes at least seven different classes of epitopes on the native structure (45).[1] Three of these antibodies were used previously to monitor the formation of intermediates and the native state during tailspike refolding *in vitro* (46). The epitopes recognized by these antibodies appeared at different stages during refolding. One of the antibodies, 236-3, in addition to recognizing *in vitro* refolding intermediates, also detected tailspike polypeptide chains in the presence of immunoprecipitated ribosomes from *in vitro* translation mixtures. However, two other antibodies, which recognized epitopes present only on later intermediates and the native trimer during refolding *in vitro*, did not detect tailspike polypeptide chains in the presence of the immunoprecipitated ribosomes. These results suggest the ribosome-associated tailspike chains contained significant, but not yet native-like, levels of structural information (46).

The methods used previously to analyze *in vivo* intermediates of tailspike folding (33) disrupted the ribosomal structures just prior to analysis. In order to determine the conformation of the nascent tailspike polypeptide chain while still attached to the ribosome, we characterized newly synthesized tailspike polypeptide chains using cell preparations from which intact ribosomes were recovered. Below we report the adoption of conformational motifs in newly synthesized, ribosome-bound tailspike polypeptide chains, prior to their release into the cytoplasm, and we compare these conformations with those adopted by tailspike polypeptide chains at the onset of *in vitro* refolding.

EXPERIMENTAL PROCEDURES

In Vitro Refolding—Tailspike was purified from P22-infected *Salmonella* cultures (see description below) as described previously (47). Protein concentrations were determined based on an absorbance value of 1.0 at 278 nm for a 1 mg/ml solution (48). Tailspike was denatured and refolded as described previously (32, 46). Briefly, 1 mg/ml tailspike was unfolded in 5 M urea, 63 mM sodium phosphate, 0.6 mM EDTA, pH 2.9, for 30 min at 25 °C. Aliquots of the unfolded protein were refolded at various time points (up to 24 h) by 50-fold dilution into 50 mM sodium phosphate, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.0. Refolding was performed at 10 °C and, as judged by SDS-PAGE,[2] resulted in ~75% of denatured chains recovering the native conformation after 24 h, consistent with previous results (32, 46). The remaining ~25% of denatured chains form non-native aggregates. After the final sample was diluted into refolding buffer, all samples were then placed on wet ice and diluted to a tailspike concentration conducive to the competition ELISA (*i.e.* a concentration where complete refolding of the protein would result in 70% antibody recognition, in order to stay within the sensitive range of the assay; see below) (46). Samples were then subjected to the competition ELISA (described below) but with an initial antigen-antibody incubation at 4 °C for only 10 min; loading and incubation of these mixtures in 96-well plates was also performed at 4 °C. Plate data were analyzed as described below and compared with the antibody recognition for an equivalent concentration of native (never unfolded) tailspike.

Cell Growth and Tailspike Expression—*S. typhimurium* strain DB7136(su^-) was grown and infected with phage P22 as described previously (47). Briefly, cells were grown at 30 °C to a density of 2×10^8 cells/ml and infected with a P22 variant, $5^-amN114/13^-amH101$ (amber mutations in genes *5* and *13* prevent formation of full-length coat and lysis proteins, respectively, thereby preventing phage head assembly and bacterial cell lysis). As a control for tailspike production, cells were infected with a P22 variant containing the aforementioned amber mutations in genes *5* and *13* as well as an amber mutation in the tailspike, $9^-amN110$, which produces only a small N-terminal fragment

[1] M. Jain, J. King, and P. L. Clark, manuscript in preparation.

[2] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

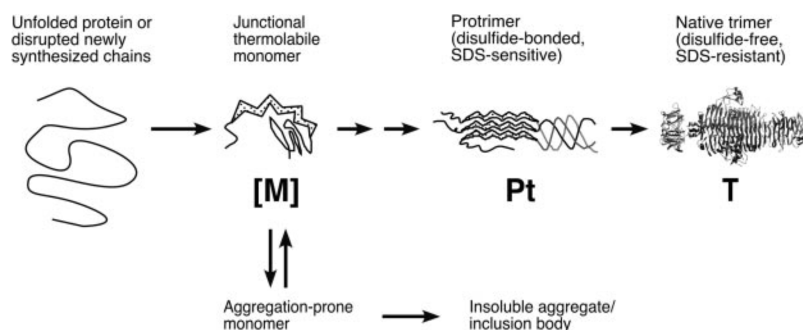

FIG. 2. **The folding pathway of released P22 tailspike chains (63).** Unfolded polypeptide chains organize first into structured monomeric intermediates (M), which can either proceed down the refolding pathway or convert into aggregation-prone monomers. All intermediates can be trapped by cold incubation of *in vitro* refolding mixtures or newly lysed cells from *in vivo* assays. Folding proceeds through a trimeric intermediate termed the protrimer (*Pt*), which migrates as a discrete band on native polyacrylamide gels (64), observed during both *in vivo* folding and *in vitro* refolding studies (34, 53). Formation of the native trimer (*T*) is slow ($t_{1/2}$ ~5 min *in vivo*; >24 h *in vitro* at 10 °C). The native trimer is resistant to unfolding by SDS and therefore appears as a distinct band on SDS-polyacrylamide gels. The folding and aggregation intermediates are sensitive to SDS-unfolding and therefore co-migrate during SDS-PAGE.

of tailspike shown previously to be unrecognized by the anti-tailspike monoclonal antibodies (45). Infected cells were cultured for 90 min at 30 °C. Cultures were chilled quickly on ice, and ice cubes of buffer R (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 150 mM KCl) were added to speed chilling. Cells were collected by centrifugation at 4000 × *g* and resuspended in buffer R containing 80 mM octyl glucopyranoside, to promote cell lysis. Cells were then frozen in liquid nitrogen and stored at −80 °C.

Isolation of Ribosome-Nascent Chain Complexes—All steps were performed on ice or at 4 °C, in order to halt tailspike polypeptide chain maturation (37). Cells were defrosted for 3 min in a cold water bath, until just thawed; this freeze/thaw cycle was sufficient to effect cell lysis. Lysates were spun for 90 s at 14,000 rpm in a microcentrifuge, and the supernatants were loaded onto 16-ml 10–30% linear sucrose gradients with a 1-ml 60% sucrose cushion. The gradients were spun at 150,000 × *g* for 7.5 h and separated into 30 0.58-ml fractions. The absorbance at 260 nm was used to measure the level of nucleic acid in each fraction, and a small portion of some fractions was also subjected to SDS-PAGE, to determine which fractions contained tailspike and ribosomal proteins.

RNase Digestion of Lysates—To determine the role of RNA-containing structures, cell cultures were split in two. One-half was treated with 50 μg/ml RNase and 50 mM EDTA (final conditions) to destabilize and digest the ribosomal particles.

Electron Microscopy of Ribosome Particles—Ribosome-containing fractions were examined using transmission electron microscopy. Fractions were dialyzed against buffer R to lower the sucrose concentration, applied to carbon-coated glow discharged 400 mesh copper grids, negatively stained with 3% uranyl acetate, blotted dry, and examined in the JEOL 1200 EX electron microscope.

Radiolabeling Nascent Polypeptide Chains—To radiolabel the newly synthesized polypeptide chains, cell cultures were pulsed for 3 min or 90 s with 3.0 μCi/ml culture ^{14}C-labeled amino acids (PerkinElmer Life Sciences) after 90 min of P22 infection. Cultures were then chased with a large volume of ice-cold unlabeled casamino acids, while simultaneously chilling the entire culture on ice, occasionally with the addition of buffer R ice cubes. Cultures were then centrifuged, resuspended, and flash-frozen as described above.

Competition ELISA Assay—Mouse anti-tailspike monoclonal antibodies were a generous gift from the lab of Dr. Michel Goldberg (Institut Pasteur); for this study, monoclonal antibodies 175-3 and 51-2 were used. These antibodies have been shown to recognize epitopes on the native tailspike trimer corresponding to a central region of the β-helix domain (α(β-helix)) and a portion of the β-coil domain that includes Glu-405 (α(Glu-405)), respectively (Fig. 1) (45).[1] An ELISA competition assay was used to detect the presence of tailspike epitopes; this was performed as described (49) with the following modifications. Briefly, aliquots of purified tailspike or refolding reaction were mixed with 0.04 μg/ml (final concentration) anti-native tailspike monoclonal antibody diluted in PBS/Tween (PBS buffer with 0.05% (w/v) Tween 20) and incubated overnight at 4 °C. Mixtures were then loaded, in triplicate, into wells of a microtiter plate coated with 1.0 μg/ml tailspike and incubated at room temperature (22 °C). After 30 min, wells were washed three times with PBS/Tween and incubated for 30 min with alkaline phosphatase-labeled goat anti-mouse IgG (Southern Biotechnology Associates). The plate was again washed three times, incubated with *p*-nitrophenyl phosphate solution (2 mg/ml *p*-nitrophenyl phosphate (Sigma) in 1 M ethanolamine, 1 mM $MgSO_4$, pH 9.8), and monitored for absorbance at 405 nm. The amount of absorbance corresponded to the amount of tailspike antibody not bound to components of the gradient fraction. Control samples with no antigen present permitted calculation of the percent of monoclonal antibody recognizing tailspike antigen in the refolding reactions. It should be noted that, under the conditions used here, the assay operated under parameters that perturbed the equilibrium established in the antigen/antibody mixtures, *i.e.* in some situations, more than 10% of the unbound antibody adhered to the well of the microtiter plate (50). Hence, data used to construct the standard curve shown in Fig. 3 cannot be used to construct a Scatchard or Klotz plot for determination of an accurate dissociation constant (50). For percent recognition values >70%, percent recognition is much less sensitive to additional amounts of native tailspike antigen present. Therefore, in Figs. 8 and 9, percent recognition values >90% have been depicted as a flat line at 100% recognition.

RESULTS

Epitope Development during in Vitro Refolding—At 10 °C, tailspike refolds very slowly ($t_{1/2}$ ~12 h) (32), and intermediates populated during refolding can be trapped for many hours by further chilling to 0 °C (37). Our assay for the conformation of these intermediates used the binding of monoclonal antibodies directed against tailspike epitopes (46). Since the refolding tailspike polypeptide chains were transient intermediates trapped by cold incubation, we were concerned about surface denaturation of these intermediates during conventional enzyme-linked immunosorbent assays. In such assays, the proteins are adsorbed to the wells of a plastic microtiter plate. To circumvent this step, we used a competition ELISA, previously developed as a method to detect conformations of folding intermediates (51). This assay involves incubating the antibody and antigen in solution, rather than attaching the antigen to the wells of a plastic plate, thereby avoiding surface binding or denaturation problems. This modification permits assaying for conformation-sensitive epitopes under physiological conditions. Excess, unbound antibody was then detected in a classical ELISA measurement, which permitted calculation of the amount of antibody bound to antigen in solution (51). Binding curves for both monoclonal antibodies to native tailspike are shown in Fig. 3. Under the conditions used for this study, these assays were both sensitive and precise within the dynamic range of 0–70% antibody recognition (see "Experimental Procedures" for details), and the experiments described below were designed to stay within this range.

We used two different monoclonal antibodies, one that recognizes a central portion of the β-helix domain (α(β-helix)) (Fig. 1), and the other that recognizes a portion of the β-helix domain located just after the dorsal fin loop domain, containing residue Glu-405 (α(Glu-405)) (Fig. 1) (45, 46).[1] Antibody binding to *in*

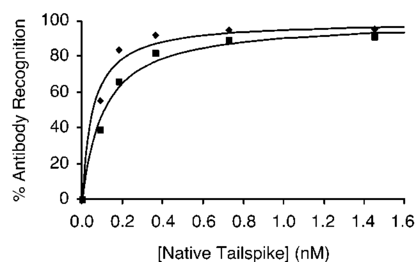

FIG. 3. **Binding curves for antibody recognition of native tailspike, for α(β-helix) (*squares*) and α(Glu-405) (*diamonds*) antibodies.** ELISA conditions were identical to those used for recognition of ribosome-bound tailspike chain epitopes and show a sensitive response to increasing concentrations of native tailspike up to ~70% antibody binding. *Lines* drawn through the points are intended as a guide for the reader.

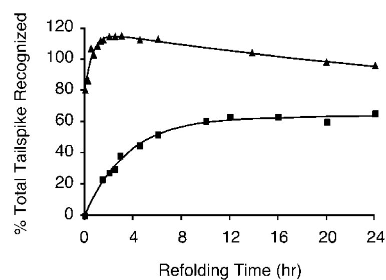

FIG. 4. **Development of tailspike epitopes during *in vitro* refolding, for α(β-helix) (*squares*) and α(Glu-405) (*triangles*) monoclonal antibodies.** Results have been normalized such that the recognition of each antibody for an equivalent concentration of native (never unfolded) tailspike is equal to 100% normalized recognition. Under the refolding conditions used here, ~75% of tailspike polypeptide chains refold to the native trimer structure after 24 h; the remaining chains are non-native (32). The α(β-helix) antibody shows minimal binding to early tailspike refolding intermediates and maximal binding at the final time point; similar results were observed previously for three other α(tailspike) antibodies (46). The α(Glu-405) antibody has higher affinity for tailspike refolding intermediates than for an equivalent concentration of native tailspike; this is confirmed by >75% binding at 24 h, indicating an affinity of this antibody for non-native tailspike conformations. *Curves* represent best fits to single (α(β-helix)) or double (α(Glu-405)) exponential equations; the rates for these folding events will be discussed in detail elsewhere.[1]

vitro tailspike refolding intermediates was performed using the conditions of Goldberg and co-workers (46). As shown in Fig 4, we detected time-dependent changes in monoclonal antibody binding over the course of the refolding reaction (24 h). For the α(β-helix) monoclonal antibody, at the earliest refolding time points the presence of the central β-helix epitope was minimal (Fig. 4). The binding of this antibody to early intermediates of refolding tailspike was similar to that reported by Friguet *et al*. (46) for three other tailspike monoclonal antibodies. Binding of the α(β-helix) antibody increases with time, reaching a maximum at the end of refolding.

For α(Glu-405) antibody recognition of *in vitro* refolding intermediates, we observed quite different results (Fig. 4). At the earliest time points for refolding, there was significant antibody recognition of the Glu-405-containing epitope. Indeed, α(Glu-405) antibody recognition reached a maximum at time points shown previously by Seckler and co-workers (52) to contain a large percentage of protrimer intermediate, instead of native state. Recognition of intermediates exceeded recognition of an equivalent concentration of native, never unfolded tailspike (120 *versus* 100% recognition), indicating that the α(Glu-405) antibody has more affinity for these non-native tailspike chains than for the native structure. In addition, whereas only ~75% of tailspike chains have reached the native state after 24 h of refolding, α(Glu-405) antibody binding was >75% of native tailspike binding at 24 h, also demonstrating affinity of this antibody for the ~25% of tailspike polypeptide chains adopting non-native conformations.

It is possible that binding of a monoclonal antibody could induce or stabilize a conformation of the ensemble of refolding tailspike polypeptide chains and therefore not accurately represent the tailspike conformational distribution at a given time point during refolding. However, the α(β-helix) antibody did not bind to tailspike chains at the earliest refolding time points, indicating that this antibody was incapable of inducing structure via binding for the early tailspike refolding intermediates. The α(Glu-405) antibody, which did bind to early tailspike refolding intermediates, may possibly induce structure formation; nevertheless, we did observe time-dependent changes in antibody binding during refolding, indicating that induced structure binding is not the only source of antibody recognition.

Preparation of Ribosome Structures—At later stages of P22 infection of *Salmonella*, transcription and translation of host cell genes are inhibited. The gene *5* coat protein and the gene *9* tailspike protein are the two major proteins synthesized within infected cells. For these experiments, we used a phage strain carrying a nonsense mutation in gene *5*. This prevents the synthesis of complete coat polypeptide chains and therefore the assembly of high molecular weight, rapidly sedimenting phage capsids. In addition, in these cells, the gene *9* tailspike polypeptide chain is the single major phage-encoded translation product.

To prepare ribosomes from P22-infected *Salmonella* cultures, we took advantage of the natural lytic effects of phage infection, caused by the production of endogenous lysozyme (see "Experimental Procedures" for details). Infected cells were incubated long enough to synthesize late-stage phage proteins. The infected cells were concentrated by centrifugation and then lysed by a single freeze/thaw cycle, allowing lysozyme to escape the membrane barrier and digest bacterial cell walls. The lysate supernatant was fractionated by sedimentation through a linear 10–30% sucrose gradient. All manipulations were performed on ice or at 4 °C, in order to trap and stabilize transient tailspike folding intermediates (34, 37, 53). Sedimentation efficiently separated ribosomal structures from other cellular components. As shown in Fig. 5*a*, absorbance peaks corresponding to nucleic acids were separated from the slowly sedimenting nucleic acids (tRNAs, degraded mRNAs, DNA fragments). The sharpest peaks correspond to 50 S and 30 S ribosomal subunits. The 70 S position, where intact ribosomes would be expected, corresponds to the leading shoulder seen in fractions 16–19. The absorbing species that sedimented faster than ribosomes were presumably polysomes. Digesting the lysates with RNase prior to sedimentation disrupted the larger 260 nm absorbing structures, consistent with their identification as ribosomes and ribosomal subunits (Fig. 5*b*).

We examined more carefully the structures sedimenting through the sucrose gradients by analyzing fractions by SDS-PAGE and detecting host and phage proteins by silver staining. Fig. 5*c* shows the distribution of proteins within several representative fractions as follows: top fractions contained many soluble proteins; a band corresponding to the 60-kDa protein of GroEL appeared at the expected sedimentation coefficient of 21 S; and low molecular weight bands corresponding to the individual ribosomal proteins appeared at 30, 50, and 70 S. A band corresponding to the full-length SDS-resistant tailspike trimer appeared near the top of the gradient, where native tailspike is known to sediment (54). In addition, a species corresponding to the apparent molecular weight of SDS-denatured tailspike chains was found throughout the ribosome-containing fractions, including those expected to contain polyribosomes. The mobility of these chains indicates that they had

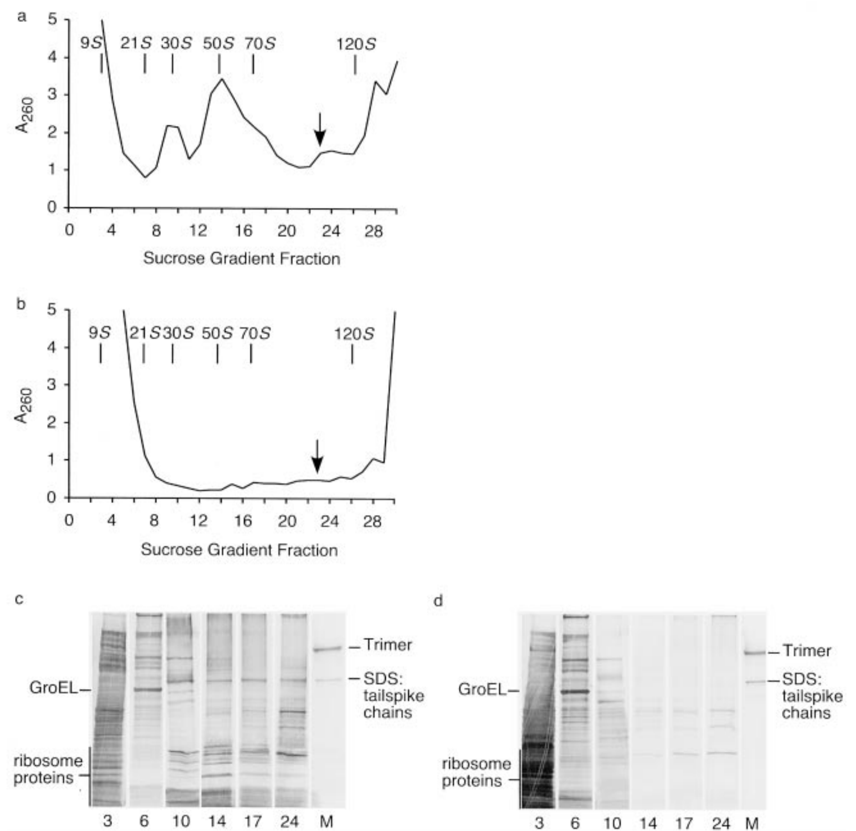

FIG. 5. **Composition of fractions from sucrose gradient sedimentation of cell lysates.** Calculated sedimentation positions for particles of different sizes are shown. *a,* absorbance of fractions at 260 nm; *b,* A_{260} measurements for a lysate digested with RNase prior to sedimentation; *c* and *d,* silver-stained SDS-PAGE lanes for select sucrose gradient fractions, from undigested (*c*) and RNase-digested (*d*) lysates. *Numbers below lanes* indicate corresponding sucrose gradient fraction. *Lane M* corresponds to tailspike standards containing SDS-resistant native trimer and SDS-sensitive (*i.e.* non-native) tailspike conformations. Also indicated are the positions of 60-kDa polypeptides from molecular chaperone GroEL and the positions of many small ribosomal proteins.

not yet reached the SDS-resistant native state at the time of cell lysis.

Fig. 5*d* shows the distribution of proteins through a sucrose gradient from a lysate digested with RNase prior to centrifugation. The small ribosomal proteins were absent from the middle of the gradient, confirming that the ribosomal subunits had been dissociated by the treatment. In addition, the band corresponding to tailspike chains was also absent from these fractions.

To confirm that the central sucrose gradient fractions contain intact ribosome particles, electron micrographs (Fig. 6*a*) were taken of negatively stained aliquots from ribosome fractions expected to contain small polysomal particles (indicated by the *arrow* in Fig. 5*a*). These images show the presence of intact 70 S ribosomes, and also many small polysomes. There are few other species present in the image fields, demonstrating the efficacy of the sucrose gradient preparation procedure. Confirming that RNase digestion destroys intact ribosomal structures, a corresponding fraction from an RNase-digested lysate (see *arrow* in Fig. 5*b*) shows no ribosome-like structures but only an occasional phage particle from the initial infection (Fig. 6*b*).

Localization of Newly Synthesized Tailspike Polypeptides with Ribosomes—As noted above, the ribosome-associated tailspike band did not migrate as native trimer in the unheated SDS gel (Fig. 5*c*), indicating that these tailspike chains had not yet reached the native state at the time of cell lysis. The tailspike polypeptide chains found in the ribosomal fractions could therefore represent one or more non-native states of tailspike as follows: newly synthesized chains associated with the ribosomes, released chains interacting nonspecifically with ribosomes, or other rapidly sedimenting forms of the tailspike, such as intracellular aggregates or inclusion body precursors (38).

To determine whether the tailspike chains present in the ribosome fractions were newly synthesized or arising from aggregates of increasing size, newly synthesized polypeptides were radiolabeled by pulsing cultures for 3 min with ^{14}C-labeled amino acids. Cultures were then chased with cold casamino acids and immediately cooled on ice, lysed, and separated through sucrose gradients; fractions from these gradients were separated on SDS-polyacrylamide gels. Quantitation of the phosphorimage from these gels shows that the tailspike chains in the ribosome fractions were labeled during the pulse (Fig. 7*a*). A significant fraction of the label was in chains

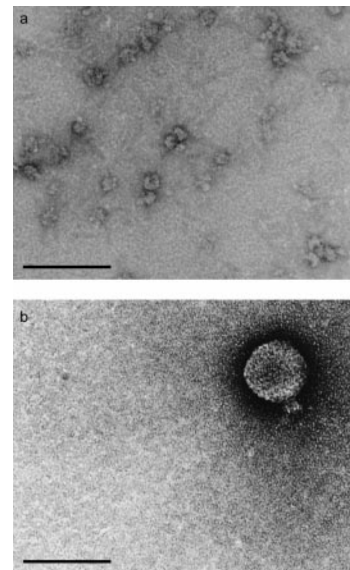

FIG. 6. **Transmission electron micrographs of dialyzed, negatively stained ribosome fractions from sucrose gradients.** *Bar* represents 0.1 μm. *a,* fraction 23 from a ribosome preparation. Intact ribosomes, ribosomal subunits, and small polysomes can be seen. *b,* fraction 23 from the cell lysate digested with RNase prior to sucrose gradient sedimentation. Fields show only background staining and input phage particles.

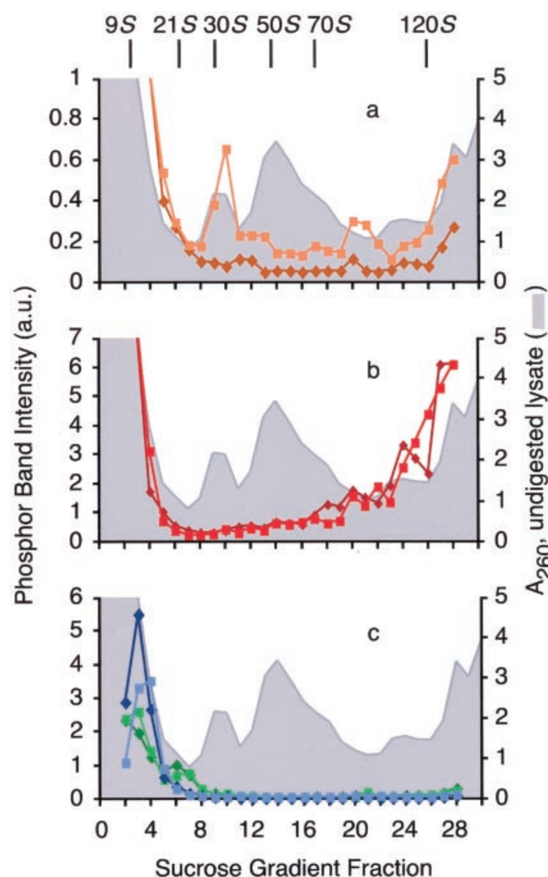

FIG. 7. **Quantitation of ^{14}C incorporation into newly synthesized polypeptide chains, from phosphorimage of SDS-polyacrylamide gels of gradient fractions from pulse-chase lysates.** Gel band quantitation is shown as *colored lines* superimposed on the *gray profile* of 260 nm absorbance for an undigested lysate, as a reference. *a,* amount of labeled SDS-sensitive (*i.e.* non-native) tailspike. *Light orange*, tailspike chains from undigested lysate; *dark orange*, tailspike chains from RNase-digested lysate. *b,* amount of labeled coat protein amber fragment. *Light red*, coat protein amber from undigested lysate; *dark red*, coat protein amber from RNase-digested lysate. *c*, amount of labeled GroEL and native tailspike trimer. *Light green*, GroEL from undigested lysate; *dark green*, GroEL from RNase-digested lysate; *light blue*, tailspike trimer from undigested lysate; *dark blue*, tailspike trimer from RNase-digested lysate.

corresponding to full-length tailspike chains. These presumably correspond to newly synthesized chains, completed or almost completed, but not yet released from the ribosome. Full-length newly synthesized chains were also detected with shorter (90 s) pulses. Partial translation products may be present but would not be expected to form discrete electrophoretic species. This distribution of translated chains *in vivo* differs somewhat from the results found for many proteins labeled using *in vitro* translation systems, where partial translation products represent a much larger fraction of the translated chains, and the rate of translation is much slower (13, 55).

As would be expected for nascent chains, the tailspike polypeptide chains no longer sedimented in the ribosome fractions when the ribosomes were digested with RNase (Fig. 7*a*). Longer chase times (20 min) also resulted in loss of the radiolabeled tailspike chains from the ribosome fractions (data not shown), indicating that the radiolabeled tailspike polypeptide chains were indeed newly synthesized and chased off of the ribosomes. Fig. 7*a* shows that the fractions containing the largest amounts of ribosome-bound tailspike chains were associated with the 30 S and small polysome fractions. The association with the 30 S subunits was unexpected, given that the exit site for newly synthesized polypeptide chains has been localized on the 50 S subunit (21, 26).

It is important to consider whether the ribosome-bound tailspike intermediate is a major populated (possibly obligate) folding intermediate or only a minor intermediate populated in a parallel pathway to native trimer formation. We detected the ribosome-bound chains using routine silver staining (Fig. 5*c*). Quantitation of the ribosome-associated tailspike bands indicates that 10–20% of all intermediate-conformation tailspike chains are located in ribosome-containing fractions, and this localization is RNase-sensitive (Fig. 5*d*). This result suggests that these chains do represent a major component of maturing chains *in vivo*.

Newly synthesized tailspike chains can proceed through two well defined pathways as follows: (*a*) the productive pathway, through the protrimer to the native trimer, or (*b*) the inclusion body pathway, through higher order multimeric species to the aggregated state (38, 56). Under the growth conditions used for these experiments, >90% of newly synthesized tailspike chains mature to native trimer (34, 60). In the experiment shown in Fig. 7*c*, we quantitated the amount of radiolabeled native tailspike trimer present before and after RNase digestion of the cell lysate. Native trimer was well resolved in the native gels from partially folded or assembled intermediates (Fig. 5*c*) (57). The amount of radiolabeled trimer present near the top of the gradient increased upon RNase digestion. Presumably, these additional radiolabeled trimers arose from ribosome-associated tailspike chains released by RNase digestion, which were then able to associate into native trimers. Our results therefore indicate that 1) a significant fraction of newly synthesized tailspike chains populate the ribosome-bound intermediate, and 2) the ribosome-bound polypeptide chains were capable of maturing to the native trimer structure.

The other major phage-encoded structural protein synthesized during late-stage infection is the 22-kDa amber fragment of the coat (gene *5*) protein (58). This amber fragment is unable to assemble into phage capsids and aggregates within cells.[3] The ^{14}C-labeled amber fragment is present throughout the gradient, presumably representing the aggregating species (Fig. 7*b*). Although digestion of the lysate with RNase eliminated the tailspike band present in the ribosome fractions, it had no affect on the coat protein amber fragment band (Fig. 7*b*). Similarly, the distribution of radiolabeled GroEL throughout the gradient was the same for both undigested and RNase-digested lysates (Fig. 7*c*).

Probing the Conformation of Newly Synthesized Tailspike Chains with Antibodies—The newly synthesized tailspike polypeptide chains found associated with the ribosomes were at or near their complete length of 666 amino acids. Since the ribosomal exit tunnel for newly synthesized polypeptides protects only 30–40 residues, most of the tailspike polypeptide chain must be outside the ribosome. To investigate the conformation of the polypeptide chain during the ribosome-associated stage of maturation, we determined the binding of the two monoclonal antibodies described above.

Fig. 8*a* shows the results of assaying gradient fractions for the presence of epitopes recognized by the α(β-helix) antibody. Top fractions showed high levels of antibody binding (>90%), saturating the sensitivity range for this assay (*i.e.* >70% antibody recognition; see "Experimental Procedures" for details). The antigen recognized in these fractions presumably represents the accumulation of native tailspike trimer. In addition, there is a significant level (with respect to the *in vitro* reaction) of tailspike β-helix epitopes detected in the ribosome-contain-

[3] C. A. Haase-Pettingell and J. King, unpublished results.

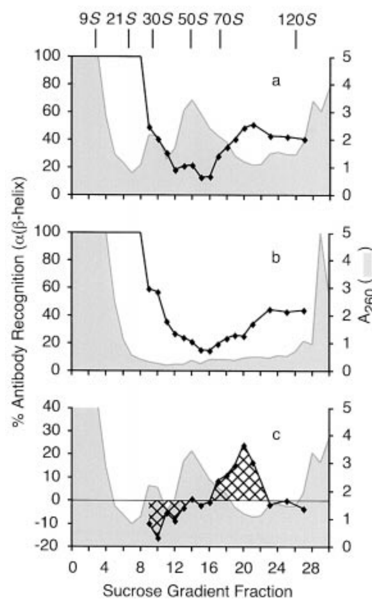

FIG. 8. **Competition ELISA binding of the α(β-helix) antibody to fractions from sucrose gradients, showing (*black line*) the level of antibody recognition, superimposed on (*gray profile*) the 260 nm absorbance of fractions.** For high levels (>70%) of antibody binding, the ELISA was less sensitive to increasing amounts of native tailspike present (Fig. 3); therefore, antibody recognition values >90% are depicted as a flat line at 100% recognition. Antibody recognition is shown for gradients from an undigested lysate (*a*), an RNase-digested lysate (*b*), and the difference (*c*) (*a* and *b*).

ing region of the gradient, including fractions corresponding to small polyribosomes.

ELISA competition assays with the α(β-helix) antibody were also performed on samples that were digested with RNase (Fig. 8*b*). As expected, the large amount of free tailspike antigen at the top of the gradient remained. The antigen levels in the polysome regions were reduced, as expected for nascent chains, but not eliminated. Subtracting the RNase-digested background binding from the antibody binding to the undigested lysate (Fig. 8*c*) yielded a positive peak centered over the 70 S small polysome position and a negative peak centered over the 30 S position. The positive peak confirms the presence of RNase-sensitive antibody binding in these fractions.

The development of the conformational epitope *in vivo* is in direct contrast to the *in vitro* results shown in Fig. 3, where we observed negligible epitope development immediately after dilution of tailspike chains from denaturant (Fig. 4). We do not yet know if these tailspike chains represent a single ribosome-bound conformation or an ensemble of intermediate states associated with the ribosome. What is clear, however, is that these newly synthesized, ribosome-bound tailspike chains adopt conformations not significantly populated by the early *in vitro* refolding intermediates.

The negative peak, although small, appeared consistently through multiple repetitions of the assay and corresponds to a significant difference in antibody recognition for 3–4 separate fractions. The negative peak indicates that there was increased antibody binding to 30 S fractions for the RNase-digested lysate *versus* the untreated lysate. The increase in antibody binding did not arise from RNase digestion of cellular components, as a control infection with a P22 variant containing a nonsense mutant in the tailspike gene *9* (see "Experimental Procedures" for details) exhibited no increase in antibody binding (data not shown). From the absorbance at 260 nm, SDS-polyacrylamide gel analysis, and pulse-chase experiments, it is clear that there were fewer 30 S subunits and newly synthesized tailspike chains present in these fractions after RNase

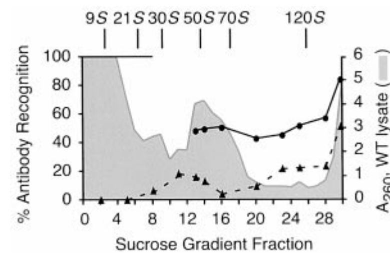

FIG. 9. **Competition ELISA binding of the α(Glu-405) antibody to sucrose gradient fractions.** Absorbance at 260 nm is shown as a *gray profile*. As for Fig. 8, recognition values >90% are depicted as a flat line at 100% recognition. The percentage of antibody recognition for lysates producing wild type tailspike (*solid line*) and the short tailspike amber fragment (*dashed line*) is shown.

digestion. Since these particles were not further dissociated, they may represent a class of 30 S-tailspike polypeptide complexes that were refractory to the RNase digestion. The increase in antigenicity suggests that the small population of 30 S subunits present after RNase digestion contained structured tailspike epitopes that were better recognized by the α(β-helix) monoclonal antibody. Our results are unable to distinguish whether the additional antibody binding was due to increased affinity between the antibody and antigen (for example, due to a rearrangement of the epitope to a conformation with more native-like structure) or increased accessibility of the antibody to the antigen epitope due to the RNase digestion procedure.

The background binding seen in the middle to lower gradient fractions for the RNase-digested lysate (Fig. 8*b*) may represent nonspecific interactions with host cell components or with other forms of the tailspike chains. This region of the gradient contains the following two classes of tailspike chains that would not be sensitive to the RNase digestion: (*a*) input phage particles from the initial infection whose tailspike chains are assembled onto the phage particle, a form which is highly antigenic (59), and (*b*) aggregated tailspike chains that are intermediates to the inclusion body state (56). In fact, the α(β-helix) antibody used in this assay was not reactive with multimeric tailspike aggregation intermediates (60). In addition, we observed intact phage particles in electron micrographs of RNase-digested gradient fractions (Fig. 6), suggesting residual phage particles from the initial infection are the cause of the background binding.

The conformations of the tailspike nascent chains were also probed with the α(Glu-405) monoclonal antibody (Fig. 1). Binding of this antibody to lysate gradient fractions yielded results similar to the binding of the α(β-helix) antibody. Antibody binding corresponded to the distribution of native trimer in top gradient fractions (again, overloading the sensitivity range of the binding assay) and fractions containing 70 S ribosomal and polysomal particles (Fig. 9). As the α(Glu-405) antibody also recognized the earliest *in vitro* refolding intermediates (Fig. 4), it is perhaps not surprising that newly synthesized, ribosome-associated tailspike chains are also recognized.

As a control for antibody binding to components other than newly synthesized tailspike chains, we also infected cells with a phage containing a nonsense mutant in the tailspike gene *9* (see "Experimental Procedures" for details). The lysate from this infection was fractionated through a sucrose gradient and assayed for α(Glu-405) antibody binding. There was a significant reduction in the level of antibody binding throughout the gradient for this lysate (Fig. 9), as would be expected for an antibody detecting only tailspike folding intermediates and native trimer in the wild type lysate. Background binding presumably represents binding to residual input phage and

nonspecific association with other cellular components.

The specific tailspike structural epitopes detected on the newly synthesized polypeptides represent conformations developed while the chains were still associated with ribosome structures. By virtue of their ribosome-bound state, these polypeptide chains by definition represent an earlier state of the polypeptide chain maturation than for released chains isolated from the cytoplasm. These conformations were clearly different than those for the early intermediates formed during *in vitro* refolding.

DISCUSSION

The tailspike polypeptide chains synthesized by phage-infected *Salmonella* cells in these experiments were translated, folded, and assembled at 30 °C. Under these conditions, there was no significant loss to aggregation (Fig. 5*d*, and data not shown), and the majority of the polypeptide chains reached the native state. However, the majority of tailspike polypeptides refolding from denaturant at 30 °C are unable to reach the native state and instead formed inactive high molecular weight aggregates (56), even though these chains refold efficiently *in vitro* at lower temperatures (4). Despite systematic searches, no clear evidence has been found for a role for prokaryotic molecular chaperones in tailspike folding *in vivo* (43, 44). An alternative hypothesis is that the translational machinery itself participates in the early stages of chain folding.

The ribosomes from P22-infected *Salmonella* were cold-trapped and recovered in association with newly synthesized tailspike polypeptide chains. Chilling *in vivo* folding or *in vitro* refolding tailspike has been shown previously to trap the transient conformations of folding intermediates (32, 34, 37), due to the unusually strong temperature dependence of tailspike folding (39). Surprisingly, the ribosome-associated tailspike polypeptides recovered from the pulse-labeling experiments were full-length, within the accuracy of the SDS gel fractionation (Fig. 5*c*). This indicates that while translation of the tailspike polypeptide may proceed quickly (~20 amino acids/s for bacterial cells), the tailspike polypeptide chain then spends the first several minutes of its existence in close association with the ribosome. These tailspike chains were not "dead ends," as pulse-chase experiments showed maturation of the labeled chains to native tailspike (data not shown), and separation from the ribosome after RNase digestion resulted in increased yields of native trimer (Fig. 7*c*). The experiments reported here do not distinguish between chains still tethered due to slow termination or terminated chains still bound to the ribosome. In either case, we would expect more than 600 amino acids of the newly synthesized chains to have emerged from the exit site but remain at the ribosome surface.

Conformations of Nascent Tailspike Polypeptide Chains— The newly synthesized, full-length tailspike polypeptide chains isolated on *Salmonella* ribosomes provided an opportunity to study the conformation of a transiently cold-trapped nascent polypeptide chain from a physiologically relevant translation system. Two monoclonal antibodies to tailspike, each of which recognizes a distinct structural epitope in the parallel β-helix of the native protein,[1] specifically recognized the ensemble of ribosome-associated tailspike polypeptide chains (Figs. 8 and 9). This indicates that the epitopes recognized by these antibodies were already present on the newly synthesized tailspike chains. Yet during *in vitro* refolding experiments, the α(β-helix) epitope was not formed at early refolding times. Thus the ribosome-bound tailspike chains appear to adopt a conformation with structure in the central region β-helix domain, not detected for early *in vitro* refolding species.

The simplest explanation for the more structured conformations of newly synthesized ribosome-bound tailspike chains (*versus* refolding intermediates *in vitro*) is that the nascent tailspike polypeptide chains can achieve a more structured conformation through direct interactions with sites on the ribosome and/or because of the vectorial nature of polypeptide synthesis *in vivo*. We are currently performing experiments to fully characterize the tailspike-ribosome interaction and to determine its effect on tailspike chain folding.

What fraction of the newly synthesized, ribosome-bound tailspike polypeptide chains adopt the recognizable β-helix epitope? If one assumes that the affinity of the α(β-helix) antibody for the ribosome-bound, transient intermediate is equal to the affinity for native tailspike, then the concentration of epitope-containing chains in these fractions was ~10 pM (Fig. 3). This concentration of tailspike chains was greater than the concentration of radiolabeled, ribosome-bound tailspike chains observed in the pulse-labeling experiments (~1 pM; Fig. 7; comparison to radiolabeled tailspike standards). An ~10-fold higher concentration of epitope *versus* radiolabeled chains indicates that, indeed, there were significant differences between the affinity of the α(β-helix) antibody for the ribosome-bound, transient conformation, and native tailspike. Without an accurate measure of the affinity of this antibody for the intermediate conformation, we are currently unable to determine accurately the fraction of radiolabeled chains that adopt the β-helix epitope. Adoption of the β-helix epitope conformation prior to ribosome release therefore may not be an obligate intermediate in the tailspike folding pathway *in vivo*; however, these results are consistent with a model where the majority of the radiolabeled tailspike chains adopt the β-helix epitope prior to dissociation from the ribosome.

In addition, it appears that the ribosome-associated conformation persists in tailspike chains isolated in association with the 30 S ribosomal subunit (Fig. 8), suggesting that an area of the ribosome possibly influencing nascent polypeptide structure could be localized at least in part on the 30 S subunit (Fig. 10). The persistence of detectable tailspike conformations on 30 S subunits provides evidence that the observed tailspike conformations do not arise due to ribosome-bound chaperone interactions, because the ribosome-associated chaperone trigger factor has been shown to interact only with the 50 S subunit (61).

The increase in tailspike monoclonal antibody binding to the RNase-digested 30 S fractions occurred despite the destruction of >90% of the 30 S particles by RNase and the corresponding loss of the majority of associated, newly synthesized tailspike chains (Figs. 5 and 7*a*). Therefore, the increase in observed antigenicity arose from a very small fraction of remaining tailspike chains, suggesting that digestion with RNase may reveal a class of 30 S-tailspike complexes that contains more reactive tailspike antibody epitopes. Possibly, digestion with RNase alters the structure of some tailspike-containing 30 S subunits in a way that also alters the conformation of the tailspike chain, increases its accessibility to the antibody, or both. The altered tailspike conformation may then protect the remaining 30 S subunits from further RNase digestion.

These findings have interesting implications for the folding of tailspike chains *in vivo*. Recognition of the newly synthesized tailspike chains by α(β-helix) antibodies suggests that the β-helix may form early during *in vivo* folding. This correlates well with results from *in vitro* refolding experiments, which indicate organization of the β-helix domain occurs early during refolding (32), and in fact, a sequence corresponding to the β-helix domain alone can adopt a β-helix structure *in vitro* (52). Formation of rungs of the β-helix domain while the polypeptide chain is still attached to the ribosome could provide crucial chain organization that predisposes the released chains for

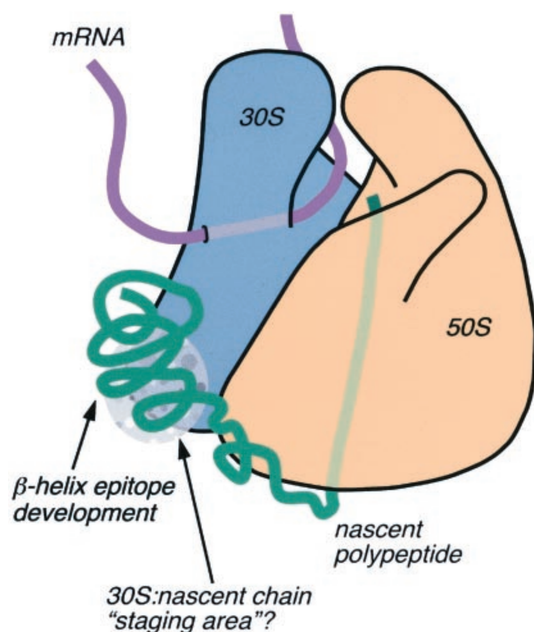

FIG. 10. **Model for the maturation of newly synthesized tailspike polypeptide chains on intact ribosomes.** Antibody binding recognition of intact ribosomal complexes suggests that portions of the nascent tailspike polypeptide chain can adopt β-helix conformations. The β-helix epitopes were also found in 30 S fractions containing tailspike chains, suggesting there may be significant interactions between the newly synthesized polypeptide and the 30 S subunit. In this study, we did not determine whether the C terminus of the tailspike polypeptide has been terminated or is still attached to the aminoacyltransferase center.

folding to the native structure, instead of aggregating. Association of newly synthesized tailspike chains with a specific site or sites on the ribosome, possibly located on the 30 S subunit, could prime the polypeptide chains for further steps of folding and assembly, in particular the formation of a conformation capable of efficient trimer formation and C-terminal strand interdigitation (Fig. 1). Certainly the tailspike folding intermediates trapped on the ribosome (the earliest *in vivo* folding intermediates trapped for tailspike) appear to have additional structural development *versus* that seen for the earliest *in vitro* refolding intermediates (Fig. 4).

Roles of the Ribosome in Nascent Chain Maturation—The presence of full-length, newly synthesized polypeptide chains on the ribosome, even after cell lysis and sucrose gradient centrifugation, suggests that these chains were relatively tightly bound by the ribosome. Such associations have not been described previously. Our experiments suggest that this association is a physiological complex, as these chains do eventually mature to native tailspike trimers. We are currently performing experiments to determine if the ribosome itself contains sites that recognize and bind partially folded polypeptide chains. Such interactions with the ribosome may protect the newly synthesized polypeptide from degradation or unproductive interactions, without relying on additional host cell systems, such as the chaperone networks (62).

As noted above, when ribosomal particles were disrupted by normal lysate manipulations or RNase digestion, significant amounts of full-length, newly synthesized tailspike polypeptide chain remained preferentially associated with remaining 30 S particles (Fig. 8*c*). This was surprising, given the accepted view that newly synthesized polypeptide chains emerge from the ribosome via an exit site on the back side of the 50 S subunit. One interpretation of this result is that upon dissociation of the active ribosomes into 30 S and 50 S subunits, a population of nascent tailspike polypeptides bound to 70 S ribosomes remain preferentially associated with the 30 S subunits, presumably through specific interactions with the 30 S subunit or some intermediary (Fig. 10). In the intact ribosome, wrapping around to reach the 30 S subunit clearly requires extension of the polypeptide chain beyond the ~30 amino acids necessary to reach the outer surface of the ribosome. Since the isolated nascent chains are full-length (more than 200 nm if extended), the surface of the 30 S subunit would be readily accessible to the nascent chain.

The adoption of structured conformations in a nascent polypeptide chain reported here might play a general role in successful protein folding *in vivo*. While tailspike polypeptide chains dwell in association with the ribosome for an unusually long time (possibly for reasons due to viral infection; see above), moderate slowing of translation, termination, and/or polypeptide chain release may permit the development of conformational preferences for a range of newly synthesized chains. For the large fraction of polypeptide chains that have no interaction with molecular chaperones during chain maturation and folding, interactions with specific sites on the ribosome and/or within the nascent chain itself could provide early folding assistance. Early ribosome-bound folding may be particularly useful during the folding of elongated protein structures that need protection from the cellular environment not provided by the nascent chain folding back on itself. Finally, formation of a partially native nascent polypeptide chain could be of particular importance for the successful folding and assembly of multimeric protein complexes, encouraging the development of structured, assembly-competent monomer chain conformations prior to nascent chain release and association.

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