The use of sarkosyl in generating soluble protein after bacterial expression

(inclusion bodies/bacterial outer membrane/actin/myosin)

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ABSTRACT Actin, like many other proteins, is highly insoluble after expression in *Escherichia coli*. In order to understand the origin of insoluble aggregates, we asked whether morphological inclusions were always correlated with insolubility. The strain expressing actin was compared to one that expresses part of the myosin tail; the latter strain yields soluble protein after various cell lysis or disruption procedures. Morphological inclusions were observed in both strains, indicating there is no obligate relationship between solubility and inclusions. Studies presented here suggest that extreme insolubility results from coaggregation of the actin with bacterial outer membrane components upon bacterial lysis. The properties of the outer membrane have been exploited in the development of nondenaturing procedures that yield soluble actin. One procedure involves the disruption of coaggregates with sarkosyl detergent (*N*-laurylsarcosine); another prevents the formation of coaggregates by lysing in the presence of sarkosyl. These methods may be useful for other proteins that become insoluble after bacterial expression.

Bacterial expression has greatly expanded the biochemical analysis of many proteins (for reviews, see refs. 1 and 2), since any mutation can be introduced into the amino acid sequence followed by the production of mutant protein in large quantities. However, the study of some proteins has been hampered by proteolysis (3, 4) or, paradoxically, by low levels of synthesis of the recombinant product (4–6). The greatest drawback of the bacterial system has been the accumulation of many expressed proteins in a highly insoluble form (for review, see ref. 7).

It has been postulated that the highly insoluble aggregates observed after lysis are composed of morphological inclusions seen in the intact bacterium and that these inclusions may be isolated and manipulated as a distinct subcellular fraction (7). Morphological inclusions have therefore been cited as evidence that highly insoluble aggregates are formed soon after synthesis of the protein (8, 9). Although immunoelectron microscopy has confirmed the presence of the expressed protein within inclusions (10, 11), the correlation of insolubility with inclusions has not been analyzed rigorously. Given the importance of this hypothesis for devising a strategy that would yield soluble protein, we compared bacteria expressing an insoluble protein (actin) to bacteria expressing a soluble protein (part of the myosin tail) for the presence of morphological inclusions. Insolubility was not correlated with the presence of inclusions. However, biochemical evidence indicated that the highly insoluble behavior of the recombinant actin was due to coaggregation with bacterial outer membrane components, an interaction that must occur upon lysis. This allowed the rational design of two

methods yielding soluble nondenatured actin, both involving the use of sarkosyl (*N*-laurylsarcosine) detergent.

MATERIALS AND METHODS

Growth of Cultures, French Press Lysis, and Sarkosyl Lysis. These were performed as described (12). The sarkosyl extraction of aggregates obtained after French press disruption is also described in ref. 12. The buffer for disruption of bacteria expressing the myosin tail fragment is the same as described (12), except that sucrose was omitted. After disruption with the French press, the sample was centrifuged at $15,000 \times g$ for 1 hr (Beckman JA-20 rotor). The supernatant was then centrifuged at $100,000 \times g$ for 1 hr (Ti50 rotor).

SDS/PAGE and Immunoblots. The preparation of samples for SDS/PAGE and the conditions for SDS/PAGE and actin immunoblots are as described (13). Western transfer of gels containing the myosin tail fragment was performed as described (14); these blots were probed with monoclonal antibody 4A1519 (kindly provided by Helen Blau, Stanford University, Stanford, CA) and visualized using goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad).

Electron Microscopy. All strains were grown as described (12), and at an absorbance at 550 nm of ≈ 0.95 the cultures were plunged into an ice-water bath for 20 min. Glutaraldehyde was added to a final concentration of 2.5%, and the cultures were incubated on ice overnight. The bacteria were postfixated in 1% OsO_4 , stained with 1% uranyl acetate, and preembedded in gelatin. The cell pellet was dehydrated in a graded series of ethanol and embedded in LX-112 (Ladd Research Industries, Burlington, VT); thin sections were poststained with uranyl acetate and lead citrate. Grids were examined on a JEOL 1200EX transmission electron microscope.

RESULTS

Bacterial Strains Expressing Soluble and Insoluble Protein Have Morphological Inclusions. Goldberg and coworkers (15) performed a detailed analysis of solubility and protein inclusions in *Escherichia coli*. They showed that when bacteria were grown in the presence of amino acid analogs, normally soluble proteins such as β -galactosidase could be pelleted at $10,000 \times g$. A protein was considered soluble if it remained in the supernatant after centrifugation at $100,000 \times g$. They also found that morphological inclusions were correlated with the accumulation of insoluble protein. The designation of expressed proteins as insoluble has been based upon the ability to be pelleted at any of several speeds ranging from $500 \times g$ to $40,000 \times g$ (3, 7, 16). In the current study, two strains expressing eukaryotic contractile proteins (actin and a fragment of myosin) were examined for the presence of morpho-

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logical inclusions. One strain expresses soluble protein (as defined above); the other expresses insoluble protein.

The bacterial strain expressing actin accumulates a full-length (42 kDa) wild-type and a truncated (29 kDa) species. After either French press or sonication disruption, between 80% and 90% of each form of actin can be pelleted by centrifugation equivalent to $15,000 \times g$ for 1 hr (13). Actin in the supernatant was pelleted at higher speeds ($150,000 \times g$ for 1 hr; data not shown). When a strain expressing part of a muscle myosin tail is disrupted in the French press, $\approx 90\%$ is soluble after a $15,000 \times g$ centrifugation for 1 hr, and almost all of the soluble fraction remains soluble after subsequent centrifugation for 1 hr at $100,000 \times g$ (Fig. 1). It was expected that a small fraction of the myosin tail fragment would pellet at low speed, since it has been observed that this protein interacts strongly with nucleic acid (R.S., unpublished observations) and some high molecular weight nucleic acid pellets at low speed (ref. 8; L.L., unpublished observations).

Intact bacteria from both strains, as well as hosts not expressing recombinant protein, were examined for the presence of inclusions. As expected, inclusions were observed in the strain expressing actin (75% of observed longitudinal sections), but they were also observed in the one expressing myosin (100% of observed longitudinal sections); no inclusions were seen in the control strains (Fig. 2). Inclusions from the two expression strains were estimated to be approximately the same size.

The morphology of the inclusions present in the two strains and their cytoplasmic polarity appeared to differ (Fig. 2). The significance of these differences is unclear. The inclusions in the myosin strain appeared to be randomly placed along the length of the cell, whereas the inclusions in the actin strain were always localized at a polar end of the cell ($n = 40$). An early study on the inclusions caused by growth in the presence of different amino acid analogs also indicated differing inclusion morphologies (17). It is unclear whether the inclusions accumulated during the expression of other proteins exhibit different morphologies, since the fixation procedures used were different (18), but some of these transformants have been reported to contain polar inclusions (19).

FIG. 1. A myosin tail fragment expressed in *E. coli* is soluble after lysis. Bacteria were lysed in the French press, and the lysate was centrifuged at low speed. The resulting supernatant was then subjected to a high speed centrifugation. (A) Coomassie-stained gel. (B) Immunoblot of the samples in A. Lane 1, total lysate; lane 2, low speed supernatant; lane 3, low speed pellet; lane 4, high speed supernatant; lane 5, high speed pellet. The arrows indicate the position of the myosin tail fragment, light meromyosin construct II. Lanes 2 and 3 have equal loadings as do lanes 4 and 5.

Actin Coaggregates with Bacterial Outer Membrane Components. Some of the proteins that are insoluble after bacterial expression are soluble and cytosolic in eukaryotes. It has therefore been postulated that conditions are different in the prokaryotic cytosol (8, 9, 19, 20). However, not all eukaryotic proteins overexpressed in *E. coli* are insoluble (refs. 7, 21, and 22; this report), and some normal prokaryotic proteins are insoluble when overexpressed (23). In one case, the same protein can be either predominantly soluble or insoluble, depending upon the vector, host strain, or method of lysis (24). Several hypotheses have been proposed to explain insolubility, including the accumulation of partially folded forms that will aggregate by hydrophobic interactions (9), the induction of the heat shock response (25), which may create an aberrant environment for protein folding, and, in the case of the cytosolic expression of membrane or secreted proteins, the absence of a maturation pathway (26).

Actin is a soluble, cytosolic protein in eukaryotes. The aggregated actin obtained after bacterial expression could only be solubilized by 8 M urea, 6 M guanidine hydrochloride, or SDS; various salts or mild chaotropic agents were ineffective (data not shown; see also ref. 27 for similar extractions on another insoluble expressed protein). An examination of the composition and behavior of actin aggregates suggested another mechanism for their extreme insolubility in addition to the ones listed above.

Wild-type *E. coli* yielded a highly insoluble low speed pellet fraction whose protein composition was identical to the low speed pellet fraction obtained from bacteria expressing actin, with the exception of the actin peptides (Fig. 3). Several lines of evidence indicated that the bacterial proteins present in both types of aggregate were outer membrane proteins. (i) Lysis and low speed centrifugation is one method for the isolation of an outer membrane fraction (28). (ii) The nonactin proteins present in pellets from the control and actin-producing strains have molecular weights corresponding to outer membrane proteins (29). (iii) The solubility characteristics of aggregated actin resembled those of an outer membrane fraction with respect to salts (30), chaotropic agents (31), and, in particular, the effects of divalent cations upon detergent solubilization (see below). (iv) Some of the outer membrane proteins undergo temperature-dependent shifts in migration during SDS/PAGE (29, 32, 33) and this property confirmed their presence in the low speed pellet fractions (Fig. 3). Protein from the control strain was heated to 70°C (Fig. 3, lane 3) or 100°C (Fig. 3, lane 4) in the presence of SDS. The band labeled with a double star in lane 3 contains two comigrating outer membrane proteins, OMP A and OMP C, which shift to the positions labeled with single stars in lane 4 (refs. 29 and 32; following the nomenclature in ref. 28). A similar mobility shift is observed with protein from the actin-producing strain (Fig. 3, lanes 1 and 2). We conclude that the insoluble aggregates consist of actin and bacterial outer membrane components.

It has been reported that in the absence of divalent cations sarkosyl can solubilize all components of bacteria except the outer membrane (34). We therefore predicted that sarkosyl would differentially solubilize the proteins in the actin aggregates. A low speed pellet obtained after French press disruption (Fig. 4, lane 2) was extracted with 1.5% sarkosyl in the presence of 1 mM EDTA and then centrifuged. All of the 29- and 42-kDa actin was in the extraction supernatant (Fig. 4, lane 3), whereas $>90\%$ of the outer membrane proteins were in the extraction pellet (Fig. 4, lane 4). Once the outer membrane components were removed, sarkosyl was not necessary for maintaining most of the actin in a soluble form (S.F., unpublished observations).

Evidence for a direct interaction between actin and outer membrane components was provided by the effects of divalent cations upon the detergent extraction of coaggregates.

FIG. 2. Bacterial strains expressing actin and a myosin tail fragment contain inclusions. Bacteria were fixed in 2.5% glutaraldehyde and examined in the electron microscope. All fields are at the same magnification. (Bar = 0.1 μ m.) Some of the inclusions are marked by arrows. (A and B) Bacteria synthesizing actin. (C) Same strain in A and B, except that the expression plasmid contains the actin coding sequence in a backward orientation. (D and E) Bacteria synthesizing the myosin tail fragment. (F) Same strain as in D and E, except that it did not contain the plasmid.

Although only the behavior of the 29-kDa actin species was evaluated in the experiments discussed below, related experiments indicated that the two forms of actin cofractionated. Divalent cations, which greatly potentiate the aggregation properties of outer membrane components (28, 34–37), prevented the extraction of either actin peptides or outer membrane components into separate fractions. In the presence of divalent cations the selectivity of sarkosyl extraction was decreased, such that some of the outer membrane protein became solubilized along with the actin (data not shown). Nonionic detergents could not be used to separate effectively the coaggregated species into different fractions even in the presence of EDTA. SDS in combination with EDTA solubilized actin and membrane proteins; the solubilization of both species was completely inhibited by divalent cations (data not shown; also ref. 37). The advantage of a

sarkosyl/EDTA treatment was that the actin could be fully separated from the outer membrane components.

Lysing Bacteria Directly into Sarkosyl Results in Soluble Actin. It seemed likely that the highly insoluble behavior of actin synthesized in bacteria resulted from its association with outer membrane components and not from its intrinsic properties. If this hypothesis is correct, such an association would occur during or after lysis. We therefore predicted that resuspension of the bacteria into a large volume of buffer and subsequent lysis with sarkosyl detergent would prevent coaggregation. Fig. 5 shows that large amounts of both actin species were soluble after lysis in 0.2% sarkosyl. The lysate was centrifuged at low speed to pellet the bacterial outer membrane fraction: the supernatant (Fig. 5, lane 2) retained 59% of the 29-kDa actin and almost all of the 42-kDa actin. Octyl glucoside was then added to sequester sarkosyl in

FIG. 3. Actin forms a coaggregate with bacterial outer membrane proteins. Bacteria were grown in LB medium and disrupted in the French press. The lysates were centrifuged at low speed, and the resulting pellets were solubilized in SDS at either 70°C or 100°C and subjected to SDS/PAGE; protein was stained with Coomassie blue. Lane 1, pellet from the strain synthesizing actin, heated to 70°C; lane 2, same pellet as in lane 1, heated to 100°C; lane 3, pellet from the control strain, heated to 70°C; lane 4, same pellet as in lane 3, heated to 100°C. The position at 70°C of two outer membrane proteins is indicated with a double star, whereas their positions at 100°C are indicated by single stars. The relative loadings of lanes 1 and 2 are not equal.

nonionic detergent micelles, divalent cations were added, and solubility was measured by performing an extended high speed centrifugation. Divalent cations are necessary for maintaining actin in its native form (38). Of the actin present in the low speed supernatant, 50% of the 29-kDa species and 60–75% of the 42-kDa species remained soluble (Fig. 5, lane 4). The sedimentation of 29-kDa actin at high speeds was inhibited by 0.8 M NaCl (data not shown) and is consistent with a process of concentration-dependent aggregation (13).

Soluble 42-kDa actin obtained by lysis in sarkosyl detergent retains several native functions: reversible polymerization, ATP-sensitive binding of myosin subfragment 1, and the ability to bind DNase I (13). When truncated actin was obtained either by sarkosyl lysis or by the treatment of actin aggregates with sarkosyl, it also exhibited the ability to bind DNase I. The ability to obtain functional actin argues against the presence of denatured protein in morphological inclu-

FIG. 4. Actin can be differentially extracted from coaggregates with sarkosyl detergent. Bacteria were grown in M10+ medium (12) and disrupted in the French press. The low speed pellet was extracted with sarkosyl/EDTA. (A) Coomassie-stained gel. (B) Immunoblot of the samples in A using an affinity-purified *Dictyostelium discoideum* actin antibody (13). Lanes 1, total lysate; lanes 2, low speed pellet; lanes 3, sarkosyl extraction supernatant; lanes 4, sarkosyl extraction pellet.

FIG. 5. Lysis directly into sarkosyl yields soluble actin. (A) Coomassie-stained gel. (B) Immunoblot of the samples in A. Bacteria were grown in M10+ medium (12) and lysis was initiated by the addition of sarkosyl. The lysate was centrifuged at low speed and the supernatant was collected. Octyl glucoside was added to the supernatant, followed by magnesium, and it was centrifuged at a speed sufficient to pellet all particles >30 S. Lanes 1, total lysate; lanes 2, low speed supernatant; lanes 3, low speed pellet; lanes 4, postribosomal supernatant; lanes 5, ribosome pellet. Each lane was loaded so as to have roughly equal amounts of 29-kDa actin, as determined by Western analysis.

sions, since the denaturation of actin is considered to be irreversible (38, 39).

DISCUSSION

The coaggregation of actin with bacterial outer membrane components may occur by a mechanism similar to the one proposed by Mitraki and King (9) for the self-aggregation of folding intermediates. Whether studied separately or as a complex, the lipopolysaccharide and protein of the outer membrane undergo extensive hydrophobic interactions and have very strong tendencies to aggregate (40). During the process of lysis, these components will fragment and display hydrophobic and anionic surfaces, acting as a trap for exposed hydrophobic or cationic surfaces on the expressed protein (see ref. 41, pp. 147–148). Once an interaction with outer membrane components has occurred, the behavior of the aggregate would reflect the highly insoluble properties characteristic of the outer membrane. This appears to be the situation with actin. Sarkosyl either can disrupt the interactions maintaining actin in coaggregates, when these have been allowed to form, or can prevent the interactions from occurring if present during lysis. Low levels of divalent cation inhibit the effect of sarkosyl in both cases, consistent with a direct interaction between actin and the outer membrane components. Other groups have noted the presence of outer membrane components in highly insoluble aggregates of overexpressed protein [ref. 42; John Tan and James Spudich (Stanford University School of Medicine), Susan Gilbert (Pennsylvania State University), and Arthur Rovner (Albert Einstein College of Medicine), personal communications], indicating that the phenomenon we describe may not be restricted to actin.

Several of the hypotheses for insolubility are not mutually exclusive and could actually occur in parallel—namely, the self-aggregation of folding intermediates, coaggregation with outer membrane components, and partially folded or degraded peptides produced by a heat shock response. Our biochemical and morphological evidence makes it difficult to extrapolate a mechanism of insolubility from the presence of morphological inclusions. In many cases, when an inclusion body fraction has been isolated from lysed bacteria, the presence of either lipopolysaccharide or outer membrane protein was not addressed. Three other proteins that form

highly insoluble aggregates after bacterial expression can be solubilized using the procedures we describe (*Dictyostelium* myosin light chain kinase, the head domain of *Drosophila* kinesin heavy chain, and the head domain of rat cardiac myosin), and the kinase has clearly been shown to maintain functional activity after such treatment [John Tan and James Spudich (Stanford University School of Medicine), Susan Gilbert (Pennsylvania State University), and Arthur Rovner (Albert Einstein College of Medicine), personal communications].

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