High Level Bacterial Expression of Uteroglobin, a Dimeric Eukaryotic Protein with Two Interchain Disulfide Bridges, in Its Natural Quaternary Structure*

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Bacterial expression of eukaryotic proteins is a tool of ever-increasing importance in biochemistry and molecular biology. However, the majority of the recombinant eukaryotic proteins that have been expressed in bacteria are produced as fusion proteins and not in their native conformation. In particular, correct formation of quaternary structures by recombinant proteins in bacterial hosts has been reported very rarely. To our knowledge, correct intracellular formation of multimeric structures containing more than one interchain disulfide bridge has not been reported so far. We have constructed three plasmids which are able to direct expression of recombinant rabbit uteroglobin, a homodimeric protein with two interchain disulfide bridges, in Escherichia coli. Among these, the plasmid pLE103–1, in which the expression of recombinant uteroglobin is controlled by a bacteriophage T7 late promoter, is by far the most efficient. With pLE103–1, recombinant uteroglobin production reached about 16% of total bacterial soluble proteins. This protein accumulated in bacterial cells in dimeric form, as it is naturally found in the rabbit uterus. Recombinant uteroglobin was purified to near-homogeneity and its N\textsubscript{H}\textsubscript{2}-terminal amino acid sequence was confirmed to be identical to that of its natural counterpart, except for 2 Ala residues the codons for which were added during the plasmid construction. This protein was found to be as active a phospholipase A\textsubscript{2} inhibitor as natural uteroglobin on a molar basis. To our knowledge, this is the first report of high level bacterial expression of a full length eukaryotic homodimeric protein with two interchain disulfide bridges in its natural, biologically active form. The plasmid pLE103–1 may be useful to explore structure-function relationships of rabbit uteroglobin. In addition, this plasmid may be useful in obtaining high level bacterial expression of other eukaryotic proteins with quaternary structure, as well as for other general applications requiring efficient bacterial expression of cDNAs.

Blastokinin (1) or uteroglobin (2) is a low molecular weight secretory protein which is found in several organs of the rabbit (for a review, see Ref. 3). The synthesis and secretion of uteroglobin are regulated by different steroid hormones in different organs (3). This protein has several biological properties, which include immunomodulatory effects, antiinflammatory properties, and an inhibitory activity on platelet aggregation (4–7). Uteroglobin is thought to play an immunomodulatory/antiinflammatory role in protecting the wet epithelia of organs which communicate with the external environment (3, 8, 9). In particular, uteroglobin has been proposed to protect the rabbit embryo from maternal immunological assault during implantation (4, 5). A uteroglobin-like protein has been recently detected in the human uterus (10, 11), respiratory tract (12), and the prostate (13). At least some of the biological effects of uteroglobin may stem from the phospholipase A\textsubscript{2} (EC 3.1.1.4) inhibitory properties of this protein (14). Because of its phospholipase A\textsubscript{2} inhibitory effect, uteroglobin can prevent liberation of arachidonic acid from membrane phospholipids, which is the first step of the arachidonate cascade, leading to the synthesis of various eicosanoids, some of which are well-known mediators of inflammation. Uteroglobin is a homodimeric protein, formed by identical subunits of 70 amino acids each, joined in antiparallel orientation by two disulfide bridges (15, 16). One of the main objectives of our present work is to study structure-function relationships in uteroglobin as a phospholipase A\textsubscript{2} inhibitor. We have recently identified a nonapeptide in α-helix 3 of uteroglobin which may be the active site, or at least a part of an active site, for the phospholipase A\textsubscript{2} inhibitory activity of uteroglobin (17). As a prelude to confirming this observation by site-directed mutagenesis, we decided to obtain high level bacterial expression of this protein. However, the structure of the protein posed a unique problem, since to our knowledge bacterial expression of multimeric eukaryotic proteins with two interchain disulfide bridges in their natural form has not been reported so far. Here, we describe the construction of three plasmids directing expression of recombinant uteroglobin in Escherichia coli. Using one of these plasmids, designated pLE103–1, we obtained high level expression of recombinant uteroglobin (about 9–11% of total bacterial soluble proteins). In this plasmid, the transcription of uteroglobin cDNA is controlled by the φ10 late promoter of bacteriophage T7. Recombinant uteroglobin in its natural dimeric form is synthesized by E. coli cells harboring pLE103–1 and bacterial lysates contain virtually only dimeric uteroglobin. The recombinant protein was purified to near-homogeneity, and its N\textsubscript{H}\textsubscript{2}-terminal amino acid sequence was found to be identical to that of its natural counterpart, except for 2 Ala residues, the codons for which were added during the plasmid construction. Recombinant uteroglobin was found to have an identical phospholipase A\textsubscript{2} inhibitory activity as that of the natural protein.
Experimental Procedures

Plasmin and Bacterial Strains—Plasmin pUG617 (18) was a gift from Dr. David Bullock (Lincoln College, Canterbury, New Zealand). Plasmin pPK233-2 (19) was kindly provided by Dr. W. Studier from Dr. David Bullock (Lincoln College, Canterbury, New Zealand). Plasmid pKK233-2 (19) was kindly provided by Dr. J. Brosius (Cold Spring Harbor, NY). E. coli strain JM109 (20) was a gift from Dr. M. Messing (University of Minnesota). E. coli strain BL21(DE3) (21, 22) was generously provided by Dr. W. Studier (Brookhaven National Laboratory, NY). "Library-efficient" competent E. coli strain HB101 (23) was purchased from Bethesda Research Laboratories (BRL). All other reagents were ultra-pure-grade from BRL.

Construction of Recombinant Plasminids pLE101, pLE102, and pLE103-1—All recombinant DNA manipulations were performed according to standard techniques (24). Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, and E. coli DNA polymerase I large fragment were purchased from Pharmacia LKB Biotechnology Inc., and strain JM109 (20) was a gift from Dr. J. Messing (University of Minnesota). Restriction endonuclease digestion was used to isolate fragments. Blunt ends were generated by treatment with DNA polymerase I "large fragment." Direct cloning of pLE101 was digested with BamHI and NcoI, extracting from pLE101 the 430-bp DNA fragment containing the whole coding sequence of plasmin and a 285-bp fragment containing the lac operator, the trc promoter and the Shine-Dalgarno region and the ATG codon was modified with a synthetic DNA fragment the sequence of the "spacer" region between phage T7, the 5' non-translated region from bacteriophage T7 gene 10, and the Shine-Dalgarno sequence from the same gene. In the synthetic DNA fragment the sequence coding for the "leader" peptide present in pre-uteroglobin mRNA was introduced. The orientation of the insert was checked by digestion of plasmid pKK233-2 with BamHI and NcoI, and the cohesive ends were made blunt by treatment with DNA polymerase I "large fragment." Direct ligation of blunt-ended uteroglobin cDNA fragment into NcoI-digested, blunt-ended pKK233-2 generated pLE102. The orientation of the insert was checked by digestion with Ascl and BamHI. For the construction of pLE103-1, the 430-bp DNA fragment was isolated by preparative agarose gel electrophoresis and ligated to a completely synthetic 89-bp BamHI-NcoI DNA fragment (prepared by OCS Laboratories, Denton, TX) containing the oct late promoter of bacteriophage T7, the 5' non-translated region from bacteriophage T7 gene 10, and the Shine-Dalgarno sequence from the same gene. In the synthetic DNA fragment the sequence of the "spacer" region between the Shine-Dalgarno region and the ATG codon was modified with respect to the wild-type gene 10 so that the ATG initiation triplet could be included in an NcoI site.

Immunoochemical Techniques—Radioimmunoassay (RIA) for uteroglobin was performed as previously described (10-13). Immunoprecipitation of bacteriophage T7 (PITG, Behring Diagnostics) early during logarithmic growth. The samples were flash-frozen in liquid N2, thawed on ice, and lysed by three cycles of sonication on ice (1 min each, setting 4.5, continuous). Recombinant uteroglobin was purified from E. coli lysate by a modification of the original method published by Nieto et al. (25) for rabbit uteroglobin. Briefly, the bacterial lysate was centrifuged at 10,000 × g for 10 min. Bacterial pellets were flash-frozen in liquid N2 in the centrifuge bottles and thawed on ice. The pellets were resuspended in a total of 10 ml of ice-cold buffer L and lysed by three cycles of sonication on ice (1 min each, setting 4.5, continuous). Two h after induction time four 1-ml samples were withdrawn from the cultures were centrifuged at 30,000 × g for 10 min. The supernatants were transferred to clean polypropylene tubes while pellets were resuspended in buffer L. Aliquots from supernatants and from resuspended pellets were assayed for uteroglobin by RIA.

Electrophoretic Determination of Disulfide Bonds in Recombinant Uteroglobin—E. coli BL21(DE3)-pLE103-1 were grown to early log-phase and induced with IPTG as described for expression experiments. Two h after induction time four 1-ml samples were withdrawn from induced and noninduced cultures, and the bacteria were pelleted by centrifugation at 12,000 × g for 1 min. For each culture two pellets were lysed directly by boiling for 10 min in SDS-PAGE sample buffer, respectively, containing 5% 3-mercaptoethanol or devoid of reducing agent.

Determination of Free Thiol Content in Natural and Recombinant Purified Uteroglobin—Free thiol content was estimated spectrophotometrically after reaction with 5,5'-dithiobis(2-nitrobenzoate) (5, 30). Briefly, samples (0.3 mg) of recombinant uteroglobin were dissolved in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing...
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The amount of expression obtained with pLF101 and pLF102 was not sufficient for preparative purposes. However, we did not observe any toxic effects of the recombinant protein on bacterial cells, which encouraged us to try to develop a more efficient expression system. We took advantage of a phage T7 late promoter. Such promoters have been previously shown to direct high level expression of recombinant proteins in E. coli (21, 22). Fig. 1B shows the construction of pLE103–1 from pLE101. In pLE103–1 the regulatory sequences originally present in pKK233–2 (19) (lac operator, trc promoter, and ribosome-binding site) have been replaced with the synthetic DNA fragment whose sequence is shown in Fig. 1B. The synthetic DNA fragment contained the 5′ late promoter of bacteriophage T7, the 5′ non-translated region of T7 gene 10, and the ribosome-binding site from the same gene. The sequence of this synthetic regulatory region was derived from the wild-type sequence which has been used by Studier and co-workers (22) in their “translation” vectors, with the exception that the two bases preceding the initiation ATG triplet were replaced with two cytosines. This replacement was made to create an NcoI site including the initiation triplet. Additionally, a BamHI site was added at the 5′ end. The plasmid obtained in this way has the same cloning sites, and should have the same possible applications, of pKK233–2 and related “ATG vectors” (16), except that expression of the recombinant protein is controlled by a more specific and very efficient viral promoter.

Expression of Recombinant Uteroglobin by pLE103–1—For expression experiments with pLE103–1, we used E. coli strain BL21(DE3)(31). In this strain, phage T7 RNA polymerase is produced upon induction with IPTG from a recombinant λ-phage which is integrated into the bacterial chromosome (21). BL21(DE3):pLE103–1 expresses recombinant uteroglobin upon induction with IPTG, and the recombinant protein is readily detectable by polyclonal anti-gel electrophoresis. Fig. 2A clearly shows the time-dependent appearance in induced bacteria of a protein band of apparent molecular weight corresponding to that of mature rabbit uteroglobin monomer. The difference in molecular weight due to the presence of the expected additional 3 residues in the recombinant protein is not apparent under these conditions. The lower molecular weight band appearing immediately below the putative recombinant uteroglobin band (Fig. 2A) may be a product of partial degradation of recombinant uteroglobin or an artifact caused by the formation of an intramolecular disulfide bridge in uteroglobin during SDS-polyacrylamide gel electrophoresis. The appearance of pure uteroglobin as a “doublet” band due to such an artifact has been described by Nieto et al. (26). The identity of the recombinant protein was confirmed by Western blot (25). That the new band appearing upon induction is recognized by anti-uteroglobin antibody is shown in Fig. 2B. Interestingly, both in the control sample of rabbit uteroglobin and in the recombinant material an immunoreactive band with apparent molecular weight of about 13,000 is present (Fig. 2B). The presence of this band is due to incomplete reduction of disulfide bonds between uteroglobin subunits under the conditions used for sample preparation, and it consistently appears when concentrated samples of pure rabbit uteroglobin are subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot. This observation gave us a preliminary indication that the recombinant protein may be present in the bacteria in dimeric form.

Quantitation of Recombinant Uteroglobin—We found that optimal expression of recombinant uteroglobin with pLE103–1 was obtained with a high concentration of ampicillin in the culture medium (200 μg/ml) and a very small initial inoculum.
(aliquots from a saturated culture were diluted 400-fold in fresh medium). These conditions were optimal also for pLE101 and pLE102. It is well known that saturated cultures of bacteria which harbor plasmid derived from pBR322 (such as the vectors described in this paper) contain large amounts of $\beta$-lactamase (21). Therefore, unless the plasmid is extremely stable in the host, it is essential to maintain a high selective pressure to avoid any growth of plasmid-free bacteria. Moreover, pLE103-1 contains the bla gene in transcriptional orientation with respect to the T7 promoter. Thus, induction with IPTG will result in transcription of a polycistronic mRNA containing the bla coding sequence, and in overexpression of $\beta$-lactamase. In fact, T7 RNA polymerase does not recognize E. coli transcriptional terminators, such as the T1 and T2 rrnB terminators present in pLE103-1 (see Fig. 1B). Under appropriate electrophoretic conditions, we have indeed observed an overexpression of a protein band of molecular weight corresponding to that of $\beta$-lactamase (data not shown). Fig. 3 shows the quantitation of recombinant uteroglobin, as determined by RIA in supernatants from bacterial lysates. The three plasmids pLE101, pLE102, and pLE103-1 were compared under identical experimental conditions, except for the host strains, i.e. JM109 for pLE101 and pLE102, and BL21(DE3) for pLE103-1. It is clear that with pLE103-1 production of recombinant immunoreactive uteroglobin is much higher (about 50-fold more than with pLE101 and 100-fold higher than with pLE102). With pLE103-1, the highest absolute concentration of recombinant immunoreactive material was reached 120 min after induction (Fig. 3A). However, when the amount of recombinant uteroglobin was expressed as µg/mg protein, the maximum level of uteroglobin was reached 90 min after induction (Fig. 3B). More than 90% of immunoreactive uteroglobin expressed was recovered in the supernatant after centrifugation at 30,000 x g. This indicates that the recombinant protein is soluble.

**Molecular Weight and Quaternary Structure of Recombinant Uteroglobin**—Fig. 4 shows the determination of molecular weight of recombinant uteroglobin by size exclusion chromatography under nondenaturing conditions. It is evident that recombinant and natural uteroglobin have an identical chromatographic behavior. Under these conditions, both proteins have an apparent molecular weight of 17,000, slightly higher than the theoretical value of 15,800. This is in agreement with the results of Nieto et al. (26) on purified rabbit uteroglobin. No immunoreactive peak indicating the presence of isolated uteroglobin subunits was observed, although uteroglobin subunits are readily recognized by our antibody in Western blots. These results seem to indicate that in lysates of induced BL21(DE3):pLE103-1 recombinant uteroglobin exists almost solely in its natural dimeric form. However, these data do not give any direct indication as to whether or not recombinant dimeric uteroglobin in bacterial lysates contains the two interchain disulfide bonds present in natural dimeric uteroglobin. In addition, the question of where the formation of disulfide bonds might take place, i.e. in the bacterial cytoplasm or in the lysate as a consequence of spontaneous oxidation, needed to be addressed. We approached these problems by an experimental strategy similar to the one used by

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**Fig. 1. Construction of the uteroglobin (UC) expression plasmids.** A, construction of pLE101 and pLE102. B, construction of pLE103-1. The sequence of pLE103-1 between the BamHI site and the HindIII site is reported above the scheme of the subcloning steps. Arrows indicate the limits of the synthetic 89-base pair BamHI-NcoI DNA fragment used for this construction. Only the restriction sites relevant for plasmid construction and the cloning sites are indicated. Pt, trc promoter; 5S, E. coli 5S rRNA gene; Z', T1 and T2 rrnB terminators; 410, 410 phage T7 promoter; UG, mature uteroglobin coding sequence from pUG617; SD, Shine-Dalgarno sequence.
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Fig. 2. Expression of uteroglobin (UG) by pLE103-1. A, expression of uteroglobin in E. coli BL21(DE3)pLE103-1 as estimated by SDS-polyacrylamide gel electrophoresis on a 15–25% gradient gel containing 0.1% SDS. Each lane was loaded with the equivalent of 50 μl of bacterial culture. Lane 1, purified rabbit uteroglobin (1 μg); lane 2, molecular weight standards (BRL, prestained); lanes 3 and 4, 1 h after induction time, non-induced culture (3) and induced culture (4); lanes 5 and 6, 2 h after induction time, non-induced culture (5) and induced culture (6); lanes 7 and 8, 3 h after induction time, non-induced culture (7) and induced culture (8). B, immunoblot of expressed recombinant uteroglobin. Each lane of a 15–25% polyacrylamide gradient gel containing 0.1% SDS was loaded with the equivalent of 50 μl of bacterial culture, and protein bands were transferred overnight to a Nitroscreen West membrane (Du Pont, 0.22-μm pore size) at 4 °C, with a current of 34 mA. Lane 1, molecular weight standards (BRL, prestained); lanes 2 and 3, 1 h after induction time, non-induced culture (2) and induced culture (3); lanes 4 and 5, 2 h after induction time, non-induced culture (4) and induced culture (5); lanes 6–7, 3 h after induction time, non-induced culture (6) and induced culture (7); lane 8, purified rabbit uteroglobin (250 ng). Note that both uteroglobin monomer and dimer bands appear to be stained (arrows).

Pollitt and Zalkin (35) for electrophoretic detection of disulfide bond formation in E. coli β-lactamase. Fig. 5 shows Western blots of total proteins from induced and non-induced E. coli BL21(DE3)pLE103-1, obtained after electrophoresis in the presence (Fig. 5a) or absence (Fig. 5b) of 5% β-mercaptoethanol in the sample buffer. In both panels, lanes 2 and 3 are samples obtained by directly lysing and boiling bacteria in sample buffer, while lanes 4 and 5 are samples obtained by treating bacteria with cold acetone and resuspending the protein pellet in iodoacetamide-containing, deaerated buffer. Fig. 5 shows that both non-alkylated and alkylated samples contain substantial amounts of both monomeric and dimeric uteroglobin when electrophoresed under non-reducing conditions. The intensity of the dimer band is drastically reduced in samples electrophoresed under reducing conditions, although the band was not completely abolished, as shown in Fig. 2B. This indicates that uteroglobin-immunoreactive material migrating as a dimer contains disulfide bond(s) which stabilize its quaternary structure. When the photographic negative obtained from the blot shown in Fig. 5b was analyzed by laser densitometry, the dimer band was found to contain approximately 33% of total uteroglobin (monomer + dimer) in non-alkylated samples and 38% in alkylated samples. This indicates that disulfide bond formation during electrophoresis under non-reducing conditions does not contribute appreciably to the amount of dimeric uteroglobin observed under these conditions. It cannot be established from these data whether the monomeric species observed under non-reducing conditions is present in the bacterial cytoplasm as free uteroglobin subunits or as non-oxidized dimers which are dissociated by boiling in the presence of SDS.

Purification of Recombinant Uteroglobin—Despite the higher complexity of the protein mixture present in lysates compared with rabbit uterine flushings, the very large head start allowed us to obtain near-homogeneous recombinant uteroglobin by a modification of the original procedure (26, Fig. 6). The chromatographic properties of recombinant uteroglobin in the columns used for purification were indistin-
samples have a higher ionic strength, pH, and concentration of SDS treated samples of non-reduced uteroglobin are subjected to SDS-tails). The band appearing at about 24 kDa in pure uteroglobin dimer is slightly higher than in non-alkylated samples. This is probably due to the different composition of the samples. In fact, alkylated samples the apparent molecular weight of both uteroglobin monomer and dimer is slightly higher than in non-alkylated samples. This is probably due to the different composition of the samples. In fact, alkylated samples have a higher ionic strength, pH, and concentration of SDS than non-alkylated samples (see “Experimental Procedures” for details). The band appearing at about 24 kDa in pure uteroglobin standard in panel b is frequently observed in our hands when concentrated samples of non-reduced uteroglobin are subjected to SDS-PAGE and Western blot, and it might represent the unstable tetrameric form of uteroglobin postulated on the basis of NMR data (37).

FIG. 5. Electrophoretic determination of disulfide bond formation in recombinant uteroglobin. a, samples electrophoresed under reducing conditions. Lane 1, molecular weight standards (BRL, prestained); lanes 2 and 3, non-alkylated samples from the non-induced culture (2) and the induced culture (3); lanes 4 and 5, alkylated samples from the non-induced culture (4) and the induced culture (5); lane 6, purified rabbit uteroglobin (0.7 µg). b, samples electrophoresed under non-reducing conditions. Lane 1, molecular weight standards (BRL, prestained); lanes 2 and 3, non-alkylated samples from the non-induced culture (2) and the induced culture (3); lanes 4 and 5, alkylated samples from the non-induced culture (4) and the induced culture (5); lane 6, purified rabbit uteroglobin (0.7 µg). Note that under non-reducing conditions in alkylated samples the apparent molecular weight of both uteroglobin monomer and dimer is slightly higher than in non-alkylated samples. This is probably due to the different composition of the samples. In fact, alkylated samples have a higher ionic strength, pH, and concentration of SDS than non-alkylated samples (see “Experimental Procedures” for details). The band appearing at about 24 kDa in pure uteroglobin standard in panel b is frequently observed in our hands when concentrated samples of non-reduced uteroglobin are subjected to SDS-PAGE and Western blot, and it might represent the unstable tetrameric form of uteroglobin postulated on the basis of NMR data (37).

FIG. 6. Purification of recombinant uteroglobin from bacterial lysate supernatant. Samples were electrophoresed using a 15% polyacrylamide gel containing 0.1% SDS. Lane a, molecular weight standards (BRL, low molecular weight standards); lane b, pure rabbit uteroglobin (4 µg); lane c, bacterial lysate supernatant (about 12 µg of protein); lane d, pooled uteroglobin-containing fractions after Sephacryl S-200 superfine chromatography (about 20 µg of protein); lane e, pooled fractions after CM-Sepharose chromatography (1.8 µg of protein); lane f, pooled fractions after Sephadex G-50 superfine gel filtration (1.8 µg of protein). Silver stain. Note that the bacterial lysate supernatant appears to be more concentrated than the Sephacryl pool. This is probably due to its high content in nucleic acid fragments (A260 > 5). Note also the presence of a very abundant band with an apparent molecular weight of about 25,000 in lane d. This band appears only after induction with IPTG and probably corresponds to overproduced β-lactamase.

guishable from those of the natural protein. The final yield of the purification was 3.2 mg of recombinant uteroglobin from 800 ml of induced bacteria, as estimated by UV absorption using the published value of 1800 for the ε280 (26). The starting material (bacterial lysate supernatant) contained a total of about 50 mg of protein, and approximately 5 µg of recombinant uteroglobin (data not shown). Therefore, the final recovery of recombinant uteroglobin can be estimated as about 64%. Identical samples of purified rabbit uteroglobin and recombinant dimeric uteroglobin (19 µM) were analyzed for the presence of free thiol groups by reaction with 5,5'-dithiobis(2-nitro)benzoate in the presence of 6 mM guanidinium chloride and showed no detectable free thiol groups. A control sample of L-cysteine (20 µM) dissolved in the same buffer at the same time as the protein samples gave an estimate of 19.4 µM free thiol groups. This indicates that in purified recombinant uteroglobin, as in natural rabbit uteroglobin, the vast majority of Cys residues are engaged in disulfide bonds.

NH2-terminal Sequence of Recombinant Uteroglobin—The NH2-terminal sequence of recombinant uteroglobin, as determined by automated Edman degradation after reduction of disulfide bridges and alkylation of Cys residues, is shown in Table I. Interestingly, only 2 Ala residues appear to precede Gly-1, instead of the expected tripeptide Met-Ala-Ala. This may indicate that the NH2-terminal Met residue of recombinant uteroglobin is removed intracellularly by an aminopeptidase.

Phospholipase A2 Inhibitory Properties of Recombinant Uteroglobin—Fig. 7 shows dose-response curves of recombinant and natural uteroglobin as phospholipase A2 inhibitors. Both proteins were tested in the range of concentrations which have been reported to be optimal for the phospholipase A2 inhibitory activity (17). It is evident that the two curves are essentially identical. This indicates that purified recombinant uteroglobin is as potent a phospholipase A2 inhibitor as the natural protein. The slightly lower percent inhibition obtained in the present study with uteroglobin, with respect to previously published data (17) is most likely due to differences in the assay procedure, particularly the change in phospholipase A2 source and batch. With the batch of phospholipase A2 currently used in our laboratory, inhibition observed with uteroglobin and other polypeptide inhibitors rarely exceeds 40%. This may be due to contaminant protease(s).

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a) NH2-Ala-Ala-Gly-Ile-Cys-Pro-Arg-Phe-Ala-His-Val-Ile-

b) NH2-Ala-Ala-Gly-Ile-Cys-Pro-Arg-Phe-Ala-His-Val-Ile-

FIG. 7. Dose response of natural and recombinant uteroglobin (UG) as inhibitors of porcine pancreatic phospholipase A2. Each point represents the average of three determinations, each performed in duplicate.
present in some batches of phospholipase A2 that may cause degradation of these inhibitors.2

**DISCUSSION**

We have obtained high level expression of recombinant uteroglobin using pLE103–1 under the control of the phage T7 ϕ 10 late promoter. The yield of recombinant protein appears to be much higher with pLE103–1 than with the pKK233–2–derived plasmids pLE101 and pLE102. Since neither E. coli strain BL21(DE3) nor JM109 are known to carry mutations in their protein degradation systems, it is unlikely that this difference could be due to the bacterial host strains. In addition, the growth curves of the three vector/host systems were not significantly different, nor were the total protein content of bacterial lysates (data not shown). Since the three plasmids are derived from pBR322 and share the same systems of replication and copy number control, differences in plasmid copy number are also unlikely. It is possible, therefore, that the striking difference in efficiency of uteroglobin expression observed between pLE103–1 and the other two plasmids could be due mainly to the higher efficiency of transcription of T7 RNA polymerase and to its high specificity for T7 promoters (21, 22).

One additional factor could be the different length of the transcripts. In fact, T7 RNA polymerase does not recognize E. coli transcriptional terminators (21) and produces from plasmids such as pLE103–1 longer transcripts than E. coli RNA polymerase, which stops at the rrnB terminators present in pKK233–2 (19, Fig. 1). The length of the RNAs transcribed by T7 RNA polymerase has been suggested to protect them from intracellular exonucleolytic degradation starting from the 3′ end, thereby increasing the half-life of these RNAs in E. coli (21).

The recombinant uteroglobin isolated from extracts of induced BL21(DE3):pLE103–1 is totally dimeric, and no free subunit could be detected. Our electrophoretic data on alkylated and non-alkylated bacterial lysates indicate that approximately 33–38% of the recombinant uteroglobin found in the bacterial cytoplasm is dimeric and that formation of interchain disulfide bonds takes place to a considerable extent in the bacterial cytoplasm. It is likely that most of the remaining reduced uteroglobin becomes rapidly oxidized in the presence of air after the lysis of bacterial cells. It should be noted that uteroglobin isolated from rabbit uterine washings is totally dimeric and oxidized and that isolation of reduced uteroglobin or free uteroglobin subunits has not been reported in the literature. Indeed, reduction of the disulfide bonds in rabbit uteroglobin does not result in dissociation of the dimer (36), and rabbit uteroglobin is completely dimeric even after reduction and carboxymethylation of Cys residues (37). This indicates that in the case of uteroglobin, although interchain disulfide bonds most likely stabilize the dimeric structure, they are not indispensable for the formation of the structure itself. In fact, crystallographic analysis has shown that besides the two disulfide bridges several other stereospecific intermolecular contacts (Van der Waals interactions and H-bonds) contribute to the stabilization of the uteroglobin dimer (15, 16). Thus, it would seem that the information needed for the correct formation of the natural quaternary structure of uteroglobin is entirely contained in the primary structure of the protein. Based on the available information, we believe that the most likely route for intracellular formation of oxidized uteroglobin dimers in E. coli involves folding of the subunits followed by rapid formation of dimers stabilized by noncovalent interactions. This, in turn, might favor the efficient formation of “correct” disulfide bonds by appropriately positioning the side chains of Cys-3 and 69′, 3′, and 69. This model might explain the apparent absence of insoluble aggregates of multimeric recombinant protein containing “incorrect” disulfide bonds, which are often observed when proteins containing cysteine are expressed in bacteria. To our knowledge, this is the first report of high level bacterial expression of a full length dimeric eukaryotic protein with two interchain disulfide bridges with intracellular formation of the natural quaternary structure. Our results demonstrate that a recombinant protein can form correct quaternary structures during overexpression in E. coli even when correct formation of two interchain disulfide bridges is essential for its structure, provided that the rate of intracellular accumulation of correctly folded subunits and the rate of association of free subunits are high enough. Non-covalent self-association of recombinant eukaryotic proteins in E. coli has been described for human tumor necrosis factor α (38) and rat liver aldehyde dehydrogenase (39). However, in the first case most of the recombinant protein appeared in the insoluble fraction due to incorrect folding (38), and in the second case the high efficiency of expression was suggested to be due to unique features of the 5′-non-translated region of the cDNA (which contained a potential prokaryotic Shine-Dalgarno sequence) and its relationship with the lac promoter present in pUC8 (39). In contrast, in pLE103–1, the 5′-non-translated region and the Shine-Dalgarno sequence are built in the vector, so that theoretically any open reading frame could be expressed in place of the uteroglobin cDNA. Until recently, it was generally believed that the intracellular environment of E. coli is not conducive to the formation of quaternary structures which require formation of interchain disulfide bridges (40). Correct folding and assembly of heterodimeric fragments of immunoglobulins after proteolytic processing of fusion precursors and secretion into the periplasmic space of E. coli has been recently described (41, 42). In one case (40), the assembly involved the formation of one interchain disulfide bridge. Our findings confirm and extend this observation further, demonstrating that (i) quaternary structures containing more than one interchain disulfide bridge can also be properly assembled in E. coli, and (ii) at least in the case of uteroglobin, correct assembly of quaternary structure can take place in the bacterial cytoplasm without the need for correct proteolytic processing of a precursor protein and transmembrane transport of the product. It should be noted that natural uteroglobin is a secretory protein which is synthesized as a precursor that naturally undergoes transmembrane transport and proteolytic processing. Our data indicate that assembly of quaternary structure in E. coli does not necessarily require the construction of a fusion precursor protein with a bacterial secretion signal sequence. Such constructions can be advantageous if secretion of correctly processed recombinant protein in the medium is achieved, but require a precise "in frame" fusion between the bacterial signal sequence and the eukaryotic coding sequence. This may require extensive manipulations on vector and/or insert DNA. All in all, our observations seem to suggest that if a sufficiently high level of intracellular accumulation of recombinant protein(s) is obtained, the possibility of formation of multimeric structures involving disulfide bridges depends essentially on the physical-chemical factors controlling the folding of the protein(s) and the interaction between subunits. Thus, it may be possible to obtain efficient bacterial expression of eukaryotic multimeric proteins other than uteroglobin, provided that (i) the vector/host system used insures a high efficiency of expression and intracellular accumulation of the product(s), and (ii) the tertiary and quaternary structure of the recombinant protein(s) are...
thermodynamically stable and the kinetics of folding and assembly are not too slow. The latter conditions obviously depend on the particular protein(s) being expressed. The results of our experiments on recombinant uteroglobin strongly support the hypothesis that the dimeric structure of recombinant uteroglobin is stabilized by two disulfide bridges identical to those of natural uteroglobin. In theory, it is possible that recombinant uteroglobin could form "inverted" dimers in which the two disulfide bridges join Cys-3 and 3', 69, and 69'. However, this possibility is highly unlikely because of the presence of several sterospecific non-covalent interactions between the two subunits (10, 16, see above). Moreover, the identical properties of recombinant and natural uteroglobin as phospholipase A2 inhibitors further support the hypothesis that the two proteins are structurally identical, with the exception of the two additional Ala residues in recombinant uteroglobin. A collaborative effort to crystallize recombinant uteroglobin and resolve its three-dimensional structure by x-ray crystallography is currently underway.

With the vector/host system used in this study, considerable overexpression of β-lactamase along with uteroglobin was observed. Simultaneous overexpression of a recombinant protein and β-lactamase has also been described with other vectors based on T7 promoters (21). Therefore, these systems are able to support overexpression of two different polypeptides and could be used for the construction of artificial operons to express heterodimeric proteins. The vector pLE103-1, described in this study, could be a useful addition to the already existing expression plasmids based on T7 promoters (21, 22). In fact, after excision of the uteroglobin coding sequence from the PsI site, pLE103-1 can be converted into a general purpose expression vector which we have denominated pLD101. The cloning sites NcoI, PstI, and HindIII give to pLE103-1 the same potential applications as "ATG vectors" (19) with the advantage of the high efficiency and specificity of the T7 promoter. In particular, the NcoI site (CCATGG) is frequently present in eukaryotic translational start sites (43). Moreover, after restriction endonuclease digestion the NcoI site can be easily "filled in" with E. coli DNA polymerase I large fragment. This process reconstitutes the ATG triplet, thereby allowing "blunt-ended" DNAs to be cloned in frame directly into the filled-in NcoI site. In addition, the presence of the PstI and HindIII sites allows "forced" or "directional" cloning. Finally, the presence of the PsI site and the relative positions of the three cloning sites allow this plasmid to be used for the construction of cDNA libraries by several different methods (45-47).

The data obtained on the biochemical properties of recombinant uteroglobin have already yielded some valuable information on the structure/function relationships of this protein. Since the recombinant protein appears to fold correctly in E. coli, the presence of the leader peptide which is physiologically present in rabbit pre-uteroglobin (34) is not necessary for the folding of uteroglobin during translation. Furthermore, the addition of 2 Ala residues at the NH2-terminus of recombinant uteroglobin does not affect its activity as a phospholipase A2 inhibitor. Preliminary data suggest that recombinant uteroglobin is also as active an inhibitor of platelet aggregation and serotonin secretion as natural uteroglobin. Taken together, our observations seem to indicate that the recombinant uteroglobin expression plasmid pLE103-1 and the recombinant protein may provide a valuable tool for site-directed mutagenesis of uteroglobin in particular, and may serve as a model for studies involving protein engineering in general.

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REFERENCES


*a J. Vostal, unpublished data.
Bacterial Expression of Dimeric Uteroglobin

High level bacterial expression of uteroglobin, a dimeric eukaryotic protein with two interchain disulfide bridges, in its natural quaternary structure.
L Miele, E Cordella-Miele and A B Mukherjee


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