The Small Subunit of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase and Its Precursor Expressed in Escherichia coli Are Associated with groEL Protein*

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The small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase is synthesized in the cytoplasm as a precursor which is transported into the chloroplast. During or after transport the precursor is processed to its mature size by removal of an amino-terminal transit peptide. Eight small subunits and eight large subunits (synthesized in the chloroplast) assemble to form the holoenzyme. We have expressed the precursor of the small subunit in *Escherichia coli* as a fusion to the carboxyl terminus of staphylococcal protein A*. The fusion protein was recovered from the bacterial lysate by chromatography on IgG-agarose. A 58-kDa protein copurified with the fusion protein in approximately equal amounts. Much less of the 58-kDa protein copurified with a fusion in which the transit peptide was deleted, and it did not copurify with protein A*. The 58-kDa protein was identified as the *E. coli* groEL gene product with antibodies directed against a homologous mitochondrial heat shock protein. This finding is particularly interesting because a chloroplast protein involved in the assembly of ribulose-1,5-bisphosphate carboxylase/oxygenase also is homologous to the groEL protein. These homologs could modulate protein-protein interactions during folding and assembly of subunits into native complexes.

The chloroplast enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) is a complex of eight large and eight small subunits (1). The large subunit (L) is synthesized in the organelle, and the small subunit (S) is synthesized in the cytoplasm as a precursor (pS) which is transported into the organelle and cleaved to its mature form (S) (2). Assembly of the L,S, holoenzyme may require the participation of another protein, ribulose-1,5-bisphosphate carboxylase/oxygenase binding protein (3, 4), which is homologous to the bacterial groEL gene product (5).

To facilitate our investigation of the transport of pS into chloroplasts, we have expressed in *Escherichia coli* several recombinant proteins which contain pS or derivatives thereof fused to the carboxyl terminus of truncated staphylococcal protein A (protein A*). Affinity chromatography of bacterial lysates on immunoglobulin G (IgG)-agarose yielded a 58-kDa protein which copurified with the fusion proteins but not with protein A* alone. This 58-kDa protein, copurifying with the fusion proteins, was identified as the groEL gene product based on its antigenic reactivity with antibodies against the *Tetrahymena thermophila* heat shock protein, hsp8 (6).

**MATERIALS AND METHODS**

**Construction of Plasmids**—For all DNA manipulations, plasmids were treated with various enzymes to generate fragments with appropriately compatible ends. The DNA was electrophoresed in low-melting agarose gels. The desired fragments were excised and then ligated in the gel as described (7). *E. coli* DH5α was transformed with the ligation mixture, and mini-preparations (8) of plasmid DNA were screened by restriction analysis.

Plasmid pAPs was constructed as follows. Plasmid pRIT2T (Pharmacia LKB Biotechnology Inc.) was linearized with the restriction enzyme SmaI and ligated with a 12-base pair NcoI linker (Pharmacia). The DNA was then digested with NcoI and PstI. Plasmid pW9 (9), containing a cDNA insert encoding wheat pS, was digested with restriction enzymes NcoI and PstI. The NcoI recognition sequence in pW9 includes the pS initiator codon. The fragment containing the cDNA was ligated between the NcoI and PstI sites in the linkered pRIT2T. In the resulting pAPs plasmid, the translational reading frame of the protein A* gene in pRIT2T is maintained through the NcoI site into the pS coding region; thus the chimeric gene encodes a protein A*–pS fusion protein.

Plasmid pAPxS was constructed by inserting a hybridized pair of oligonucleotides which encode the sequence, Asn-Ser-Ile-Glu-Gly-Arg-Thr, between the unique EcoRI and NcoI sites of pAPs. Factor X, is expected to cleave the fusion protein between arginine and threonine. Information concerning these cloning steps, cleavage of the fusion protein, and transport of bacterially synthesized pS will be presented elsewhere. A similarly cleavable fusion of the αcrI protein and β-globin was previously expressed in *E. coli* by Nagai and Thøgersen (10).

Plasmid pAXS, encoding a protein A*–S fusion, was constructed by digesting pAPxS with NcoI, end-filling with Klenow polymerase, digesting with *SphI* (corresponding to the pS processing site), blunting the ends with mung bean nuclease, and recircularizing the large fragment.

Plasmid pAPxSd52-72 was constructed utilizing a pS cDNA subcloned into a derivative of pSP65 (Promega) from which the DraII sites had been deleted. The plasmid was digested with DraII, trimmed with mung bean nuclease, then digested with *BstI*, and finally religated. The NcoI/PstI fragment from this plasmid was ligated into pAPxS which was digested with the same enzymes. The fusion protein expressed from this plasmid contains a deletion in the mature portion of pS from Pro25 (the fifth residue of S1 to Ala52).

**Preparation of Fusion Proteins**—Plasmid pRIT2T contains the λ P6 promoter upstream from the protein A* gene. Initial experiments revealed that *E. coli* DH5α could serve as host to the plasmid despite the absence of a gene for a repressor in the chromosome or plasmid. Furthermore, protein A* or fusion protein was expressed in DH5α at a greater level than in *E. coli* N430-1 transformed with the same plasmid induced at 42 °C. Strain N430-1 contains the αcrI857 gene encoding a temperature-sensitive repressor (11).
Protein A' or fusion protein was prepared essentially as described (12). A 0.5-liter culture was grown in rich medium at 37 °C to stationary phase and then chilled on ice. All subsequent manipulations, with the exception of affinity chromatography, were carried out at 4 °C. The cells were pelleted, raised in 50 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.1 mg/ml leupeptin, and lysed by sonication. Cell debris was pelleted by centrifugation at 10,000 × g and the supernatant was applied to 2.5 ml of loosely packed IgG-agarose prepared using Reacti-gel (6×) (Pierce Chemical Co.) and rabbit IgG according to the manufacturer's recommendations. The mixture was rocked continuously for 30 min at room temperature, and then the matrix was washed batchwise three times with a large excess of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. Protein A' or fusion protein was eluted from the column with three washes of 3 ml of 0.5 M acetic acid adjusted to pH 3.4 with ammonium acetate. The eluted batches were pooled and lyophilized. The lyophilized fusion protein appears to be insoluble in aqueous buffers unless they contain denaturants such as urea or SDS.

The recovery of all fusion protein in the lysate was accomplished by starting with a 6-ml culture and affinity-purifying protein A' and fusions as above so the amount of affinity column was not limiting. In this case the proteins were eluted with 0.1 M glycine/HCl, pH 2.4. Some IgG was removed from the column by this procedure.

Gel Electrophoresis and Western Blotting—Samples representing equal portions of lysates (and unbound fractions from the quantitative purification) and samples containing approximately equal amounts of protein A' or fusion protein (or samples representing equal portions of affinity-purified fractions from the quantitative affinity purification) were fractionated by SDS-PAGE (13). Identical sets of lanes in the gel were either stained with Coomassie Blue or electrophoretically transferred to nitrocellulose membranes. Blotted membranes were probed with antibodies raised against T. thermophila hsp58 according to the method of Burnette (14) and subjected to autoradiography.

RESULTS AND DISCUSSION

E. coli DH5α transformed with the recombinant plasmid pApS, pAXpS, or pAXpSd52-72 expressed the fusion protein at a moderate level, approximately 2.5 mg/liter of culture. Protein A' alone and protein A'-S were expressed at a much higher level than fusions which contain the pS transit peptide. Polypeptides corresponding to protein A' (30-kDa polypeptide in Fig. 1, top panel, lane 1) and protein A'-S fusion (45-kDa polypeptide in lane 2), but not to fusions containing the transit peptide, are evident in the stained gel of total soluble proteins from DH5α transformed with the respective recombinant plasmids.

Analysis of the affinity-purified proteins in SDS-PAGE revealed that a 58-kDa protein copurified in approximately equal amounts with fusions containing the transit peptide and to a lesser extent with protein A'-S (compare lanes 2, 3, and 5 to lane 4 in Fig. 2, top panel). The 58-kDa protein did not copurify with protein A' expressed from the parent vector (lane 1). The 66-kDa polypeptide in affinity-purified fractions copurified in proportion to the amount of cell lysate applied to the column (Fig. 3, top panel). This protein apparently has an affinity for IgG-agarose, and it was not further characterized. Other smaller polypeptides associated with the affinity-purified fusions but not protein A' alone are likely to be breakdown products of the fusions that retain one or more of the six IgG-binding domains of protein A'. These polypeptides probably arise in E. coli as a result of endogenous proteolytic activities.

Antibodies directed against the T. thermophila hsp58 cross-react with a single E. coli 58-kDa protein, the groEL gene product (6). Other evidence indicates that hsp58 and the groEL protein are homologous. Both the T. thermophila hsp58 (6) and the groEL protein (15) can be isolated as 14-subunit homooligomers having unusual 7-fold radial symmetry, and the genes for the yeast hsp58 homolog and groEL have a high degree of sequence similarity (16).

Antibodies raised against T. thermophila hsp58 were used to probe Western blots of cell lysates and affinity-purified fractions from E. coli which expressed protein A' or fusion protein (Figs. 1 and 2, bottom panels). One predominant signal was observed in each lysate at a position corresponding to a 58-kDa polypeptide. The signal is slightly stronger in lysates from E. coli that expressed a fusion compared to the signal in the lysate of E. coli that expressed protein A'. Thus, expression of a fusion which contains pS or S may enhance synthesis of groEL protein.

Western blotting of affinity-purified fractions revealed that the copurifying 58-kDa protein is indeed groEL protein, and the intensity of the signals correlates with the intensity of the stained bands. Since no signal was detectable in the affinity-purified fraction of the lysate from E. coli producing protein A' alone, we conclude that the groEL protein has a strong affinity for pS or S and not protein A' or IgG-agarose. The low degree of association of groEL protein with protein A'-S could result from the high level of expression of protein A'-S if the amount of fusion exceeds the amount of available groEL protein.

Protein A' and fusions to pS and S were purified from small cultures so that all of the protein A' or fusion in the lysate would be recovered. The lysates, unbound fractions, and affinity-purified fractions were analyzed by SDS-PAGE and Western blotting as before (Fig. 3). The amount of groEL protein that copurified with fusions to pS and S was equal indicating that the total amount of groEL associated with each fusion is the same (compare lanes 8 and 9). As before, groEL protein did not copurify with protein A' expressed alone (lane 7). A fraction of the groEL protein was in the

![Fig. 1. SDS-PAGE of total soluble proteins in the lysates of E. coli which express protein A’ or a fusion protein and the autoradiograph of the Western blot probed with anti-hsp58. Top panel, extracts of E. coli transformed with the plasmid pRIT2T (lane 1), pAPs (lane 2), pAXpS (lane 3), pAXS (lane 4), or pAXpSd52-272 (lane 5) were separated by SDS-PAGE and stained with Coomassie Blue. Molecular weight markers are at the left. Bottom panel, a set of lanes from SDS-PAGE identical to those stained in the top panel was transferred to a nitrocellulose membrane. The blotted membrane was probed with antibodies to the T. thermophila hsp58 and subjected to autoradiography. Only the region in the autoradiograph corresponding to the mobility of the 58-kDa protein is shown.](image-url)
groEL Protein Associates with a Chloroplast Protein

unbound fraction which suggests that a fraction of groEL protein is unavailable for binding to pS or S. This fraction may be sequestered from the fusion by binding to E. coli constituents having a greater affinity for groEL protein. This is not surprising in view of the absolute requirement of functional groEL for E. coli viability (17). Since protein A' and protein A'-S were expressed at a higher level in E. coli than protein A'-pS, we speculate that the availability of groEL protein limits the accumulation of fusion proteins which contain the pS transit peptide. In attempts to express unfused pS in E. coli we have detected only S in the extracts (data not shown), indicating that pS is a good substrate for host proteases. Some of the pS expressed as a fusion may be protected from proteases by its association with groEL protein. Expression of protein A'-S evidently is not limited by the availability of groEL protein presumably because it is not degraded as efficiently by host proteases.

Precursors probably are unfolded during translocation into the endoplasmic reticulum, the mitochondrion, and the chloroplast (18). Refolding of newly imported proteins inside the organelle and their assembly into macromolecular complexes may be promoted by a protein having protein folding/unfolding activity. This function was suggested for the mitochondrial hsp58 (6). In chloroplasts the ribulose-1,5-bisphosphate carboxylase/oxygenase binding protein appears to mediate the assembly of ribulose-1,5-bisphosphate carboxylase/oxygenase, and both large (3) and small (19) subunits have been shown to associate with the binding protein. Assembly of Anacystis nidulans LsSs ribulose-1,5-bisphosphate carboxylase/oxygenase as well as the Rhodospirillum rubrum Ls enzyme expressed in E. coli is facilitated by overexpression of groEL and groES (20). Like hsp58, ribulose-1,5-bisphosphate carboxylase/oxygenase binding protein is homologous to the groEL protein although there is as yet no evidence of enhanced expression of the binding protein as a result of heat shock. The groEL protein and its homologs may recognize exposed hydrophobic surfaces in unfolded proteins as postulated for hsp70 (21).

At least two newly synthesized bacterial proteins, chloramphenicol acetyltransferase and the precursor of β-lactamase (pre-β-lac), transiently associate with the groEL protein in vitro (22). Dissociation of the groEL complex with chloramphenicol acetyltransferase or pre-β-lac, as well as the post-translational translocation of pre-β-lac into inverted vesicles, occurred in the presence of ATP, but not in the presence of a nonhydrolyzable analog of ATP. Similarly, the ribulose-bisphosphate carboxylase/oxygenase binding protein complex with large subunits dissociates in the presence of ATP but not a nonhydrolyzable analog of ATP (23). Since the binding protein binds S (19), as does groEL protein, and since groEL protein is involved in refolding or assembly of a wide range of proteins (22, 24, 25), the ribulose-bisphosphate carboxylase/oxygenase binding protein may also have a broad specificity of protein binding. A generalized activity of protein binding might be involved in the assembly of proteins other than the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase. Experiments designed to test this possibility presently are under way.

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