Assessment of Plant Chaperonin-60 Gene Function in Escherichia coli*

(Received for publication, April 14, 1992)

Lynn P. Cloney, Diana R. Bekkaoui, Maureen G. Wood, and Sean M. Hemmingsen‡

From the Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Saskatchewan S7N 0W9, Canada

Brassica napus chaperonin-60a and chaperonin-60b genes expressed separately and in combination produce three novel Escherichia coli strains: α, β, and αβ. In β and αβ cells, the plant gene products assemble efficiently into tetradecameric cpn60₁₄ species, including novel hybrids containing both bacterial and plant gene products. The levels of authentic groEL₁₄ are reduced in these cells (Cloney, L. P., Wu, H. B., and Hemmingsen, S. M. (1992) J. Biol. Chem. 267, 23327-23332). The assembly of cpn60₁₄ species contains authentic 10-kDa polypeptides (groES₇) and a homotetradecamer of 60-kDa polypeptides (groEL₁₄). After assembly into tetradecameric cpn60₁₄ species, including authentic groEL₁₄ are lower in cells in which efficient assembly of plant chaperonin polypeptides occurs, any increase in the level of cyanobacterial rubisco assembly can be attributed specifically to activity contributed by the plant polypeptides. In αβ cells, p60P-αβ accumulates but is either not assembled into cpn60₁₄ or forms a stable binary complex with groEL₁₄ (groEL₁₄-p60P-αβ). In β and αβ cells, the plant polypeptides assemble efficiently into cpn60₁₄, species, the majority of which are novel hybrid species composed of both bacterial and plant cpn60 polypeptides. The cpn60₁₄ species formed in these cells may or may not include the authentic plant and bacterial forms. As a result, β and αβ cells contain (a) increased levels of total cpn60₁₄, (b) decreased levels of authentic groEL₁₄, and (c) decreased levels of groEL polypeptide present in cpn60₁₄ species of any composition (1).

In the current study, we assessed whether the cpn60₁₄ species containing plant polypeptides are functional as molecular chaperones in E. coli. Genetic experiments have demonstrated that the assembly of cyanobacterial rubisco (EC 4.1.1.39) in E. coli requires the functions of both groEL₁₄ and groES; and further that assembly is limited by normal levels of expression of the groE operon. Increased groE operon expression results in increased assembly and accumulation of cyanobacterial rubisco (2). We have tested the effect of expression of plant chaperonin genes on the capacity of E. coli cells to assemble cyanobacterial rubisco. Since the levels of authentic groEL₁₄ are lower in cells in which efficient assembly of plant chaperonin-60 polypeptides occurs, any increase in the level of cyanobacterial rubisco assembly can be attributed specifically to activity contributed by the plant polypeptides.

**MATERIALS AND METHODS**

Bacterial Growth Conditions—E. coli DH5α cells (14) harboring the plasmids indicated were cultured as described (1). Induction of Ptrc and Plac was as described in the text.

Extraction of Proteins from E. coli—E. coli cells were recovered by centrifugation and resuspended in the buffer used for rubisco activity assays. Cell suspensions were passed through a French pressure cell at 6,000 p.s.i. Soluble protein extracts were defined as the supernatants recovered after centrifugation, 12,000 × g for 15 min, and desalting on Sephadex G-25. Total protein extracts were as previously described (1).

Electrophoresis and Immunoblot Analysis—The analysis was as described (1). Anti-rubisco antiserum has been described (1).

Construction of Brassica napus Chaperonin-60 Expression Vec-

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 306-975-5242; Fax: 306-975-4839.

The abbreviations used are: cpn, chaperonin(s); rubisco, ribulose-P₂ carboxylase.

---

Vol. 267, No. 32, Issue of November 15, pp. 23333-23336, 1992
Printed in U.S.A.
tors—pOF39 (15) was cut with SspI and ligated with a linker to incorporate NeoI and HindIII sites while maintaining the original N terminus of groES. It was cut with HindIII and BamHI, and the fragment which carried the groES gene was ligated to pTZ19R (Pharmacia LKB Biotechnology Inc.) that had been cut with HindIII and BamHI. A second NeoI site was introduced at the initiator Met codon of groEL by site-directed mutagenesis. The resulting plasmid was cut with NeoI, and the fragment that carried the groES gene was cloned into pKKα or pKKβ (1) that had been cut with NeoI. The resulting plasmids were pKKESα and pKKESβ. pKKβ was cut with ScaI, and the fragment which carried the groES gene was cloned into pKKn or pKKB (1) that had been cut with NcoI. The resulting plasmids were pACYCa, pACYCa0, pACYCES, pACYCESn, pACYCESB, and pACYCESnB. pACYCESnB is illustrated (Fig. 1).

Assay of Rubisco Activity—Rubisco activity of soluble protein extracts was assayed as described (18) with modifications. Final conditions in 200-μl assays were: 10–400 μg of protein, 100 mM Tris-Cl, 20 mM MgCl₂, 1 mM EDTA, 1 mM 2-mercaptoethanol, 25 mM NaHCO₃, (14CO₂) specific activity of 1.85 Bq/nmol, 0.4 mM ribulose bisphosphate and NaH₂¹⁰O and were terminated after 10 min.

RESULTS AND DISCUSSION

Effect of Expression of Plant Chaperonin Genes in E. coli on the Capacity to Chaperone the Assembly of Cyanobacterial Rubisco—Plasmids designed to direct the synthesis of one or both of the mature B. napus cpn60 polypeptides (pACYCAα, pACYCAβ, pACYCαβ, and pACYC as control) were introduced into E. coli cells already carrying a plasmid-born cyanobacterial rubisco operon (pDB50 (19)). Transcription of the plasmid-encoded chaperonin and rubisco genes were inducible by isopropylthiogalactoside. The rates of synthesis of the rubisco large and small subunit polypeptides are expected to be identical in each strain. The accumulation of rubisco activity should therefore reflect the capacity of each strain to assemble the enzyme from the available subunits.

In the absence of isopropylthiogalactoside, low levels of rubisco activity were detectable in all cells harboring pDB50. Upon addition of isopropylthiogalactoside, the rates of cell proliferation and the rubisco activity levels recovered varied depending on the plasmid present and on the stage of growth at which isopropylthiogalactoside was added. Therefore, in the following experiments, gene expression was induced when each culture reached an apparent optical density of 0.6–0.8.

α, β, and αβ cells were analyzed 4 h after induction for rubisco activity (Fig. 2A, lanes α, β, and αβ), the presence of immunoreactive LαSβ, rubisco (Fig. 2B), and the accumulation of rubisco large subunit polypeptides (Fig. 2C). Co-expression of the plant cpn60 genes with the rubisco operon markedly affected the accumulation of active rubisco. Nondenaturing polyacrylamide gel electrophoresis analysis confirmed that the relative levels of LαSβ present were consistent with the rubisco activities measured. Thus, assessment of rubisco activity is a valid measure of the accumulation of assembled rubisco enzyme.

![Fig. 1. Schematic representation of expression vector pACYCESαβ](image)

In E. coli. The cyanobacterial rubisco operon and plasmid-encoded chaperonin genes were co-induced in late logarithmic phase. Four hours after induction, total and soluble protein extracts were made. A, rubisco activity expressed as nmol of CO₂ fixed/min for a constant cell number (per A unit). B, soluble proteins resolved by nondenaturing polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with anti-rubisco. Positions of migration of LαSβ, rubisco holoenzyme and cpn60αβ are indicated. C, polypeptides in total protein extracts resolved by SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with anti-rubisco. Position of migration of rubisco large subunit polypeptide (total LSU) is indicated. Also indicated is an unidentified, immunoreactive band (band X) as an indication of relative loadings in each lane. All strains except the negative control contained pDB50. The control strain also contained pACYC, and each of the other strains also contained the indicated chaperonin genes on pACYC-based vectors.

![Fig. 2. Analysis of cyanobacterial rubisco gene expression in E. coli](image)
The levels of total large subunit polypeptide recovered showed a similar pattern of variation as did rubisco activity and immunoreactive L8S protein. Apparently, large subunit polypeptides that were not successfully assembled were subject to proteolysis. It has been reported that cells that assembled increased levels of cyanobacterial rubisco due to increased expression of the \textit{groE} operon contain unchanged levels of rubisco large subunit polypeptides (2). From this, it was concluded that \textit{groE} proteins do not influence the stability of large subunit polypeptides. Our results indicate that the plant chaperonins can influence polypeptide stability. Thus, the conditions of our experiments and those reported previously had different effects on the fate(s) of polypeptides that failed to assemble correctly.

We observed that rubisco accumulation after gene induction varied with time in a strain-dependent manner. Therefore, in an independent experiment, \(\alpha\), \(\beta\), and \(\alpha\beta\) and control cells were harvested 2, 4, 6, and 24 h after induction, and rubisco activity was assayed. 

Rubisco activity was expressed as nmol of CO\(_2\) fixed/min for a constant cell number (per A unit). Rubisco activity increased levels of cyanobacterial rubisco due to increased expression of the \textit{groE} operon when \(\alpha\) cells was similar to that in control cells except after 24 h when higher levels were present in \(\alpha\) cells. Increased rubisco accumulation after 24 h in \(\alpha\) cells was, however, not consistently observed. Thus, p60\(^{\text{cpn60}}\), when it was present alone, did not significantly increase the capacity of \(\alpha\) cells to chaperone the assembly of rubisco. In marked contrast, rubisco accumulation in \(\beta\) cells exceeded that in \(\alpha\) cells at all times. The increase was 2-fold at 2 h, and 4-fold at 24 h after induction. Thus, when present alone, p60\(^{\text{cpn60}}\) contributes significantly to the assembly of rubisco in \(E.\ coli\). When both p60\(^{\text{cpn60}}\) and p60\(^{\text{cpn60}}\) were present together, there was a dramatic decrease in rubisco assembly at all times. The accumulation of active cyanobacterial rubisco differed by 50-fold in \(\beta\) and \(\alpha\beta\) cells.

**Effect of Expression of Plant Chaperonin Genes on the Growth of \(E.\ coli\) Cells**—It might be expected that effects on chaperonin function that resulted in decreased capacity to chaperone the assembly of cyanobacterial rubisco would be generally reflected in the overall chaperonin capacity of that cell. If so, \(\alpha\beta\) cells, which display a severe reduction in the capacity to assemble rubisco, might also be limited for chaperonin functions required for cell viability and growth. Induction of gene expression in \(\alpha\beta\) cells during late logarithmic phase growth did not result in a major change in their growth rate, although these cells did reach a slightly lower stationary phase cell density (Fig. 3B). Induction of gene expression at the time of cell inoculation, however, dramatically altered \(\alpha\beta\) cell growth (Fig. 4A). An extended lag phase was observed under these conditions. Compared with control cells, \(\alpha\beta\) cells required an additional 5 h to reach an apparent absorbance of 0.5. Thus, under these conditions of gene induction, in addition to being dysfunctional for rubisco assembly, \(\alpha\beta\) cells were deficient for some function(s) required for cell proliferation.

Since \textit{in vitro} studies have implicated chaperonin function in the assembly of both chloramphenicol acetyltransferase and \(\beta\)-lactamase \(20, 21\), the effect of the presence or absence of chloramphenicol and ampicillin on the growth of \(\alpha\beta\) cells...
was tested. The extended lag phase of αδ cells when gene expression was induced at inoculation was independent of the presence of these antibiotics (not shown). The extended lag phase cannot, therefore, be attributed to the inability of these cells to chaperone the assembly or secretion of these enzymes that confer antibiotic resistance. Some unidentified activity required for cell proliferation is apparently affected by co-expression of cpn-60α and cpn-60β under these conditions.

Effects of groES Gene Dose—It is anticipated that a chaperonin (cnp10) exists in the plastid, but the gene encoding it has not yet been identified (22). Thus, it is not possible to assess the effect of the presence of a plant cnp10 homolog on plant cpn60 function. In vitro studies of chaperonin function suggest that the capacity to assemble rubisco might be affected by the relative levels of cpn60α and cpn10β in the cell. Therefore, to assess the effect of different levels of cnp10β on the ability of plant cpn60 to chaperone the assembly of rubisco in E. coli, a second series of plasmids was designed to include a copy of the E. coli cnp10α gene, groES. Each of these plasmids (pACYCES, pACYCESα, pACYCESβ, and pACYCESαβ) was introduced into cells that contained pDB50. Expression of the plasmid copies of groES were inducible by isopropylthiogalactoside (not shown). The accumulation of rubisco in these cells is presented in Figs. 2 and 3.

Increased levels of groES in ESα cells led to a decrease in their capacity to assemble rubisco, as compared with control cells. Increased levels of groES in ESβ cells had little or no effect on rubisco assembly at early times as compared with β cells. At later times, however, ESβ cells had accumulated much less rubisco than β cells. Increased levels of groES in ESα cells resulted in a greatly decreased capacity to assemble rubisco at all times as compared with α cells. Remarkably, increased levels of groES in ESαβ cells relieved the suppression of cyanobacterial rubisco assembly that had been observed in αβ cells. Furthermore, ESαβ cells had a greater capacity to assemble rubisco at all times than control cells. Thus, p60groES and p60groEL when present together can assemble into active cpn60α species that chaperone the assembly of cyanobacterial rubisco, but a co-ordinate increase in the level of groES is required for this activity.

Although the suppression of rubisco assembly seen in αβ cells was relieved by elevated expression of groES as in ESαβ cells (Fig. 3A), the extended lag phase observed when gene expression was co-induced at the time of inoculation (Fig. 4B) was not relieved. Thus, although this is a convenient assay for chaperonin function in vivo, the capacity to assemble and accumulate cyanobacterial rubisco does not appear to represent the full range of normal chaperonin functions in E. coli.

Implications—Assessment of the assembly of active cyanobacterial rubisco in E. coli has provided a sensitive in vivo assay for heterologous chaperonin function. Greatly enhanced chaperonin function attributable to the activity of assembled plant cpn60 polypeptide has been demonstrated in αβ cells. Since the majority of cpn60α species in these cells contain both plant and bacterial gene products, it would seem that the enhanced activity is attributable to the hybrid species. The hybrid species also appear to be active with respect to normal groELα functions required for cell viability and growth. These two observations suggest that some functions of p60groES and groEL polypeptides may be interchangeable.

There is evidence that cpn60α species formed in β and αβ cells have different requirements for groES function. GroES function is limiting for cyanobacterial rubisco assembly in αβ cells but not in ESαβ cells. This suggests that the cpn60α species that form in β cells is apparently not limited by the levels of groES polyepptide present. In fact, an increase in groES expression over that present in β cells leads to decreased rubisco assembly, as in ESαβ cells. This suggests that p60groES function may not require groES function. It is tempting to suggest that multiple cpn60α species may exist in the chloroplast and that the requirements for the function of chloroplast groES homologues may differ between them. Differential requirements for groES function have been described for the folding of different polyepptides both in vivo and in vitro (20, 22–26).

Acknowledgments—We are grateful for the assistance of Gina Feist and Darwin Reed and for critical comments of R. John Ellis, Saskia van der Vies, Bill Wu, and Mohan Balasubramanian. We thank Costa Georgopoulos and Saskia van der Vies for providing materials.

REFERENCES

1. Clooney, L. P., Wu, H.-B., and Hemmingsen, S. M. (1992) J. Biol. Chem. 267, 23267-23272.
2. Goloshchikoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 337, 44-47.
3. Ellis, R. J., and van der Vies, S. M. (1991) Annu. Rev. Biochem. 60, 321-350.
4. Ellis, R. J. (1990) Semin. Cell Biol. 1, 1-9.
5. Ellis, R. J., van der Vies, S. M., and Hemmingsen, S. M. (1989) Biochem. Soc. Symp. 55, 145-153.
6. Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988) Nature 333, 330-334.
7. Ellis, R. J., and Hemmingsen, S. M. (1989) Trends Biochem. Sci. 14, 339-342.
8. Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) J. Bacteriol. 171, 1379-1385.
9. Pushkin, A. V., Tauprun, V. L., Sokoljova, N. A., Shobun, V. V., Evstigneeva, Z. G., and Kravtsov, V. I. (1982) Biophys. Biochem. Acta 704, 155-158.
10. Tauprun, V. L., Boekems, E. J., Samsonidze, T. G., and Pushkin, A. V. (1991) FEBS Lett. 289, 205-206.
11. Hemmingsen, S. M., and Ellis, R. J. (1986) Plant Physiol. 80, 269-276.
12. Martel, R., Clooney, L. P., Pelcher, L. E., and Hemmingsen, S. M. (1990) J. Biol. Chem. 265, 181-187.
13. Ohtaka, C., Nakamura, H., and Ishikawa, H. (1992) J. Bacteriol. 174, 3869-3874.
14. Hanahan, D. (1983) J. Mol. Biol. 166, 557-560.
15. Fayet, O., Louarn, J.-M., and Georgopoulos, C. (1986) Mol. Genet. Dev. 122, 430-445.
16. Zumstein, L., and Wang, J. C. (1986) J. Mol. Biol. 191, 333-340.
17. Chang, A. C. Y., and Cohen, S. N. (1978) J. Bacteriol. 134, 1141-1156.
18. van der Vies, S. M., Bradley, D., and Gatenby, A. A. (1986) EMBO J. 5, 2439-2444.
19. Bradley, D., van der Vies, S. M., and Gatenby, A. A. (1986) Philos. Trans. R. Soc. Lond. B Biol. Sci. 313, 447-458.
20. Laminet, A. A., Ziegelhoffer, T., Georgopoulos, C., and Püllchun, A. (1990) EMBO J. 9, 2313-2319.
21. Bokharskaia, E. S., Liisian, N. M., and Girshovich, A. S. (1988) Nature 338, 335-337.
22. Lubken, T. H., Gatenby, A. A., Donaldson, G. K., Lorimer, G. H., and Viitanen, P. K. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7855-7867.
23. Georgopoulos, C., and Argi, D. (1990) Semin. Cell Biol. 1, 19-22.
24. Buchner, J., Schmidt, M., Foeh, M., Jaenicke, R., Rudolph, R., Schmid, F. X., and Kieffaber, T. (1991) Biochemistry 30, 1586-1591.
25. Koppel, P., Litwinska, E., Argi, D., and Georgopoulos, C. (1990) Gene (Amst.) 88, 19-25.
26. Nordan, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 13044-13049.