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The Predominant Protein in an Aphid Endosymbiont is Homologous to an *E. coli* Heat Shock Protein

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Abstract

Bacterial endosymbionts, harbored by aphid fat body cells called bacteriocytes, are indispensable for the reproduction of the host insect. *In vivo* labeling of the endosymbiont with ^{35}S-methionine in the presence of inhibitors of host cell protein synthesis detected only a single radioactive polypeptide of symbionin with a molecular mass of 63 kDa. We localized symbionin in the endosymbiont using antiserum raised against it and show that it is homologous to groEL protein, a heat shock protein of *E. coli* essential for cell viability and bacteriophage capsid assembly. Symbionin may be related to chloroplast and mitochondrial chaperonins, a class of molecular chaperons, required for the assembly of polypeptides into oligomeric complexes.

Abbreviations: SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis

1. Introduction

Aphid species (Homoptera; Aphidoidea) harbor prokaryotic endosymbionts in their bacteriocytes, huge polyploid cells differentiated for this purpose. The aphid enbryos growing parthenogenetically in their mother's ovariole are infected with the symbionts at an early stage of development, and the symbionts

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are thereby transmitted through generations of the host insect without having a free-living stage (Buchner, 1965). In fact, the aphid endosymbiont cannot replicate outside of the bacteriocyte (Lanham, 1968), and similarly,these symbionts are indispensable for the reproduction of the host insect (Kloft, 1977). It has been also suggested that in this endosymbiosis not only low molecular weight metabolites but also proteins are exchanged between endosymbiont and host, similar to the exchange between cell organelles and the nucleus-cytoplasm in an eukaryotic cell (Ishikawa, 1984a; 1989).

In an effort to study such host-symbiont interactions in the bacteriocyte of the pea aphid *Acyrthosiphon pisum*, we suggested that the primary symbiont *in vivo* in the host cell synthesizes essentially the only protein, symbionin (Ishikawa, 1982a, 1984b). This forms a striking contrast with the observation that the symbiont is capable of synthesizing at least several hundred proteins when taken out of the bacteriocyte and incubated *in vitro* (Ishikawa, 1982a). Since symbionin is not only abundant in the endosymbiont but also synthesized highly preferentially by the endosymbiont *in vivo* (Ishikawa, 1982a), it may be a key protein in the maintenance of this symbiotic system. Symbionin is an acidic polypeptide with a molecular weight of about 63,000 when determined on 2D-PAGE (Ishikawa, 1984b).

In the present study, we have purified symbionin from the aphid tissues and characterized its chemical and physical properties. Also, to understand biological functions of symbionin we have searched *E. coli* total proteins for a protein homologous to symbionin. The search was justified by the reported close similarity between the rRNAs of the primary endosymbiont and *E. coli* (Unterman et al., 1989).

2. Materials and Methods

Insect materials

An established parthenogenetic clone of pea aphids, *Acyrthosiphon pisum* (Harris) was maintained on young broad bean plants, *Vicia faba* (L.) at 15°C in a long-day regime with 18 hr light-6 hr dark (Ishikawa, 1982b). The insect materials were stored at −80°C until used.

Isolation of Endosymbionts

Pea aphid endosymbionts were isolated to the method described by Harrison et al. (1989).

Purification of symbionin

Frozen tissues of the pea aphid were homogenized in 0.15 M K-phosphate buffer, pH 6.4, containing 0.3 M KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and centrifuged at 13,700 g for 20 min. Ammonium sulfate was added to the supernatant, and the proteins precipitated between 40% and 50% saturation were recovered as a crude fraction of symbionin. This precipitate was dissolved in 50 mM K-phosphate buffer (pH 6.8), and the solution was dialyzed against the same buffer and then applied to a column of hydroxyapatite. The proteins adsorbed to hydroxyapatite column was eluted with a linear gradient of 50 mM to 250 mM phosphate. SDS-PAGE (Laemmli, 1970) of proteins in the column fractions indicated that most of the symbionin eluted between 120 mM and 160 mM phosphate. The symbionin fractions from the hydroxyapatite column were combined and then dialyzed against 10 mM Tris-HCl (pH 7.2) containing 0.2 M NaCl. the protein was adsorbed to a column of DEAE-Sephacel in the same buffer and symbionin was eluted from the column with a linear gradient of 200 mM to 350 mM NaCl in 10 mM Tris-HCl (pH 7.2). Fractions between 250 mM and 300 mM NaCl contained electrophoretically pure symbionin.

Identification of purified symbionin

Since no biological activity of symbionin had been known, purification of this protein was performed on the basis of its molecular weight on SDS-PAGE. To further identify the purified protein, the protein was mixed with the total aphid proteins and separated two-dimensionally (O'Farrell, 1975). When the total aphid proteins are separated by 2D-PAGE, the spot due to symbionin is easily identified in accordance with the previous results (Ishikawa, 1984b). The spot of symbionin alone became much denser when the purified protein was added, indicating that the protein was really symbionin. About 2.5 mg of purified symbionin was recovered from 50 g of total tissues of the pea aphid.

Purification and identification of GroEL protein

Lysate of *E. coli* LE392 was concentrated by precipitation with 45% saturated ammonium sulfate and then the proteins were centrifuged in a linear gradient of glycerol underlayered with CsCl as described by Hendrix (1979). Fractions containing the groEL protein, which was identified by SDS-PAGE, were combined, and further purified in the purification procedure for symbionin as described above.

For identification for the groEL protein, a 5.2 kb EcoRI-SmaI fragment containing the entire groEL-coding region from the *E. coli groE* operon was recombined into a plasmid vector pUC19 (Yanish-Perron, 1985). The recombinant plasmid pOTKG1 was expressed in *E. coli* JM109. Proteins from the transformed and non-transformed cells were probed with the anti-symbionin anti-serum (see below).

Preparation of anti-symbionin antiserum

Symbionin recovered through a hydroxyapatite column was further purified by SDS-PAGE, and the cell band containing symbionin was excised. The gel piece was homogenized in a minimal volume of 10 mM Na-phosphate (pH 7.4) containing 0.14 M NaCl. The homogenate was mixed with the equal volume of Freund's complete adjuvant, and the emulsion obtained was used as antigen. The antigen was injected hypodermically and intravenously into a male Japan White rabbit that had been raised and kept in a clean room. The blood obtained from the immunized rabbit was clotted overnight at 4°C, and the antiserum was obtained by centrifugation and stored at −80°C.

Immunoblot analysis

Proteins were resolved by SDS-PAGE or 2D-PAGE and transferred to nitrocellulose membrane. Immuno-reactive proteins were probed with anti-symbionin antiserum using biotinylated anti-rabbit IgG and avidin/biotinylated horseradish peroxidase (ABC Reagent, Vector). Bound peroxidase-conjugated antibody was visualized using 4-chloro-1-naphthol/hydrogen peroxidase (Yolken et al., 1983).

Immuno-cytochemistry

Insect tissues that had been pre-fixed in paraformaldehyde, post-fixed in OsO_4, and embedded in a mixture of EM-Spurr set (Nissin EM), were sectioned at 0.5 μm thick on a ultramicrotome (Spurr, 1969). The tissue sections were probed for symbionin with the anti-symbionin antiserum as described above (Yolken et al., 1983; Hsu et al., 1981).

Determination of N-terminal amino acid sequence of symbionin

Symbionin was purified through a DEAE-Sephacel column and its N-terminal amino acid sequence was determined by an Applied Biosystem 470A gas-phase microsequencer with an average repetitive yield of 98.4% in Edman

degradation. The PTH-amino acid obtained from each sequencing cycle was identified by an Applied Biosystem 120A on-line PTH-amino acid analyzer.

3. Results

Antiserum raised against symbionin

Since the starting material for the purification of symbionin was the whole tissue of the pea aphid, it was necessary to show that the purified symbionin was a component of the endosymbiont, as had been suggested in the previous studies (Ishikawa, 1982a; 1984b). For this purpose, proteins from the isolated endosymbionts and the aphid whole tissue were subjected to immunoblot analysis using antiserum raised against the purified symbionin (Fig. 1). It was

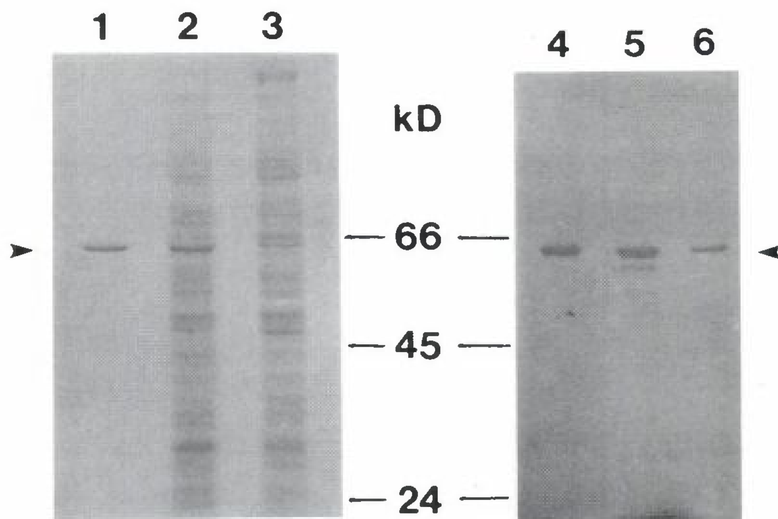

Figure 1. Specificity of antiserum raised against symbionin. Proteins were separated by SDS-PAGE and stained with Coomassie-blue (lanes 1–3) or immunoblotted using the anti-symbionin antiserum (lanes 4-6). 1, 4, Purified symbionin (1.3 μg); 2, 5, proteins from isolated endosymbionts (135 μg). Arrow-heads indicate the positions of symbionin.

found that the anti-symbionin antiserum reacted on a protein shared by the endosymbiont and whole tissue with the same molecular mass as that of symbionin. It was also evident that under the present conditions no other protein in the whole tissue was cross-reactive to the anti-symbionin antiserum. When the proteins from isolated endosymbionts were analyzed, the antiserum seemed to cross-react on some other polypeptides smaller than symbionin (Fig. 1, lane 5). It is likely that these polypeptides were degradation products of symbionin produced during isolating the endosymbionts (Hara et al., 1990).

Immuno-cytochemistry of aphid bacteriocyte

To further test the locality of symbionin and the specificity of antiserum prepared against it, histological sections of the aphid bacteriocyte were stained with the antiserum using an indirect enzyme method (Yolken et al., 1983; Spurr, 1969). Figure 2 represents a whole image of the pea aphid bacteriocyte sectioned at 0.5 μm thick. It was apparent that in the bacteriocyte the endosymbionts were stained specifically and exclusively with the anti-symbionin antiserum (Fig. 2). When sections of the bacteriocyte were treated with preimmune serum in the same manner, no endosymbiont was stained (data not shown).

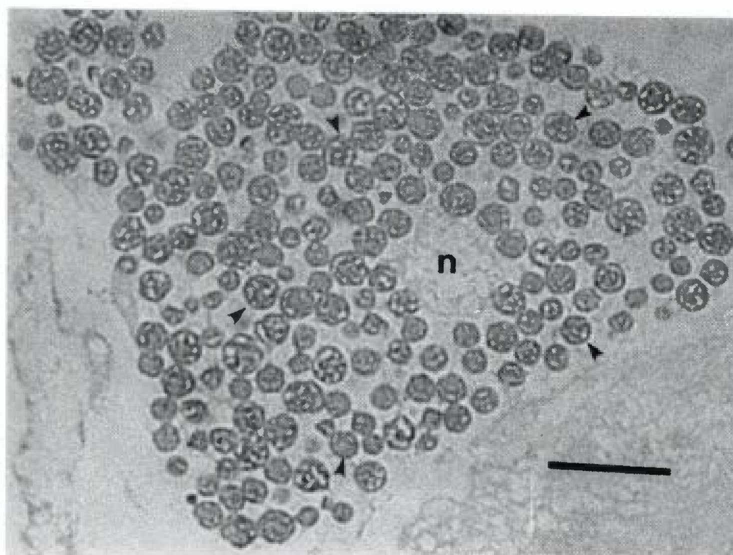

Figure 2. Immuno-cytochemistry of an aphid bacteriocyte using the anti-symbionin antiserum. A whole body of the pea aphid was fixed first in paraformaldehyde and next in OsO_4 and embedded in a mixture of EM-Spurr set (NSA, DER-736, ERL-4206, S-1; S-1; Nissin EM) (Spurr, 1969). Sections at 0.5 μm thick were made using a Porter-Blum MT2B ultramicrotome. A section representing a whole image of the bacteriocyte was stained with the anti-symbionin antiserum by an indirect enzyme method (Yolken et al., 1983). Bar represents 10 μm. Arrow-heads indicate endosymbionts. n, nucleus of the bacteriocyte.

Immunoblotting of E. coli *proteins with anti-symbionin antiserum*

To determine the origin and properties of symbionin, *E. coli* total proteins resolved by 2D-PAGE were probed with the antiserum raised against symbionin. As a result, it was indicated that *E. coli* contained a protein highly reactive to the antiserum. The *E. coli* protein was rich in amount and, like symbionin itself (Ishikawa, 1984b), spotted at about 63 kDa and pH 5.8 on 2D-PAGE (Fig. 3). When *E. coli* total proteins were probed with pre-immune

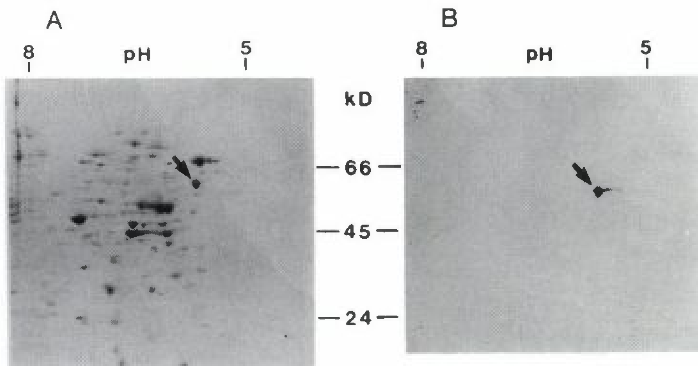

Figure 3. *E. coli* protein reactive to the anti-symbionin antiserum. A, Coomassie blue-staining of total proteins of *E. coli*; B, immunoblotting of the same sample using the anti-symbionin antiserum. Arrows indicate the positions of the immuno-reactive protein.

serum from the same rabbit in the same manner, no immune reactive protein was noticeable (data not shown).

In an attempt to identify this *E. coli* protein, we took notice of the similarity of symbionin in molecular structure (Hara and Ishikawa, 1990) as well as molecular mass and pI on 2D-PAGE (Fig. 3) to the *E. coli* groEL protein, a heat shock protein essential for cell viability and the assembly of bacteriophage capsids (Sternberg, 1973, Georgopoulos et al., 1973; Zweeig and Cummings, 1973).

To examine the similarity, the groEL protein was purified from *E. coli* LE392 by the method of Hendrix (1979) and subjected to immunoblotting with the anti-symbionin antiserum as a probe. As shown in Figs. 4A and B, the groEL protein separated by glycerol gradient and SDS-PAGE was strongly reactive to the anti-symbionin antiserum. The same protein separated by native polyacrylamide gel electrophoresis (Davis, 1964) was also immuno-reactive to the antiserum (data not shown).

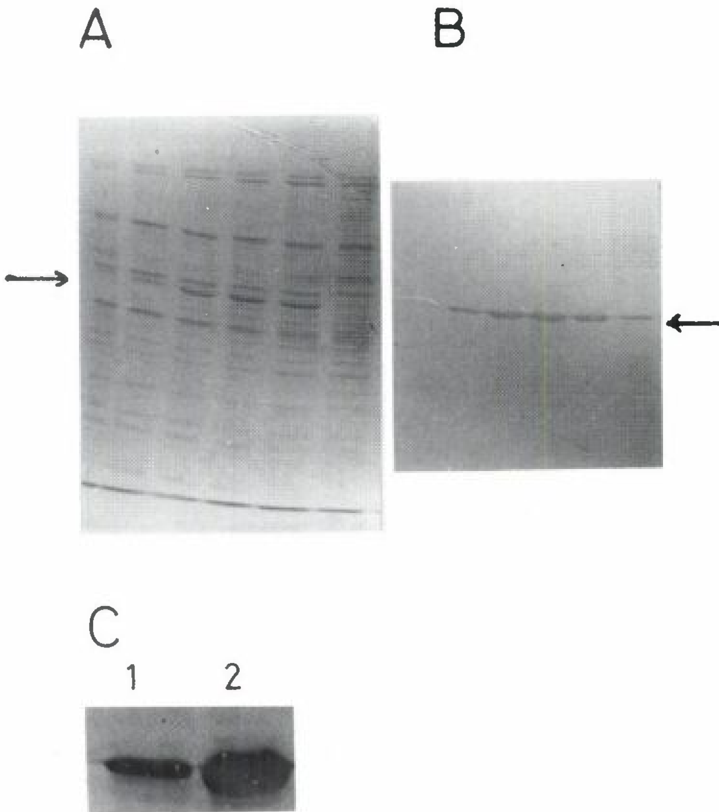

Figure 4. Purification and identification of the groEL protein reactive to the anti-symbionin antiserum. Lysates of *E. coli* LE392 were concentrated by precipitation with 45% saturated ammonium sulfate. Proteins in the lysate were centrifuged in 10%–30% linear gradient of glycerol underlayered with CsCl at a density of 1.6 g/cm^3 at 25,000 rpm for 16 hr in a Hitachi SW25 rotor. Fractions containing the groEL protein were subjected to SDS-PAGE. A, Coomassie blue-staining of groEL-containing fractions separated by glycerol density gradient; B, immunoblotting of the same sample using anti-symbionin antiserum. Arrows indicate the positions of groEL protein. To identify the immuno-reactive protein, an EcoRI-SmaI fragment containing the entire groEL-coding region from the *E. coli groE* operon was recombined into a plasmid vector pUC19 (Yanish-Perron et al., 1985), and the recombinant plasmid pOTKG1 was expressed in *E. coli* JM109. About 1 μg of proteins from non-transformed (lane 1) and transformed cells (lane 2) were separated by SDS-PAGE and immunoblotted with the anti-symbionin antiserum. The panel C represents a relevant part of the photograph.

To further identify the *E. coli* protein reactive to the anti-symbionin antiserum as the groEL protein, the recombinant plasmid pOTKG1 containing the entire groEL-coding region was expressed in *E. coli* JM109 and the proteins were probed with the antiserum. It was evident that the over-expressed protein in *E. coli* JM109/pOTKG1 was highly reactive to the anti-symbionin antiserum (Fig. 4C). Because no other *E. coli* protein was reactive to the antiserum, it was concluded that the groEL protein is immunologically related to symbionin.

N-terminal amino acid sequence of symbionin

To estimate the extent of the similarity of symbionin to the groEL protein, symbionin was purified through a DEAE-Sephacel column and its N-terminal amino acid sequence was determined by Edman degradation. The N-terminal forty amino acid sequence of symbionin was aligned with that of the groEL-coding region (Fig. 5) (Hemmingsen et al., 1988). It was suggested that, as far as the N-terminal forty amino acids were concerned, symbionin differed from the groEL protein in only three positions.

```
     1                                   10                                          20
a    Ala-Ala-Lys-Asp-Val-Lys-Phe-Gly-Asn-Glu-Ala-Arg-Ile-Lys-Met-Leu-Arg-Gly-Val-Asn-
b     -   -   -   -   -   -   -   -   -  Asp  -   -  Val  -   -   -   -   -   -   -

     21                                  30                                          40
a    Val-Leu-Ala-Asp-Ala-Val-Lys-Val-Thr-Leu-Gly-Pro- X -Gly-Arg-Asn-Val-Val-Leu-Asp-
b     -   -   -   -   -   -   -   -   -   -   -   -  Lys  -   -   -   -   -   -   -
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Figure 5. N-terminal amino acid sequence of symbionin in comparison with that of the groEL protein. Symbionin was purified through a DEAE-Sephacel column and its N-terminal amino acid sequence was determined as described under Methods. a, N-terminal amino acid sequence of symbionin, the amino acid position that could not be identified being denoted by X; b, N-terminal region of predicted amino acid sequence based on the nucleotide sequence of the *gro*EL open reading frame (Hemmingsen et al., 1988), only the amino acids that differ from those of symbionin being shown (identities denoted by hyphens).

4. Discussion

One major finding in the present studies is that symbionin, essentially the only protein synthesized by an aphid endosymbiont *in vivo* is highly homologous to the *E. coli* groEL protein. While in the present paper we demonstrated the similarity between the two proteins based on mobility on 2D-PAGE (Fig. 3), immunogenicity (Fig. 4) and the N-terminal amino acid sequence (Fig. 5), the two are also closely related to one another in terms of the amino acid composition, molecular weight and native structure (Hara and Ishikawa, 1990). In the present paper, we also demonstrated that symbionin is really a component of the aphid endosymbiont biochemically (Fig. 1) and cytochemically (Fig. 2) using the antiserum raised against symbionin.

One of the recent findings that is significant from the aspect of cell evolution is that eukaryotic cell organelles contain assembly factors homologous to the *E. coli* groEL protein (Hemmingsen et al., 1988; Chen et al., 1989; Reading et al., 1989; Ellis, 1987; Goloubinoff et al., 1989). The assembly factors include the Rubisco (ribulose-bisphosphate carboxylase) subunit-binding protein of chloroplasts (Barraclough and Ellis, 1980) and a mitochondrial heat-shock protein HSP60 required for the assembly into oligomeric complexes of proteins imported into the mitochondrial matrix (Chen et al., 1989; Reading, 1989). These factors as well as the groEL protein are members of 'chaperonin' class of molecular chaperones (Hemmingsen et al., 1988; Ellis, 1987). The present finding that symbionin is homologous to the groEL protein, therefore, raises the possibility that the protein preferentially produced by the aphid endosymbiont is also a chaperonin. In this context, however, it should be emphasized that the anti-symbionin antiserum did not cross-react on the mitochondrial chaperonins under the present conditions used for immunoblot analysis (Fig. 1) and immuno-cytochemistry (Fig. 2). These results suggest, on one hand, that the symbionin used as an antigen was free of the mitochondrial chaperonins, and, on the other, that symbionin is not as closely related to organellar chaperonins as to the groEL protein. This suggestion is compatible with the result that the yeast mitochondrial HSP60 and the groEL protein share only 53% amino acid similarity (Reading et al., 1989).

It has been suggested that chaperonins occur in all prokaryotes examined and are associated with mitochondria and chloroplasts in widely diverged eukaryotes (McMullin and Hallberg, 1988). It follows that the presence of a chaperonin-related protein in the prokaryotic endosymbiont is not unexpected. It is rather unexpected that this symbiont's protein is highly preferentially synthesized when the symbiont is housed by the bacteriocyte (Ishikawa, 1982a;

1984b). It is a matter of speculation what causes the endosymbiont to synthesize this protein, symbionin, selectively under the conditions. There are two possibilities that are not necessarily incompatible with each other. One is relevant to the fact that chaperonins belong to a class of stress proteins (Hemmingsen et al., 1988). It is possible that the endosymbiont selectively synthesizes symbionin *in vivo* because it is under stress of a certain kind. The other possibility is raised in connection with the observation that the endosymbiont *in vivo* imports many polypeptides synthesized by the host's ribosomes (Ishikawa, 1984a; 1989; 1990). It is conceivable that the aphid endosymbiont *in vivo* synthesizes symbionin to use this protein, just like in the cell organelles (Hemmingsen et al., 1988), to mediate assembly processes of polypeptides imported from the outside (Ellis and Hemmingsen, 1989).

In the light of the endosymbiosis theory, the eukaryotic cell organelles are descendants of certain endosymbionts (Margulis, 1970; 1981). The organelles in widely diverged eukaryotes seem to require chaperonins to assemble polypeptides imported from the cytoplasm (Ishikawa, 1990). In the meantime, the aphid endosymbiont, when housed by an eukaryotic cell, bacteriocyte synthesizes preferentially symbionin, a protein related to a chaperonin, the groEL protein. These facts,taken together, may suggest that the endosymbionts, in common, require the proteins of this class in order to get along under the intracellular conditions.

One important difference between organellar chaperonins and symbionin seems to reside in the locality of their syntheses. While known organellar chaperonins are nuclear-encoded and synthesized by the cytoplasmic ribosomes (Hemmingsen et al., 1988; Chen et al., 1989), symbionin is synthesized by the endosymbiont's ribosomes (Ishikawa, 1982a; 1984b), though the locality of its gene is still unclear (Ishikawa, 1984a).

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