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DIFFERENTIAL PROTEIN PROFILES REFLECT THE DIFFERENT LIFESTYLES OF SYMBIOTIC AND APOSYMBIOTIC *ANTHOPLEURA ELEGANTISSIMA*, A SEA ANEMONE FROM TEMPERATE WATERS

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Summary

Mutualistic associations are prevalent in virtually all environments yet relatively little is known about their complex biochemical and molecular integration and regulation. The endosymbiosis between cnidarians such as the sea anemone *Anthopleura elegantissima* and the photosynthetic dinoflagellate *Symbiodinium californium*, in which the algal symbionts are housed in vacuoles within animal endodermal cells, is an ideal model for the study of highly integrated associations at the biochemical and molecular levels. This study describes differential protein synthesis between symbiotic *A. elegantissima*, collected from environments with high levels of light in the intertidal zone and *A. elegantissima* that naturally lack symbionts (aposymbiotic), collected from nearby deep-shade habitats. Two-dimensional gel electrophoresis profiles of both steady-state and newly synthesized proteins were compared between the two types of animals using scanning densitometry and image analysis. Symbiotic and aposymbiotic animals share a majority of proteins; however, striking differences in several abundant proteins in steady-state profiles occur. Two proteins are unique to symbiotic animals, one at 32 kDa with an isoelectric point (pI) of 7.9 and another at 31 kDa, pI 6.3. Levels of six proteins with an apparent molecular mass of 25 kDa and pI values ranging from 4.8 to 5.5 are greatly enhanced in aposymbiotic animals. Furthermore, profiles of newly synthesized proteins from symbiotic animals contain a unique cluster of proteins ranging from 25 to 30 kDa and pI 6.6 to 6.9. These marked differences in protein profiles must be a reflection either of underlying differences in the regulation of gene expression or in post-translational modification of common proteins. Identifying the symbiosis-specific products present in *A. elegantissima* and identifying the inter-partner signaling and cues that result in differential expression will provide an insight into the understanding of these highly integrated associations.

Key words: symbiosis, *Anthopleura elegantissima*, symbiosis-specific protein synthesis, cnidarian/algal symbiosis.

Introduction

Symbiotic associations between two or more unrelated organisms are found throughout all ecosystems, where they encompass a spectrum of lifestyles ranging from mutualism to parasitism and from extracellular to intracellular relationships. A critical question in the effort to understand the nature of these relationships is whether, and to what extent, the intimate associations between hosts and symbionts result from the interplay between the products of their respective genomes. There is a rich literature describing this interplay between parasitic symbionts and their hosts (see, for example, Buchmeier and Heffron, 1990; Abshire and Neidhardt, 1993; Kantengwa and Polla, 1993). However, there is little information regarding the genetic regulation of mutualistic relationships except in the case of nitrogen-fixing bacteria and their higher plant hosts, where 50 genes in the host plant (nodulin-encoding genes) and 50 genes in the bacterium (*nod* genes) have been identified as essential to the success of the dialogue or 'reciprocal molecular conversation' in establishing a stable, functional root nodule symbiosis (Fisher and Long, 1992; Werner *et al.* 1994).

The importance of symbiotic relationships in biology has led us to undertake an investigation of the biochemical basis of the endosymbiosis between cnidarians and their photosynthetic dinoflagellate algae. Endosymbioses between invertebrates from several phyla and eukaryotic photosynthetic algae occur throughout the marine environment. The algae are thought to contribute significantly to host nutrition by the translocation of reduced organic carbon to the host in return for high concentrations of inorganic nitrogen and an environment safe from herbivores (Falkowski *et al.* 1984). The most significant of these associations is that between members of the phylum Cnidaria, including sea anemones and stony corals, and the dinoflagellate *Symbiodinium* spp. (see, for example, McNally *et al.* 1994). The abundance of these associations in many

tropical and some temperate ecosystems makes *Symbiodinium* an important primary producer and a major contributor to global carbon fixation (Mann, 1982; Muscatine and Weis, 1992).

To date the mechanisms of signaling, regulation and maintenance of these associations at the biochemical and molecular level are largely unexplored. One strategy for uncovering this molecular interplay is to focus on specific genes and gene products that are thought to play key roles in the symbiosis. Another strategy is to describe differences in patterns of proteins synthesized in symbiotic *versus* aposymbiotic animals, thereby leading to the identification of genes and proteins that are unique or enhanced in the association. There is an elegant precedent for this descriptive approach in studies of both parasitic (e.g. Buchmeier and Heffron, 1990; Abshire and Neidhardt, 1993) and mutualistic (e.g. Legocki and Verma, 1980; Govers *et al.* 1985) associations, and it is this method that we have employed in the examination of symbiosis-specific protein production in *Anthopleura elegantissima*, a temperate anemone that harbors the dinoflagellate *Symbiodinium californium* (Banaszak *et al.* 1993). *A. elegantissima* is a major sessile colonial invertebrate inhabiting the rocky intertidal zone of western North America, where it forms large clonal mats up to several meters in width (Buchsbaum, 1968). Animals occurring in sun-exposed locations harbor *S. californium* within their endodermal cells (symbiotic animals), giving them a deep brown color, whereas animals occurring in caves or rock crevices lack symbiotic algae and appear white (aposymbiotic animals; Fig. 1). In this study we demonstrate, using two-dimensional polyacrylamide gel electrophoresis, differential protein production between symbiotic and aposymbiotic *A. elegantissima.*

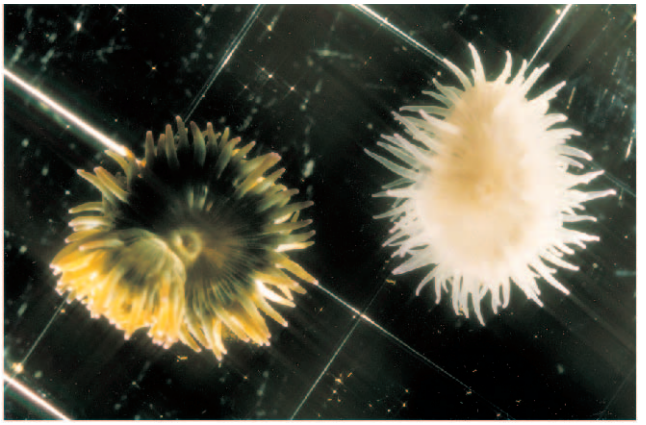

Fig. 1. Photograph of a symbiotic (left) and aposymbiotic (right) *Anthopleura elegantissima* with oral disc diameters of approximately 2 cm. Brown animals were collected from areas in the intertidal zone subjected to full ambient sunlight while white animals were found in areas in the deep shade.

Materials and methods

Maintenance of experimental organisms

Specimens of *Anthopleura elegantissima* (Brandt) were collected near Hopkins Marine Station, in Pacific Grove, CA, and maintained for short periods in tanks of running sea water at ambient temperature (approximately 15 °C). Naturally occurring aposymbiotic animals were collected from the intertidal zone underneath the pilings of the neighboring Monterey Bay Aquarium. Light levels in the deep shade of the building averaged from 40 to $<10\,\mu$mol quanta $m^{-2}\,s^{-1}$. Symbiotic animals were collected from rocks adjacent to the shaded overhang, where animals were exposed to full ambient sunlight, with values of up to $2000\,\mu$mol quanta $m^{-2}\,s^{-1}$ (Zimmerman *et al.* 1994). In the laboratory, aposymbiotic animals were kept in the dark in tanks of running sea water at ambient temperature, whereas symbiotic animals were kept in tanks in full sunlight at ambient temperature. Animals were not fed and were killed no more than 4 days after collection. Experimentally derived aposymbiotic animals were generated by placing symbiotic animals in the dark in running sea water for over 1 year. At the end of this period, the animals appeared white. These animals were fed once or twice weekly with either adult brine shrimp or chunks of frozen squid. Experimentally derived aposymbiotic animals destined for sampling were starved for 1 week prior to homogenization.

Tentacle clips of symbiotic, naturally occurring aposymbiotic and experimentally derived aposymbiotic animals were taken to quantify algal cell numbers in host tissue. The tentacles were homogenized in sea water in microfuge tubes with a plastic micro-pestle, and the homogenates were centrifuged at $900\,\boldsymbol{g}$ in a tabletop centrifuge for 1 min to pellet the algae. The animal supernatant was decanted, and its protein concentration was determined colorimetrically (Bradford, 1976). The algae were resuspended in sea water and quantified using a hemocytometer, and algal cell numbers were normalized to animal protein content.

Preparation of A. elegantissima *homogenates and freshly isolated algal extracts*

Oral discs from *A. elegantissima*, which contain the majority of symbiotic algae and lack the thick mesoglea present in column tissue, were excised and minced with a razor blade. The tissue was then placed in a hand-held Teflon/glass tissue grinder and homogenized on ice in a homogenization buffer composed of $40\,$mmol l^{-1} Tris HCl, $10\,$mmol l^{-1} ethylenediamine tetraacetic acid and $1\,$mmol l^{-1} each of the protease inhibitors phenyl methylesulfonyl fluoride (PMSF), pepstatin, chymostatin and leupeptin, at pH 7.4. Homogenates were then centrifuged for 20 min at $15\,000\,\boldsymbol{g}$ in a microfuge at 4 °C to pellet algae and animal debris. Pellets were inspected for evidence of damaged or lysed algae, and none was detected. The resulting supernatants, containing soluble animal protein, were kept on ice and the concentration of protein was determined (Bradford, 1976).

To determine whether the extraction efficiency between the symbiotic and aposymbiotic animals was the same, the ratio of soluble animal protein to total animal protein was measured and compared between symbiotic states. Animals were

homogenized as described above and centrifuged at 2000 ***g*** for 2 min to pellet the algae from the symbiotic homogenates. The resulting supernatants, containing both soluble and insoluble animal protein, were divided into two parts. To the first sample, sodium dodecyl sulfate (SDS) was added to a final concentration of 1 %, in order to extract most insoluble protein. To the second, an equal volume of distilled water was added. Both samples were centrifuged at 15 000 ***g*** in a microfuge as described above with the resulting supernatants containing total protein for the sample containing SDS and soluble protein for the sample lacking SDS. Protein concentration was determined according to the method of Bradford (1976).

Cell extracts from *S. californium*, freshly isolated from *A. elegantissima*, were obtained by placing 15–20 symbiotic anemones in a blender with 125 ml of filter-sterilized sea water (FSW) and homogenizing them until completely ground (approximately 1 min). The resulting homogenates were centrifuged for 5 min at 900 ***g*** in a tabletop centrifuge to pellet the algae. The large algal pellets were rinsed three times in FSW and centrifuged each time at 900 ***g*** for 2 min. The pellets were resuspended in 50 mmol l^{-1} Tris, 0.45 mol l^{-1} NaCl, 1 mmol l^{-1} PMSF, 0.02 % SDS, 10 mmol l^{-1} dithiothreitol (DTT), pH 7.4, and shaken vigorously and re-pelleted as before. This procedure resulted in clean algal preparations that were free of contaminating animal debris. The algae were resuspended in the homogenization buffer (see above) and lysed by vortexing with glass beads, as described in detail in Weis (1991). The cell extracts were centrifuged at 15 000 ***g*** in a microfuge at 4 °C for 10 min to pellet cell wall debris. The resulting homogenates were deep orange in color. The concentration of protein in the extracts was determined (Bradford, 1976).

^{35}S labeling of A. elegantissima

Symbiotic and aposymbiotic anemones, approximately 1 cm in oral disc diameter, were placed in 10 ml beakers containing 7 ml of FSW and held in a recirculating water bath at 15 °C and allowed to adjust to their surroundings for 1 h prior to the addition of isotope. Symbiotic animals were exposed to 250 μmol quanta m^{-2} s^{-1} of light, which is above the saturation point for photosynthesis in isolated algae from *A. elegantissima* (G. Muller-Parker, personal communication), for the duration of the experiment, whereas aposymbiotic animals were kept in the dark. After the 1 h pre-incubation, 1.85×10^5 Bq of [^{35}S]methionine (Amersham) was added to each vessel and the animals were incubated for an additional 4 h. Each animal was then rinsed thoroughly three times in 10 ml of FSW followed by 10 ml of FSW containing 8 mmol l^{-1} methionine. Animals were then homogenized as described above.

Polyacrylamide gel electrophoresis (PAGE)

For one-dimensional gels, animal and algal proteins were resolved using PAGE in the presence of SDS, under reducing conditions on 12.5 % resolving gels (methods modified from Laemmli, 1970). Two-dimensional PAGE was performed on the Multiphor II System from Pharmacia, according to the manufacturer's instructions (methods modified from O'Farrell, 1975). Samples were loaded onto either small-format (110 mm long) or large-format (180 mm long) precast isoelectric focusing gel strips (Pharmacia), with a pH gradient of 3–10. 35 μg of protein was loaded per sample when comparing steady-state proteins, and equal counts, usually 2×10^5 to 5×10^5 cts min^{-1}, were loaded when comparing ^{35}S-labeled proteins. Duplicate gels were run for each sample. Following electrophoresis in the first dimension, the strips were equilibrated for 20 min in a 50 mmol l^{-1} Tris HCl buffer, pH 6.8, containing 6 mol l^{-1} urea, 2.5 mmol l^{-1} DTT, 12 mmol l^{-1} iodoacetamide, 30 % glycerol and 0.01 % SDS. Electrophoresis in the second dimension was performed on small- or large-format gels, 80 mm with an 8 % to 18 % gradient or 150 mm with a 12 % to 14 % gradient respectively (precast from Pharmacia). Gels of steady-state proteins were silver-stained according to the manufacturer's instructions (methods modified from Heukeshoven and Dernick, 1986). Two-dimensional gel protein standards (Biorad) were resolved on a separate gel, and in each sample, for calculations of apparent molecular masses and isoelectric points (pI). Gels of ^{35}S-labeled proteins were left unstained, fixed in 40 % methanol, 10 % acetic acid for 30 min, and then enhanced with a chemiluminescence autoradiography enhancer (Resolution from Electron Microscopy Sciences) according to the manufacturer's instructions. Following enhancement, gels were dried and exposed to Kodak XAR-5 film at −70 °C. Stained gels and autoradiograms were scanned on an Imagemaster desktop scanner (Pharmacia) and analyzed using Imagemaster software (Pharmacia). Imagemaster scanned a calibration strip with each gel scan and, from this calibration, gel spots were assigned optical density (OD) values ranging from 0.1 to 2.0.

Immunoblots

For immunoblotting, proteins were electrophoretically transferred from unstained gels onto nitrocellulose membranes overnight at 12 °C at a constant current (20 mA) in a 25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 20 % methanol, 0.01 % SDS buffer, pH 8.3 (modified from Towbin *et al.* 1979). Immunoblots were developed with a chemiluminescence detection system (Renaissance Kit, Dupont NEN Research Products). Membranes were first blocked for 1 h at room temperature (22 °C) in 20 mmol l^{-1} Tris, 0.5 mol l^{-1} NaCl, 0.1 % Tween 20, pH 7.5 (TTBS), containing 5 % powdered milk. Following this, they were incubated in a 1:1000 dilution of primary antibody, either rabbit anti-*Chaetoceros gracilis* peridinin–chlorophyll protein (PCP; Orellana *et al.* 1988; gift from G. J. Smith, UCLA) or rabbit anti-*Chaetoceros gracilis* ribulose bisphosphate carboxylase oxygenase (rubisco; Orellana *et al.* 1988; Orellana and Perry, 1992; gift from G. J. Smith) in TTBS for 1 h at room temperature, rinsed in 20 mmol l^{-1} Tris, 0.5 mol l^{-1} NaCl, pH 7.5 (TBS), and subsequently incubated in a 1:1000 dilution of secondary antibody, goat anti-rabbit IgG conjugated to horseradish

peroxidase (Sigma) in TBS. Following a rinse in TBS, the immunoblots were developed according to the manufacturer's directions.

Analysis of free methionine pools in host tissue

Oral discs from animals were homogenized and the resulting extracts were centrifuged as described above. Amino acids present in the supernatant were immediately analyzed by HPLC using a reversed-phase C_{18} column according the methods of Pregnall *et al.* (1984). The quantity of methionine present was calculated from a methionine standard curve and values were normalized to animal wet mass.

Results

Contrasting algal cell numbers in symbiotic versus *aposymbiotic anemones*

Symbiotic and aposymbiotic *A. elegantissima* differ markedly both in their location in the intertidal zone (Buchsbaum, 1968) and in their appearance (Fig. 1), resulting from the vastly differing quantities of symbiotic algae within their tissues. Tentacle clips of symbiotic animals revealed an endoderm full of unicellular brown *S. californium* at a concentration of $1.25\times10^6\pm0.48\times10^6$ cells mg^{-1} animal protein (mean ± S.D., N=5), whereas those from naturally occurring aposymbiotic animals showed no algae in their endoderm (N=5). Despite their prolonged incubation in the dark and their bleached appearance, experimentally derived aposymbiotic animals still contained 1058 ± 218 cells mg^{-1} animal protein (mean ± S.D., N=5).

Comparison of one-dimensional profiles of symbiotic and aposymbiotic anemones and isolated algae

In order to expose the underlying differences in protein synthesis between the symbiotic and non-symbiotic state, we compared steady-state and newly synthesized protein profiles from symbiotic and aposymbiotic anemone tissue using both one-dimensional and two-dimensional PAGE. A comparison of steady-state protein profiles between symbiotic and aposymbiotic animals should reveal gross differences in relatively abundant structural proteins. Structural proteins present exclusively in symbiotic animals might include proteins linked to protective pigments that shield the algae from high light levels or damaging ultraviolet light and cytoskeletal proteins that anchor the symbionts in place within the endodermal cells. One-dimensional SDS–PAGE of symbiotic and aposymbiotic anemone tissue revealed similar protein profiles (Fig. 2A), but with certain marked differences between the two states. For example, a major band at 31 kDa in the symbiotic animal homogenates (arrow, Fig. 2A), which accounts for 5 % of the total protein loaded (determined by densitometry), was all but absent in aposymbiotic animal homogenates and in homogenates of isolated *S. californium*. Other bands at 30, 26 and 19.5 kDa were also present in symbiotic but absent from aposymbiotic homogenates. Conversely, bands unique to aposymbiotic homogenates were detected at 27, 18 and 15 kDa. These differences in profiles were consistent in each of nine comparisons made between symbiotic and aposymbiotic animals.

It is possible that differential protein profiles could be due to differential extraction of proteins during the homogenization of the anemones rather than to real differences between samples. To determine whether extraction efficiency in symbiotic and aposymbiotic animals was the same, we compared the ratio of soluble animal protein to total animal protein between symbiotic states. The soluble protein:total protein ratios were 0.597±0.042 (mean ± S.D.; N=8) for symbiotic animals and 0.592±0.039 (N=8) for aposymbiotic animals. These ratios were not significantly different (t-test, P>0.1), indicating that, when using our homogenization procedure, protein extraction efficiency does not differ between aposymbiotic and symbiotic animals.

Although care was taken to prevent algal cell damage or lysis during animal homogenization, it is possible that differences between symbiotic and aposymbiotic animals could be due in part to algal protein contamination in symbiotic animal tissue. To test for possible algal contamination in symbiotic protein profiles, we probed for the presence of two abundant *Symbiodinium* proteins, PCP and rubisco, in both algal and animal homogenates using immunoblots (Fig. 2B,C respectively). In algal profiles, anti-PCP labeled a strong band at 16 kDa, an apparent molecular mass consistent with dinoflagellate PCP (reviewed in Prezelin, 1987), and anti-rubisco labeled a weak band at 43 kDa, an apparent molecular mass approximately consistent with a wide range of marine phytoplankton rubiscos (Orellana and Perry, 1992). However,

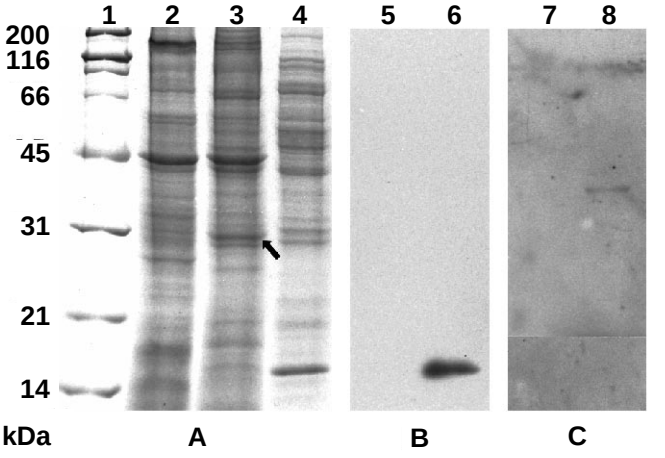

Fig. 2. One-dimensional SDS–PAGE and immunoblots comparing homogenates of symbiotic and aposymbiotic *A. elegantissima* and of freshly isolated symbiotic *Symbiodinium californium*. All lanes except the standard lane contain 15 μg of protein. A one-dimensional gel stained with Coomassie Blue is shown in A; lane 1, molecular mass standards; lane 2, aposymbiotic anemone; lane 3, symbiotic anemone; lane 4, isolated *S. californium*. Immunoblots using anti-PCP and anti-rubisco antibodies shown in B and C, respectively; lanes 5 and 7, symbiotic anemone; lanes 6 and 8, isolated *S. californium*. The arrow highlights the strong 31 kDa band present exclusively in symbiotic anemone homogenate (see text for details).

neither antiserum labeled any bands in symbiotic animal profiles. The failure to detect bands in symbiotic homogenates, even with probes for two of the most abundant algal proteins, suggests that algal protein did not contaminate symbiotic animal homogenates.

It is possible that the observed differences between symbiotic and aposymbiotic animals is due not to the presence or absence of algae but to genetic differences between the two populations. Protein profiles of experimentally derived aposymbiotic animals, animals that were formerly symbiotic, were compared with protein profiles from symbiotic and naturally occurring aposymbiotic animals to determine whether differential patterns were related to the presence of algae in the animal tissues or to genetic differences between symbiotic and aposymbiotic animals. The one-dimensional protein profiles of experimentally derived aposymbiotic anemone tissue differed very little from those of naturally occurring aposymbiotic animals; most notably, the 31 kDa band so prominent in symbiotic animal homogenates was also very faint in experimentally derived aposymbiotic animals (Fig. 3A). Likewise, two-dimensional steady-state protein profiles from the two types of aposymbiotic animals revealed very similar patterns (details from these gels are shown in Fig. 3B). Comparison of spot quantities from these representative gels as detected by image analysis showed that approximately 90 % of the spots were shared between sample types (Table 1). In other studies, symbiotic and aposymbiotic *A. elegantissima* from the same clone, the former growing in high levels of light and the latter in deep shade, have been observed in the field (Buchsbaum, 1968), suggesting that animals can be genetically similar or even identical and exist in different states of symbiosis. Genetic studies, such as DNA fingerprinting, would be useful in definitively determining the relatedness of neighboring symbiotic and aposymbiotic animals.

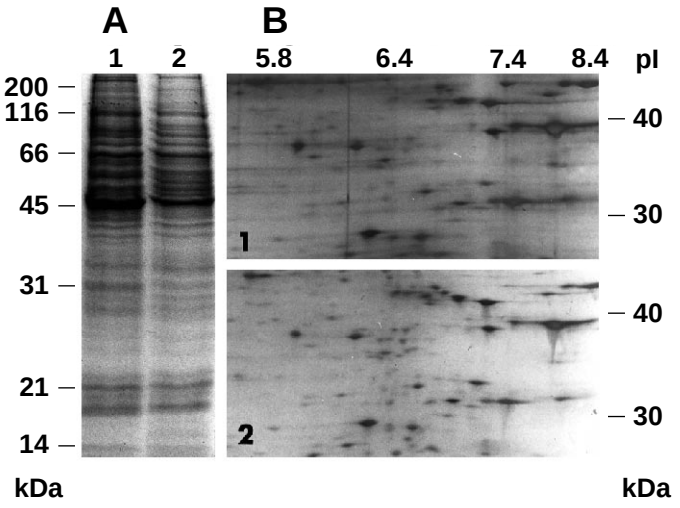

Fig. 3. One-dimensional and two-dimensional PAGE gels comparing naturally occurring and experimentally derived aposymbiotic animals. (A) One-dimensional gel, lanes 1 and 2 are naturally occurring and experimentally derived aposymbiotic animals respectively. 15 μg of protein was loaded per lane and the gel was stained with Coomassie Blue. (B) Two-dimensional gels, panels 1 and 2 are naturally occurring and experimentally derived aposymbiotic animals respectively. 35 μg of protein was loaded per gel and gels were silver-stained.

Comparison of two-dimensional steady-state protein profiles from symbiotic and aposymbiotic anemones

To obtain a more detailed comparison of symbiotic and aposymbiotic animal homogenates, protein profiles were generated by two-dimensional PAGE. Comparisons were made between three symbiotic and three aposymbiotic animals. Duplicate gels of each sample were run to check for reproducibility of the spot patterns. Profiles proved to be highly consistent both when comparing duplicate gels of the same sample and when comparing between different samples of the same state, either symbiotic or aposymbiotic (Table 2). When comparing within a sample type, approximately 90 % of the spots were the same (Table 2). Representative steady-state large-format gels (180 mm×150 mm) of symbiotic and

Table 1. *Comparison of spot numbers from two-dimensional gels of steady-state proteins in naturally occurring and experimentally derived aposymbiotic* Anthopleura elegantissima

	Naturally occurring aposymbiotic	Experimentally derived aposymbiotic
Total spot number	488	512
Spots common to both gels (%)	92	88
Unique spots (%)	8	12

Detail of these gels is shown in Fig. 3.

Table 2. *Comparisons of spot numbers from two-dimensional gels of steady-state proteins in symbiotic and aposymbiotic* Anthopleura elegantissima *samples*

	sym1/sym2	apo1/apo2	sym1/apo1*	sym2/apo2	sym3/apo3
Total spot number	443/428	496/480	443/496	428/480	440/514
Spots common to both gels (%)	89/92	90/93	62/55	61/55	67/57
Unique spots (%)	11/8	10/7	38/45	39/45	33/43

Sym and apo refer to profiles from symbiotic and aposymbiotic animals respectively.
*This pair of gels is shown in Figs 4–6.

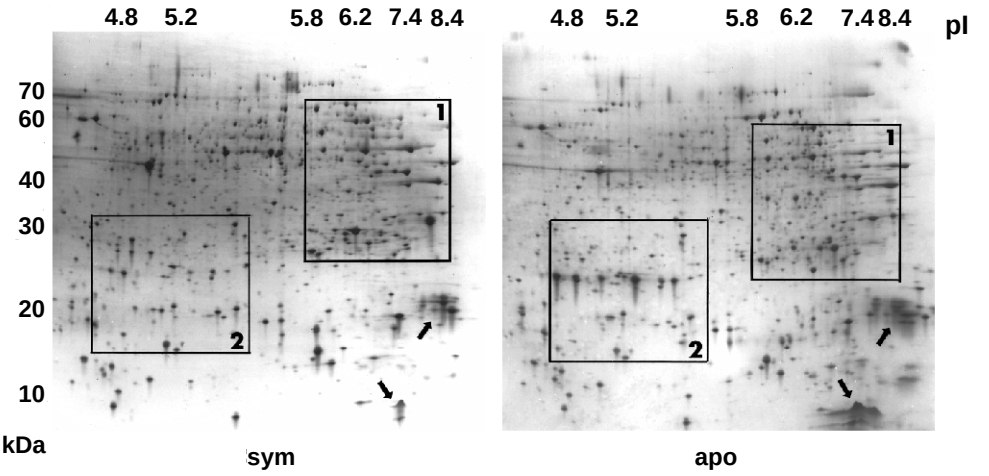

Fig. 4. Silver-stained two-dimensional PAGE gels of steady-state proteins from symbiotic (sym) and aposymbiotic (apo) anemone homogenates. Both gels contain 35 μg of protein. Boxes 1 and 2 are detailed in Figs 5 and 6 respectively. Arrows highlight two clusters of spots that are enhanced in aposymbiotic compared with symbiotic gels.

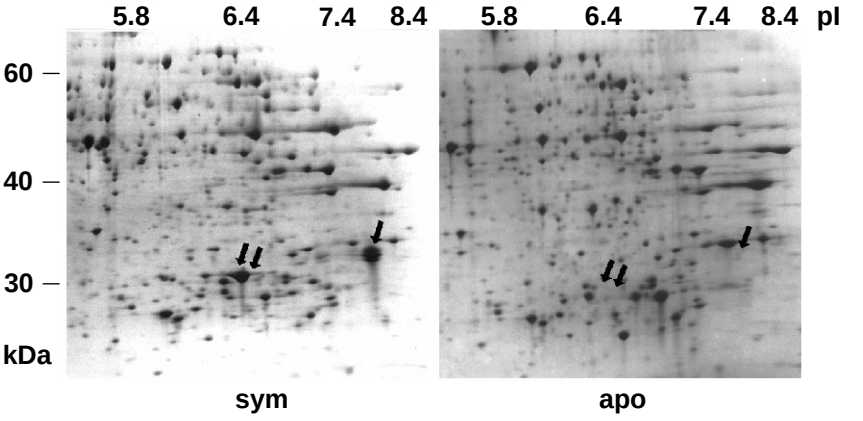

Fig. 5. Detail of two-dimensional PAGE gels of steady-state proteins from symbiotic (sym) and aposymbiotic (apo) anemones; box 1, Fig. 4. The single arrows highlight a protein with an apparent molecular mass of 32 kDa and a pI of 7.9 that is abundant in the symbiotic but almost missing from the aposymbiotic profile, and the double arrows highlight a protein with an apparent molecular mass of 31 kDa and a pI of 6.3, also abundant in symbiotic profiles but absent from aposymbiotic profiles.

aposymbiotic animal homogenates are shown in Fig. 4. The total number of spots, as detected and analyzed by image analysis, for these two gels and for two other pairs are given in Table 2. Aposymbiotic profiles consistently contained approximately 10 % more spots than symbiotic profiles. Although the majority of spots, 55–67 %, were common to both states, a considerable number in each of the profiles were unique to either the symbiotic or aposymbiotic state.

Among the many differences between the symbiotic and aposymbiotic state were several abundant proteins whose appearance in two-dimensional gels corresponded to differences described above in one-dimensional gels. To illustrate these differences, higher magnifications of the boxed areas in Fig. 4 are shown in Fig. 5 (box 1) and Fig. 6 (box 2). Fig. 5 shows that two large spots in the symbiotic profile were absent or highly attenuated in the aposymbiotic profile. One spot having an apparent molecular mass of 32 kDa and a pI of 7.9, had an optical density (OD) of 1.204 in the symbiotic but only 0.150 in the aposymbiotic profile (single arrow). This 32 kDa spot closely corresponds to the 31 kDa band in one-dimensional gels that was very strong in symbiotic but almost absent in aposymbiotic profiles. Upon purification by gel filtration, the native protein has an apparent molecular mass of 32 kDa (V. M. Weis and R. P. Levine, unpublished results).

A second spot in Fig. 5, with an apparent molecular mass of 31 kDa and a pI of 6.3, had an OD of 0.369 in the symbiotic profile but was absent from the aposymbiotic one (double arrow). This spot corresponds to the unique symbiotic band in one-dimensional gels at 30 kDa. The apparent molecular mass and isoelectric point of this protein are markedly similar to those of the enzyme carbonic anhydrase. Indeed, when two-dimensional gel standards containing bovine carbonic anhydrase as one of the markers were added to symbiotic animal homogenate, the carbonic anhydrase marker exactly overlaid this spot. In preliminary studies, an anti-human carbonic anhydrase polyclonal antibody strongly labels this spot on immunoblots (V. M. Weis and R. P. Levine, unpublished data).

There were also several abundant proteins in aposymbiotic two-dimensional profiles that were highly attenuated in corresponding symbiotic ones. Fig. 6 shows a series of six large spots in aposymbiotic homogenates, at an apparent molecular mass of 25–25.5 kDa and ranging in pI from 4.8 to 5.5, that are almost absent in symbiotic homogenates. Whereas the ODs in the aposymbiotic profile range from 0.184 to 0.618, in symbiotic profiles they range from 0 to 0.175. These spots may represent a single protein species that contains several isoforms of differing isoelectric point. Other groups of proteins that are enhanced in aposymbiotic compared with symbiotic profiles occurred in two clusters, one at an apparent molecular

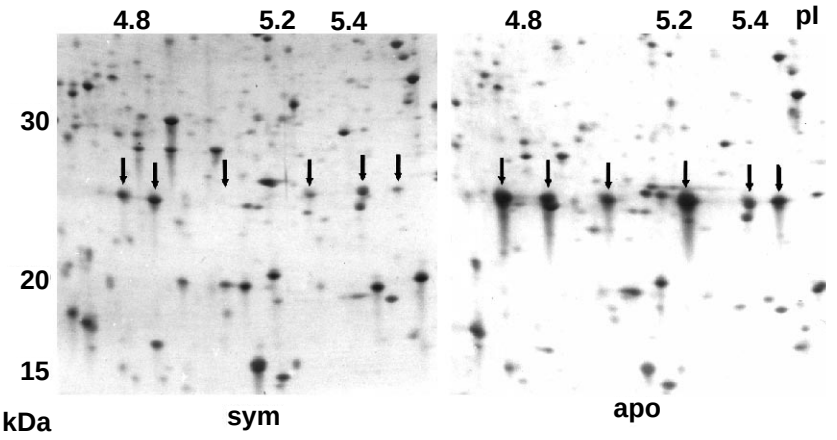

Fig. 6. Detail of two-dimensional PAGE gels of steady-state proteins from symbiotic (sym) and aposymbiotic (apo) anemones; box 2, Fig. 4. Arrows highlight six proteins with an apparent molecular mass of 25 kDa and pI values ranging from 4.8 to 5.5 that are abundant in aposymbiotic profiles but highly attenuated in symbiotic profiles.

mass of 18–21 kDa and a pI of 8.0–8.5 and the other at an apparent molecular mass of 8–10 kDa and a pI of 7.0–7.6 (arrows, Fig. 4).

Free methionine pool sizes in symbiotic and aposymbiotic anemone tissue

It was important to measure the free methionine pools in symbiotic and aposymbiotic animals to determine that potential differences in the uptake of [^{35}S]methionine in protein profiles would be due to differences in protein synthesis rather than to differences in methionine pool size. Two samples were analyzed for free methionine in symbiotic and aposymbiotic animals. Symbiotic animals contained 0.033 and 0.035 μmol g^{-1} wet mass compared with 0.034 and 0.035 μmol g^{-1} wet mass for aposymbiotic animals. These values corresponded well with the methionine pool size of 0.070 μmol g^{-1} wet mass measured in *A. xanthogrammica*, a congener to *A. elegantissima* (Male and Storey, 1983). These results suggest that the symbiotic state does not affect free methionine pool size and, therefore, relative differences in [^{35}S]methionine labeling between symbiotic state can be attributed to differential protein synthesis.

Comparison of two-dimensional profiles of newly synthesized proteins from symbiotic and aposymbiotic anemones

The comparison of newly synthesized proteins between symbiotic and aposymbiotic anemones might reveal differences in proteins with high turnover rates but low relative abundance, such as enzymes and signaling proteins that would function in the molecular crosstalk between host and symbiont. Representative two-dimensional profiles of [^{35}S]methionine-labeled proteins from symbiotic and aposymbiotic animal homogenates are shown in Fig. 7, with the total number of spots for these two gels and another pair of gels given in Table 3. Unlike the steady-state profile comparisons, gels of newly synthesized proteins from the two states contained approximately the same number of total spots. In addition, they also contained a greater percentage of their spots in common, 59–68 %, when compared with the steady-state gels. Most notable among the newly synthesized proteins unique to the symbiotic profiles was a cluster of six proteins ranging in apparent molecular mass from 25 to 30 kDa and in pI from 6.6 to 6.9 (single arrows in Fig. 7). Although the exact apparent molecular mass and pI values of these six proteins varied slightly between symbiotic samples, they were always among the darkest spots in the profiles. Furthermore, these six proteins were never detected in any aposymbiotic samples. This strong protein cluster is not a prominent feature of the steady-state symbiotic profile (Fig. 4), suggesting that it does not consist of abundant, long-lived proteins, but rather contains a set of products with a high turnover rate. Interestingly the 32 kDa, pI 7.9 protein so prominent in steady-state profiles in symbiotic tissue was almost absent in profiles of newly synthesized proteins (double arrow in Fig. 7). Preliminary studies of the purified protein have shown from its amino acid composition that it is methionine-poor (V. M. Weis and R. P. Levine, unpublished data). This would result in a weak ^{35}S signal. Alternatively, the protein could be highly stable and have a low turnover rate, resulting in only a small amount of synthesis during the incubation period.

Table 3. *Comparisons of spot numbers from two-dimensional gels of newly synthesized proteins in symbiotic and aposymbiotic* Anthopleura elegantissima

	sym4/apo4*	sym5/apo5
Total spot number	90/86	102/89
Spots common to both gels (%)	62/65	59/68
Unique spots (%)	38/35	41/32

Sym and apo refer to profiles from symbiotic and aposymbiotic animals respectively

*This pair of gels is shown in Fig. 7.

Discussion

This study is the first in the field of cnidarian–algal symbiosis to document differential protein production in the symbiotic *versus* aposymbiotic state. We have demonstrated (1) that differences in protein profiles are due to differential protein production in host animal tissue and not to contamination from algal proteins that might be present in symbiotic animal homogenates; (2) that two-dimensional

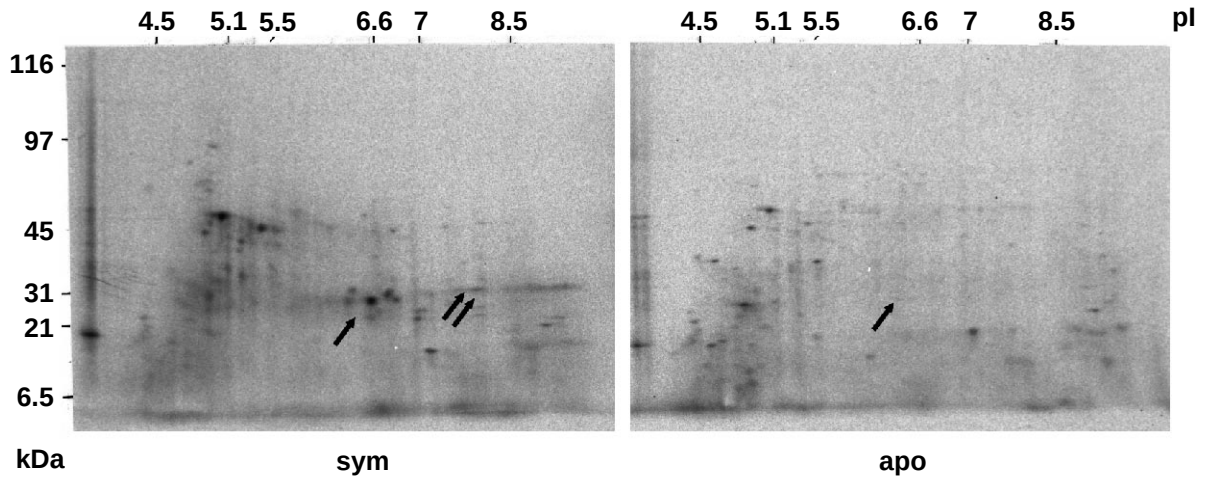

Fig. 7. Autoradiogram of two-dimensional PAGE gels of newly synthesized, ^{35}S-labeled proteins from symbiotic (sym) and aposymbiotic (apo) anemone homogenates. Single arrows indicate the presence in symbiotic profiles and absence from aposymbiotic profiles of a prominent cluster of proteins with an apparent molecular mass ranging from 25 to 30 kDa and pI values of 6.6–6.9. Double arrows highlight the presence of the 32 kDa, pI 7.9, protein that is so abundant in steady-state symbiotic profiles but barely visible in profiles of newly synthesized proteins.

PAGE profiles of steady-state proteins from symbiotic and aposymbiotic anemones have a majority of proteins in common but that numerous differences between the two states are present including unique proteins in symbiotic profiles at 32 kDa, pI 7.9, and 31 kDa, pI 6.3, and a group of six proteins whose synthesis is enhanced in aposymbiotic profiles at 25 kDa, ranging from pI 4.8 to pI 5.5; and (3) that two-dimensional PAGE profiles of newly synthesized proteins also have a majority of proteins in common, but symbiotic profiles contain a unique cluster of proteins around 27 kDa and pI 6.7. The comparative two-dimensional gel approach has proved to be not only highly repeatable but capable of demonstrating even subtle differences between the symbiotic and aposymbiotic state.

These results suggest that the presence of algal symbionts in *A. elegantissima* directly affects host biochemistry. Furthermore, differential protein production must be an indication either of underlying differences in the regulation of the expression of genes that both symbiotic and aposymbiotic animals have in common or of differential post-translational modifications of protein products that result in the differing patterns. The identification of these genes and gene products and an understanding of their function in the symbiosis is the obvious next step in exploring the nature of cnidarian–algal associations.

Studies are under way to identify and characterize the abundant 32 kDa, pI 7.9, protein that is greatly enhanced in symbiotic anemones. The other abundant protein that is unique to symbiotic steady-state profiles, with an apparent molecular mass of 31 kDa and a pI of 6.3, shares these characteristics with the enzyme carbonic anhydrase. Carbonic anhydrase has been studied in some detail in symbiotic cnidarians (Weis *et al.* 1989; Weis, 1991, 1993). High levels of carbonic anhydrase activity occur in symbiotic compared with non-symbiotic species of tropical cnidarians. In addition, carbonic anhydrase, which is thought to aid in inorganic carbon delivery to the algae for photosynthetic carbon fixation, is induced in the tropical anemone *Aiptasia pulchella* upon infection of aposymbiotic animals with algae. Carbonic anhydrase activity in *A. elegantissima* from the Puget Sound is high in symbiotic compared with aposymbiotic anemone homogenates (V. M. Weis and G. Muller-Parker, unpublished data).

Evidence of symbiosis-specific protein synthesis in cnidarian–algal associations

In addition to the demonstration of carbonic anhydrase induction in symbiotic cnidarians described above, there is another example in cnidarian–algal symbioses of specific changes in protein production in the animal host resulting from the presence of the algae. Symbiotic *A. elegantissima* animal tissue contains high levels of superoxide dismutase compared with the levels in aposymbiotic anemones, possibly as an adaptation to neutralize damaging superoxide radicals produced during photosynthesis by its algal symbionts (Dykens and Shick, 1982, 1984).

Furthermore, there are well-described biochemical, physiological and developmental phenomena resulting from the interactions of the two partners that must be orchestrated and controlled by changes in the expression of suites of genes and their encoded proteins. For example, metamorphosis in the jellyfishes *Cassiopeia xamachana* and *Mastigias papua* is induced by the presence of symbiotic algae (Sugiura, 1964; Trench, 1979). The non-symbiotic asexual polyp phase will not metamorphose to produce the symbiotic sexual jellyfish until it is infected with the algae. In another example, symbiotic algae supply host animals with large quantities of reduced organic carbon, mostly in the form of glycerol and alanine (Muscatine, 1967; Trench, 1971). The pathways that handle these compounds, thereby delivering them to the Krebs cycle, are likely to be enhanced in symbiotic compared with non-

symbiotic cnidarians. Still completely undescribed, however, is the inter-partner communication and signaling that must occur both during the onset of the association and throughout its continuing maintenance and the genes and proteins that effect this interplay. In addition, the cytoskeletal matrix that anchors the algal symbionts in the host endodermal cells is likely to consist of proteins that are unique to or enhanced in symbiotic animals. The differential protein synthesis that we describe in this study probably results from the unique or enhanced expression of genes in some or all of these symbiosis-specific phenomena.

Symbiosis-specific gene expression and protein production during the onset of symbiosis

The protein profiles of symbiotic and aposymbiotic *A. elegantissima* provide snapshots taken at the extreme ends of a dynamic process that must occur during the initiation and establishment of the symbiosis. Examining the changes in gene expression that take place during the infection process, by examining both changing patterns of protein production and changes in the expression of specific genes, will provide valuable insight into this largely unexplored process. Interestingly, we have been unable to infect naturally occurring aposymbiotic animals despite trying, over the course of a year, several different infection methods including incubation in the presence of symbiotic animals and injection of isolated algae into the coelenteron. Buchsbaum (1968) reports similar difficulties in infecting aposymbiotic animals in her studies on *A. elegantissima*. However, experimentally derived aposymbiotic animals do become infected after injection with algae or incubation in the light in the presence of other symbiotic animals (V. M. Weis and R. P. Levine, unpublished data). This contrast in infection potential between naturally occurring and experimentally derived aposymbiotic animals suggests that there is a 'developmental window' for algal infection over a limited period of the life history of *A. elegantissima*. Adult animals spawn aposymbiotic gametes that form uninfected planulae (Siebert, 1974). The larvae must, therefore, acquire symbionts anew with each generation. The challenge will be to capture the initiation and establishment processes during natural anemone development and to examine the changes in gene expression and protein production that result.

In conclusion, this study demonstrates that the presence or absence of symbiotic algae in *A. elegantissima* is associated with differential protein production that underlies the highly contrasting lifestyles and appearance of symbiotic and aposymbiotic anemones. The identity of these differing products and how they function in the symbiosis will be critical to understanding the nature of this endosymbiosis. Furthermore, identifying the signals that trigger differential expression will begin to uncover the complex communication process in which the animal and algal partners must engage during the regulation of their highly integrated relationship.

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