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Nitric Oxide and Cnidarian-Dinoflagellate Symbioses: Pieces of a Puzzle[1]

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SYNOPSIS. The presence of nitric oxide synthase (NOS) activity is demonstrated in the tropical marine cnidarian *Aiptasia pallida* and in its symbiotic dinoflagellate algae, *Symbiodinium bermudense*. Enzyme activity was assayed by measuring the conversion of arginine to citrulline. Biochemical characterization of NOS from *Aiptasia* was characterized with respect to cellular localization, substrate and co-factor requirements, inhibitors, and kinetics. In response to acute temperature shock, anemones retracted their tentacles. Animals subjected to such stress had lower NOS activities than did controls. Treatment with NOS inhibitors caused tentacular retraction, while treatment with the NOS substrate L-arginine inhibited this response to stress, as did treatment with NO donors. These results provide a preliminary biochemical characterization of, and suggest a functional significance for, NOS activity in anthozoan-algal symbiotic assemblages.

INTRODUCTION

Nitric oxide (NO)

Over the past several years, NO has been recognized as a molecule of fundamental significance in mediating a variety of interactions between cells. It plays important roles as a weapon in the cellular defenses of metazoans (Nathan and Hibbs, 1991), as a modulatory molecule in the chemosensory modality of olfaction (Breer and Shepherd, 1993), as a mediator of a variety of secretory processes, including those for the export of molecules such as amino acids and peptides, and ions such as bicarbonate (Schmidt and Walter, 1994), and, most famously if not most importantly, as a regulator of vascular processes (Burnett *et al.*, 1992). Although the role of NO in these phenomena has been best characterized in vertebrates, and usually in mammals, NO is implicated in the chemosensory responsiveness of aquatic invertebrates from taxa as diverse as cnidarians (Colasanti *et al.*, 1995), molluscs (Elphick *et al.*, 1995*a*) and arthropods (Elphick *et al.*, 1995*b*). Furthermore, each of the listed functions can be seen as being of potential fundamental importance in the establishment, maintenance, and termination of endosymbiotic relationships such as those that exist between anthozoans and photosynthetic dinoflagellates.

NO differs from many molecules used in cellular signaling in several fundamental aspects. Because it passes through cellular membranes, it cannot be stored in vesicles and exocytosed upon demand. Rather than being made in advance, NO must be generated on an as-needed basis. Furthermore, due to the transparency of biological membranes to this molecule, its many effects are not mediated by non-covalent, spatially complementary interactions with cell surface receptors. Instead, NO exerts its effects by covalently interacting with molecular oxygen, the superoxide anion, transition metals, and thiol groups (Stamler, 1994). Proteins that complex with metal ions or that contain thiol groups are thus candidates for the sites at which NO can exert a physiologically meaningful function.

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Nitric oxide synthase (NOS)

The fact that NO must be produced on an as-needed basis has led to extensive studies of NOS, the enzyme(s) responsible for the generation of this molecule. Although it is now known that there are multiple isoforms of NOS, they are all currently grouped under the single Enzyme Commission number 1.14.13.39. The first NOS to be isolated (Bredt and Snyder, 1990) and cloned, sequenced, and functionally expressed (Bredt *et al.*, 1991) came from a mammalian neural source (rat cerebellum). To perform its function of converting L-arginine to citrulline and NO, this NOS required as cofactors calmodulin, NADPH, and flavin mononucleotides. Analysis of cDNA and deduced amino acid sequences demonstrated that this enzyme had a high degree of resemblance to cytochrome P-450 reductase, an enzyme involved in electron transfer reactions that also requires flavins and NADPH as cofactors.

Subsequently, NOSs from other tissue types and other organisms have been functionally and molecularly characterized. As enough data have accumulated from mammalian sources, comparisons have shown that the functional, antigenic, and sequential similarities between the various NOSs are more striking than the differences (Nathan and Xie, 1994). Enough differences exist, however, to group the NOSs into two categories. One category, which includes the neuronal NOS first characterized by Bredt and colleagues, is constitutively expressed, and is calmodulin dependent. The other category is inducible (or, more accurately, perhaps, subject to dramatic up-regulation) and is not as dependent on calmodulin (Marietta, 1994). Sequential homologies are stronger within constitutive and inducible categories than between them, but the isoforms are still clearly structurally similar. The striking functional and structural conservatism of the NOSs that have been characterized to date make it plausible to hypothesize that many of the NOSs from nonmammalian sources may be similar to them. Studies of NOSs from lower vertebrates (Liepe *et al.*, 1994), molluscs (Elphick *et al.*, 1995*a*), and arthropods (Elphick *et al.*, 1995*b*) so far seem to corroborate this hypothesis.

Chemical mediation of cnidarian-algal symbioses

From the earliest recognition of the existence of cnidarian-algal symbioses, it was hypothesized that the partners in the relationship exchanged chemicals (reviewed by Shick, 1991). Throughout this century, there have been numerous demonstrations of such metabolic interchanges, which have been summarized in a number of recent reviews (Trench, 1971*a*, *b*, *c*; Muscatine and Porter, 1977; Cook, 1983; Shick, 1991). Much of this work has focussed on the transfer of photosynthetically-generated carbohydrate from algal symbiont to cnidarian host. Movement of nitrogen from host to symbiont, usually in the form of ammonium, and the transfer of amino acids between the partners have also been studied. Of particular interest are studies by McCauley (1986, 1987, 1988, 1991) demonstrating that *Chlorella*, the genus of algae that live in symbiosis with *Hydra viridissima*, can internalize exogenous amino acids, and that amino acid transport rates are higher in algae that have been freshly isolated from a symbiotic host than they are in algae that have been maintained in culture. In a related line of investigation, Ferrier (1992) showed that the free amino acid pool of symbiotic dinoflagellate algae (*Symbiodinium*) freshly isolated from the sea anemone host *Aiptasia pallida* was dominated by the basic amino acid arginine, while this amino acid was present in only very low concentrations in conspecific algae that had been maintained in culture.

Compared to studies of nutritional fluxes, the study of chemical interactions between host and symbiont that are fundamentally informational in nature has a shorter and more recent history. Fitt (1984, 1985) has demonstrated that amino acids, presumably host-derived, can serve as chemoattractants for free-living cells of *Symbiodinium microadriaticum* (as all strains of this dinoflagellate were then called); his results can be interpreted as a demonstration of host-to-symbiont chemical communication. A number of investigators have sought to identify

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and characterize a host-derived chemical or mixture of chemicals, known as host factor (HF), that can induce symbionts to export photosynthetically-generated carbohydrate. Their studies have produced a body of contradictory results, with HF being described as being both heat stable and heat labile (Muscatine, 1967; Muscatine *et al.*, 1972; Sutton and Hoegh-Guldberg, 1990), as being both a large and a small molecule (Cook and Orlandini, 1992), as being both present and absent in dinoflagellate-free anemones (Cook and Orlandini, 1992), and as both enhancing and not enhancing carbon fixation (Trench, 1971*c*; Masuda *et al.*, 1994). Gates *et al.* (1995) demonstrated that a crude aqueous extract of tissue from the coral *Pocillopora damicornis* both enhances the fixation of carbon by, and induces the export of fixed carbon from, *Symbiodinium* isolated either from *P. damicornis* or from the sea anemone *Aiptasia pulchella.* They characterized the amino acid composition of this crude extract, and demonstrated that a synthetic free amino acid mixture based upon this composition was capable of mimicking the carbon fixation and exporting effects of the crude extract. In similar experiments, Bester *et al.* (1997) demonstrated that an amino acid mixture qualitatively and quantitatively representative of the free amino acid composition of *A. pallida* is capable of converting the low-capacity, high-affinity amino acid transport of free-living, cultured *Symbiodinium bermudense* cells to the high-capacity, low-affinity transport characteristic of cells of *S. bermudense* that have been freshly isolated from a symbiotic relationship. Experiments such as these have clearly demonstrated that identified chemical signals from the host can induce changes in the cellular physiology of photosynthetic symbionts. The ways in which these signals are transduced by the algae remain to be elucidated. Also unknown is whether chemicals that function as signals, rather than as nutrients, pass from the symbionts to the host organism.

Research goal

The goal of our research is to advance our understanding of the chemical communication between the members of cnidarian-dinoflagellate symbioses. In the following sections, we describe work done to date to test the hypothesis that NO may be an important signal molecule in these assemblages.

MATERIALS AND METHODS

Chemicals

Radiolabeled L-arginine (^{3}H, 40Ci/mmol) was obtained from New England Nuclear, and the liquid scintillation cocktail UltimaGold was from Packard Instrument Corporation. All other reagents were from Sigma Chemical Company.

Organisms

Samples of *A. pallida* were collected from Walsingham Pond, Bermuda. Anemones were maintained at 25°C in 100 ml glass petri dishes in low nutrient seawater (collected from a depth of 10 m in the Sargasso Sea, 175 km southeast of Bermuda) with a 12 hr light/dark photoperiod; they were acclimatized for at least 48 hr before use. Individuals of 5 mm oral disc diameter or above were used for experiments. Symbiotic specimens of *Symbiodinium bermudense* were isolated from *A. pallida* by mechanical homogenization of the symbiotic assemblage, with subsequent removal of the algae from the homogenate by repeated centrifugation and filtration. Algae were ultimately suspended in sterile F/2 medium (Guillard, 1975). Cultured specimens of *S. bermudense* were obtained from the Provasoli-Guillard National Collection of Marine Phytoplankton, and maintained at a concentration of 250,000 cell/ml in sterile F/2 medium at 26°C with a 12 hr photoperiod. These cultures were originated from algae isolated from specimens of *A. pallida* that had been collected from Walsingham Pond, Bermuda.

Determination of NOS activity

Individual anemones were rinsed with 2 ml and homogenized in an equal volume of homogenization buffer (HB: 50 mM HEPES, 1 mM EDTA, pH 7.4) using a 15-ml glass Potter homogenizer. The homogenate was centrifuged for 10 min at 16,000 RCF and the resulting supernatant collected. NOS activity was determined by monitor-

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ing conversion of ^{3}H-arginine to ^{3}H-citrulline using a modification of the method of Bredt and Snyder (1989), as described by Morrall *et al.* (2000). Briefly, 100 μl of homogenate supernatant was brought to 200 μl with HB. The incubation was initiated by adding 100 μl of HB containing cofactors and radiolabel. Final concentrations of cofactors in the incubation were: 1 mM NADPH, 1.25 mM $CaCl_2$, 1 mM dithiothreitol, 10 μM tetrahydrobiopterin, 10 units/ml of calmodulin, and 20 μM ^{3}H-L-arginine. Following incubation at 25°C for 30 min, reactions were terminated by addition of 900 μl ice-cold stop solution (100 mM HEPES, 10 mM EDTA, pH 5.5). Entire incubation volumes were applied to a 1-ml column of Dowex AG50WX–8 (Na^+ form) and ^{3}H-citrulline was eluted with 3 ml ice-cold H_2O with pulse centrifugation at 1,310 × g. A 1-ml aliquot of eluant was mixed with 5 ml Ultima Gold, and ^{3}H-citrulline quantified by liquid scintillation spectrophotometry. Enzyme activity is expressed as pmoles ^{3}H-citrulline formed per μg protein per minute incubation, with protein being quantified by means of the dye-binding assay of Bradford (1976), using bovine serum albumin as standard. The results obtained are expressed as means ± SD of determinations on multiple anemones; typically, each data point represents NOS activity from three specimens. Kinetics of the conversion of arginine to citrulline were investigated with substrate concentrations in the range 0.01–1,000 μM. V_{max} and K_m values were derived by Lineweaver-Burke analysis. Potential inhibitors of NOS activity were included in incubation media at concentrations indicated in legends to figures.

To determine NOS activity in *Symbiodinium*, algae, either isolated from anemones or taken from culture, were first rinsed with ice-cold HB. They were then sonicated in 1 ml of HB, in an ice-jacketed 1.5-ml microcentrifuge tube with a Heat Systems sonicator. The sonicate was centrifuged for 10 min at 16,000 RCF and the resulting supernatant collected. NOS activity was then determined as described above.

Effects of temperature stress on sea anemone behavior and NOS activity

The short-term effects of the acute thermal stressing of animals on NOS activity was investigated by incubating anemones at either 17°C for 120 min, or 33°C for either 30 or 120 min, prior to homogenization and assay; results were compared with those obtained from control animals maintained at 25°C. Long-term effects were investigated by incubating anemones in water that was slowly warmed over a period of several days, prior to homogenization and assay. In both experiments, anemone behavior was observed during these incubations.

Effects of NOS substrate, NOS inhibitors, and NO donors on sea anemone behavior

Effects of NOS inhibitors and NO donors on anemone behavior were examined by observing anemones that were incubated in seawater containing 1 mM concentrations of either the NOS substrate L-arginine, the NOS inhibitor N^G-monomethyl-L-arginine (NMMA), or the NO donor S-nitrosoglutathione (SNOG), at both 25°C and 28°C. Control incubations contained no additions.

RESULTS

Preliminary observations: NOS activity in anemones

In preliminary time-course experiments, the generation of ^{3}H-citrulline was linear with time for up to 40 min using standard reaction mixtures containing 20 μM arginine (data not shown). In subsequent experiments, incubations were standardized at 30 min. The relationship between protein concentration and citrulline generation was linear up to 25 μg protein per assay (Fig. 1; Morrall *et al.*, 2000). Activity was completely abolished by prior heating of the homogenate to 60°C for 2 min (data not shown). The NOS activity of whole and cytosolic homogenate fractions were essentially equal, indicating a predominantly cytosolic location for the enzyme activity (Fig. 2). The kinetics of this enzymatic activity are characterized by a K_m of 19.04 μM and a V_{max} of 2.96 pmol/min/μg protein (Fig. 3; Morrall *et al.*, 2000).

The arginine analogues N^G-monomethyl-

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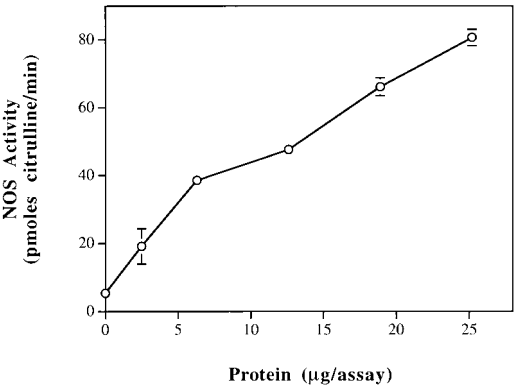

FIG. 1. The effect of protein concentration on production of ^{3}H-citrulline. Points represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means (from Morrall *et al.*, 2000, with permission from Elsevier Science).

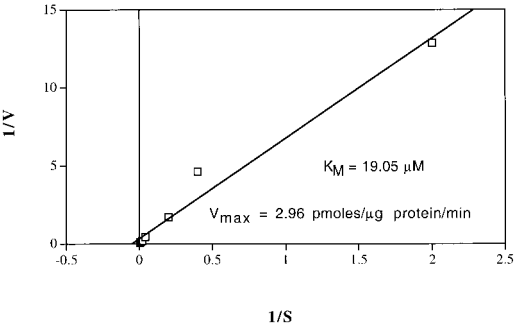

FIG. 3. Kinetic parameters of *A. pallida* NOS activity. Production of ^{3}H-citrulline was measured as a function of ^{3}H-arginine concentration. The Lineweaver-Burke transformation of the resulting data is displayed, along with the resulting estimates of K_M and V_{max} values (from Morrall *et al.*, 2000, with permission from Elsevier Science).

L-arginine acetate (L-NMMA), D-NMMA, N^G-nitro-L-arginine methyl ester (L-NAME), and N^G-nitro-L-arginine (L-NNA), and the arginase inhibitors L-valine and L-ornithine were pre-incubated with anemone cytosol for 10 min at up to 1 mM concentrations, after which NOS activity was determined. The effects of treatment with these potential inhibitors are shown in Figure 4 (Morrall *et al.*, 2000). Formation of ^{3}H-citrulline decreased with increasing concentrations of L-NMMA (Fig. 5; Morrall *et al.*, 2000). Concentrations of L-NMMA of 500 and 1,000 μM reduced citrulline activity by 70% and 75% respectively, while at inhibitor concentrations below 500 μM, little inhibition was evident.

NOS activity levels were optimized by the addition of exogenous NADPH, were unaffected by addition of exogenous tetra-

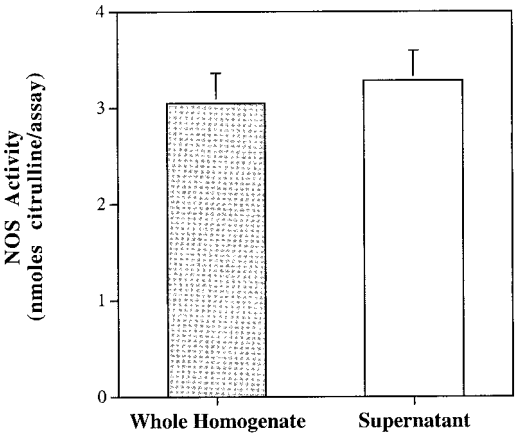

FIG. 2. Localization of NOS activity. Homogenates of *A. pallida* were split into two fractions, one of which was assayed directly (Whole Homogenate), while the other was centrifuged to remove membranous material. Bars represent the means and standard errors of triplicate determinations.

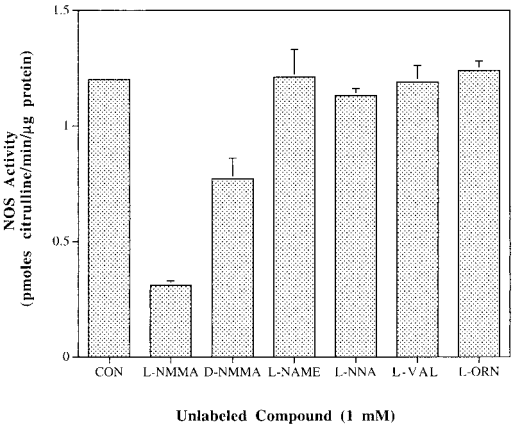

FIG. 4. Effect of arginine analogues and arginase inhibitors on the production of ^{3}H-citrulline. *A. pallida* homogenates were pre-incubated in 1 mM concentrations of each of the indicated compounds for 10 min prior to addition of ^{3}H-arginine and cofactors. Bars represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means (from Morrall *et al.*, 2000, with permission from Elsevier Science).

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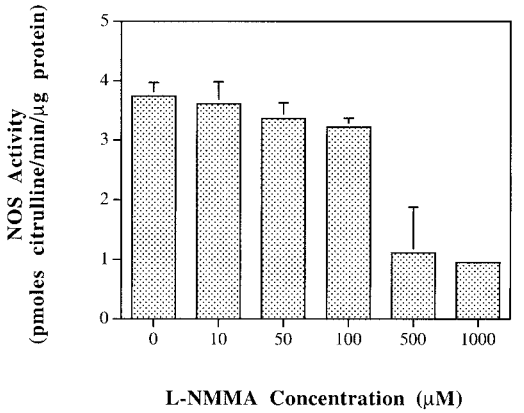

FIG. 5. Effect of increasing concentrations of L-NMMA on ^{3}H-citrulline production. The indicated concentrations of L-NMMA were added to aliquots of cytosol 10 min prior to assays, which were then performed as described in Materials and Methods. Bars represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means (from Morrall *et al.*, 2000, with permission from Elsevier Science).

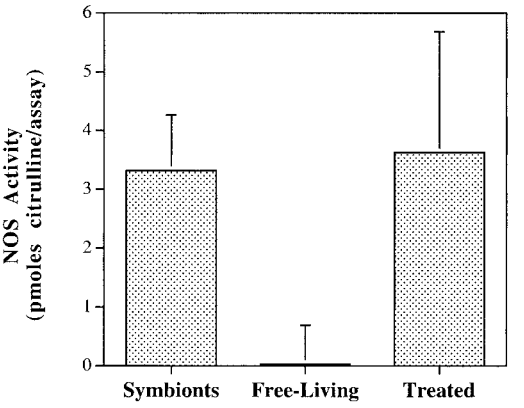

FIG. 6. NOS activity of sonicates of *S. bermudense.* Each bar represents the means and standard errors of triplicate determinations of NOS activity in sonicates of 10^6 algal cells. The bar labeled 'symbionts' represents activity in cells that were freshly isolated from *A. pallida.* The bar labeled 'free-living' represents activity in cells that had been maintained in a symbiosis-free culture in F/2 medium (Guillard, 1975). The bar labeled 'treated' represents activity in free-living cultured cells that had been pre-incubated in a mixture of free amino acids that represents the free amino acid composition of *A. pallida.*

hydrobiopterin, dithiothreitol, or calmodulin, and were reduced by the addition of exogenous $CaCl_2$ (Morrall *et al.*, 2000).

NOS activity in zooxanthellae

Algal cells that had been freshly isolated from *A. pallida* possessed NOS activity, whereas cells that had been maintained in non-symbiotic conditions (in culture) had little or no ability to convert arginine to citrulline (Fig. 6). However, if cultured zooxanthellae were preincubated for 48 hr in an amino acid mixture that mimicked the free amino acid concentration of *A. pallida* (Bester *et al.*, 1997), when assayed they had NOS activity comparable to cells that had just been removed from a symbiotic relationship (Fig. 6).

Effect of temperature stress on NOS activity

Transferring anemones directly from 25°C water to 33°C resulted in a pronounced retraction of the animals' tentacles. Upon homogenization and assay, either 30 min or 120 min after temperature change, NOS activities were significantly lower in temperature-stressed animals than they were in unstressed controls (Fig. 7). The same phenomena of tentacular retraction and lowered NOS activities were seen when animals were subjected to cooler, 17°C water (Morrall *et al.*, 1998).

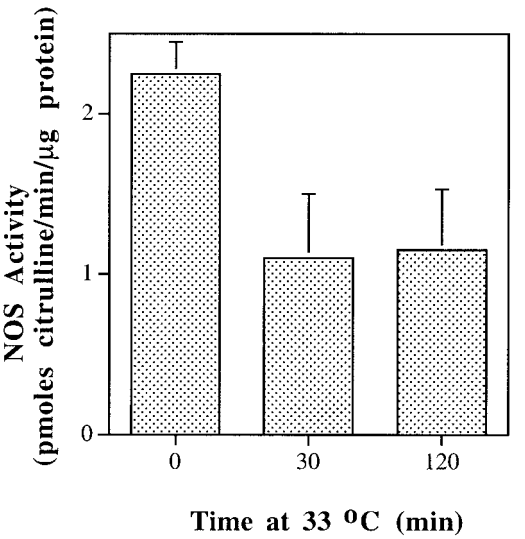

FIG. 7. Effect of an 8°C increase in temperature, for the indicated periods of time, on NOS activity in *A. pallida.* Bars represent the means and standard errors of triplicate determinations.

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TABLE 1. *The effect of L-NMMA, L-arginine and SNOG on* Aiptasia *behavior.**

Condition	25°C	28°C
Control	Fully expanded tentacles.	Tentacles and column contracted. Stubby. Small oral disc.
L-NMMA	Tentacles retracted and more flaccid than control.	Some tentacles fully retracted into column. Oral disc very small.
L-arginine	Tentacles fully expanded. Wide oral disc—not visibly different from control.	Tentacles and column fully expanded. Oral disc widely expanded.
SNOG	Tentacles fully expanded. Wide oral disc—not visibly different from control.	Tentacles and column fully expanded. Oral disc widely expanded.

* Incubations were performed and observations were made as described in *Materials and Methods.* The indicated compounds were present at 1 mM concentrations.

Effects of NOS substrate, NOS inhibitors, and NO donors on anemone behavior

The effects of incubation in the NOS substrate L-arginine, in the NOS inhibitor L-NMMA, and the NO donor SNOG are reported in Table 1. At the ambient temperature of 25°C, organisms incubated in L-NMMA were more retracted than either control anemones or anemones incubated in L-arginine or SNOG. Under conditions of elevated temperature, control anemones retracted their tentacles, whereas animals incubated with either L-arginine or SNOG appeared as fully extended as did animals at ambient temperature.

DISCUSSION

The results presented here and in the work of Morrall *et al.* (2000) indicate that NOS activity is present in the tissue of the tropical anemone *A. pallida*. The activity of the enzyme when measured by the formation of ^{3}H-citrulline from ^{3}H-arginine indicated a basal level in whole body homogenate of 0.50–1.16 pmol/min/μg protein (Morrall *et al.*, 2000), which is similar to levels previously reported in rat heart homogenate using similar methods of detection (Giraldez and Zweier, 1998). The possibility that formation of ^{3}H-citrulline was occurring through the activity of arginase and ornithine transcarbamylase, which can indirectly synthesize L-citrulline from L-arginine (Blachier *et al.*, 1991) was ruled out by the lack of inhibition seen with the arginase inhibitors L-valine and L-ornithine. This is an important consideration when working with a whole body homogenate in which multiple and related enzymatic pathways may be present. The K_m of 19.05 μM calculated for the enzyme was higher than previously reported values of 2.9 μM for mammalian constitutive (Pollock *et al.*, 1991) and 2.5 μM for invertebrate inducible (Colasanti *et al.*, 1995) forms of the enzyme. In addition, the V_{max} value obtained was lower than other reports at 2.96 pmol/min/μg protein. The differences in purification state of the enzyme preparations used in our studies and in those of other workers may account for some of these quantitative differences, and may in fact make comparisons of kinetic parameters from different studies of only marginal value.

The fact that enzyme activity was not affected by pre-treatment with bacterial lipopolysaccharide (Morrall *et al.*, 2000), taken together with the cytosolic location, suggest that the enzyme activity bears some similarity to mammalian neuronal NOS (nNOS). The rank order of inhibition seen for competitive arginine analogues is in agreement with this, although the relative potency of L-NMMA and L-NNA as inhibitors, especially of constitutive nNOS, are reportedly highly dependent on experimental conditions (Klatt *et al.*, 1994). Formation of ^{3}H-citrulline was inhibited by L-NMMA in a dose-dependent manner, with residual activity remaining even in the presence of high concentrations of this inhibitor. This lack of complete inhibition even when L-NMMA is present at concentrations high-

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er than the competing substrate arginine, has been noted by others (Griffiths and Gross, 1996).

Comparison of enzyme activity with and without cofactor addition (Morrall *et al.*, 2000) indicates that 75% activity can be achieved without exogenous cofactor addition (assuming maximum activity is achieved when full cofactor mix is added). These findings suggest that endogenous cofactors are present in the cytosolic fraction in concentrations sufficient to support close to maximal activity. These findings are similar to those of Elphick *et al.* (1993), who found that NOS activity in locust brain extracts was reduced to 80% of the maximum when NADPH was omitted. The reduction in enzyme activity seen when $CaCl_2$ was added is a striking finding, and has been noted by other researchers; Radomski *et al.* (1991) showed an approximately 30% reduction in activity on addition of 200 μM Ca^{2+}. This was interpreted as evidence of the presence of a calcium independent NOS enzyme. Alternatively, such observations are also consistent with an enzyme possessing a biphasic response to this divalent cation. Experiments designed to determine which of these interpretations is correct need to be conducted. In addition, the apparent lack of calmodulin dependency shown in this work warrants further investigation using specific calmodulin inhibitors.

The functional similarities between the invertebrate enzyme reported here and mammalian NOS enzymes is worth noting, separated as they are by 500 million years of evolution. However, a direct comparison to mammalian constitutive and inducible isoforms may be inappropriate. Recent phylogenetic evidence indicates that the gene duplication preceding the evolution of an immunological role for iNOS in mammalian species may have occurred after the divergence of vertebrates and invertebrates (Hughes, 1998). Diversity of function and regulation would then have arisen convergently in these groups.

Although the results presented here give no direct information on the functional role of NOS in *A. pallida*, it is possible to speculate on the likely role of the enzyme based on studies of other invertebrate species. The demonstration by Morrall *et al.* (2000) that NOS activity, as localized by NADPH diaphorase (NADPHd) histochemistry, is distributed throughout the outer epithelial layer as well as in cells of the gastrodermal layer is compatible with a broad involvement of NOS in both sensory and motor activities. Elofsson *et al.* (1993) found only non-selective staining in *Aiptasia* sp. and in another cnidarian, *Hydra oligactis*, and suggested that the distribution of NADPHd positive cells might be restricted to invertebrates having more developed nervous systems. However, in many invertebrate species in which NOS has been studied, the enzyme appears in the central and peripheral nervous system. In molluscs, NO is apparently involved in muscle cell physiology (Martinez, 1995). In addition, individual nitrergic neurons in both the buccal ganglion and cerebral ganglion of *Lymnaea stagnalis* are directly involved in that mollusc's feeding behavior (Korneev *et al.*, 1998, 1999; Park *et al.*, 1998). The polyplacophorans show NADPHd staining only in buccal muscle cells. In other phyla, activity is apparent in the neurons innervating these muscles, which may indicate a switch in the site of NO-mediated control of muscle motility. NOS activity has been shown to be a controlling factor in the olfactory feeding response of the cnidarian *Hydra vulgaris* (Colasanti *et al.*, 1997). The response consists of tentacular writhing and mouth opening and is similar to the feeding response seen in *A. pallida.*

Many of the effects of NO in invertebrates, including phenomena as diverse as control of smooth muscle activity (Elphick and Melarange, 1998), olfaction (Müller and Hildebrandt, 1995), feeding (Colasanti *et al.*, 1995), and learning (Robertson *et al.*, 1996), are thought to be brought about by its activation of soluble guanylyl cyclase (sGC), which, by generating the intracellular second messenger cyclic GMP (cGMP), can control a variety of cellular functions. The NO-sGC-cGMP pathway has been demonstrated in a number of animal taxa, including invertebrates such as cnidarians (Colasanti *et al.*, 1995), annelids (Leake and Moroz, 1996), molluscs (Jacklet and

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Koh, 1999), nematodes (Bascal *et al.*, 1995), arthropods (Müller, 1997), and echinoderms (Elphick and Melarange, 1998), as well as vertebrates (Ishii *et al.*, 1989). It remains to be seen if the NO-sGC-cGMP signaling pathway is involved in physiological responses of *A. pallida* to NO.

To our knowledge, the demonstration of NOS activity in the alga *Symbiodinium bermudense* is the first time that such activity has been described in symbiotic dinoflagellates. It is important to note that the activity induced in cultured zooxanthellae by incubation with a host-mimicking mixture of amino acids cannot be interpreted as activity emanating from residual anemone tissue that could possibly be present in incubations of zooxanthellae freshly isolated from a symbiotic relationship. It is clear that much more work is needed to fully characterize algal NOS activity before its functional significance can be understood with any confidence. If, however, as our bioassays indicate, NO is involved in the extension of this cnidarian's tentacles, it is tempting to speculate that, since the symbiotic algae are present in the highest densities in the anemone's tentacles, the NO that they generate may contribute to this behavior. Pearse (1974*a*, *b*) showed that zooxanthellate specimens of the anemone *Anthopleura elegantissima* expanded their tentacles in conditions of moderate light, and retracted them in bright light or in darkness, whereas azooxanthellate anemones were indifferent to light. Further experiments could test the hypothesis that symbiont-generated NO may be exerting a significant degree of control over the host's behavioral responses to light, an environmental factor of extreme importance to this photosynthesizing symbiotic assemblage.

In conclusion, we offer this characterization of NOS activity in the tropical marine anemone *A. pallida* as an addition to our knowledge of NOSs in invertebrates. Furthermore, the demonstration of NOS activity in the animal's symbionts, coupled with bioassays suggesting that NO may be involved in mediating behavioral aspects of the cnidarian-algal symbiotic assemblage, point towards a new role for the multifaceted signaling molecule NO. As we collect and put into place more pieces of this puzzle, we believe that an interesting and attractive picture of the ways in which NO is involved in cnidarian-algal symbioses will be assembled.

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