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Amino Acid Content of Zooxanthellae Freshly Isolated from *Pocillopora damicornis*[1]

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ABSTRACT: Total amino-N content and glutamine to glutamate ratios (gln : glu) were determined in zooxanthellae freshly isolated from colonies of the coral *Pocillopora damicornis* (Linnaeus) incubated in ambient seawater or in seawater supplemented with ammonium to give a final concentration of 20 or 50 μM. Addition of ammonium did not change total amino-N content but did increase gln : glu from 0.25 to 0.47–0.48, suggesting that ammonium was directly utilized by the symbiotic zooxanthellae. Gln : glu in zooxanthellae from corals maintained in seawater "stripped" of ammonium fell to 0.18. Sizes of pools of most free amino acids in zooxanthellae from *P. damicornis* were roughly two to five times those of zooxanthellae from the temperate sea anenome *Anemonia viridis*, but the latter, which is not believed to be N-limited, exhibited higher gln : glu ratios. These data indicate that gln : glu is a sensitive measure of the response of symbiotic zooxanthellae to exogenous dissolved nitrogen, but despite an increase in gln : glu when seawater is supplemented with ammonium, it cannot be concluded that individual zooxanthellae are normally N-limited.

Several studies have shown that addition of ammonium causes an increase in population density of coral symbionts (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Dubinsky et al. 1990, Stambler et al. 1991, Muller-Parker et al. 1994*b*). However, although increase in population density as a response to elevation of supply of dissolved inorganic nitrogen (DIN) is taken as an indication that the growth rate of populations of symbiotic zooxanthellae is nitrogen-limited (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Szmant et al. 1990), so far little attention has been paid to physiological and biochemical parameters that are indicative of nutrient status in microalgae. Cook and D'Elia (1987) have shown that uptake of the ammonium analog ^{14}C-methylammonium by freshly isolated zooxanthellae was indicative of nitrogen-limited metabolism, but other parameters remain to be investigated. The Hawaii Institute of Marine Biology workshop provided the opportunity to measure both elemental composition of zooxanthellae (Muller-Parker et al. 1994*a*) and size and composition of zooxanthellar amino acid pools.

Size of amino acid pools has been used to determine nitrogen status in a variety of microalgae, including *Chlorella* symbiotic with green hydra (Ohmori et al. 1984, Dortch et al. 1985, McAuley 1987, 1992, Flynn et al. 1989). In particular, it has been pointed out that the ratio of glutamine to glutamate content (gln : glu) is very sensitive to nitrogen status, because ammonium is assimilated into glutamine via glutamine synthetase (GS) (Flynn et al. 1989, Flynn 1990, 1991). In nitrogen-limiting conditions, glutamine levels fall, but glutamate pools appear to be conserved, perhaps because the latter play a central role in aminating reactions. Davidson et al. (1992), studying batch culture growth of the marine phytoplankter species *Isochrysis galbana* Parke, found a drop in gln : glu upon

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exhaustion of the extracellular N source. A similar drop in gln : glu was recorded in cells of a strain of symbiotic *Chlorella* transferred to N-free medium (McAuley 1992). Changes in zooxanthellar glutamine and glutamate pools in response to addition of DIN to the intact symbiosis may also indicate whether or not zooxanthellae directly assimilate DIN.

Here, amino acid levels were measured in zooxanthellae from the coral *Pocillopora damicornis* (Linnaeus) to determine whether change in ammonium concentration caused perturbation of zooxanthellar amino acid pools. The effect of ammonium addition on total pool size and on glutamine : glutamate was determined, and composition of amino acid pools was compared with that of zooxanthellae isolated from the temperate-water symbiosis *Anemonia viridis*.

MATERIALS AND METHODS

The collection and maintenance of colonies of *Pocillopora damicornis* in seawater supplemented with different levels of ammonium or "stripped" by passage through a flume containing macroalgae are described by Stambler et al. (1994). Zooxanthellae were isolated from colonies of *P. damicornis* using a Water Pik (Johannes and Wiebe 1970) and cleaned by centrifugation as previously described (Muller-Parker et al. 1994*b*).

Total amino acid content was estimated from extracts of duplicate aliquots of zooxanthellae. Pellets were extracted with 5% TCA for 1 hr at room temperature, neutralized with NaOH, and amino acid-N content was estimated by a modification of the ninhydrin method of Wylie and Johnson (1962). Absorbance of samples was measured at 570 nm using a spectrophotometer (Hewlett Packard HP 8452A) and compared with that of known amounts of glycine.

Contents of amino acid pools were measured by high performance liquid chromatography (HPLC). Then 100 μl of zooxanthellae suspension (containing between 5×10^4 and 2×10^5 cells) were extracted by adding 400 μl absolute ethanol. Extracts were dried by evaporation and resuspended in 12.5 μM αamino butyric acid (AABA, used as internal standard). Aliquots were prederivatized with *o*-phthaldialdehyde and separated on a Waters Resolve 5 μm C18 column using a methanol : sodium-acetate stepped gradient (Jones et al. 1981). Sample injections were performed using a Rheodyne injection valve equipped with a 20-μl sample loop; the gradient was generated by an HPLC pump (LKB model 2150) and LC controller (model 2152) with a flow rate of 0.8 ml min^{-1}. Derivatized amino acids were detected using a Milton Roy Fluoromonitor III equipped with a 418-nm cut-off filter, and peak areas were integrated with a Spectra Physics Chromjet integrator. Amounts of amino acids were calculated from the fluorescence of samples of 12.5-μM standards run on the same day, corrected for differences in detector sensitivity by reference to the fluorescence of AABA internal standards.

Anemonia viridis was maintained in filtered seawater from St. Andrews Bay, Fife, Scotland, at 15°C in constant light (60 μmol photons m^{-1} sec^{-1}) and were fed to repletion on chopped whitefish meat once per week. Ammonium content of the seawater varied between 2 and 5 μM, higher than that of surface seawater from Kaneohe Bay in which control corals were maintained (Stambler et al. 1994), but lower than levels in supplemented seawater treatments. Zooxanthellae were isolated by homogenization of clipped tentacles, washing three times by centrifugation, and filtration through two layers of 60-μm mesh plankton netting followed by a final washing by centrifugation. Microscopic examination revealed that the resulting pellet was essentially free of animal contamination, particularly nematocysts, but preliminary experiments showed that the animal fraction of the homogenate contained high levels of glycine and taurine, and even small amounts of contamination could give falsely high values for pools of those amino acids in isolated zooxanthellae. To ensure that only amino acids on zooxanthellar internal pools were measured, samples were filtered onto Whatman GF/C filter papers using low vacuum pressure, washed with 20 ml seawater, and extracted in 80% ethanol for amino acid analysis. This

procedure removed contaminating animal material but did not harm the zooxanthellae.

Statistical analysis was performed by one-way analysis of variance using Minitab (Minitab Inc., State College, PA) statistical software.

RESULTS

Total Amino Acids

Measurement of total amino acid content (as amino acid-N) showed no difference between control coral heads (incubated in untreated seawater from Kaneohe Bay) and those incubated in seawater supplemented with 20 or 50 μM ammonium (Table 1). Ammonium treatment did not increase amino acid pools of zooxanthellae in terms of total N content. Indeed, two measurements of total amino acid content of zooxanthellae from colonies incubated in "stripped" seawater showed that this value was higher than in control corals.

Glutamine : glutamate Ratios

Gln : glu ratios were compared in zooxanthellae isolated from colonies in "stripped" or ambient seawater with those from colonies treated with ammonium (Table 1). Ratios in zooxanthellae from untreated corals were between 0.14 and 0.34, but ratios in zooxanthellae from ammonium-treated corals were considerably higher, between 0.30 and 0.58 in zooxanthellae from corals treated with 20 μM ammonium and between 0.34 and 0.57 from corals treated with 50 μM ammonium. Conversely, zooxanthellae isolated from corals incubated in "stripped" seawater exhibited reduced gln : glu ratios (0.14–0.24, mean = 0.18) compared with controls.

Zooxanthellae isolated from corals incubated with either 20 or 50 μM ammonium showed elevated gln : glu ratios within 2 weeks. This is consistent with observations that supply of ammonium caused changes in algal biomass parameters within the first 2 to 4 weeks of addition of ammonium (Muller-Parker et al. 1994*b*). In the case of zooxanthellae from *P. damicornis* treated with seawater containing 20 μM ammonium, elevation in gln : glu was a result of increase in glutamine rather than decrease in glutamate. Glutamine levels increased steadily over the 6 weeks of treatment for which these measurements were made. Comparison of summed means of all 20-μM treatments with controls showed that glutamine levels were significantly increased by ammonium treatment, and there was also a less significant increase in glutamate levels (glutamate, $F = 3.67$, $df = 10$, $P < 0.10$; glutamine, $F = 15.62$, $df = 10$, $P < 0.005$). If it is assumed that ammonium assimilation proceeded via GS, increased amounts of glutamine would be predicated from an increased supply of ammonium, and increased gln : glu in zooxanthellae suggests that they were able to take up ammonium directly and assimilate it into amino acids.

Zooxanthellae from *P. damicornis* incubated in the 50-μM ammonium treatment exhibited increased glutamine levels but decreased glutamate levels compared with controls, although because of high variation in treated samples, in neither case was the difference significant (glutamate, $F = 1.26$, $df = 9$, $P < 0.294$; glutamine, $F = 1.75$, $df = 9$, $P < 0.222$). Although the resulting gln : glu was similar to that of zooxanthellae from *P. damicornis* incubated in 20 μM ammonium, other workers have noted that *P. damicornis* treated at the higher ammonium level exhibited signs of stress (Høegh-Guldberg 1994, Muller-Parker et al. 1994*b*).

Amino Acid Pool Composition

To my knowledge, no analyses of amino acid pools of coral zooxanthellae have been published. Table 2 shows the size of intracellular pools of 16 amino acids from five replicate extracts of zooxanthellae from *P. damicornis* colonies incubated in ambient seawater. Histidine could not be measured because its peak co-resolved with an unknown nonprotein amino acid that has been found in a number of marine phytoplankton species (Flynn and Flynn 1992). As might be expected from its role in primary amino

TABLE 1

TOTAL AMINO ACID-N CONTENT AND GLUTAMINE TO GLUTAMATE RATIOS OF ZOOXANTHELLAE FRESHLY ISOLATED FROM *Pocillopora damicornis*

TREATMENT	TOTAL AMINO ACID-N (PG PER CELL)	GLUTAMATE (FMOL PER CELL)	GLUTAMINE (FMOL PER CELL)	GLN:GLU
Ambient seawater control				
Avg.	0.265	11.12	2.80	0.25
SE	0.017	1.13	0.5	
n	5	5	5	5
20 μM ammonium, 2 weeks				
Avg.	0.277	11.32	5.46	0.48
SE	0.003	1.15	0.79	
n	2	2	2	2
20 μM ammonium, 4 weeks				
Avg.	0.257	15.94	6.89	0.43
SE	0.017	0.53	2.23	
n	2	2	2	2
20 μM ammonium, 6 weeks				
Avg.	0.283	14.70	7.96	0.55
SE	—	0.39	0.29	
n	1	2	2	2
20 μM ammonium, 8 weeks				
Avg.	0.269	—	—	—
SE	0.105	—	—	—
n	2			
50 μM ammonium, 2 weeks				
Avg.	0.263	8.68	4.04	0.45
SE	—	1.30	1.61	
n	1	2	2	2
50 μM ammonium, 6 weeks				
Avg.	0.334	9.65	3.30	0.34
SE	—	—	—	
n	1	1	1	1
50 μM ammonium, 8 weeks				
Avg.	0.224	9.08	4.98	0.56
SE	0.035	4.43	2.39	
n	2	2	2	2
"Stripped," 2 weeks				
Avg.	—	18.29	3.43	0.20
SE	—	2.53	0.30	
n		2	2	2
"Stripped," 6 weeks				
Avg.	—	12.13	2.03	0.17
SE	—	—	—	
n		1	1	1
"Stripped," 8 weeks				
Avg.	0.420	12.91	2.20	0.18
SE	0.026	1.52	0.16	
n	2	2	2	2

acid metabolism, glutamate was the largest component of the total amino acid pool. Amounts of amino acids in free pools were similar to those in zooxanthellae isolated from two species of reef-forming coral in the Caribbean (P.J.M. and V. J. Smith, unpubl. data), but were generally higher than those of zooxanthellae freshly isolated from 1-day starved *Anemonia viridis* (a temperate-water symbiosis). Only glycine, methionine, and taurine levels were similar in the two types of zooxanthellae; *Anemonia* symbionts con-

TABLE 2

Free Amino Acid Content of Zooxanthellae Freshly Isolated from *Pocillopora damicornis* and from *Anemonia viridis*

	Pocillopora ($n = 5$)		*Anemonia* ($n = 4$)	
FREE AMINO ACID	FMOL PER CELL	% TOTAL	FMOL PER CELL	% TOTAL
asp	3.72 ± 0.60	6.93	0.77 ± 0.06	3.30
glu	10.45 ± 0.99	19.45	3.02 ± 0.39	12.87
asn	1.03 ± 0.14	1.92	0.20 ± 0.01	0.85
ser	5.53 ± 1.50	10.29	1.55 ± 0.10	6.60
gln	2.74 ± 0.48	5.10	4.36 ± 0.29	18.53
gly	4.12 ± 0.58	7.66	4.79 ± 1.67	20.39
thr	1.20 ± 0.08	2.23	0.64 ± 0.19	2.73
arg	3.56 ± 0.47	6.62	0.30 ± 0.06	1.29
tau	3.92 ± 0.98	7.29	4.05 ± 1.16	17.23
ala	5.15 ± 0.82	9.59	1.25 ± 0.15	5.33
tyr	1.77 ± 0.20	3.29	0.45 ± 0.04	1.90
met	0.36 ± 0.19	0.68	0.37 ± 0.02	1.56
val	3.15 ± 0.71	5.86	0.76 ± 0.13	3.22
phe	1.69 ± 0.28	3.15	0.46 ± 0.07	1.96
iso	1.23 ± 0.35	2.30	0.23 ± 0.01	0.99
leu	3.10 ± 0.58	5.77	0.29 ± 0.01	1.24
Total	52.72 ± 7.00	100.00	23.50 ± 3.72	100.00

Note: Aliquots of cells were extracted in 80% ethanol, resuspended in distilled water containing 12.5 μM AABA as internal standard, and stored at −40°C for HPLC. Figures are means ± SE; number of determinations are given in parentheses.

tained higher levels of glutamine. The gln : glu ratio in *Anemonia* symbionts was also high: 1.44 compared with 0.26 in zooxanthellae isolated from coral heads incubated in untreated seawater and 0.48 in zooxanthellae from coral heads incubated in 20 μM ammonium. This did not appear to be a result of recent host feeding, because levels remained unchanged during 2 weeks starvation of hosts (unpubl. data).

DISCUSSION

Incubation of colonies of the coral *Pocillopora damicornis* in seawater containing elevated levels of ammonium did not alter total amino acid content of the symbiotic zooxanthellae. This is consistent with the findings of Achituv et al. (1994), who found that protein content of zooxanthellae showed no consistent trend related to ammonium supplementation. However, ammonium supplementation of the seawater in which colonies were incubated did increase the glutamine : glutamate ratio of zooxanthellae. This suggests that the nitrogen metabolism of the zooxanthellae was directly affected by increased levels of ammonium in the seawater, because if it is assumed that assimilation of ammonium proceeds via GS, then elevated levels of ammonium would lead to elevated levels of glutamine. This conclusion is supported by the findings of Muller-Parker et al. (1994*a*), who measured an increase in elemental N content and C : N ratios of zooxanthellae, but not animal tissue of *P. damicornis* incubated in 20 μM ammonium. Zooxanthellae also responded to ammonium supplementation by increased chlorophyll contents (Muller-Parker et al. 1994*a*) and mitotic indices (Høegh-Guldberg 1994).

Although zooxanthellae respond directly to ammonium supplementation in a number of ways, it is not yet clear if they are normally nitrogen limited in the symbiosis. Flynn

(1990, 1991) suggested that gln : glu ratios of <0.2 may be indicative of nitrogen-limited growth in microalgae. Here, mean gln : glu ratios of zooxanthellae isolated from *P. damicornis* ranged from 0.18 ("stripped"), 0.25 (controls), and up to 0.56 (ammonium supplemented). Only zooxanthellae from colonies maintained in "stripped" seawater, which were exposed only to ammonium produced by host catabolism, could be considered N-limited by Flynn's (1990, 1991) criteria. Zooxanthellae isolated from *Anemonia viridis*, which has a symbiont population density similar to that measured in *P. damicornis* (unpubl. observations; Muller-Parker et al. 1994*b*), possessed higher gln : glu than zooxanthellae isolated from *P. damicornis*, even after coral colonies had been incubated in 20 or 50 μM ammonium for 6–8 weeks. Growth of populations of *A. viridis* symbionts does not appear to be N-limited, because expulsion of zooxanthellae by the host is continuous in normal conditions, and both mitotic index and levels of free amino acids do not fall during 2 weeks host starvation in the light (unpubl. observations).

Although availability of ammonium may limit population density of zooxanthellae, as shown by increase in numbers of zooxanthellae per unit area in colonies treated with supplemented seawater (Muller-Parker et al. 1994*b*), tight recycling of N within the symbiosis may mean that although overall population size may be N-limited, individual zooxanthellae may not be physiologically N-limited. Nitrogen status of individual symbionts depends not only upon flux of DIN through the symbiosis, but also upon the population density of symbionts, rates of flux through symbiont amino acid pools (dependent upon rates of protein synthesis and amino acid catabolism), and ability of symbionts to utilize stored N. More observations are required before it can be determined whether or not individual cells of zooxanthellae symbiotic with *P. damicornis* are physiologically N-limited. In particular, it would be of interest to measure changes in amino acid pools of cultured zooxanthellae grown under varying conditions of N-limitation to provide baseline data for comparison with freshly isolated symbionts. However, these preliminary results show that HPLC analysis of amino acid pools of zooxanthellae freshly isolated from corals is a useful and sensitive method to determine response of zooxanthellae to changes in supply of DIN to the symbiosis.

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