Ectoaminopeptidase specificity and regulation in Antarctic marine pelagic microbial communities

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ABSTRACT: Ectoaminopeptidase activities of pelagic marine microbial communities were investigated on several research cruises in the western Antarctic Peninsula region from 1992 to 1994, using the fluorogenic substrate analogue L-leucyl-β-naphthylamine. $K_m$ values at $in situ$ temperature were comparable to those observed by other investigators for a variety of aquatic environments. Competitive inhibition by dipeptides of a variety of amino acids showed that the aminopeptidases present were broadly specific; no strong tendency toward greater affinity for hydrophobic or hydrophilic amino acids was observed. Seawater cultures (1.0 µm filtrate) were inoculated with a variety of monomeric organic compounds and incubated for 24 to 72 h prior to activity determination; histidine and phenylalanine were found to consistently inhibit aminopeptidase expression. It is hypothesized that auxotrophy for histidine and phenylalanine may be widespread in these assemblages, giving rise to high levels of constitutive, nonspecific aminopeptidase activity which results in significant respiration of the more common amino acids. Whatever the exact mechanism, aminopeptidase activity is strongly affected by the specific compounds present and not simply by the carbon-to-nitrogen ratio.

KEY WORDS: Aminopeptidase · Enzyme specificity · Enzyme regulation · Antarctica

INTRODUCTION

Hydrolytic ectoenzymes are of great importance to aquatic microorganisms because a significant fraction of the organic matter in natural waters is present in polymeric form (Sharp 1973, Amon & Benner 1994). Fluorogenic substrate analogues have facilitated the measurement of ectoenzyme activity in natural aquatic habitats (Hoppe 1983, 1963). Among the enzymes most commonly studied, exopeptidases commonly called 'leucine aminopeptidase' appear to be ubiquitous in the sea and play a significant role in microheterotrophic substrate acquisition and biogeochemical cycling (Somville & Billen 1983, Fontigny et al. 1987, Billen 1991, Hoppe 1991, Hoppe et al. 1993, Christian 1993, Christian & Karl 1995a, Martinez et al. 1996). Exopeptidases catalyze the hydrolysis of polymeric amino acids (dipeptides to polypeptides) to monomers. There are many unanswered questions about this class of enzymes in natural microbial communities, including the kinetic response (natural variability and response to natural, ambient substrate), specificity and diversity (one broadly specific enzyme or several highly specific ones), metallic ion requirements and other cofactors, regulation of expression (constitutive or induced), and localization of the enzyme on the cell surface. Furthermore, this enzyme class plays a significant but as yet poorly quantified role in the carbon and nitrogen nutrition of the microbial community, and more generally in nutrient cycling in the pelagic realm. In this paper we present the results of several experiments that address outstanding questions about the nature of the enzyme or enzymes, and, indirectly, the organisms expressing it.

The Palmer Long-Term Ecological Research (LTER) grid extends for 1000 km along the western side of the Antarctic Peninsula, from the edge of the continent to just beyond the shelf break (Quetin & Ross 1992, Smith et al. 1995). Studies of bacterioplankton in this region have shown that their biomass, relative to that of phytoplankton, is less than in other marine and fresh waters (Karl et al. 1991, 1996). The Palmer LTER region
is an upwelling area, with high inorganic nutrient concentrations ([NO$_3^-$] > 20 μM), except during the seasonal phytoplankton blooms, and low organic nutrient concentrations (dissolved organic carbon and nitrogen concentrations are generally less than 70 and 4 μM respectively). Most of the experiments presented here were conducted when [NO$_3^-$] was >20 μM, implying dissolved organic nitrogen concentrations <4 μM (Karl et al. 1996).

Ribosomal rRNA evidence has suggested that Archaea may represent a larger fraction of the microbial community in surface waters in the Antarctic Peninsula region than elsewhere in the ocean (DeLong et al. 1994, Massana et al. in press). Ectoenzyme activities measured in whole water samples cannot be definitely attributed to particular groups of microorganisms. It is probably reasonable to assume that the majority of activity is associated with prokaryotes, but too little is known about Archaea to make informed speculation about their role relative to Bacteria. We therefore refer to enzymatic activity as associated with microbial communities rather than any particular group.

Spatial and temporal variations of in situ leucine aminopeptidase activity in the western Antarctic Peninsula region have been extensively documented (Christian & Karl 1992, 1993, 1994, 1995a, Christian 1995). In this paper we address several unresolved questions about these enzymes. Firstly, $K_m$ determinations show values consistent with those observed in temperate environments (67 to 132 μM) at in situ temperature, but somewhat higher at temperatures above 10°C. Secondly, competitive inhibition of hydrolysis of the model substrate L-leucyl-$\beta$-naphthylamine by dipeptides of a variety of amino acids suggests that hydrolysis of various amino acid linkages is carried out by broadly specific enzymes rather than by a suite of highly specific ones. Finally, ectoaminopeptidase expression appears to be regulated by the availability of specific amino acids even in the presence of abundant C and N in highly labile form. Enzyme expression appears to be repressed by several amino acids known to be rare in seawater (histidine and phenylalanine).

**MATERIALS AND METHODS**

**Study area.** Experiments were conducted in the Antarctic Peninsula region of the Southern Ocean, in the Palmer LTER study area (Quetin & Ross 1992, Smith et al. 1995). RV 'Polar Duke' (PD) and RVIB 'Nathaniel B. Palmer' (NBP) cruise designations were: PD92-09 (November 1992), NBP93-02 (March–May 1993), PD94-01 (January–February 1994) and PD94-12 (December 1994).

**Ectoenzyme assays.** The method of using fluorogenic substrate analogues as tracers for ectoenzymatic activity in natural waters was introduced by Hoppe (1983) and Somville & Billen (1983); a useful summary is given by Hoppe (1993). The substrate analogue used was L-leucyl-$\beta$-naphthylamine (LLBN) (Somville & Billen 1983, Christian & Karl 1995a). The enzyme(s) hydrolyzing LLBN is referred to as leucine aminopeptidase (EC 3.4.1.1) and abbreviated as LAPase. $\beta$-Naphthylamine external fluorescence standards were prepared as described by Christian & Karl (1995b). Fluorescence was determined in a Perkin-Elmer LS-5B spectrophotometer with quartz cuvettes. Excitation and emission wavelengths were 337 and 411 nm respectively, with 10 nm bandwidths.

LLBN was added to 6 ml of seawater and incubated for 12 to 24 h. All incubations were conducted in the dark. In experiments where seawater cultures were incubated with supplemental N compounds prior to activity determination (regulation experiments), LLBN was added at saturating concentration (1 mM). Samples not analyzed immediately following the incubation were poisoned with 0.1 ml of a saturated solution of mercuric chloride (final concentration ~4 mM) to stop the reaction, and stored frozen (Christian & Karl 1995b). Mercuric chloride precipitates excess LLBN which was removed by filtration (0.2 μm, Gelman Accrodisc®) or centrifugation prior to fluorescence determination.

Fluorescence was corrected for nonenzymatic hydrolysis and background fluorescence of the substrate analogue by subtracting the fluorescence of control samples with mercuric chloride added at time zero (Christian & Karl 1995b). Activity in nmol l$^{-1}$ h$^{-1}$ was calculated as $(F_t - F_0)/A/t$, where $F_0$ is the control fluorescence, $A$ is the concentration in mM given by 1 fluorescence (arbitrary) unit and $t$ is the incubation time in hours.

**Specificity.** Specificity experiments were conducted by incubating freshly collected seawater with substratating concentrations of LLBN and dipeptides of various amino acids. All of the dipeptides used were homodipeptides except Ser-Leu (serine N-terminal). Experiments were conducted in both kinetic (with several concentrations of both LLBN and the competing dipeptide) and nonkinetic fashion (with a single concentration of each). In the nonkinetic experiments the concentrations of the dipeptides were approximately 3 times the LLBN concentration. In the kinetic experiments the concentrations were chosen to span the region of saturation response, where competitive effects are most strongly displayed.

**Sample preparation for seawater cultures.** Seawater cultures for ectoenzyme regulation experiments were prepared by filtering freshly collected seawater to
incubate picoplankton in the absence of grazers prior to LAPase activity determination. Seawater was filtered through 1.0 µm polycarbonate membrane filters (Nuclepore) using an enclosed polycarbonate collection vessel (Nalgene). This apparatus was rinsed thoroughly with 10% HCl, then rinsed 2 or 3 times with distilled water and 2 or 3 times with sample water. Organic substrates were added at concentrations much lower than the concentration of LLBN added in the LAPase assay to exclude potential effects of product inhibition.

**Data analysis.** The Henri-Michaelis-Menten equation, and the expanded Henri-Michaelis-Menten equation

\[ V = \frac{V_{\text{max}} S}{K_m + \frac{I}{K_I} + S} \]  

where \( I \) is the concentration of a second, competing substrate and \( K_I \) is the equilibrium constant for its binding to the enzyme, were fit to the data using a nonlinear least-squares (Gauss-Newton) algorithm based on Tarantola (1987) and Johnson & Faunt (1992). This algorithm will converge upon the values of the model parameters that minimize the sums of squares of the residuals, which is equivalent to the maximum likelihood value if the standard assumptions of least-squares are met (errors Gaussian and uncorrelated, errors in independent variable negligible, sufficient data to give representative sample of errors). As with all nonlinear curve-fitting routines, initial parameter estimates must be reasonable for convergence to occur. In most cases values derived from linear transformations (e.g. Lineweaver-Burk) using model I linear regression were adequate.

**RESULTS**

**Temperature and concentration response**

The apparent \( K_m \) for LAPase was in the range of 48 to 218 µM over all temperatures assayed (-1.7 to +20°C) and 67 to 132 µM at \textit{in situ} temperature (-1.7 to +2°C) (Fig. 1). Values at \textit{in situ} temperature fall within the range observed by other investigators for LAPase in seawater (Hoppe 1983, Somville & Bilhen 1983, Fontigny et al. 1987). Values obtained at higher temperatures (i.e. temperatures typical of the \textit{in situ} temperatures in the temperate waters where most other investigators have determined \( K_m \) for LAPase) are somewhat higher. Maximum values were not observed in individual experiments, but a composite of several experiments suggests a maximum at -15°C (Fig. 1). A minimum at 4 to 6°C was observed several times. This cannot be shown to be statistically significant with these data, but it appeared consistently enough to suggest that it was not purely stochastic variability. Other results regarding temperature responses of LAPase in Antarctic microbiota are given by Christian & Karl (1995a).

**Specificity**

The specificity of the enzyme or enzymes hydrolyzing LLBN was investigated in austral autumn 1993 (NBP-9302) by competitive inhibition experiments with selected dipeptides. All of the dipeptides used showed some evidence of inhibition, including D-leucine (Fig. 2). Differences among the various amino acids added were small, and no clear order of relative affinities was observed (Fig. 2).

Double-reciprocal (Lineweaver-Burk) plots followed, in most cases, the pattern expected for competitive inhibition (Fig. 3A). The values of \( K_m \) and \( V_{\text{max}} \) calculated by nonlinear least-squares are suggestive of competitive inhibition, with \( K_m \) increasing in the presence of a competing dipeptide (data not shown). However, the \textit{a posteriori} variances of the model parameter estimates were generally large, and in some cases \( V_{\text{max}} \) also increased. The values of \( V_{\text{max}}, K_m \) and \( K_I \) estimated from the expanded Henri-Michaelis-Menten equation (Eq. 1) predicted the measured activities reasonably well in most cases (Fig. 3B).

**Regulation by organic and inorganic nitrogen sources**

The effects of a variety of nitrogen compounds on LAPase expression were examined by incubating seawater supplemented with these compounds for 24 to
Fig. 2. Rate of LLBN (L-leucyl-b-naphthylamine) hydrolysis in the presence of potentially competing dipeptides of various amino acids. (A) Dipeptide concentrations are 80 μM and LLBN concentration is 35 μM. (B) Dipeptide concentrations are 148 μM and LLBN concentration is 49 μM. All amino acids are L-forms except where specified; there are 2 replicates for each treatment.

Fig. 3. (A) Example double reciprocal (Lineweaver-Burk) plot showing competitive inhibition of L-leucyl-b-naphthylamine (LLBN) hydrolysis by L-Glu-L-Glu. Substrate concentrations (S) are in μM and activities (V) in nmol l⁻¹ h⁻¹. Lines are model I linear regression. (B) Rate of LLBN hydrolysis in the presence of L-Glu-L-Glu (symbols), shown relative to values predicted by expanded Henri-Michaelis-Menten equation (Eq. 1) with model parameters estimated by nonlinear least-squares (curves).

74 h prior to ectoenzyme activity determination, preliminary experiments in November 1992 (PD92-09) showed large increases in LAPase activity over 50 h in filtered (1.0 μm) seawater. Increases similar to those in unsupplemented controls occurred in the presence of supplemental nitrogen as NH₄⁺ (200 μM) or amino acids (glycine and proline added at 100 μM each) (Fig. 4). Therefore, nitrogen availability alone is a poor predictor of LAPase expression.

On subsequent cruises in January–February 1994 (PD94-01) and December 1994 (PD94-12), the regulatory effects on LAPase of a wider variety of nitrogen compounds, mostly amino acids, were tested. Histidine, and to a lesser extent phenylalanine, significantly repressed LAPase expression, relative to glycine or glucose added at identical molar concentrations of C and N (additional N added as NH₄Cl where required) (Fig. 5). Other amino acids including tyrosine and leucine had no effect even at concentrations that should provide a more than adequate source of organic N in a highly labile form (data not shown). The effect of incubation with added histidine was highly consistent and reproducible (Table 1). It was observed in unfiltered seawater (i.e. in the presence of grazing microzooplankton), but the percent difference, relative to the controls, was less than in 1.0 μm filtered seawater (Table 1).

**DISCUSSION**

Aminopeptidase activity has been shown to be widespread if not ubiquitous in the marine environment and among marine Bacteria (Christian 1995, Christian & Karl 1995a, Martinez et al. 1996), but little is known...
about the enzyme or enzymes involved. We have referred to the enzyme(s) hydrolyzing LLBN as 'leucine aminopeptidase' (LAPase), implying an enzyme that most rapidly cleaves leucine residues but with relatively broad specificity (Delange & Smith 1971). Broadly specific enzymes should be evolutionarily advantageous in this context (ectoenzymes whose function is to get nutrients from the medium). There is some suggestion in the competitive inhibition experiments described that LAPases in these microbial communities may have greatest affinity for acidic residues (Fig. 2B), as for example aminopeptidases A (Delange & Smith 1971). However, this result is not statistically significant. In addition, competitive inhibition of LLBN hydrolysis by these dipeptides demonstrates only that they bind to the active site; it is only assumed that they are hydrolyzed.

A variety of N-terminal aminopeptidases have been isolated from Bacteria (e.g. Prescott & Wilkes 1966, Vogt 1970, Prescott et al. 1971). *Escherichia coli* has at least 5, of which 3 have broad specificity (Miller 1987). The basic structure of these enzymes is likely to be evolutionarily ancient and common to diverse types of organisms (cf. Kim & Lipscomb 1994). That such an enzyme is broadly distributed among native aquatic prokaryotes is suggested by the similar $K_m$ values observed in widely separated regions of the ocean (Somville & Billen 1983, Fontigny et al. 1987, Christian

**Table 1. Percent inhibition of LAPase activity by added histidine (His), relative to unamended controls. Samples filtered (1.0 μm) unless otherwise noted.**

<table>
<thead>
<tr>
<th>Date</th>
<th>Preincubation (h)</th>
<th>[Histidine] (μM)</th>
<th>LAPase (% of control)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan 1994</td>
<td>24</td>
<td>80</td>
<td>52</td>
<td>(His + heat-sterilized brown ice), vs unamended control</td>
</tr>
<tr>
<td>Jan 1994</td>
<td>72</td>
<td>80</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Jan 1994</td>
<td>48</td>
<td>80</td>
<td>49</td>
<td>(His + heat-sterilized brown ice), vs ice only</td>
</tr>
<tr>
<td>Jan 1994</td>
<td>48</td>
<td>80</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Dec 1994</td>
<td>48</td>
<td>10</td>
<td>51</td>
<td>Unfiltered seawater</td>
</tr>
<tr>
<td>Dec 1994</td>
<td>48</td>
<td>10</td>
<td>65</td>
<td>Bloom conditions, high chlorophyll</td>
</tr>
<tr>
<td>Dec 1994</td>
<td>48</td>
<td>10</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>
ined 44 seawater isolates for LAPase activity and did not find any lacking it. Another possible interpretation is that His, at these concentrations, is toxic to many of the microorganisms present, and that the decline in LAPase simply reflects decline in the number of active cells. This (admittedly improbable) explanation cannot be entirely overlooked, as we did not determine cell numbers, which could not in any case exclude this possibility, as dead or dying cells may be included in the total count (Zweifel & Hagstrom 1995). Another possibility is that many of the microorganisms present are auxotrophic for other amino acids as well, but that LAPase is not repressed by additions of these because of high natural background concentrations. However, concentrations of monomeric amino acids are generally low in seawater (e.g. Tupas et al. 1994).

Our results contrast strongly with those of Chrost (1991), who found that glucose, acetate and an amino acid mixture (which included neither histidine nor phenylalanine) repressed LAPase activity in a temperate eutrophic lake. By contrast, our results are consistent with those of Rivkin et al. (1991), who found little or no increase in bacterial growth as determined by thymidine uptake with addition of monomeric compounds such as glucose, glutamic acid and glycine, but not necessarily with their interpretation. They hypothesized that Antarctic bacterioplankton are 'substrate sufficient' and that growth is limited by some other factor, such as temperature. An alternative explanation suggested by our results is that the communities used in their experiments were in fact limited by substrate availability, but the compounds they added were not the ones required to increase growth rates.

Because of the consistency of repression of LAPase by histidine, we advance the hypothesis that a large fraction of the Bacteria and/or Archaea in our study area are auxotrophic for this amino acid. Auxotrophy for amino acids is widespread among Bacteria (Guirard & Snell 1962). Of 15 strains isolated from seawater by Ostroff & Henry (1939), only 5 could grow with ammonium as the sole nitrogen source. Two hyperthermophilic heterotrophic Archaea (one from each of the major kingdoms within the Archaea) isolated from deep-sea hydrothermal sulfide deposits were auxotrophic for 11 different amino acids (Hoaki et al. 1993).

There are several observations for which the auxotrophic hypothesis suggests explanations. Amino acid respiration appears to be a major pathway for C and N cycling in pelagic marine ecosystems of the Western Antarctic Peninsula (Tupas et al. 1994). If histidine requirements cause LAPases to be expressed at higher levels than are required to satisfy basic carbon and nitrogen requirements, the flux of amino acids to the cell should exceed that required for biosynthesis (which is limited by availability of amino acids not synthesized), so that a substantial fraction is respired.
Widespread respiration of amino acids would help to explain why the ratio of aminopeptidase activity to that of glucosidases is higher in Antarctica than elsewhere (Christian & Karl 1995a).

The results presented here can be summarized as follows. Firstly, values of $K_m$ for LAPase were generally consistent with those from other aquatic environments. Secondly, enzymes hydrolyzing LLBN appear to be broadly specific. Hydrolysis of the fluorogenic substrate analogue was not more strongly inhibited by L-Leu-L-Leu than by dipeptides of other amino acids, and Lineweaver-Burk plots characteristic of competitive inhibition were obtained in some cases. Finally, expression of this enzyme(s) appears to be regulated by availability of amino acids known to be rare in seawater (histidine and phenylalanine). It is highly probable that growth and metabolism of heterotrophic prokaryotes is regulated by the mixture of compounds available and not simply by C or N availability. The hypothesis of auxotrophy for these amino acids is advanced to explain the latter observation. These observations suggest important future areas for research on biosynthesis and growth regulation in aquatic prokaryotes.

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