Purification, Characterization, and Sequencing of an Extracellular Cold-Active Aminopeptidase Produced by Marine Psychrophile *Colwellia psychrerythraea* Strain 34H

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The limited database on cold-active extracellular proteases from marine bacteria was expanded by successful purification and initial biochemical and structural characterization of a family M1 aminopeptidase (designated ColAP) produced by the marine psychrophile *Colwellia psychrerythraea* strain 34H. The 71-kDa enzyme displayed a low optimum temperature (19°C) and narrow pH range (pH 6 to 8.5) for activity and greater thermolability than other extracellular proteases. Sequencing of the gene encoding ColAP revealed a predicted amino acid sequence with the highest levels of identity (45 to 55%) to M1 aminopeptidases from mesophilic members of the γ subclass of the *Proteobacteria* and the next highest levels of identity (35 to 36%) to leukotriene A₄ hydrolases from mammalian sources. Compared to mesophilic homologs, ColAP had structural differences thought to increase the flexibility for activity in the cold; for example, it had fewer proline residues, fewer ion pairs, and a lower hydrophobic residue content. In addition to intrinsic properties that determine enzyme activity and stability, we also investigated effects of extracellular polymeric substances (EPS) from spent culture medium of strain 34H on ColAP activity at an environmentally relevant temperature (0°C) and at 45°C (the maximum temperature for activity). In both cases, ColAP stability increased significantly in the presence of EPS, indicating the importance of considering environmentally relevant extrinsic factors when enzyme structure and function are investigated.

Perennially cold environments comprise a significant portion of Earth's biosphere (~80%), due largely to the presence of a predominantly cold ocean (<5°C) (2). The vast numbers of cold-adapted microorganisms which successfully inhabit these regions, which are referred to as psychrophilic organisms (optimal growth temperature [T_{opt}], $\leq 15^\circ\text{C}$; maximum growth temperature, $\leq 20^\circ\text{C}$) (45) and psychrotolerant organisms (organisms able to grow at low temperatures, but with a T_{opt} range of 20 to 35°C) (8), play vital roles in global elemental cycles and in the mineralization of organic matter. To do so, they must possess enzymes with sufficient activity to catalyze chemical reactions at low in situ temperatures.

Despite the environmental and physiological significance of these microorganisms, the mechanisms that allow enzymatic activity in the cold are currently not well understood. Low temperatures lead to exponential decreases in chemical rates, as described by the Arrhenius equation, and also tend to increase the compactness of proteins, thus limiting the conformational breathing necessary for catalysis (49). Despite these challenges, cold-active enzymes have evolved. Compared to mesophilic enzymes, these enzymes display three general distinguishing characteristics: a higher specific activity (k_{cat}) or a catalytic efficiency (k_{cat}/K_m) at temperatures between 0 and 30°C; a lower T_{opt} for activity; and limited stability in the presence of thermal increases and denaturing agents (24).

In this paper, we describe purification and initial biochemical and structural characterization of a cold-active, extracellu-

lar, family M1 aminopeptidase (designated ColAP) produced by the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H. All cultured members of the genus *Colwellia* are cold adapted if they are not psychrophilic (7, 8). Representatives have been isolated from Arctic and Antarctic seawater and sea ice (6, 7, 38), as well as from cold deep-sea environments (20). Because the majority of organic matter in marine environments is composed of high-molecular-weight compounds that are largely unavailable for direct uptake by heterotrophic bacteria, the hydrolytic activity of extracellular enzymes plays a crucial role in bacterial acquisition of dissolved organic matter (as described by Vetter et al. [58]). The role of this activity is thought to be particularly important in low-temperature environments, in which bacterial activity is believed to require higher levels of dissolved organic matter than the bacterial activity in warmer environments requires (48). Of the classes of extracellular enzymes that have been examined, proteolytic activity has consistently been found to be the dominant activity throughout the marine environment (14, 34, 53), indicating its importance in the transformation and bacterial acquisition of nitrogen-rich organic compounds. However, few extracellular proteases from marine organisms have been characterized in order to better understand the mechanisms that enable their activity in situ (21). Aside from their environmental and ecological importance, cold-active proteolytic enzymes are also useful for investigations of the structural basis of protein stability and offer great potential for biotechnological applications (10, 27). We compared the activity and structural characteristics of the newly purified enzyme ColAP to the activities and structural characteristics of homologous enzymes from mesophilic organisms in an attempt to clarify features unique to cold activity. We also examined the poten-

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tial stabilizing effect on ColAP of extracellular polymeric substances (EPS) extracted from spent culture medium of strain 34H in order to begin to address the importance of extrinsic factors in facilitating the activity of cold-active extracellular enzymes in the environment.

MATERIALS AND METHODS

Bacterial strain, culture conditions, and crude enzyme concentration. The marine psychrophile *C. psychrerythraea* strain 34H belonging to the γ subclass of the *Proteobacteria* (γ -proteobacteria) was isolated from surficial sediments of the continental shelf off northeast Greenland (79°43'N, 16°14'W) from a depth of 305 m (33). The strain was purified by the dilution-to-extinction approach by using marine broth 2216 (Difco Laboratories), the same medium used for routine culturing in this study. The empirically observed temperature growth range of this organism is -6 to 19°C , and the T_{opt} for growth is 8 to 9°C (35); the highest levels of proteolytic activity in the culture medium are observed at low growth temperatures (-1 to 2°C) during the late log to early stationary phase.

For protease purification studies, a logarithmic-phase culture was inoculated into 20 liters of marine broth 2216 at 2°C in aerated (bubbled air passed through a $0.2\text{-}\mu\text{m}$ -pore-size filter) and stirred 25-liter carboys. Cells were harvested at the late log phase as determined by measuring the optical density at 600 nm. To obtain the extracellular fraction, the culture was centrifuged at $3,400 \times g$ for 20 min at 4°C and then filtered through a $0.45\text{-}\mu\text{m}$ -pore-size Gelman membrane capsule. The extracellular extract was concentrated approximately 25-fold above a 10,000-Da spiral-wound membrane cartridge by using a tangential-flow ultrafiltration unit. The filtration and concentration steps were performed at 2°C . The concentrated extract was amended with 10% glycerol and frozen at -80°C for future studies.

Enzyme assays. Proteolytic activity in the crude extracellular extract was assayed by using the fluorescently tagged substrate analog L-leucine 7-amido-4-methylcoumarin (MCA-L) (Sigma) to measure leucine aminopeptidase (LAPase) activity in an artificial seawater (ASW) buffer (0.4 M NaCl, 9 mM KCl, 26 mM MgCl_2 , 28 mM MgSO_4 , 5 mM TAPSO [*N*-[Tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropanesulfonic acid]; pH 7.2). Briefly, 25 to 250 μl of a suitable dilution of enzyme solution was added to 2.5 ml of ASW buffer. Substrate was added at a saturating concentration (200 μM), as determined by substrate saturation experiments. Activity was measured in duplicate samples during time course experiments with a Perkin-Elmer LS-5B spectrofluorometer at excitation and emission wavelengths of 355 and 440 nm, respectively.

During purification, activity was assayed routinely with MCA-L in ASW buffer. LAPase activity was also measured by using 200 μM Ala-Ala-Pro-Leu *p*-nitroanilide (AAPL *p*-nitroanilide) as the substrate in ASW buffer during time course experiments with a spectrophotometer at 410 nm with an extinction coefficient of $8,480\text{ M}^{-1}\text{ cm}^{-1}$, as described above. General proteolytic activity was assayed by using the macromolecular substrate azocasein in a reaction mixture containing 300 μl of 1% (wt/vol) azocasein in ASW buffer and 200 μl of enzyme solution. The reaction was terminated after several hours by adding 500 μl of 10% trichloroacetic acid. The activity of the supernatant fluid was measured with a spectrophotometer at 366 nm with an extinction coefficient of $900\text{ M}^{-1}\text{ cm}^{-1}$ after precipitated protein was removed by centrifugation at $13,000 \times g$ for 2 min. All activity assays were performed at 20°C unless indicated otherwise.

Protein quantification. Protein was quantified by the method of Bradford by using bovine serum albumin as the standard, as outlined in the Bio-Rad protein assay protocol (9).

Separation of extracellular proteins. Proteins in the crude extracellular extract were initially separated by fast protein liquid chromatography by using a calibrated gel filtration column (Superose 12; Amersham Pharmacia) equilibrated with 10 mM Tris buffer containing 0.3 M NaCl (pH 7.3) eluted at a flow rate of 1.0 ml min^{-1} .

Enzyme purification. Most enzyme purification steps were performed with buffers chilled to $\sim 0^\circ\text{C}$ by using ice baths; the only exception was the hydroxyapatite column elution step, which was performed at 20°C due to precipitation of the elution buffer at low temperatures.

(i) **Ion-exchange chromatography.** The frozen, crude extracellular extract was thawed and buffer exchanged with 20 mM piperazine- N,N' -bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.75) at 0°C by using an Amicon pressure concentrator equipped with a YM10 membrane filter. The sample was loaded at a flow rate of 1 ml min^{-1} onto a Q-HP anion-exchange column (bed volume, 1 ml; Amersham Pharmacia) equilibrated with the same buffer. The column was washed with 5 ml of loading buffer. Bound proteins were eluted with an 18-ml gradient of NaCl as follows: 3 bed volumes of 0 to 200 mM NaCl, 10 bed volumes of 200 to 300 mM NaCl, 2 bed volumes of 300 to 400 mM NaCl, and 3 bed volumes of 400 to 1,000 mM NaCl. The flow rate was 1.0 ml min^{-1} . One-milliliter fractions were col-

lected, placed immediately on ice, and assayed for LAPase activity as described above. One peak of activity eluted between 200 and 237 mM NaCl.

(ii) **Hydroxyapatite chromatography.** The active fractions from the first ion-exchange step were combined and diluted 10-fold in chilled 10 mM Na_2HPO_4 buffer (pH 7.2). The sample was loaded at a rate of 0.75 ml min^{-1} onto a hydroxyapatite column (bed volume, 1 ml; Bio-Rad) equilibrated with the same buffer. The column was washed with 3 bed volumes of loading buffer. Bound proteins were eluted with a 17-ml gradient by using 400 mM Na_2HPO_4 buffer (pH 6.8) as follows: 15 bed volumes of 0 to 300 mM Na_2HPO_4 and 2 bed volumes of 300 to 400 mM Na_2HPO_4 . One-milliliter fractions were collected, stored on ice, and assayed for LAPase activity; one peak of activity eluted between 116 and 155 mM Na_2HPO_4 .

(iii) **Ion-exchange chromatography.** Active fractions from the hydroxyapatite column were combined and diluted 10-fold in chilled 20 mM PIPES buffer (pH 6.75). The sample was loaded at a rate of 1.0 ml min^{-1} onto a Resource Q anion-exchange column (bed volume, 1 ml; Amersham Pharmacia) equilibrated with the same buffer. The column was washed with 5 bed volumes of loading buffer. Bound proteins were eluted with an 18-ml gradient of NaCl as follows: 3 bed volumes of 0 to 200 mM NaCl, 10 bed volumes of 200 to 300 mM NaCl, 2 bed volumes of 300 to 400 mM NaCl, and 3 bed volumes of 400 to 1,000 mM NaCl. One-milliliter fractions were collected, kept on ice, and assayed for LAPase activity; one peak of activity eluted between 210 and 230 mM NaCl. Pure protein was either used immediately or frozen at -80°C for future analysis.

Electrophoresis. Fractions from the various fast protein liquid chromatography purification steps were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis by using denaturing 12% polyacrylamide gels. SYPRO Ruby protein gel stain (Molecular Probes) was used for protein visualization. The method used for SDS-PAGE was essentially the method described by Laemmli (41).

Extraction of EPS. *C. psychrerythraea* strain 34H was grown at 2°C and harvested at the late log phase, as determined by optical density at 600 nm, to obtain a crude EPS extract. Extraction was performed as previously described (3, 19), with the following modifications. Cells were removed from the late-log-phase culture by gentle centrifugation at $1,500 \times g$ for 50 min at 2°C and subsequent filtration of the supernatant through a GFF filter. Three volumes of chilled 100% ethanol was added to the filtered supernatant, which was then incubated overnight at 2°C . EPS formed a precipitate that was collected by centrifugation at $10,000 \times g$ for 20 min. The ethanol wash and centrifugation steps were repeated three times. To remove low-molecular-weight polysaccharides, the final pellet was dissolved in distilled water and dialyzed for 48 h at 2°C by using Spectra Por dialysis tubing (2,000- to 3,500-Da cutoff). The resulting dialysate was frozen at -20°C for future use. EPS was quantified by the colorimetric phenol-sulfuric acid method (22).

Enzyme characterization. Michaelis-Menten kinetic parameters for the activity of the purified enzyme, which was later determined to be a member of the M1 family of aminopeptidases and was designated ColAP, were determined from substrate saturation assays at -1 , 9 , and 19°C in ASW buffer by using MCA-L at various concentrations as the substrate. Values for the maximum velocity and half-saturation coefficient (K_m) were determined by plotting the substrate concentration versus the initial velocity of each reaction and subjecting the data to nonlinear regression analysis (with the statistical software package SigmaStat). The activation energy (E_a) of ColAP was calculated from the slope ($-E_a/R$) of an Arrhenius plot of specific activity of the enzyme ($\ln k_{\text{cat}}$) versus the reciprocal of temperature (in Kelvin).

To determine the temperature range and T_{opt} for activity of the enzyme, $0.1\text{ }\mu\text{g}$ of purified ColAP was incubated in duplicate at -1 , 4 , 7.5 , 12 , 16 , 19 , 26 , 28 , 35 , and 46°C for 2 h in ASW buffer with 200 μM MCA-L as the substrate. Thermal stability was determined by incubating $0.1\text{ }\mu\text{g}$ of ColAP in duplicate at 30 , 40 , 45 , and 50°C for up to 45 min, as well as at 0°C for 45 h, in ASW buffer. At 0 and 45°C , ColAP was also incubated with an environmentally relevant concentration of EPS ($\sim 33\text{ }\mu\text{g of C ml}^{-1}$) (39). Fractions incubated at the higher temperatures (30 to 50°C) were first placed on ice to stop the inactivation reaction; the residual activity in each case was then measured after equilibration of the sample at 20°C .

To determine the effect of pH on activity, $0.1\text{ }\mu\text{g}$ of ColAP was assayed in duplicate at 20°C at pH values ranging from 5.0 to 9.0 in buffer-amended ASW by using 200 μM MCA-L as the substrate. The following buffers were used at a concentration of 20 mM: acetic acid (pH 5.0); morpholineethanesulfonic acid (MES) (pH 6.0); PIPES (pH 6.5 and 7.0); TRIZMA [2-amino-2-(hydroxymethyl)-1,3-propanediol] (pH 7.5, 8.0, and 8.5); and AMPPO [N-1,1-dimethyl-2-hydroxyethyl]-3-amino-2-hydroxypropanesulfonic acid] (pH 9.0).

For inhibition and metal studies, ColAP was incubated with different inhibitors (EDTA, phenylmethylsulfonyl fluoride, dithiothreitol) and divalent ions (CaCl_2 , MgCl_2 , MnCl_2 , and ZnCl_2) for 1 h on ice in 20 mM Tris buffer containing 0.4 M NaCl (pH 7.2). Residual activity was measured at 20°C by using MCA-L as the

FIG. 1. Superose 12 gel filtration chromatography profile of the proteolytic activity in a crude extracellular extract from a late-log-phase culture of *C. psychrerythraea* strain 34H at 2°C. A 500- μ l sample of concentrated extract (see text) was gravity loaded onto a column which was preequilibrated with 10 mM Tris–0.3 M NaCl (pH 7.3), eluted with the same buffer at a rate of 1.0 ml min⁻¹, and collected in 1-ml fractions. Fractions were assayed for proteolytic activity as described in Materials and Methods by using the following substrates: MCA-L (■), AAPL *p*-nitroanilide (□), and azocasein (◆). The arrows indicate the positions of molecular masses (in kilodaltons).

substrate. To determine if ColAP activity was chloride ion dependent, the activity in 20 mM Tris buffer lacking NaCl was also determined. The number of concentrations tested (concentration range, 0.1 to 10 mM) for each inhibitor or ion was limited by enzyme availability.

In addition to MCA-L, AAPL *p*-nitroanilide, and azocasein (see above), the substrate preference of ColAP was determined in ASW buffer at 20°C by using the following substrates at a concentration of 200 μ M: L-alanine 7-amido-4-methylcoumarinylamide trifluoroacetate salt; L-arginine 7-amido-4-methylcoumarin; L-aspartic acid β -7-amido-4-methylcoumarin; L-proline 7-amido-4-methylcoumarin hydrobromide; and L-serine 7-amido-4-methylcoumarin HCl (all obtained from Sigma).

N-terminal amino acid sequencing. The N-terminal sequence of the purified enzyme was obtained by electroblotting SDS–12% PAGE gels loaded with ColAP onto polyvinylidene difluoride membranes, which were subsequently stained with Coomassie blue R-250. An automated Edman-type (23) analysis was

TABLE 1. Purification of ColAP, a cold-active M1 aminopeptidase from *C. psychrerythraea* strain 34H

Purification step	Total protein (mg)	Total activity (U) ^a	Sp act (U mg ⁻¹)	Purification (fold)	Yield (%)
Culture supernatant	6.2	3.3	0.54	1.0	100
Sepharose Q	0.72	2.1	2.9	5.4	64
Hydroxyapatite	0.0054	1.0	185	340	30
Resource Q	0.0032	0.80	250	460	24

^a One unit was defined as degradation of 1 nmol of MCA-L min⁻¹.

performed with excised protein bands by using a Perkin-Elmer Applied Biosystems model 494 Procise protein sequencer with an online 140C PTH amino acid analyzer. Electrophoresis and protein sequencing were performed by the Iowa State University Protein Facility.

Determination of predicted amino acid sequence. The N-terminal sequence of ColAP was used to perform a BLAST search of the genome sequence of *C. psychrerythraea* strain 34H available at The Institute for Genomic Research website (<http://www.tigr.org>). A 100% match was determined for ORF01786, which encodes a putative family M1 aminopeptidase with a predicted molecular mass of 71,273 Da.

Sequence analysis. Similar sequences from other organisms were identified by BLAST sequence analysis by using the GenBank database at the National Center for Biotechnology Information. Predicted amino acid sequences were aligned with CLUSTAL X and then optimized by eye with Se-Al. Phylogenetic trees were constructed from the alignments with the program TREE-PUZZLE 5.0. An analysis of the predicted amino acid sequence of ColAP was performed with the ExPASy computer facilities, particularly the ProtParam program available at <http://www.expasy.ch>. For comparative purposes, similar analyses were performed for several putative family M1 aminopeptidases from mesophilic members of the γ -proteobacteria with high levels of sequence identity to ColAP (45 to 55% as determined by BLAST results).

Three-dimensional modeling and analysis. Starting with a PSIBLAST alignment (1) and then using MSA Clustal analysis (31), we determined that there was 33% amino acid identity and 57% amino acid similarity between ColAP and human leukotriene A₄ (LTA₄) hydrolase, a bifunctional enzyme that displays both fatty acid hydrolysis and aminopeptidase activities whose crystal structure has been determined. Because of the high sequence similarity with ColAP relative to the similarities to other proteins with known structures, the crystal structure of LTA₄ hydrolase (54) was chosen as a template for initial construction of a three-dimensional (3D) model of ColAP and selected mesophilic M1 aminopeptidases. Following construction of the initial comparative model, a structure prediction was performed by de novo methods (51). Template selection, 3D modeling, and structural analysis were performed by using RAMP software available at the 3D protein modeling server <http://protinfo.compbio.washington.edu>. Ionic and aromatic interactions were determined by using cut-off distances of 4.0 and 6.0 Å between interacting groups, respectively. The solvent-accessible surface area was determined with a probe radius of 2.0 Å.

Nucleotide sequence accession number. The GenBank accession number of the *C. psychrerythraea* strain 34H ColAP gene sequence is AY302752.

RESULTS

Production of extracellular proteases by strain 34H. The results of gel filtration chromatography of concentrated extracellular extracts indicated that *C. psychrerythraea* strain 34H produces several extracellular proteolytic enzymes with different specificities (Fig. 1). Three peaks of azocasein digestion were observed in column eluant fractions corresponding to molecular masses of 71 to 83, ~43, and ~22 kDa. Additional peaks of MCA-L and AAPL *p*-nitroanilide hydrolysis were also observed; in each case they corresponded to a molecular mass of 71 to 83 kDa.

Purification of ColAP. ColAP was purified to homogeneity (Fig. 2), as summarized in Table 1. Purification was difficult, even after we paid attention to the temperature, due to the sensitivity of the enzyme to standard purification procedures,

FIG. 2. SDS-PAGE analysis with 12% polyacrylamide gels of the fractions obtained during purification of ColAP from the extracellular extract. Lane 1, marker proteins (relative molecular masses [in kilodaltons] are indicated on left); lane 2, extracellular extract; lane 3, Sepharose Q eluate; lane 4, hydroxyapatite eluate; lane 5, Resource Q eluate (see Materials and Methods and Table 2).

TABLE 2. Kinetic parameters for purified ColAP

Assay temp (°C)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	E_a (kJ mol ⁻¹) ^a
-1	0.043 ± 0.006	43 ± 22	1.0	
9	0.13 ± 0.02	58 ± 31	2.2	71 ± 1.5
19	0.36 ± 0.06	72 ± 34	5.0	

^a E_a was calculated from an Arrhenius analysis of the k_{cat} data.

such as variations in the pH and the salt concentration. Ion-exchange and hydroxyapatite columns resulted in greater retention of ColAP activity than the other techniques tested (e.g., hydrophobic interaction chromatography). Hydroxyapatite techniques resulted in significant purification (~340-fold increase in specific activity), while the final ion-exchange step consistently removed a 50-kDa contaminant (barely visible in Fig. 2). The purification procedure resulted in a relatively high yield (24%) with a ~460-fold increase in specific activity. The molecular mass of purified ColAP was ~71 kDa as determined by both SDS-PAGE and gel filtration analysis, indicating that ColAP is a monomeric enzyme.

Kinetic parameters and thermostability of activity. The highest specific activity (k_{cat}) for ColAP (0.36 s⁻¹) was observed at ~19°C (Table 2). The enzyme showed no significant activity at 46°C, but 12% of the maximal activity was still observed at the lowest temperature tested, -1°C (Fig. 3). The half-saturation coefficient (K_m) for ColAP was lowest at -1°C (43 μM) and increased with increasing temperature (to 72 μM at 19°C) (Table 2). The highest k_{cat}/K_m value for ColAP (5.0 s⁻¹ mM⁻¹) was also observed at 19°C, and 44% of this value retained at 9°C (the T_{opt} for growth of strain 34H); 20% of the value was retained at -1°C, an environmentally relevant temperature (Table 2). The activation energy for cold-active ColAP was 71 ± 1.5 kJ mol⁻¹ (Table 2).

Thermostability of activity. ColAP exhibited great sensitivity to heat, and the calculated half-lives were ~67, 38, 10, and 5 min at 30, 40, 45, and 50°C, respectively (Fig. 4A). In the

FIG. 3. Activity of ColAP when 0.1 μg of the enzyme was incubated at various temperatures for 2 h with 200 μM MCA-L as the substrate. The values indicate specific rates of hydrolysis as determined by duplicate time course experiments; the error bars indicate 95% confidence intervals. The 100% specific activity value was 0.36 s⁻¹.

FIG. 4. Thermostability of ColAP at various temperatures in the presence (open symbols) or absence (solid symbols) of EPS (33 μg of C ml⁻¹) extracted from spent culture medium of *C. psycherythraea* strain 34H at different temperatures. (A) 50°C (●), 45°C (■ and □), 40°C (▲), and 30°C (●); (B) 0°C. The values in panel A are averages based on two independent experiments. The enzyme (0.1 μg) was incubated in ASW buffer at the various temperatures for the times indicated; the residual activity was measured at 20°C.

presence of EPS extracted from spent culture medium, the stability of ColAP increased by a factor of 17 at 45°C, and the observed half-life was 170 min (Fig. 4A). At 0°C (Fig. 4B), the half-life of ColAP in ASW buffer alone was about 18 h, while no detectable loss of activity was observed in the presence of EPS after incubation for 45 h. Furthermore, at both 45 and 0°C, the initial activity appeared to be higher (by a factor of 1.3 to 2.7) in the EPS-amended fractions than in the ASW controls. No ColAP activity was detected in the EPS extract itself.

Effect of pH on activity. ColAP exhibited a narrow pH range for activity, and the maximal activity was observed at neutral pH. At pH 6.0 and 8.5, only 10 and 5% of the maximal activity was retained, respectively, while no activity was detected at pH 5.0 or 9.0. No recovery of activity was observed when the preparation was returned to neutral pH.

Effects of inhibitors and ions on enzyme activity. Of the potential inhibitors tested (Table 3), phenylmethylsulfonyl fluoride (a serine protease inhibitor) did not affect enzyme activity significantly, while 10 mM dithiothreitol (a reducing agent) and all concentrations of EDTA (a metal-chelating agent)

TABLE 3. Effects of different compounds on ColAP activity

Buffer or compound ^a	Concn (mM)	ColAP activity (%)
Control buffer		100 ± 7
Inhibitors		
Phenylmethylsulfonyl fluoride	0.1	91 ± 20
Dithiothreitol	1	75 ± 1
	10	0
EDTA	1	0
	5	0
	10	0
Divalent cations		
ZnCl ₂	0.1	0
	1	0
	10	0
CaCl ₂	1	84 ± 5
	10	76 ± 13
MgCl ₂	1	100 ± 15
	10	122 ± 10
MnCl ₂	10	0
Control buffer without NaCl		0
ASW buffer		189 ± 15

^a Purified ColAP (0.1 µg) was incubated with various compounds for 1 h on ice in control buffer (20 mM Tris, 0.4 M NaCl; pH 7.2); residual activity was measured at 20°C.

tested completely inhibited ColAP activity. Of the divalent cations tested, Zn²⁺ and Mn²⁺ strongly inhibited activity (Table 3), while Ca²⁺ was slightly inhibitory. Mg²⁺ stimulated ColAP activity at a concentration of 10 mM or higher (equivalent to the concentration found in seawater), as shown by the value for ASW buffer in Table 3. Activity also appeared to be strongly dependent upon chloride salts; no activity was seen in 20 mM Tris buffer lacking NaCl (Table 3).

Substrate preference. Purified ColAP displayed the highest levels of activity with methylcoumarin substrates containing alanine and arginine residues and intermediate levels of activity with leucine (Table 4). Lower levels of activity were observed with serine, while no detectable activity was observed with the cyclic and acidic residues proline and aspartic acid. Activity was also observed with AAPL *p*-nitroanilide and the macromolecular substrate azocasein (Table 4).

Sequence analysis and protein modeling. The predicted amino acid sequence of ColAP (35) was examined to determine its similarity to sequences available in the National Center for Biotechnology Information database. ColAP exhibited the highest overall levels of amino acid identity (45 to 55%) with putative M1 aminopeptidases from mesophilic members of the same group of bacteria (γ -proteobacteria) and the next

TABLE 4. Relative activities of ColAP with various substrates at pH 7.2 and 20°C

Substrate	Hydrolysis rate (µmol min ⁻¹ mg ⁻¹)	Relative activity (%)
L-Leucine 7-amido-4-methylcoumarin	0.33 ± 0.030	100
L-Alanine 7-amido-4-methylcoumarin	1.2 ± 0.080	364
L-Arginine 7-amido-4-methylcoumarin	1.1 ± 0.10	333
L-Aspartic acid β-7-amido-4-methylcoumarin	— ^a	0
L-Proline 7-amido-4-methylcoumarin	—	0
L-Serine 7-amido-4-methylcoumarin	0.020 ± 0.015	6.1
AAPL <i>p</i> -nitroanilide	0.040 ± 0.030	
Azocasein	0.13 ± 0.030	

^a —, no detectable activity.

FIG. 5. Phylogenetic analysis of ColAP, based on its deduced amino acid sequence. Sequences were aligned by using the CLUSTAL X and Se-Al programs; phylogenetic trees were constructed by using the program TREE-PUZZLE 5.0. *Lactobacillus helveticus* was used as an outgroup for this analysis. The sequence sources and GenBank accession numbers (if available) are as follows: *Cavia porcellus* (guinea pig), reference 44; *Homo sapiens*, AAA36176; *Lactobacillus helveticus*, reference 13; *C. psychrerythraea* strain 34H, AY302752; *Shewanella oneidensis* MR-1, AAN55048; *Xanthomonas axonopodis* pv. citri 306, AAM35534; *X. campestris* pv. campestris ATCC 33913, AAM42755; and *Xylella fastidiosa* 9a5c, AAF84297.

highest levels of identity (35 to 36%) with LTA₄ hydrolases from various sources (Fig. 5). The results of a multiple-sequence alignment indicated that there was perfect conservation of the putative substrate binding site (GGMEN), the zinc binding motif (HEXXH-X₁₈-E), and catalytic residues involved in aminopeptidase activity (Glu 301 and Tyr 386 in ColAP, which are thought to act as a general base and a proton donor, respectively [4, 54, 59]).

Biocomputational sequence analysis revealed amino acid identities distributed throughout the entire ColAP sequence that corresponded to secondary structural elements. As a result, the structural model of ColAP (35) had three domains that together created a cleft containing a catalytic zinc site similar to that of LTA₄ hydrolase (54).

In our analysis of the sequences and models of ColAP and its mesophilic equivalents (Table 5) we focused on structural features believed to increase the structural flexibility usually associated with low-temperature enzyme activity. In ColAP the number of proline residues, which affect the backbone flexibility and thus the local mobility of the chain, is less than the numbers of proline residues in the other enzymes. The number of arginine residues, which have the potential to form multiple ion pairs and H bonds, is less than the number of lysine resi-

TABLE 5. Summary of structural parameters of ColAP, potentially involved in adaptation to low temperatures, and M1 aminopeptidases from mesophilic members of the γ -proteobacteria

Enzyme	Pro content (no. of residues)	Arg/(Arg + Lys) ratio	% of total residues			Theoret- ical pI	GRAVY index ^a	No. of ion pairs	Solvent-accessible surface area		
			Nonpolar residues (A, F, I, L, M, P, V, W)	Polar residues (C, G, N, Q, S, T, Y)	Charged residues (D, E, H, K, R)				% Nonpolar residues	% Polar residues	% Charged residues
<i>C. psychrerythraea</i> ColAP	25	0.35	43.8	32.8	23.4	5.25	-0.343	15	28.3	29.7	42.0
Mesophile M1 aminopeptidases											
<i>Shewanella oneidensis</i> MR-1	32	0.56	45.2	35.8	19.0	5.75	-0.257	17	30.9	37.3	31.8
<i>Xanthomonas axonopodis</i>	43	0.42	47.6	29.7	22.7	5.28	-0.282	19	34.5	23.9	41.6
<i>Xanthomonas campestris</i>	44	0.40	47.9	29.9	22.2	5.46	-0.261	18	34.4	25.7	39.9
<i>Xylella fastidiosa</i>	34	0.43	46.6	31.0	22.4	6.49	-0.178	18	32.2	26.8	41.0

^a GRAVY, grand average of hydropathicity.

dues, as shown by the decrease in the Arg/(Arg + Lys) ratio. ColAP is also characterized by having a lower content of hydrophobic residues (A, F, I, L, M, P, V, and W) and a higher content of charged residues (D, E, H, K, and R) than the other enzymes. Parameters resulting from biocomputation of the complete amino acid sequences of ColAP and its mesophilic homologs with the ProtParam tool indicated that the theoretical pI and grand average of hydropathicity (40) of ColAP are relatively low (5.25 versus 5.28 to 6.49 and -0.343 versus -0.282 to -0.178, respectively), suggesting that the hydrosolubility of ColAP is higher and that this enzyme has a better interaction with the solvent. The results of 3D modeling also support these findings; the solvent-accessible surface of ColAP has a lower number of hydrophobic residues and a higher number of charged residues than the surfaces of its counterparts from mesophilic γ -proteobacteria. Modeling analysis further suggested that ColAP has fewer ion pairs (15 versus 17 to 19), which leads to a decrease in the number of intrinsic stabilizing bonds. The enzymes did not differ in other indices thought to confer low-temperature activity, such as the aliphatic index (36) and aromatic interactions (data not shown).

DISCUSSION

In this study, we developed a successful protein purification scheme that allowed us to investigate the cold activity of an extracellular aminopeptidase (ColAP) produced by a marine psychrophile that represents a bacterial genus that is known to occur in a variety of cold marine environments. *C. psychrerythraea* strain 34H is particularly useful as a model organism for cold adaptation studies because the sequence of its whole genome was recently obtained (B. Methe, M. Lewis, B. Weaver, J. Weidman, W. Nelson, A. Huston, J. Deming, and C. Fraser, unpublished data; <http://www.tigr.org>). In silico analysis of the whole genome sequence has suggested that strain 34H is capable of producing many more extracellular proteolytic enzymes than the enzymes observed in this study; at least 90 open reading frames have been classified as having roles related to protein and peptide degradation, and 51 of the proteins encoded by these open reading frames contain predicted signal peptides which suggest transport from the cytoplasm and a possible role in extracellular protein degradation (35).

Inhibition studies with EDTA confirmed that the ColAP enzyme that we purified from the exudates of strain 34H is a

metalloprotease, while sequence analysis suggested that zinc is required for activity of this enzyme. Zinc, however, inhibited ColAP activity at the concentrations tested. Although the mechanism is currently unknown, similar results have been obtained for other zinc-dependent aminopeptidases, such as thermolysin and LTA₄ hydrolase, when they have been incubated with zinc at a concentration greater than that required for activity (molar ratios greater than 1) (32, 60). In contrast, other salts common in seawater either stimulated ColAP activity (magnesium) or appeared to be required for activity (chloride). More detailed studies of chloride effects on ColAP may reveal stimulation mediated via an anion binding site, given other relationships between ColAP and LTA₄ hydrolase, an enzyme in which chloride stimulation obeys saturation kinetics (29).

ColAP also resembles LTA₄ hydrolase in terms of substrate preference; both enzymes appear to prefer arginine and alanine substrates (29). The observed hydrolytic activity against AAPL *p*-nitroanilide and azocasein suggests that ColAP has both endopeptidase and exopeptidase activities. The fact that the specific activities of LTA₄ hydrolase with synthetic substrates, such as nitroanilide and β -naphthylamide derivatives of amino acids, are significantly lower than the specific activities with dipeptides and tripeptides (28) may help explain the observed low specific activity of ColAP with synthetic methylcoumarin substrates. Further work is needed to determine the specific activities of ColAP with dipeptide and tripeptide substrates.

Activity characterization revealed that the T_{opt} for ColAP activity (19°C) is low relative to the T_{opt} s of previously described extracellular proteases (whether they were purified from mesophilic, psychrotolerant, or psychrophilic bacteria), which generally have been found to exhibit maximal activity at temperatures between 30 and 60°C (10, 21). In agreement with the current view that low-temperature enzymatic activity is associated with reduced structural stability, ColAP exhibited a narrow pH range (pH 6.0 to 8.5) for activity compared to the pH ranges of previously characterized cold-active proteases (pH 5 to 11) (46, 50). Furthermore, in the absence of stabilizing agents, ColAP displayed a thermolability equal to or greater than the thermolabilities of other extracellular proteases from psychrophilic and psychrotolerant sources (11, 42, 46).

However, the lifetime of a cell-free enzyme in a salt solution lacking other dissolved or particulate organic compounds does

not have physiological or environmental relevance. Many microorganisms in natural environments live in assemblages that form organic-rich flocs and biofilms (15); the highest concentrations of cell-free enzymes in cold marine environments are usually found in such organic-rich sites, including detrital aggregates (34), sediments (57), and sea ice (30). These enzymes are likely to be embedded in organic matrices or to be associated with high-molecular-weight materials (such as EPS) found in phytodetritus and sea ice (39). Our observation that EPS has significant effects on the half-life of ColAP, especially at 0°C, suggests that EPS may have an important stabilizing role for ColAP in an environmental context. Although we did not characterize the components of our EPS extract or the enzyme-stabilizing mechanism, the results of previous studies suggest that EPS (as well as compatible solutes) act nonspecifically via preferential exclusion mechanisms that create an entropically unfavorable state, thus favoring the folded state over the unfolded state of proteins (55, 56). The hydrated property of EPS may also act as a buffer that maintains extracellular enzyme activity during local chemical and osmotic changes (18), while it also protects enzymes from proteolysis. Furthermore, marine polymer gels (such as EPS) have been found to sequester high levels Mg^{2+} and Ca^{2+} relative to the levels in the surrounding seawater (12), which may further stabilize the structure and stimulate the activity of metalloenzymes such as ColAP.

Regardless of the molecular mechanisms, the longer ColAP lifetime at 0°C mediated by the presence of EPS easily matches or exceeds the observed generation times of microbial assemblages in samples of Arctic seawater (43) and sinking particles (35). The stabilizing effect of EPS may facilitate the selection of cold-active extracellular enzymes by enhancing their benefits to the producing (and other nearby) organisms over several microbial generations. By helping to maintain enzyme function, whether in low- or high-thermal-energy environments (26), extrinsic factors such as EPS may increase the range of extreme environments that bacteria can inhabit successfully.

At the intrinsic level, the current working hypothesis is that cold-active enzymes have increased flexibility in certain parts of their structures to accommodate the substrates and molecular movement necessary for catalysis at low temperatures, a flexibility that also results in reduced structural stability (17). As found in other studies (16, 25), the observation that all amino acids putatively involved in the reaction mechanism are strictly conserved in ColAP when this enzyme is compared to its mesophilic homologs suggests that molecular adaptation to cold lies elsewhere in the protein structure. The numerous structural differences observed when the sequence and 3D model of ColAP were compared to the sequences and 3D models of its homologs are consistent with increased protein flexibility and the observed low-temperature activity and reduced stability of ColAP. Whether such differences are indeed responsible for the enhanced structural flexibility that is thought to allow activity at low temperatures remains to be determined mechanistically. When subjected to statistical analysis, purported rules for amino acid substitution in different thermal classes of enzymes have yielded insignificant results (5, 47); this is not surprising, since the stabilization energies of mesophilic and extremophilic enzymes differ by the equivalent of only a few noncovalent interactions (37). Also, the taxonomic differences among source organisms when enzymes

from different thermal regimens are compared increase the likelihood of variable selective pressures and random genetic drift in the enzyme sequence (52). To better determine structural features necessary for or unique to activity at low temperatures, a larger database developed with techniques that enable direct comparison of proteins with few structural differences (52) is needed. The availability of the genome of the marine psychrophile *C. psychrerythraea* strain 34H should enable evaluation of concomitant gene expression and potential interactions between cold-active enzymes and extrinsic factors, such as chaperonins and compatible solutes, as well as the EPS that we included in this study. Further characterization of cold-active enzymes in the presence of such extrinsic factors should provide a better understanding of how these enzymes function in the environment, thus providing insight into the effects of selection pressures on their structure and function.

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