THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 20, Issue of May 15, pp. 12109–12115, 1998

Printed in U.S.A.

Characterization of the C-terminal Propeptide Involved in Bacterial Wall Spanning of α-Amylase from the Psychrophile *Alteromonas haloplanctis**

(Received for publication, October 22, 1997, and in revised form, March 6, 1998)

Georges Feller‡§, Salvino D'Amico‡, Abderrafi M. Benotmane‡, Fabian Joly‡, Jozef Van Beeumen¶, and Charles Gerday‡

From the ‡Laboratory of Biochemistry, Institute of Chemistry B6, University of Liege, B-4000 Liege and the ¶Laboratory of Protein Biochemistry and Protein Engineering, University of Gent, B-9000 Gent, Belgium

The antarctic psychrophile *Alteromonas haloplanctis* secretes a Ca^{2+}- and Cl^--dependent α-amylase. The nucleotide sequence of the *amy* gene and the amino acid sequences of the gene products indicate that the α-amylase precursor is a preproenzyme composed by the signal peptide (24 residues), the mature α-amylase (453 residues, 49 kDa), and a long C-terminal propeptide or secretion helper (192 residues, 21 kDa). In cultures of the wild-type strain, the 70-kDa precursor is secreted at the mid-exponential phase and is cleaved by a nonspecific protease into the mature enzyme and the propeptide. The purified C-terminal propeptide displays several features common to β-pleated transmembrane proteins. It has no intramolecular chaperone function because active α-amylase is expressed by *Escherichia coli* in the absence of the propeptide coding region. In *E. coli*, the 70-kDa precursor is directed toward the supernatant. When the α-amylase coding region is excised from the gene, the secretion helper can still promote its own membrane spanning. It can also accept a foreign passenger, as shown by the extracellular routing of a β-lactamase-propeptide fusion protein.

Most Gram-negative bacteria actively secrete proteins to the extracellular medium in amounts sometimes comparable to those achieved by Gram-positive bacteria or by yeast. Polypeptides secreted by Gram-negative bacteria include biodegradative enzymes, toxins, and pathogenicity factors that have important industrial or medical applications. As a result, there is now considerable interest in the elucidation of the molecular mechanisms that allow a polypeptide to initiate a journey in the cytoplasm and its subsequent routing to a specific cellular compartment. These studies have highlighted the remarkable diversity of the targeting processes involved in bacterial secretion (1–4).

Proteins secreted by the major secretory pathway cross the bacterial wall in a two-step mechanism via the periplasm. Exoproteins taking this two-step route possess a N-terminal signal peptide and use the general *sec* machinery for inner membrane translocation. Transport across the outer membrane in the second step requires a secretory apparatus encoded by large gene clusters, which are either specific or common to several exoproteins. Polypeptides transported via the hemolysin-type secretory pathway cross the cell envelope by a single-step process without periplasmic intermediates. Proteins targeted through this pathway have no N-terminal signal sequence and show *sec*-independent translocation to the medium. However, they require the assistance of accessory proteins, encoded by genes contiguous to the exoprotein gene, which are presumed to form a "pore" or an intermembrane channel. Secretion signals essential for translocation are located in the C-terminal part of these proteins.

All proteins transported by these pathways do not contain sufficient internal information to reach the external medium without assistance. By contrast, the unusual secretion system of gonococcal IgA proteases employs a two-step route, but after *sec*-dependent translocation of the inner membrane, the periplasmic intermediate is directed to the outer membrane by a C-terminal propeptide, which is subsequently cleaved by autolysis of the enzyme precursor (5–7). Propeptides are not uncommon but are usually found in the N-terminal sequence of proteases; they are essential for acquisition of the final folding of the active enzyme (8, 9).

α-Amylase from the antarctic psychrophile *Alteromonas haloplanctis* has been extensively analyzed in the context of enzyme adaptations to catalysis at low temperatures (10–13). We found that its secretion is assisted by a C-terminal propeptide. Unlike other propeptides, the C-terminal domain of the α-amylase precursor has no intramolecular chaperone function but constitutes an autonomous secretion signal that can be purified from culture supernatant after proteolytic processing. We report here structural and functional analyses of this C-terminal secretion helper.

EXPERIMENTAL PROCEDURES

Purification of α-Amylase, Propeptide, and Protease—The antarctic bacteria *A. haloplanctis* A23 was grown at 4 °C for 3–5 days in 1-liter Erlenmeyer flasks containing 400 ml of broth (16 g/liter Bactotryptone, 16 g/liter yeast extract, 20 g/liter NaCl, 10 g/liter sea salts, 30 g/liter maltose, pH 7.6) run at 250 rpm. After concentration and diafiltration (10, 11), the culture supernatant was loaded on a DEAE-agarose column (2.5 × 40 cm) equilibrated in 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5, and eluted with a NaCl linear gradient (500 ml–500 ml, 0–0.8 M NaCl). The propeptide was eluted in front of the gradient, the protease at 0.3 M NaCl, and the α-amylase at 0.5 M NaCl. The latter was further purified as described (11).

Fractions containing the propeptide were brought to 4 M NaCl and loaded on a Phenyl-Sepharose CL-4B column (1.5 × 20 cm), subsequently washed with a decreasing gradient (40 ml) from 4 to 0 M NaCl in 50 mM Tris-HCl, pH 7.5, at room temperature. Proteins were eluted with a gradient (40 ml) of 0–10% (v/v) isopropanol containing 2 mM

* This work was supported by EU Network Contract ERBCHCT 940521, Concerted Action BIO4-CT95-0017, and Program Grant BIO4-CT96–0051; by Ministère de l'Education, de la Recherche et de la Formation, Concerted Action ARC93/98-170; and by Région Wallonne-Direction Générale des Technologies, Convention 1828. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X58627.

§ To whom correspondence should be addressed. Tel.: 32-4-366-33-43; Fax: 32-4-366-33-64; E-mail: gfeller@ulg.ac.be.

PMSF.[1] Fractions containing the propeptide were then loaded on a DEAE-agarose column (1.5 × 40 cm) equilibrated in 20 mM Tris-HCl, 2 mM PMSF, pH 7.5, and eluted with a gradient of 0–0.2 M NaCl (500 ml). An Ultrogel AcA 54 column (2.5 × 100 cm) eluted with 20 mM Tris-HCl, pH 7.5, was used as the last chromatographic step.

The protease recovered from the first fractionation of the crude supernatant was further purified by Ultrogel AcA 54 and DEAE-agarose chromatography under the above-mentioned conditions, except that PMSF was omitted. The last purification step was carried out on a Beckman System Gold chromatograph fitted with an FPLC anion exchange column (Hydropore-AX, Rainin, 1 × 10 cm) eluted with a gradient of 0–0.2 M NaCl in 5 mM Tris-HCl, 15% (v/v) isopropanol, pH 7.5. For further experiments, the purified proteins were conditioned in the appropriate buffers by gel filtration on PD10 columns.

Mutagenesis of the amy Gene—An expression vector for the α-amylase precursor (pαH12) was constructed by ligating the *Hpa*I site located 60 nucleotides upstream from the initiation codon of the *amy* gene to the *Sma*I site of the pUC12 polylinker. This construction was used as template for subsequent PCR and inverse PCR amplifications by Vent^R DNA polymerase (New England Biolabs) using optimized conditions described elsewhere (14).

The vector pαH12WT* encoding for the recombinant mature α-amylase was constructed by PCR amplification of the *amy* gene using a silent sense primer and the mutating antisense primer 5′-CCTCTAGATTCATGAGGCAGAACTG-3′, which introduces a stop codon and a *Xba*I site after the mature enzyme coding sequence. The mutations were returned in the template using a *Pvu*II-*Xba*I restriction fragment. Deletion of the α-amylase coding region in pEPCT was carried out by inverse PCR of pαH12 using silent primers, 24 nucleotides in length, ending at codon GCT for Ala-30 (antisense primer) and starting at codon AAT for Asn-448 (sense primer). The amplification product was purified (QIAquick PCR purification kit, Qiagen), phosphorylated by T4 polynucleotide kinase, and circularized by ligation before transformation. Deletion of the *amy* gene in plasmid pEPST1 was produced by *Pst*I digestion of pαH12 and recircularization of the vector, retaining only a coding sequence ending at Ala-30.

The β-lactamase-propeptide fusion in pBLACT was performed by inverse PCR on a construction made of a propeptide coding region (*Eco*RV-*Xho*I) cloned downstream from the *bla* gene (*Xba*I-*Sph*I) of *Psychrobacter immobilis* (15) in the polylinker of pSP73 (Promega). Amplification used an antisense primer ending at codon AAC for the C-terminal Asn-362 from β-lactamase and a sense primer starting at codon AAT for Asn-448 from α-amylase. The hybrid coding sequence was then introduced in the kanamycin-resistant vector pBGS18+ (16) at restriction sites (*Xba*I-*Sph*I) identical to those of the cloned wild-type *bla* gene. The sequence of these constructions was checked by double-strand sequencing on an ALF DNA sequencer (Amersham Pharmacia Biotech).

Production and Purification of the Recombinant Enzymes—Recombinant proteins were expressed in *Escherichia coli* RR1 under the constitutive *lacZ* assistance (no isopropyl-1-thio-β-D-galactopyranoside induction) at 18 °C in a medium containing 16 g/liter Bactotryptone, 16 g/liter yeast extract, 5 g/liter NaCl, 2.5 g/liter K_2HPO_4, 0.1 μM $CaCl_2$, 100 mg/liter ampicillin. The recombinant precursor and mature α-amylase were purified using the protocol developed for the wild-type enzyme, except that concentration of the supernatant by ammonium sulfate at 70% saturation was required before the first chromatographic step.

Enzyme Assays—α-Amylase assay was carried out at 25 °C using 3.5 mM EPS (Boehringer Mannheim) as substrate and excess (23 units/ml) of α-glucosidase as coupling enzyme in 100 mM Hepes, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.1. Activities toward the synthetic substrate were recorded in a thermostated Uvikon 860 spectrophotometer (Kontron) and calculated on the basis of an absorption coefficient for 4-nitrophenol of 8,980 M^{-1} cm^{-1} at 405 nm (17). The kinetic parameters k_{cat} and K_m were determined by the initial velocity method using a nonlinear regression computer fit of the saturation curves.

The standard assay of β-lactamase was carried out at 25 °C with 300 μM nitrocefin (Glaxo Group Research) as the substrate in 50 mM phosphate buffer, pH 7.0 (18). Protease assays using azocasein as the substrate were performed as described previously (19).

Analytical Procedures—Dissociation constants K_d for chloride and calcium were determined by activation kinetics following Cl^- or Ca^{2+} titration of the apo-enzymes and fitting the saturation curves by a nonlinear regression analysis of the Hill equation, as described previously (11, 12). DTNB titration was carried out in 100 mM Tris, 1 mM EDTA, 1 mM DTNB, 8 M urea, pH 8.0, using an absorption coefficient for 2-nitro-5-thiobenzoate of 13,600 M^{-1} cm^{-1} at 412 nm (20).

Circular dichroism spectra were recorded in a 0.2-cm path length cell under constant nitrogen flush using a Jobin Yvon CD6 dichrograph. N-terminal amino acid sequences were determined using a pulsed-liquid-phase protein sequencer (Procise 492, Applied Biosystems, Perkin-Elmer Division, Foster City, CA) fitted with an on-line phenylthiohydantoin analyzer. C-terminal amino acid sequences were obtained on a Procise 494CT sequencer (Applied Biosystems, Perkin-Elmer Division) equipped for alkylthiohydantoin analysis. Electrospray ionization mass spectrometry of protein samples was performed on a BIO-Q electrospray mass spectrometer (Micromass, Altrincham, United Kingdom).

FIG. 1. **Nucleotide sequence and derived amino acid sequence of the C-terminal propeptide from the α-amylase precursor.** The propeptide cleavage site is indicated. Amino acids confirmed by N- and C-terminal sequencing are *underlined*, and the four cysteine residues are *double-underlined*. Amino acids are *numbered*, starting at the N-terminal residue of the native precursor after signal peptide cleavage. Nucleotide numbering corresponds to the sequenced genomic DNA fragment (GenBank/EMBL accession number X58627).

RESULTS

Sequence of the amy Gene and Characterization of the Gene Products—The cloning of the *amy* gene from *A. haloplanctis* in *E. coli*, its nucleotide sequence, and the deduced amino acid sequence of the native α-amylase have been reported previously (10). The updated nucleotide sequence of the 3′ region of the gene is shown in Fig. 1 along with the corresponding open reading frame. These new data indicate that the *amy* gene encodes an α-amylase precursor composed of 669 amino acid residues. N- and C-terminal amino acid sequences of the native α-amylase secreted by *A. haloplanctis* allow the location of three distinct functional domains of the precursor: (i) the peptide signal made of 24 residues, (ii) the mature enzyme composed of 453 residues with a M_r value of 49,340, and (iii) a large C-terminal propeptide composed of 192 residues (Fig. 1). In-

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; EPS, 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCR, polymerase chain reaction; AHP, *A. haloplanctis* protease.

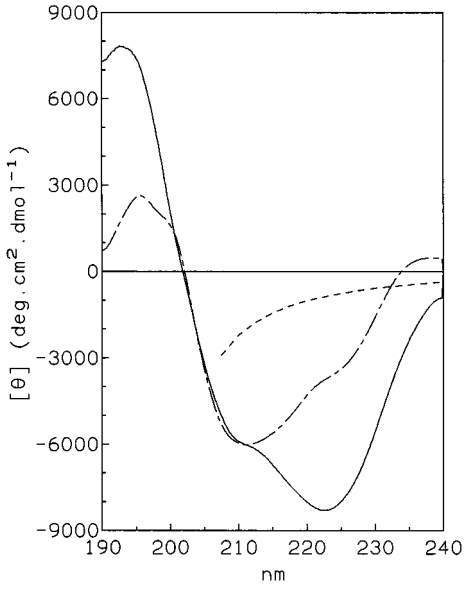

FIG. 2. **Far-UV circular dicroism spectra of α-amylase and its propeptide.** Spectra of the propeptide (*long dash*) and of α-amylase (*line*) were taken in 5 mM NaH_2PO_4, pH 7.0. Propeptide in the presence of 8 M urea (*dashed*). Data are expressed in terms of the mean residue ellipticity, Θ.

spection of *A. haloplanctis* culture supernatants revealed the occurrence of a 21-kDa protein, which was further purified to homogeneity. N- and C-terminal amino acid sequences identified this component as the α-amylase propeptide and confirmed the unique cleavage site between Ser-453 and Ser-454 (Fig. 1). Electrospray mass spectrometry yielded an M_r value of 21,518 ± 1.5, in excellent agreement with that deduced from the gene (21,519). However, electrophoretic mobility on SDS gels leads to overestimated values (see Fig. 5). The propeptide is a slightly acidic protein, having a pI = 5.0 determined under non-denaturing conditions. It contains four cysteine residues. Sulfhydryl titration by DTNB of both the native and the denatured protein in 8 M urea indicates that there is no free thiol group, demonstrating the occurrence of two disulfide linkages. There is no significant difference in the amino acid molar ratios of both native α-amylase and the cleaved C-terminal domain. A search through the GenBank/EMBL data banks failed to reveal any significant homology with known proteins or translated nucleotide sequences, except with the C-terminal region (34% identity in 200 amino acid overlap) of the hypothetical α-amylase from the nematode *Caenorhabditis elegans* (Swissprot P91982). To date, only the nucleotide sequence of *C. elegans* α-amylase is known, but multiple sequence alignment with the translated protein indicates that it also belongs to the Ca^{2+}- and Cl^--dependent α-amylase family. Its C-terminal extension (200 residues predicted) is possibly involved in secretion or it could serve to anchor the enzyme to the eukaryote membrane.

Secondary structure prediction algorithms (GCG 9.0) suggest a high content of β-sheet-forming residues, with several possible amphipathic β-sheets as indicated by hydrophobic moment analysis. This is further emphasized by the far-UV circular dichroism spectra of the secretion helper (Fig. 2). Secondary structure analysis of CD spectra (21) correctly estimated the α-helix and the β-sheet content (20% and 30%, respectively) of the known α-amylase three-dimensional structure (22) taken as reference. CD spectra of the propeptide contrasted with those of α-amylase and were typical of a β-pleated protein (50–60%) with a low α-helical content (~10%). Addition of urea abolished the CD signals and confirms the existence of secondary structure organization of the isolated propeptide.

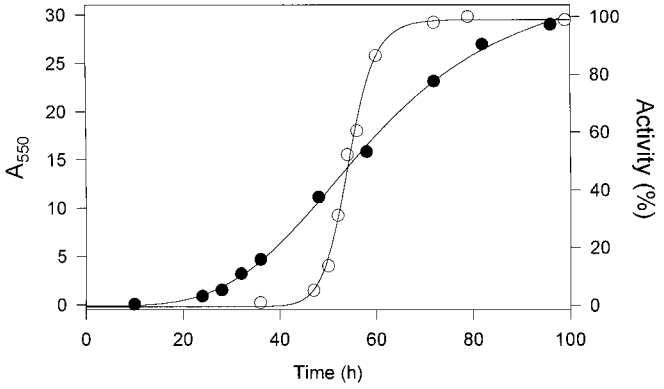

FIG. 3. **Growth of *A. haloplanctis* and α-amylase production.** Growth of the psychrophilic bacteria at 4 °C (●) and α-amylase activity in the culture supernatant (○).

Expression of the amy Gene in A. haloplanctis—*A. haloplanctis* is a psychrophilic bacterium efficiently growing at near-zero temperatures (generation time of 4.0 h at 4 °C) and reaching cell densities as high as 5×10^{10} cells/ml after 100 h of growth (Fig. 3). Addition of maltose up to 3% results in a 200-fold induction of α-amylase expression. Fig. 3 also shows that the amylolytic activity is sharply produced in the supernatant during the exponential growth phase. Rabbit antibodies raised against α-amylase and the propeptide were used to study expression of the *amy* gene in the wild-type strain. Western blot analysis of samples taken at all growth stages (Fig. 4) reveals that α-amylase is expressed in the culture supernatant as a 70-kDa precursor, which further dissociates into the mature enzyme and the free propeptide. The level of extracellular activity correlates with the production of the precursor rather than with the appearance of the mature enzyme, already suggesting that the precursor is active. Neither the precursor nor its two products have been detected in cell pellets.

Expression of the Recombinant Precursor in E. coli—In order to study the maturation mechanism, the precursor was expressed in *E. coli.* The *amy* gene was cloned downstream from the *lacZ* promoter of pUC12, resulting in the production of 60–100 mg of precursor/liter in the host culture supernatant. N- and C-terminal amino acid sequence determinations indicated that the signal peptide is correctly cleaved in *E. coli* and that no additional post-translational cleavage occurred. This recombinant precursor is fully active (see below), and a native conformation of the propeptide is expected, taking into account the extracellular location of the gene product.

When incubated in wild-type conditions (4 °C in sterile broth or in 10 mM imidazole and the medium salts), the purified precursor is stable for weeks. By contrast, addition of filter-sterilized aliquots of *A. haloplanctis* culture supernatants initiated its cleavage into α-amylase and the propeptide. This demonstrates the requirement for an extrinsic factor (*i.e.* a protease) in the maturation process.

Proteolytic Maturation of the α-Amylase Precursor—Only one proteolytic enzyme was detected in *A. haloplanctis* culture supernatants. This 45-kDa protease (AHP) is a metalloenzyme (60% inhibition by excess EDTA) from the serine-protease family (98% inhibition by 2 mM PMSF). AHP has a broad specificity and readily hydrolyses macromolecular substrates such as precipitated casein or azo-labeled casein. The N-terminal amino acid sequence of AHP (S-T-P-N-D-P-P-F-D-D-Q-S-Y-Y-E-Q-A-G-) shows strong homology with some other microbial Ser proteases such as those from *Bacillus thuringiensis* (accession no. JN0369) and *Dichelobacter nodosus* (no. L18984) and, notably, with the protease from a mesophilic *Alteromonas* strain (no. D38600).

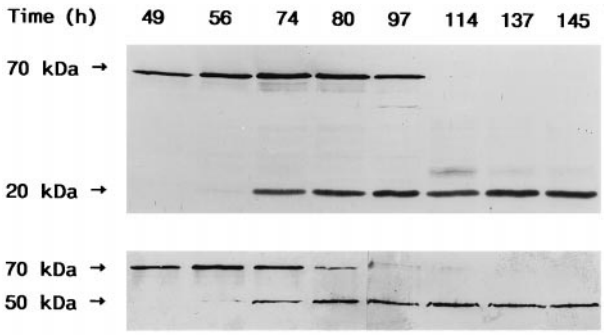

FIG. 4. **Secretion and maturation of the α-amylase precursor in *A. haloplanctis* culture supernatant.** Western blots of cell-free supernatant samples corresponding to the culture shown in Fig. 3. Sampling time and antigen molecular mass are indicated. *Upper panel*, antigen detection using rabbit IgG anti-propeptide. *Lower panel*, antigen detection by rabbit IgG anti-α-amylase. Secondary antibodies were alkaline phosphatase-conjugated anti-rabbit IgG.

When the purified AHP and the recombinant precursor are mixed in the *in vivo* ratio (≤1/10 in sterile broth), cleavage into α-amylase and the propeptide occurs at a rate similar to that recorded in *A. haloplanctis* cultures (Fig. 5). Furthermore, the N-terminal sequence of the *in vitro* processed propeptide is identical to that of the wild-type propeptide, demonstrating that AHP is the extrinsic factor required for the maturation of the precursor.

Linker Susceptibility to Proteolysis—The specific cleavage of the propeptide by the nonspecific AHP protease prompted us to test the action pattern of other nonspecific proteases such as Pronase, proteinase K, and subtilisin. As shown in Fig. 5, these proteases preferably cleaved the precursor in the linker region between α-amylase and the propeptide. Proteolytic cleavage by *E. coli* proteases was also noted during expression of the recombinant precursor. Indeed, about 5% of the produced enzyme is cleaved before starting the purification procedure. N-terminal sequence of the propeptide processed in *E. coli* reveals that the cleavage site is displaced between Thr-455 and Glu-456 (Fig. 1). A M_r value of 21,331 for this propeptide is predicted from the nucleotide sequence, in perfect agreement with the electrospray ionization mass spectrometric analysis (21,330 ± 4).

It is concluded that the linker region between α-amylase and the propeptide probably consists of a disordered, solvent-exposed loop, prone to various proteolytic attacks. This is also supported by the lack of defined electron density for the last five residues in the x-ray structure of *A. haloplanctis* α-amylase (22).

The Propeptide Has No Foldase Activity—The foldase activity generally associated with propeptides (8, 9) has been probed by removing the propeptide sequence from the *amy* gene and introducing a stop codon after Ser-453, the last residue of the mature wild-type enzyme. Properties of the wild-type and recombinant α-amylases as well as those of the recombinant precursor are compared in Table I. It is shown that both the kinetic and ion-binding parameters are identical in the three related enzymes. All the cysteine residues of the native α-amylase and of the propeptide are engaged in disulfide linkages. Thus, the lack of significant free sulfhydryl groups, as detected by DTNB titration, also confirmed the absence of misfolded species in the recombinant enzymes.

Heterologous Secretion in E. coli—Involvement of the propeptide in the secretion pathway of *A. haloplanctis* α-amylase has been analyzed by genetic modification of the *amy* gene and its expression in *E. coli*. Its function of secretion helper during translocation across the outer bacterial wall is well illustrated in Fig. 6. When the *amy* gene (pαH12) is expressed

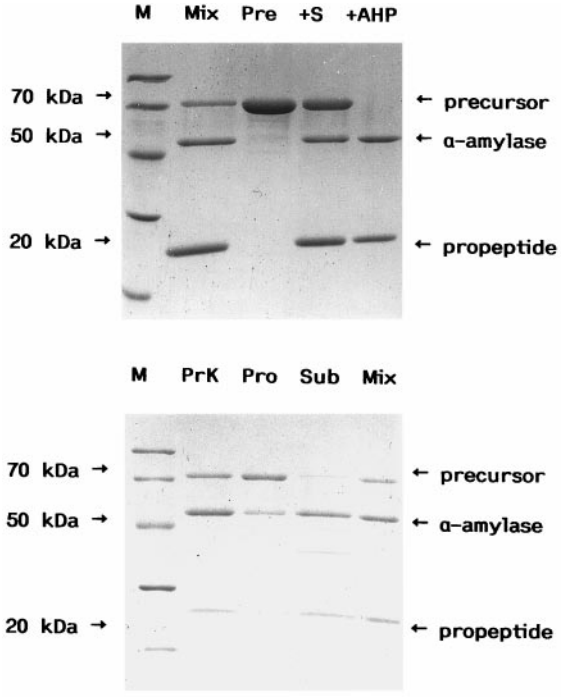

FIG. 5. ***In vitro* maturation of the recombinant α-amylase precursor.** *Upper panel*, 12% SDS-PAGE of protein standards (*M*, from the top: phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; and soybean trypsin inhibitor, 21.5 kDa); *Mix*, mixture of the purified recombinant precursor and of wild-type α-amylase and its propeptide; *Pre*, recombinant α-amylase precursor incubated at 4 °C in sterile culture medium for 1 week; *+S*, as for *Pre* with 10% volume of sterile cell-free supernatant from *A. haloplanctis* culture; *+AHP*, as for *Pre* with 5% (w/w) purified serine-protease from *A. haloplanctis*. *Lower panel*, maturation by nonspecific proteases. 12% SDS-PAGE of *M* and *Mix* (see *upper panel*); incubation of the precursor at room temperature with 0.1% proteinase K for 60 min (*PrK*), 1% Pronase for 160 min (*Pro*), or 1% subtilisin for 120 min (*Sub*). Western blot using IgG anti-propeptide (data not shown) confirmed the propeptide release.

in *E. coli*, the extracellular targeting of the precursor follows the bacterial growth curve. At the end of the exponential growth phase (≈30 h), about 80% of the total enzyme production is found in the cell-free supernatant of *E. coli*, 15% is found in the periplasmic space, and 5% remains cell-associated as determined by osmotic shocks (data not shown). The same results were obtained when the *amy* gene expression was reduced 100-fold (in *E. coli* BL21 expressing the LacI repressor), showing that the appearance of the precursor in the medium is not the result of its overexpression. By contrast, removal of the propeptide sequence (pαH12WT*) leads to periplasmic accumulation of the recombinant α-amylase (followed by its release into the medium during cell lysis). It is concluded that the propeptide efficiently assists outer membrane translocation in *E. coli*. The outer membrane integrity has been checked by monitoring the β-lactamase activity in the cell-free supernatants (β-lactamase is a periplasmic enzyme encoded by the plasmid vector and is responsible for the antibiotic resistance). Fig. 6 shows that heterologous secretion of the α-amylase precursor (pαH12) induces outer membrane damage in *E. coli* as indicated by the release of β-lactamase in the medium, by the slight growth inhibition and early cell lysis.

Self-secretion of the Propeptide—The autonomous outer membrane insertion of the propeptide was analyzed by removing the α-amylase coding region from the *amy* gene (pEPCT). In order to target the modified gene product into the periplasmic space, the peptide signal and the 30 N-terminal amino acids, which can form the export initiation domain (23, 24), were not

TABLE I
Kinetic parameters, dissociation constants, and free thiol groups for the wild-type and the recombinant α-amylases

α-Amylase	k_{cat}	K_m	k_{cat}/K_m	$K_{d(Cl-)}$	$K_{d(Ca)^{2+}}$	Cysteines[a]	Free thiol
	s^{-1}	μM	$s^{-1} \cdot \mu M^{-1}$	mM	M	mol^{-1}	mol^{-1}
Wild-type	780 ± 25	174 ± 8	4.6	5.9 ± 0.2	2.10^{-8}	8	0.03
Native, recombinant (pαH12WT*)	792 ± 34	168 ± 14	4.7	6.1 ± 0.2	2.10^{-8}	8	0.05
Precursor, recombinant (pαH12)	781 ± 39	171 ± 8	4.6	6.2 ± 0.2	2.10^{-8}	12	0.06

[a] Cysteines from the amino acid sequence.

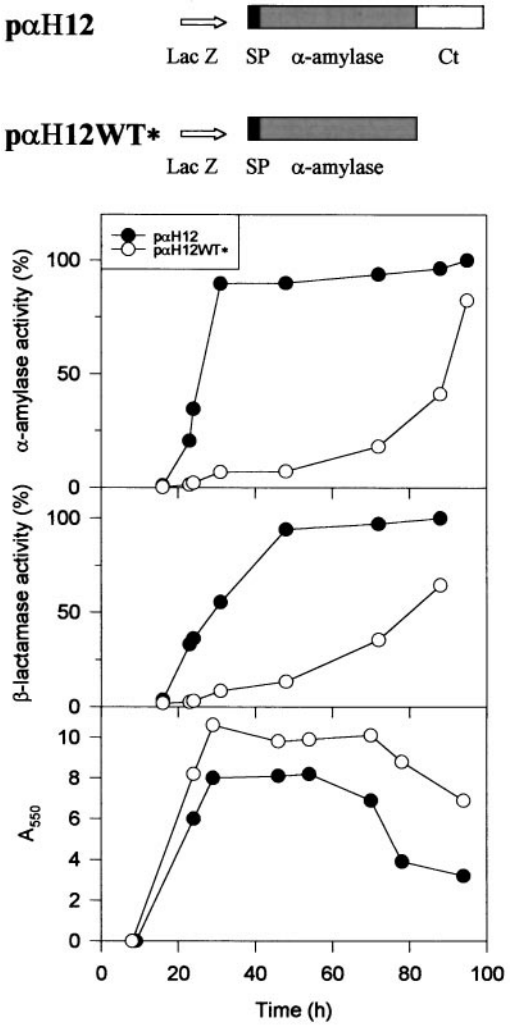

FIG. 6. **Expression of the precursor and of the recombinant native α-amylase in *E. coli*.** *Upper panel*, gene constructs encoding the 70-kDa precursor (pαH12) and the native α-amylase (pαH12WT*) devoid of the propeptide coding sequence. *SP*, signal peptide; *Ct*, C-terminal propeptide. *Lower panel*, α-amylase and β-lactamase activity in *E. coli* culture supernatants (activity is expressed as percent of the maximal activity recorded in the cell-free supernatant of *E. coli* (pαH12)) and bacterial growth at 18 °C (A_{550}).

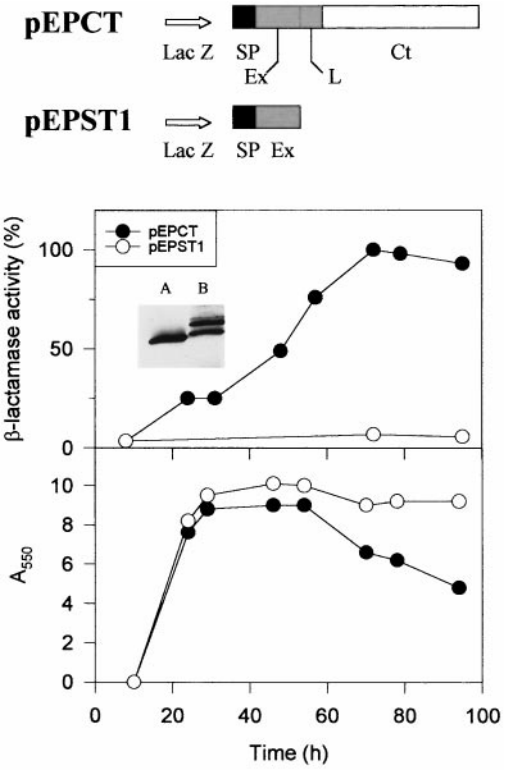

FIG. 7. **Expression of the propeptide in *E. coli*.** *Upper panel*, gene construct encoding the propeptide (*Ct*) preceded by the signal peptide (*SP*), the export initiation domain (*Ex*), and the linker (*L*) in pEPCT. The control construct pEPST1 only encodes signal peptide and export initiation domain. *Lower panel*, β-lactamase activity in *E. coli* culture supernatants (activity is expressed as percent of the maximal activity recorded in the cell-free supernatant of *E. coli* (pEPCT)) and bacterial growth at 18 °C (A_{550}). *Inset*, Western blot of the purified wild-type propeptide (*A*) and of the cell-free supernatant of *E. coli* (pEPCT) after 30 h (*B*) using IgG anti-propeptide for detection.

deleted. In addition, the six last C-terminal residues of the mature α-amylase were also conserved. Indeed, sequence alignment with other chloride-dependent α-amylases from insects and vertebrates shows that the bacterial enzyme is six residues longer at the C terminus. These residues can therefore belong to the linker region with the propeptide and were not deleted. The control construction (pEPST1) is identical but only encodes for the peptide signal and the 30 first α-amylase residues.

When the pEPCT construct is expressed in *E. coli*, two gene products recognized by IgG anti-propeptide already appear in the supernatant during the exponential growth phase and further accumulate in the extracellular medium (Fig. 7). As shown by Western blots, one compound corresponds to the wild-type propeptide, whereas the second has a slightly higher molecular mass (±3.5 kDa). It is likely that the propeptide is expressed with the export initiation domain and that cleavage at the linker by *E. coli* proteases further occurs in the periplasm, as already noted for the complete precursor. Autonomous propeptide translocation also induces outer membrane damage as indicated by β-lactamase leakage, whereas the control vector encoding for the export initiation domain alone does not affect the host cells.

β-Lactamase Targeting to the Extracellular Medium—The ability of the α-amylase propeptide to export a foreign passenger was tested by the construction of a β-lactamase-propeptide protein fusion. The class C β-lactamase from the Gram-negative bacterium *P. immobilis* A5 (15) was selected because (i) it is also a heat-labile psychrophilic enzyme; (ii) like other β-lactamases, the recombinant enzyme accumulates in the periplasmic space of *E. coli*; and (iii) it is devoid of disulfide bonds that may impair outer membrane translocation in *E. coli* as reported previously (25), although the α-amylase contains four disulfide bonds but is secreted efficiently.

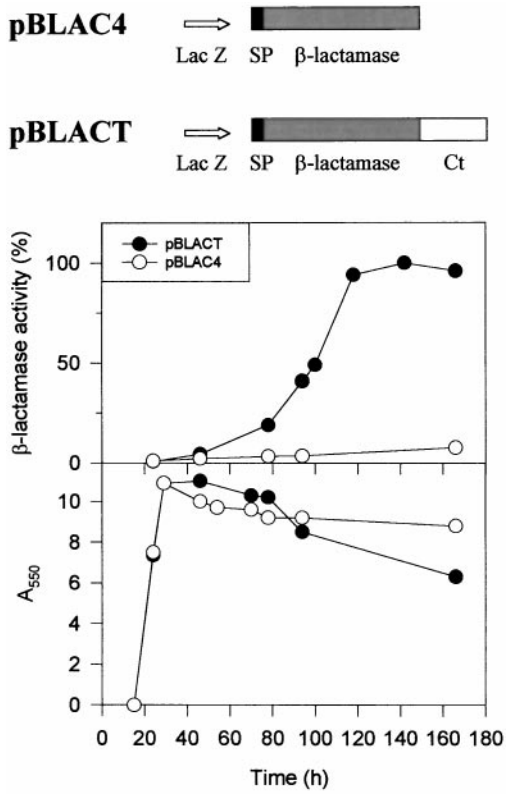

FIG. 8. **Expression of the β-lactamase-propeptide fusion protein in *E. coli*.** *Upper panel*, gene constructs encoding the wild-type β-lactamase (pBLAC4) and its fusion with the propeptide (pBLACT). *Lower panel*, β-lactamase activity in *E. coli* culture supernatants (activity is expressed as percent of the maximal activity recorded in the cell-free supernatant of *E. coli* (pBLACT)) and bacterial growth at 18 °C (A_{550}).

The coding sequence of the propeptide and of the six C-terminal linker residues were fused to the *bla* gene by inverse PCR and cloned in a kanamycin-resistant vector (pBLACT). The control vector pBLAC4 is identical but only carries the wild-type *bla* gene. The β-lactamase-propeptide fusion remains catalytically active and provides the usual ampicillin resistance; its specific activity on nitrocefin in clear periplasmic extracts is similar to that of the wild-type β-lactamase prepared in the same conditions. Unlike the native β-lactamase, which remains periplasmic, the β-lactamase-propeptide fusion appears in the cell-free supernatant as shown in Fig. 8. However, the extracellular targeting of the fusion is delayed when compared with α-amylase (pαH12), indicating a less efficient translocation process. No cleavage of the fusion protein was detected in the supernatant by Western blots using IgG anti-propeptide but mild proteolytic treatment using proteinase K, Pronase, and *A. haloplanctis* serine protease allowed to remove the C-terminal propeptide (data not shown).

DISCUSSION

In most enzymes possessing an N- or C-terminal propeptide, truncation of the prosequence precludes correct folding of the associated enzyme leading to inactive polypeptides. A foldase activity or an intramolecular chaperone function has been therefore attributed to propeptides (8, 9). We have shown that *A. haloplanctis* α-amylase produced in the presence or in the absence of the propeptide has the same catalytic and ion binding properties (Table I). Owing to the stringent structural requirements for functional substrate and ion binding site formation, it can be safely concluded that the enzyme synthesized without the propeptide is properly folded and, therefore, that its C-terminal domain is definitely not an intramolecular chaperone.

By contrast, the propeptide is involved in the translocation across the outer bacterial wall, as evidenced by its autonomous translocation and the extracellular targeting of the α-amylase precursor in *E. coli* (Figs. 6 and 7). In the latter, propeptide-assisted membrane spanning induces outer membrane alterations, which are likely to be due to differences in outer wall composition (especially in lipids) between the mesophilic *E. coli* and the psychrophilic *A. haloplanctis*. The purified propeptide has a high β-sheet content (Fig. 2). Additionally, it is interesting that the 15 last residues of the propeptide can form an amphipathic β-sheet ending with a C-terminal phenylalanine, which are essential features for the correct assembly of most bacterial outer membrane proteins such as PhoE (26).

The following two-step secretion pathway of *A. haloplanctis* α-amylase can be proposed. The 200-fold induction of α-amylase production suggests the occurrence of a maltose-regulated promoter as already reported for some other microbial α-amylases (27). After gene induction and initiation of the translation, the nascent α-amylase precursor undergoes the classical *sec*-dependent inner membrane translocation, as evidenced by the occurrence of a cleavable signal peptide. The periplasmic intermediate then inserts into the outer wall via its C-terminal propeptide in a way probably similar to other bacterial outer membrane proteins. However, the next specific events involve translocation of the α-amylase domain across the outer wall and the extracellular release of the uncleaved precursor in a native conformation. The last step requires the assistance of the nonspecific AHP protease in order to remove the C-terminal secretion helper by cleavage at the easily accessible linker region. As the precursor is not detected in *A. haloplanctis* cell pellets, the post-transcriptional events leading to secretion seem very fast, without accumulation of detectable cell-associated intermediates. The propeptide-assisted mechanism of membrane spanning (through or beside a possible β-barrel) and the translocation driving force remain unknown.

To our knowledge, few other bacterial exoenzymes possessing a C-terminal propeptide have been reported: *Neisseria* IgA proteases (5–7), *Thermus aquaticus* aqualysin I protease (28), *Serratia marcescens* SSP protease (29), *Helicobacter pylori* VacA cytotoxin (30), *Lysobacter enzymogenes* alkaline phosphatase (31), *E. coli* and *Shigella* virulence proteins EspC (32) and VirG (33), and *Bordetella pertussis* pertactin (34). According to the available data, the extracellular routing of these enzymes and of α-amylase should follow the same main steps of the above mentioned secretion pathway. However, the propeptide from *A. haloplanctis* α-amylase precursor is unusual because it remains associated to the precursor in the external medium, it requires external proteolytic assistance for cleavage, it can be recovered from supernatants, and it has no intramolecular chaperone function.

Acknowledgments—We are grateful to the Institut Français de Recherche et de Technologie Polaire for the support and facilities offered at the Antarctic station Dumont d'Urville during earlier stages of this work. We also thank B. Samyn for expertise in the C-terminal amino acid analysis, as well as N. Gerardin-Otthiers and R. Marchand for expert technical assistance.

REFERENCES

1. Pugsley, A. P. (1993) *Microbiol. Rev.* **57,** 50–108
2. Salmond, G. P. C., and Reeves, P. J. (1993) *Trends Biochem. Sci.* **18,** 7–12
3. Gennity, J. M., and Inouye, M. (1991) *Curr. Opin. Biotechnol.* **2,** 661–667
4. Hirst, T. R., and Welch, R. A. (1988) *Trends Biochem. Sci.* **13,** 265–269
5. Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T. F. (1987) *Nature* **325,** 458–462
6. Klauser, T., Krämer, J., Otzelberger, K., Pohlner, J., and Meyer, T. F. (1993) *J. Mol. Biol.* **234,** 579–593
7. Klauser, T., Pohlner, J., and Meyer, T. F. (1993) *BioEssays* **15,** 799–805
8. Shinde, U., and Inouye, M. (1993) *Trends Biochem. Sci.* **18,** 442–446

9. Eder, J., and Fersht, A. R. (1995) *Mol. Microbiol.* **16,** 609–614
10. Feller, G., Lonhienne, T., Deroanne, C., Libioulle, C., Van Beeumen, J., and Gerday, C. (1992) *J. Biol. Chem.* **267,** 5217–5221
11. Feller, G., Payan, F., Theys, F., Qian, M., Haser, R., and Gerday, C. (1994) *Eur. J. Biochem.* **222,** 441–447
12. Feller, G., le Bussy, O., Houssier, C., and Gerday, C. (1996) *J. Biol. Chem.* **271,** 23836–23841
13. Aghajari, N., Feller, G., Gerday, C., and Haser, R. (1996) *Protein Sci.* **5,** 2128–2129
14. Cease, K. B., Potcova, C. A., Lohff, C. J., and Zeigler, M. E. (1994) *PCR Methods Appl.* **3,** 298–300
15. Feller, G., Zekhnini, Z., Lamotte-Brasseur, J., and Gerday, C. (1997) *Eur. J. Biochem.* **244,** 186–191
16. Spratt, B. G., Hedge, P. J., te Heesen, S., Edelman, A., and Broom-Smith, J. K. (1986) *Gene* (*Amst.*) **41,** 337–342
17. Rauscher, E., Neumann, U., Schaich, E., von Bülow, S., and Wahlefeld, A. W. (1985) *Clin. Chem.* **31,** 14–19
18. O'Callaghan, C., Morris, A., Kirby, S., and Shingler, A. (1972) *Antimicrob. Agents Chemother.* **1,** 283–288
19. Davail, S., Feller, G., Narinx, E., and Gerday, C. (1994) *J. Biol. Chem.* **269,** 17448–17453
20. Habeeb, A. (1973) *Anal. Biochem.* **56,** 60–65
21. Provencher, S. W., and Glöckner, J. (1981) *Biochemistry* **20,** 33–37
22. Aghajari, N., Feller, G., Gerday, C., and Haser, R. (1997) *Protein Sci.* **7,** 564–572
23. Rasmussen, B. A., and Silhavy, T. J. (1987) *Genes Dev.* **1,** 185–196
24. Anderson, H., and von Heijne, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 9751–9754
25. Klauser, T., Pohlner, J., and Meyer, T. F. (1992) *EMBO J.* **11,** 2327–2335
26. Struyvé, M., Moons, M., and Tommassen, J. (1991) *J. Mol. Biol.* **218,** 141–148
27. Virolle, M. J., Long, C. M., Chang, S., and Bibb, M. J. (1988) *Gene* (*Amst.*) **74,** 321–334
28. Kurosaka, K., Ohta, T., and Matsuzawa, H. (1996) *Mol. Microbiol.* **20,** 385–389
29. Ohnishi, Y., Nishiyama, M., Horinouchi, S., and Beppu, T. (1994) *J. Biol. Chem.* **269,** 32800–32806
30. Schmitt, W., and Haas, R. (1994) *Mol. Microbiol.* **12,** 307–319
31. Au, S., Roy, K. L., and von Tigerstrom, R. G. (1991) *J. Bacteriol.* **173,** 4551–4557
32. Stein, M., Kenny, B., Stein, M. A., and Finlay, B. B. (1996) *J. Bacteriol.* **178,** 6546–6554
33. Suzuki, T., Lett, M.-C., and Sasakawa, C. (1995) *J. Biol. Chem.* **270,** 30874–30880
34. Charles, I., Fairweather, N., Pickard, D., Beesley, J., Anderson, R. Dougan, G., and Roberts, M. (1994) *Microbiology* **140,** 3301–3308