Proteolysis in Heterocyst-Forming Cyanobacteria: Characterization of a Further Enzyme with Trypsin-Like Specificity, and of a Prolyl Endopeptidase from *Anabaena variabilis*

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Dedicated to Prof. Achim Trebst on the occasion of his 65th birthday

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Anabaena variabilis, Cyanobacterium, Heterocysts, Proteolytic Enzymes, Prolyl Endopeptidase

Soluble extracts of the cyanobacterium *Anabaena variabilis* ATCC 29413 and an engineered mutant that lacks an intracellular protease cleaving after Lys and Arg (Maldener, Lockau, Cai, and Wolk, Mol. Gen. Genet. **225,** 113–120 (1991)) were separated by ion exchange chromatography, and protease profiles determined using azocasein, Nα-benzoyl-D,L-arginine-4-nitroanilide and N-carbobenzoxy-glycyl-L-proline-4-nitroanilide as substrates. A second enzyme cleaving at the carboxyl side of lysine and arginine, and a prolyl endopeptidase were detected, enriched and characterized. Both proteolytic enzymes appear to be located in the periplasm.

Introduction

Under aerobic conditions, certain filamentous cyanobacteria like *Anabaena* are able to fix molecular nitrogen in heterocysts, cells specialized to provide an anaerobic environment for this process [1]. An early step in the differentiation of vegetative cells to heterocysts is an increase in the rate of intracellular proteolysis [2–4], which can be observed when cultures grown in a medium containing a fixed nitrogen source such as ammonia or nitrate are transferred to a nitrogen-free medium ("nitrogen stepdown"). Nitrogen stepdown induces nearly synchronous heterocyst differentiation. The proteolysis degrades some proteins that are abundant in vegetative cells only, like ribulose-bisphosphate carboxylase, phycobiliproteins and subunits of photosystem II, and is believed to provide amino acids for the synthesis of proteins that are specific for or abundant in heterocysts, like nitrogenase [1, 5].

The enzymes responsible for the enhanced proteolysis are not known with certainty and the mechanism of their regulation is not well understood. An inhibitor of translation, chloramphenicol, has been reported to inhibit the enhancement of proteolysis when supplied 2 h before nitrogen stepdown [6]. One of the candidate enzymes, a calcium-dependent protease with a specificity like trypsin [3, 7], has been inactivated by site-directed mutagenesis in two strains of *Anabaena* [8]. This mutation has no detectable consequences on the physiology of the cyanobacteria.

Using mutant IM 141 of *Anabaena variabilis,* which lacks the calcium-dependent protease, we here show that the cyanobacterium contains a second soluble protease that cleaves at the carboxyl side of Lys and Arg, like trypsin. In the course of purification of this enzyme, a prolyl endopeptidase was detected. Both enzymes may be located in the periplasm. The extractable activity of the enzymes was not influenced by conditions promoting heterocyst formation, *i.e.* nitrogen deprivation of the growth medium.

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Abbreviations: BAPNA, Nα-benzoyl-D,L-arginine-4-nitroanilide; DEAE-, diethylaminoethyl-; EGTA, ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; NA, 4-nitroanilide; TLCK, Nα-4-toluenesulfonyl-L-lysine chloromethyl-ketone; *Z*-Gly–Pro–NA, N-carbobenzoxy-glycyl-L-proline-4-nitroanilide; 50 T 8, 50 mM Tris, adjusted to pH 8.0 with HCl.

Enzymes: Aminopeptidase M (EC 3.4.11.2); prolyl endopeptidase (EC 3.4.21.26); trypsin (EC 3.4.21.4).

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Materials and Methods

Sources

The cyanobacterium *Anabaena variabilis* ATCC 29413 strain FD [9], used as the wild type, was obtained from Prof. C. P. Wolk (East Lansing, Michigan). Its engineered mutant IM 141, which lacks a calcium-dependent protease, is described in ref. [8]. Substrates and inhibitors were purchased from Bachem (Heidelberg), Boehringer (Mannheim), Fluka (Neu-Ulm), Serva (Heidelberg) and Sigma (Deisenhofen), [^{3}H]diisopropyl fluorophosphate from Amersham (Braunschweig), SDS-PAGE standards and Biogel P-200 from Bio-Rad (München), Phenyl-Sepharose CL-4B, Poly Buffer Exchanger 94 and Polybuffer 74 from Pharmacia (Freiburg), DE-52 from Whatman. Aminopeptidase M (EC 3.4.11.2) from porcine kidney microsomes was purchased from Sigma.

Crude extracts

Anabaena variabilis and its mutant IM 141 were grown photoautotrophically with N_2 or, where indicated, NH_4NO_3 as nitrogen source in continuously illuminated batch culture as described [7, 10]. At the end of the logarithmic growth phase, cells were harvested by centrifugation (20 min at $2000 \times g$), washed once with water and stored at −20 °C as a concentrated, frozen suspension in 50 mM Tris, adjusted to pH 8.0 with HCl ("50T8"). Aliquots of 100 ml were thawed, cells disrupted by cavitation (9×1 min with 1 min intervals, ice-water cooling, Branson Sonifier B-12, ½″ horn, setting 8) and the homogenate centrifuged for 30 min at $48{,}000 \times g$. The pellet was resuspended in 100 ml of 50T8 and cavitated and centrifuged as before. The combined supernatants served as crude extracts.

Preparation of heterocysts

Approx. 1 l of wild type culture grown on N_2 was harvested by centrifugation (20 min at $4000 \times g$), washed twice with TEM buffer (50 mM Tris, 25 mM EDTA, 25 mM mannitol, pH 8.1). The filaments were incubated for 30 min in 20 ml of TEM buffer containing 1 mg/ml of egg white lysozyme, collected by centrifugation, resuspended in 25 ml TEM buffer and sonicated for approx. 20 min in an ultrasonic cleaning bath (Sonorex RK 100, Bandelin, Berlin, F.R.G.) filled with ice-cold water. This treatment distroyed nearly all vegetative cells, as followed in a phase contrast microscope. An aliquot of 5 ml was saved as the source of total cell extract. Heterocysts were collected from the remaining suspension by a 20 min centrifugation at $300 \times g$, washed twice with 20 ml TEM (5 min centrifugation at $150 \times g$) and resuspended in 5 ml TEM. Cell-free extracts of total cells and of heterocysts were obtained by sonication of the two 5 ml aliquots for 1.5 min/ml (Branson Sonifier B-12, conical microtip, setting 4, ice/water cooling) followed by a 30 min centrifugation at $48{,}000 \times g$.

Enzyme assays

Hydrolysis of chromogenic substrates: These were dissolved as described [7, 11]. The initial rate of appearance 4-nitroaniline was determined photometrically at 405 nm or (in an Aminco DW 2 dual wavelength photometer) at 405 minus 470 nm in a solution containing 0.1 M Tris (pH 8.0) and usually 0.5 mM of the chromogenic substrates, $T = 37$ °C. An extinction coefficient of 9.8 $mM^{-1} \times cm^{-1}$ was used. The reaction was started with enzyme extract.

Hydrolysis of azocasein: Assays contained 0.26% (w/v) azocasein in 90 mM Tris (pH 8.0) and were started with enzyme extract. The temperature was 30 °C. At various times for up to 4 h, aliquots were mixed with ⅓ volume of 40% (w/v) trichloroacetic acid, 30 min thereafter centrifuged at $8000 \times g$ for 2 min and the absorbance of supernatants determined at 340 nm. Controls were run without azocasein and without extract, respectively.

Estimation of protein

The "Micro BCA Protein Assay Reagent" from Pierce was used. Bovine serum albumin served as standard. Before assay, protein of the samples was precipitated by the deoxycholate/trichloroacetic acid procedure [12].

Profiles of soluble proteases

Crude extracts from 1.5 l cultures of *A. variabilis* and of its mutant IM 141, grown with N_2 or NH_4NO_3 as the nitrogen source to the late logarithmic phase of growth, were prepared as described above and dialyzed for 2 h against 5 mM Tris, pH 8.5, 1 mM $CaCl_2$. The supernatants of a 15 min centrifugation at $48{,}000 \times g$ were applied to columns of DE-52 (bed

volume of 30 ml, 1.5 cm diameter) equilibrated with 5 mM Tris, pH 8.5, 1 mM $CaCl_2$. The columns were developed first with 10 ml of equilibration buffer, then with a linear salt gradient (5 mM Tris, pH 8.5, 1 mM $CaCl_2$ to 50 mM Tris, pH 8.5, 1 mM $CaCl_2$, 1.2 M NaCl; total gradient volume = 120 ml). Fractions of 6 ml were collected and assayed for hydrolysis of BAPNA and of azocasein.

Purification of the protease with trypsin-like specificity

The purification started with crude extract prepared from 60 l of culture of mutant IM 141 grown on N_2. All purification steps were carried out at 0–6 °C. Activity of the protease was assayed with 0.5 mM BAPNA as the chromogenic substrate under the conditions described above.

Ammonium sulfate precipitation: The extract was fractionated by addition of solid $(NH_4)_2SO_4$ to final concentrations of 20, 40 and 70% followed by 30 min centrifugations at 12,000 × *g*. The pellet of the 70% step was dissolved in a minimal volume of 50 T 8 and dialyzed against this buffer overnight, followed by a 30 min centrifugation at 12,000 × *g*.

Ion exchange chromatography: Dialyzed extract containing up to 5 g of protein was applied to a column of DE-52 (560 ml bed volume, 5 cm diameter, equilibrated with 50 T 8). The column was washed with 0.5 l 50 T 8, followed by a linear gradient of 0.6 l from 0 to 1.2 M NaCl in 50 T 8. The protease started to elute towards the end of the gradient. Elution was continued with 1.2 M NaCl in 50 T 8 until the activity was completely recovered.

Hydrophobic interaction chromatography: Active fractions from DE-52 chromatography were combined, solid NaCl added to 2 M final concentration, and applied to column of Phenyl-Sepharose CL-4 B (50 ml bed volume, diameter 1.6 cm, equilibrated with 2 M NaCl in 100 mM Tris, pH 8.0). After washing with 150 ml equilibration buffer, the protease was eluted with a linear, descending gradient from equilibration buffer to 10 mM Tris (pH 8.0) with a total volume of 0.5 l. The protease eluted as a broad peak approximately in the middle of the gradient.

Chromatofocussing: Active fractions from the Phenyl-Sepharose column were combined and concentrated in a dialysis bag imbedded in solid polyethylene glycol (average MW = 20,000) to *ca.* 5 ml. Following a 2 h dialysis against 25 mM L-histidine (pH 6.2 with HCl), the sample was applied to a column of Poly Buffer Exchanger 94 (bed volume 11 ml, equilibrated with the histidine buffer). After washing with 20 ml of the histidine buffer, the enzyme was eluted with 110 ml of an 8-fold dilution of Polybuffer 74 (adjusted to pH 4.0 with HCl). The protease eluted at pH 4.2 to 4.0, well separated from the main protein peak. Since the protease was unstable under these conditions, the fractions were brought to a pH value of 8 to 8.5 by addition of aliquots of 0.5 M Tris (pH 8.5).

Size exclusion chromatography: This was carried out on a column of Bio-Gel P 200 (200 ml bed volume, 2.5 cm diameter) in 100 mM Tris, pH 8.0. Bovine serum albumin, ovalbumin and chymotrypsinogen served as standards.

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate was performed on slab gels containing 15% acrylamide as described [7]. Native PAGE was carried out on 13.5 cm long, 2 mm thick slab gels. The stacking gel contained 4%/0.11% acrylamide/N,N′-methylene bis-(acrylamide) in 62 mM Tris (pH 6.7 with HCl). The separating gel consisted of 10%/0.27% acrylamide/N,N′-methylene bis-(acrylamide) in 10% glycerol/375 mM Tris (pH 8.9 with HCl). The electrode buffer was 50 mM Tris/380 mM glycine (pH 8.3). Electrophoresis was carried out at room temperature, initially at 100 V. When the tracking dye, bromphenol blue, entered the separating gel, the voltage was increased to 150 V.

Staining for protease activity: Each sample was applied to two tracks of the "native" polyacrylamide slab gel. After electrophoresis, the gel was cut into two halfs and one half fixed for 1 h with 10% trichloroacetic acid plus 20% methanol, followed by staining with Coomassie Brilliant Blue R-250 [13]. The other half was incubated at 37 °C with 0.5 mM BAPNA or 0.125 mM *Z*-Gly–Pro–NA in 100 mM Tris, pH 8.0 and periodically scanned at 395 nm for 4-nitroaniline formation. The area showing nitroaniline formation was cut out, the gel piece placed on a track of a 2 mm thick polyacrylamide slab gel containing SDS, overlayed with sample buffer containing 2% SDS [14], electrophoresed and stained with Coomassie.

Analysis of [^{3}H]diisopropyl fluorophosphate incorporation

Incubation, SDS-PAGE and fluorography were carried out as described [7].

Results and Discussion

Profiles of soluble proteases in crude extracts from *Anabaena variabilis* and its mutant IM 141, grown on N_2 as nitrogen source, are shown in Fig. 1.

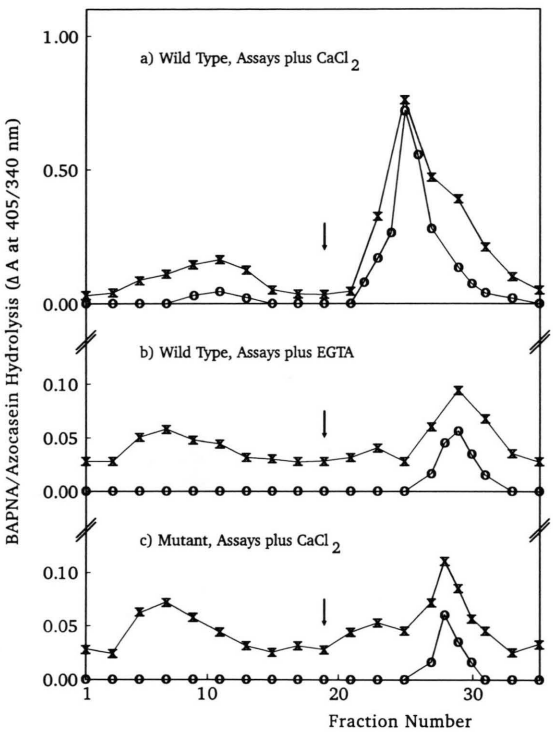

Fig. 1. Protease profiles of wild type (a, b) and of mutant IM 141 (c) of *Anabaena variabilis* ATCC 29413 strain FD grown on N_2 as nitrogen source. Crude extracts were dialyzed and chromatographed on columns of DEAE-cellulose. Fractions were assayed for hydrolysis of Nα-benzoyl-D,L-arginine-4-nitroanilide (o) and of azocasein (x). Assays contained 0.5 mM $CaCl_2$. In (b), the fractions of (a) were used, but $CaCl_2$ in the assays was replaced by 0.5 mM ethyleneglycol-bis-(2-aminoethyl-ether)-N,N,N′N′-tetraacetic acid to inhibit Ca^{2+}-dependent protease(s). Azocasein was hydrolyzed also without extract; this background was not subtracted so as to give an impression of the "signal-to-noise ratio" in these experiments. Note the expanded absorbance scales in b and c. Arrows indicate the beginning of the salt gradient. Rates are given as $\Delta A_{405\,nm}$ (ml extract × min)$^{-1}$ for BAPNA and $\Delta A_{340\,nm}$ (ml extract × 240 min)$^{-1}$ for azocasein, respectively. Further details are described in the text.

Extracts were separated by ion exchange chromatography on DEAE-cellulose and the fractions analyzed for hydrolysis of BAPNA (a chromogenic substrate for proteases cleaving at the carboxyl side of arginine) and of the "general" protease substrate azocasein. In extracts of the wild type, cleavage of both these substrates was largely Ca^{2+}-dependent (compare parts a and b of Fig. 1). When the wild type extract was assayed in the presence of EGTA so as to inhibit the Ca^{2+}-dependent protease, the profile was similar to that of the mutant extract assayed with $CaCl_2$ (compare Fig. 1 b and c). The differences in both profiles were within the range of column-to-column variations and are not considered to be significant. The results with mutant extracts were essentially identical when $CaCl_2$ in the assays was replaced by EGTA. Results indistinguishable from those of Fig. 1 were obtained with extracts prepared from filaments grown on NH_4NO_3 or from filaments harvested 5 h after transfer from medium containing NH_4NO_3 to medium free of combined nitrogen ("nitrogen stepdown", data not shown). This suggests that the extractable activity of soluble proteases detected with BAPNA or azocasein does not change upon transfer of *Anabaena* to a medium promoting the formation of heterocysts. Of course, this does not exclude changes in the activity of proteolytic enzymes that are membrane-associated or have a different substrate specificity or require special assay conditions.

Three peaks hydrolyzing BAPNA were evident in crude extracts from the wild type of *Anabaena* (Fig. 1 a, b): One in the flow-through of the column around fraction 11, a large one around fraction 25, and a third one (peaking at fractions 28–29) clearly visible in the Ca^{2+}-free assays and in extracts from the mutant. The first two peaks are both from the Ca^{2+}-dependent protease characterized previously [7, 8]: They are Ca^{2+}-dependent and absent in the mutant. Furthermore, when the first or the second peak were rechromatographed on the DEAE column under the same conditions, the splitting into two peaks was again observed.

Purification of the Ca^{2+}-independent, BAPNA-hydrolyzing enzyme

The enzyme was purified from mutant IM 141 of *Anabaena variabilis*. The mutant lacks the Ca^{2+}-dependent protease. The individual steps, described

Table I. Purification steps of the protease with trypsin-like specificity. Enzymic activity was assayed at 37 °C with 0.5 mM Nα-benzoyl-arginine-4-nitroanilide as substrate. Details are given under Materials and Methods.

Step	Protein [mg]	Specific activity nmol [mg protein^{-1} min^{-1}]	Yield [%]	Purification [-fold]
1. Crude extract	16630	0.12	100	1
2. $(NH_4)_2SO_4$ precipitate	2252	0.58	65	4.8
3. DE-52 chromatography	580	2.81	82	23.4
4. Hydrophobic interaction	51	25.4	65	212
5. Chromatofocussing	3.9	210	41	1750

under Materials and Methods, are summarized in Table I. Activity was assayed with the chromogenic substrate BAPNA. Ion exchange chromatography on DEAE-cellulose somewhat increased total activity when compared to the preceding step. This column may remove inhibitory substance(s), *e.g.* peptides. After the final chromatofocussing column, the yield was 40%, the purification 1700-fold. During purification, no evidence was obtained for additional enzymes hydrolyzing BAPNA.

Analysis of the active fractions from the chromatofocussing column by SDS-PAGE showed polypeptide bands with apparent molecular masses of 70, 42, 40, 37.5 and 25 kDa of roughly equal staining intensity with Coomassie R-250 (not shown). Attempts to further purify the protease by HPLC chromatography on ion exchange (AX-300) and on hydrophobic interaction (BU-300) columns failed to remove any of these peptides. The enzyme did not bind to the affinity media arginine–agarose, lysine–agarose and aprotinin–agarose.

Using BAPNA as substrate, enzymic activity of the protease was detected after electrophoresis in non-denaturing polyacrylamide gels as a single, fairly sharp band (Fig. 2). When that area of the gel was excised and analyzed by SDS-PAGE, the 70, 37.5 and 25 kDa peptides were still present (not shown). In size exclusion chromatography (see Materials and Methods), the enzyme migrated slightly slower than the monomer of BSA, suggesting a native mass around 60 kDa (not shown). Incubation of the enzyme preparation with [^{3}H]diisopropyl fluorophosphate prior to SDS-PAGE, under conditions successfully employed with the propyl endopeptidase (described below) and previously with the calcium-dependent protease of *Anabaena* [7], did not label any of the polypeptides detectably. It thus remains unclear which of the three polypeptides are constituents of the protease.

The enriched enzyme hydrolyzed azocasein (compare Fig. 1). The specificity of the protease was characterized with a range of chromogenic substrates (Table II). The enzyme cleaved at the carboxyl sides of Arg and Lys, *i.e.* with a specificity like trypsin. The affinity of the enzyme to substrates containing a C-terminal Lys was lower than to those with an Arg at this position (Table II). The enzyme was strongly inhibited by the trypsin inhibitor TLCK and by the inhibitors of serine proteases, leupeptin and antipain (Table III), suggesting its identification as a serine protease. It was, however, only

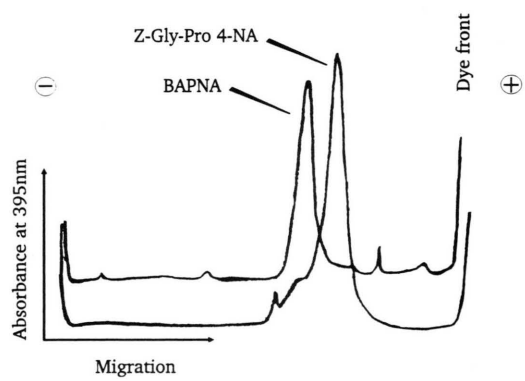

Fig. 2. Activity staining with Nα-benzoyl–Arg 4-nitroanilide (BAPNA) and with N-carbobenzoxy-Gly–Pro 4-nitroanilide (*Z*-Gly–Pro 4-NA) in a non-denaturing polyacrylamide gel. Active fractions from the chromatofocussing column (BAPNA) and from the Phenyl-Sepharose column (*Z*-Gly–Pro 4-NA) were combined and approx. 20 μg of protein subjected to non-denaturing PAGE. The appropriate parts of the gel were then incubated at room temperature with BAPNA or *Z*-Gly–Pro 4-NA and scanned at 395 nm for 4-nitroaniline formation as described under Materials and Methods. Both traces are from the same slab gel.

Table II. Substrate specificity of the purified protease. For the determination of relative rates, all substrates were added from stock solutions of 10 mM in dimethyl sulfoxide to a final concentration of 0.4 mM (0.125 mM in the case of N-carbobenzoxy-Gly-Pro-NA) in the assays. The absorbance of the reaction mixtures at 405 nm was determined immediately after mixing and after a 1 h incubation at 37 °C. The rate with Nα-benzoyl-Arg-NA was set to 100%, the level of detection estimated to <5% of that rate. For the determination of K_M values, the concentration of substrates was varied in the range of 0.008 to 0.5 mM. Initial rates were determined (see Materials and Methods) and the K_M values determined from Lineweaver-Burk plots. *Abbreviations:* NA, 4-nitroanilide; tosyl, 4-toluenesulfonyl.

Substrate	Relative rate [%]	K_M [mM]
Nα-Benzoyl-Arg-NA	100	0.05
Nα-Benzoyl-Lys-NA	190	0.275
N-Tosyl-Gly-Pro-Arg-NA	30	0.1
N-Tosyl-Gly-Pro-Lys-NA	90	0.25
Leu 4-NA	not detected	
N-Succinyl-Ala-Ala-Pro-Leu-NA		
N-Succinyl-Ala-Ala-Pro-Phe-NA		
N-Carbobenzoxy-Gly-Pro-NA		
N-Succinyl-Tyr-Leu-Val-NA		
S-Benzoyl-Cys-NA		

partially inhibited by 4-aminobenzamidine and the trypsin inhibitor from soybean, and by phenylmethane sulfonylfluoride (Table III).

Detection of a prolyl endopeptidase

When the substrate specificity of the protease having trypsin-like characteristics was determined as described above, it was observed that active fractions of the DEAE column hydrolyzed *Z*-Gly-Pro-NA, but those of the final chromatofocussing column (Table II) did not. A reinvestigation of the DEAE cellulose step with *Z*-Gly-Pro-NA as substrate showed that the activity cleaving at the carboxyl side of Pro eluted mostly after the trypsin-like protease, but the elution profiles overlapped (Fig. 3). The Phenyl-Sepharose column did not separate both activities. In the chromatofocussing step, the enzyme eluted around pH 4.4, *i.e.* at a higher pH value than the trypsin-like enzyme, but the recovery of activity was less than 5%, probably because of instability under these conditions. In non-denaturing PAGE, the presumptive prolyl endopeptidase migrated faster

Table III. Effects of various inhibitors on the activity of the trypsin-like protease and of the prolyl endopeptidase. The purified enzymes were incubated with the listed inhibitors under the conditions and for the times described previously [7, 11] in a volume of 0.8 ml. Thereafter, 0.2 ml Nα-benzoyl-Arg 4-nitroanilide (for the protease with trypsin-like specificity) or N-carbobenzoxy-Gly-Pro 4-nitroanilide (prolyl endopeptidase) were added to final concentrations of 0.4 and 0.125 mM, respectively, and the initial rates of hydrolysis determined spectrophotometrically as described under Materials and Methods. The trypsin-like enzyme was purified as shown in Table I. The source of the prolyl endopeptidase were fractions from the DE-52 ion exchange step (Fig. 3). *Abbreviations:* TLCK, Nα-toluenesulfonyl-L-lysine chloromethylketone; TPCK, N-4-toluenesulfonyl-L-phenylalanine chloromethylketone.

Inhibitor	Final concentration	Remaining rate (% of control) Trypsin-like protease	Prolyl endopeptidase
TLCK	0.5 mM	3	82
TPCK	0.5 mM	68	73
Phenylmethanesulfonyl fluoride	2.3 mM	75	8
Leupeptin	100 μg/ml	19	87
Antipain	50 μg/ml	14	78
Trypsin inhibitor from soybean	100 μg/ml	75	78
4-Aminobenzamidine	1.0 mM	98	89
N-Ethylmaleimide	1.0 mM	88	–[a]
$HgCl_2$	0.1 mM	95	50
E-64	100 μg/ml	97	48
Pepstatin	10 μg/ml	97	55
EGTA	1.0 mM	85	93

[a], not determined.

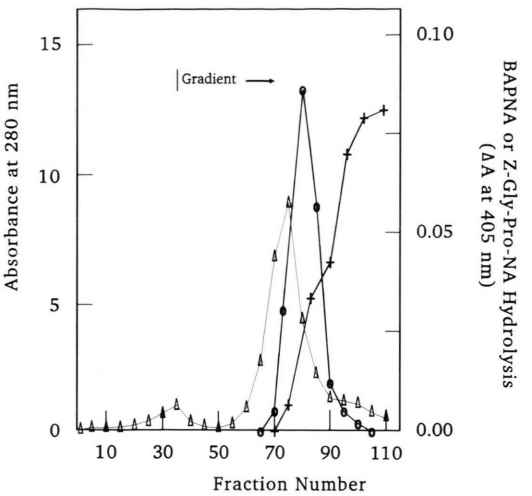

Fig. 3. Separation of the trypsin-like protease (○) and of the prolyl endopeptidase (+) of *Anabaena* by ion exchange chromatography. The DE-52 step of the purification procedure is shown. Rates are given as $\Delta A_{405\,nm}$ (ml extract × min)$^{-1}$. The assay volume was 1.0 ml. For further details, see text. Symbols: (○) hydrolysis of Nα-benzoyl-Arg 4-nitroanilide; (+) hydrolysis of N-carbobenzoxy-Gly-Pro 4-nitroanilide; (△), absorbance at 280 nm.

than and was well separated from the trypsin-like enzyme (Fig. 2).

For an initial characterization of the peptidase, fractions from the DEAE column (Fig. 3) that lacked the trypsin-like activity were collected and further subjected to chromatography on Phenyl-Sepharose, as described in Materials and Methods. In size exclusion chromatography (see Materials and Methods), the enzyme activity migrated like bovine serum albumin, suggesting a native molecular mass around 67 kDa (not shown). SDS-PAGE resolved up to 8 polypeptides in the active fractions. Of those, the largest one with an apparent mass of 70 kDa was the only one labelled by [^{3}H]diisopropyl fluorophosphate (not shown). A strong labelling by [^{3}H]-diisopropyl fluorophosphate in this molecular mass region has been observed before with crude extracts of *Anabaena* [7]. Together with the inhibitor data described below, this suggests that the enzyme is a monomer of about 70 kDa, similar to most known prolyl endopeptidase (compare [15]).

Of the chromogenic substrates tested (Table II), the presumptive prolyl endopeptidase liberated 4-nitroaniline from *Z*-Gly-Pro-NA only. However, when a substrate with an internal proline residue such as N-succinyl-Ala-Ala-Pro-Leu-NA was used, with L-leucine-aminopeptidase (4 mU) added to the reaction mixture, 4-nitroaniline was formed. The rationale of this assay is that internal cleavage of the peptides makes an unblocked amino terminus accessible to the aminopeptidase (see ref. [11] for an outline). Azocasein was not degraded. This specificity and the reaction with [^{3}H]diisopropyl fluorophosphate support an identification of the enzyme as a prolyl endopeptidase (compare [15]). However, unlike *e.g.* the enzymes from *Flavobacterium* and from lamb kidney [16, 17], it was readily inhibited by phenylmethane sulfonylfluoride (Table III). As nearly all known prolyl endopeptidases are serine proteases that react with diisopropyl fluorophosphate [15, 18–21], this may reflect minor differences in the active site structure. Prolyl endopeptidases are considered to be ubiquitous in vertebrates. It is of note that in an extensive survey of microorganisms, such an activity has previously been detected in just a few bacteria [22]. Its physiological role in prokaryotes is not known. Recently, the presence of an unusual prolyl endopeptidase in chloroplast thylakoids has been reported; the enzyme is believed to specifically degrade a subunit of photosystem II [23]. Site-directed mutagenesis will be the method of choice to probe the function of the prolyl endopeptidase in *Anabaena*.

Hydrolysis of BAPNA and of Z-Gly-Pro-NA by extracts of heterocysts

Extracts of heterocysts and of total cells (*i.e.* vegetative cells plus heterocysts) of *Anabaena* were prepared as described under Materials and Methods. As the inclusion of EDTA in the medium for heterocysts preparation tends to inhibit the Ca^{2+}-dependent protease irreversibly (W. Lockau, unpublished), only the Ca^{2+}-independent hydrolysis of BAPNA and the prolyl endopeptidase were assayed under standard conditions in the presence of 2 mM EGTA. In a typical experiment, heterocyst extract hydrolyzed BAPNA at a rate of 1.6 and *Z*-Gly-Pro-NA at a rate of 16.1 nmol × mg^{-1} protein × min^{-1}, whereas total cell extracts showed rates of 2.4 and of 17.5 nmol × mg^{-1} protein × min^{-1}, respectively. Thus, both proteolytic enzymes described here appear to be expressed in both these cell types at similar levels.

Table IV. Hydrolysis of Nα-Benzoyl – Arg 4-nitroanilide and of N-Succinyl – Gly – Pro 4-nitroanilide by intact and sonicated cells of *Anabaena variabilis* FD and its mutant IM 141. Cultures were grown photoautotrophically with N_2 as nitrogen source as described under Materials and Methods, harvested by centrifugation, washed once and resuspended in 0.1 M Tris (pH 8.1) containing 1 mM $CaCl_2$. Assays were run in that buffer containing either 0.5 mM Nα-benzoyl – Arg 4-nitroanilide (BAPNA) or 0.125 mM N-succinyl – Gly – Pro 4-nitroanilide (*Z*-Gly – Pro – NA) as substrates at 20 °C in a stirred cuvette. Formation of 4-nitroaniline was followed at 405 minus 470 nm in an Aminco DW 2 spectrophotometer. The rates are mean values from two independent experiments, with maximal deviations of ± 20% from the mean. Where indicated, cells were broken by sonication for 20 sec/ml under the conditions described under Materials and Methods, and used without further treatment. Activity was not detected in the used growth medium with any of the substrates.

Strain	Substrate	Rate		
		Intact cells	Sonicated cells	Stimulation by sonication
Wild type	BAPNA[a]	0.5	26.9	54-fold
	BAPNA[b]	0.6	0.8	1.3-fold
	Z-Gly – Pro – NA	1.5	3.1	2.1-fold
IM 141	BAPNA	1.0	1.1	1.1-fold
	Z-Gly – Pro – NA	2.7	5.4	2.0-fold

[a] Rate sensitive to 2 mM EGTA.
[b] EGTA-insensitive rate.
Rates are given in nmol 4-nitroaniline × (mg protein × min)$^{-1}$.

Hydrolysis of BAPNA and of Z-Gly – Pro – NA by filaments of the wild type and of mutant IM 141

Proteases involved in heterocyst differentiation are most likely intracellular enzymes. An attempt was made to localize the here described enzymes in the cells of *Anabaena* by use of small chromogenic substrates. Such molecules are expected to enter the periplasmic space of the cyanobacterium, as its outer membrane has been shown to contain pores with a suggested exclusion limit of about 2 kDa for globular molecules [24]. The rates of hydrolysis of these substrates by intact cells and by cells opened by sonication were compared. The intracellular [3, 7], Ca^{2+}-dependent protease served as a reference (EGTA-sensitive rates of BAPNA hydrolysis of the wild type cells, see Table IV). The activity of this enzyme was stimulated more than 50-fold by sonication. By contrast, the EGTA-insensitive BAPNA hydrolysis and the hydrolysis of *Z*-Gly – Pro – NA were stimulated by a factor of 1 to 2 only, both in the wild type of *Anabaena* and in mutant IM 141 (Table IV). This is taken as evidence that the two proteolytic enzymes described here are at least in part located in the periplasm of *Anabaena;* a periplasmic location has been suggested for the prolyl endopeptidase of *Flavobacterium* [20].

Conclusions

Two new constitutive proteolytic enzymes have been characterized in extracts of *Anabaena,* namely an endoprotease with a specificity like trypsin, and a prolyl endopeptidase. Both enzymes appear to be located in the periplasmic space of the cyanobacterium. Their involvement in heterocyst differentiation is questionable.

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