Cyanophycin Synthetase-Like Enzymes of Non-Cyanobacterial Eubacteria: Characterization of the Polymer Produced by a Recombinant Synthetase of Desulfitobacterium hafniense

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Some bacterial genomes were found to contain genes encoding putative proteins with considerable sequence homology to cyanophycin synthetase CphA of cyanobacteria. Such a gene from the Gram-positive, spore-forming anaerobe Desulfitobacterium hafniense was cloned. Expression in Escherichia coli resulted in the formation of a polydispers copolymer of aspartic acid and arginine, with a minor amount of lysine, of about 30 kDa molecular mass. In contrast to cyanophycin, this polymer was water-soluble. The structure of the polymer formed by the synthetase from Desulfitobacterium hafniense was studied by enzymatic degradation with the cyanophycin-specific hydrolase cyanophycinase, and by chemical and mass-spectroscopic analyses. Despite of the differences in solubility, indicating that both polymers cannot be completely identical, the chemical structure was found to be very similar to that of cyanophycin. The results suggest that the use of cyanophycin-like polymers as a nitrogen-rich reserve material is not restricted to cyanobacteria, and that such polymers may not necessarily be stored in granules.

Introduction

Cyanophycin (multi-l-arginyl-poly-l-aspartic acid) is a protein-like reserve polymer that consists of aspartic acid and arginine in an nearly equimolar ratio. The amino acids are arranged in a unique structure: the aspartic acid residues form a poly-(α-aspartate) backbone, with their β-carboxyl groups linked to the α-amino groups of arginine residues by isopeptide bonds (Simon and Weathers, 1976; Simon et al., 1980). Cyanophycin has been reported to occur exclusively in cyanobacteria, where the polymer is found in membraneless granules deposited in the cytoplasm and in the so-called polar plugs of heterocysts (reviewed in Allen, 1984; Simon, 1987). The polymer has unusual solubility properties. It is insoluble at neutral pH and physiological ionic strength but is soluble in diluted acids and bases. The molecular mass of the polydispers compound ranges over 20–100 kDa (Simon, 1971).

Cyanophycin is synthesized non-ribosomally in an ATP-dependent elongation reaction catalyzed by cyanophycin synthetase CphA (Simon, 1976; Ziegler et al., 1998). It is degraded by a special peptidase, the cyanophycinase, but is resistant to other endoproteases and exopeptidases (Allen, 1984; Simon, 1987; Richter et al., 1999). Both cyanophycin synthetase (CphA) and cyanophycinase (CphB) have been purified and characterized, and their genes have been sequenced for a number of cyanobacteria. The cyanophycin synthetase consists of a dimer of a single polypeptide of approximately 100 kDa (Ziegler et al., 1998; Aboulmagd et al., 2001). The enzyme can be expressed in Escherichia coli in functionally active form, which results in the formation of large amounts of cyanophycin deposited in granules.

The polymer isolated from the transgenic cells differs from cyanophycin from cyanobacteria mainly in its size. It is smaller (approximately 25–35 kDa)
and contains, in addition to arginine, a small amount of lysine as basic amino acid (Ziegler et al., 1998; Aboulmagd et al., 2000). The solubility properties are unaffected by these modest changes in molecular composition.

Hitherto it has been considered that cyanophycin is unique for cyanobacteria. However, genome sequencing showed that ORFs with considerable homology to cyanobacterial genes (named cphA) encoding cyanophycin synthetase are present in a number of non-cyanobacterial eubacteria. We have expressed one such gene from the Gram-positive, spore-forming anaerobe Desulfitobacterium hafniense (Christiansen and Ahring, 1996) in Escherichia coli. The transgenic cells produced a polymer which, unlike cyanophycin, is highly water-soluble. This communication describes the purification of the polymer and studies on its structure. The data presented indicate that the compound, despite of the difference in solubility, is remarkably similar to cyanophycin with regard to amino acid composition and chemical structure.

Materials and Methods

Recombinant DNA techniques

Standard recombinant DNA techniques (Sambrook et al., 1989) or minor variations thereof were used. Restriction enzymes and DNA ligase were purchased from New England Biolabs, LB agar and LB broth from Difco Laboratories. Restriction fragments and PCR products were purified with High Pure PCR Product Purification Kit (Roche Diagnostics). Plasmid DNA was purified with High Pure Plasmid Isolation Kit (Roche Diagnostics). Sequence analyses were performed using the software of Heidelberg Unix Sequence Analysis Resources (HUSAR).

Template DNA preparation, polymerase chain reaction and cloning of the synthetase gene

Cells of Desulfitobacterium hafniense, strain designation DSM 10664, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig) in lyophilized form. Cells were suspended in 50 mM Tris, 10 mM EDTA, 600 mM NaCl (adjusted to pH 8.0 with HCl), and were subsequently treated with lysozyme (final concentration 3 mg ml⁻¹, 2 h at 37 °C with occasional shaking) and proteinase K (final concentration 1 mg ml⁻¹; 10 min at 50 °C). DNA was purified therefrom with the High Pure PCR Template Purification Kit (Roche Diagnostics) following the instructions of the supplier. The synthetase gene was ampliﬁed by polymerase chain reaction (PCR) using Pfu DNA Polymerase (Promega) and the supplied reaction buffer. The oligonucleotide 5’-GGACAAATATCCATATGGAGA TACTGA AAATCC-3’ (WL254) served as forward primer, 5’-GGCGATAGCGTGCAGACTAGTGCC-3’ (WL255) as reverse primer. The primers were designed according to the sequence of contig3152 of the Desulfitobacterium hafniense genome project (DOE Joint Genome Institute, http://www.jgi.doe.gov) complementary to the positions at bps 5575–5607 (WL254) and bps 8262–8238 (WL255). Variations from the genomic sequences (given in italics) inserted additional NdeI and SalI sites (underlined) upstream and downstream of the coding region of the synthetase gene. The product of the PCR reaction was digested with NdeI/SalI, ligated into the NdeI/XhoI-sites of expression vector pET-19b (Novagen) and cloned in E. coli strain DH5α (Clontech). The cloned fragment was analyzed by DNA sequencing (Agowa GmbH, Berlin). The obtained sequence differed in one position from the nucleotide sequence reported for contig3152. The base change causes one amino acid exchange in the expressed protein, when compared to the amino acid sequence deduced from the published sequence (see Results and Discussion). The recombinant plasmid contains the bps 5589–8252 of contig3152, i.e. the entire coding region of the synthetase gene starting with bp 1 and ending 6 bps after the natural stop codon. As the fragment was cloned in frame behind the His-tag coding region of pET-19b, the protein is expressed in fusion with an N-terminal His₁₀-tag.

Expression of the synthetase in E. coli and purification of its reaction products

Plasmid DNA isolated from the transgenic DH5α strain was transformed to transform cells of the E. coli expression strain BL21(DE3) (Novagen). Selection was performed on LB agar supplemented with glucose (10 g l⁻¹) and ampicillin (125 μg ml⁻¹). Several colonies were used to inoculate 100 ml LB medium containing glucose (10 g l⁻¹) and ampicillin (125 μg ml⁻¹). The culture was grown at 37 °C to an OD₆₀₀
of approximately 1.0. Cells were harvested by centrifugation (5000 × g, 10 min, 24 °C) and used to inoculate 400 ml double-strength LB medium with 250 µg ml⁻¹ ampicillin. Cells were incubated at 16 °C under vigorous shaking (300 rpm) for 18 h, harvested, washed with and resuspended in 20 mM Tris-HCl, pH 8.0, and stored at −20 °C. For preparation of the polymer, cells were thawed and desintegrated by sonication under ice/water cooling. The supernatant of a centrifugation step (30,000 × g, 15 min, 4 °C) served as crude extract. It was incubated for 15 min at 60 °C in a water bath in order to denature proteins. Insoluble material was sedimented by centrifugation (30,000 × g, 15 min, 4 °C). To the supernatant, proteinase K (Roche Diagnostics) was added to a final concentration of 200 µg ml⁻¹ and the assay incubated overnight at 60 °C. The polymer was then precipitated by addition of ice-cold ethanol to a final concentration of 75% (v/v) and centrifugation (14,000 × g, 15 min, 4 °C). The pellet was washed with acetone (centrifugation as before), briefly dried in vacuo, dissolved in 25 mM ammonium acetate (pH 5.2) and loaded onto a Resource Q anion exchange chromatography column (Pharmacia; 1 ml bed volume) equilibrated and run with 25 mM ammonium acetate (pH 5.2) at a flow of 1 ml min⁻¹. The polymer passed through the column without retardation. The eluate was collected and lyophilized.

Digestion of the polymer with cyanophycinase and analysis of reaction products by thin layer electrophoresis

One mg of the lyophilized polymer was dissolved in 200 µl 50 mM ammonium bicarbonate (pH 7.9) and incubated overnight with approximately 5 µg purified recombinant cyanophycinase from Synechocystis sp. PCC 6803 (Richter et al., 1999) at 37 °C. The assay mixture was lyophilized twice and redissolved in 50 µl water. Two µl of 10 mM aqueous solutions of β-Asp–Arg, (prepared as in Berg et al., 2000) and α-Asp–Arg (Bachem), and 2 µl of the products of the digestion with cyanophycinase were loaded onto cellulose TLC plastic sheets (Merck). The sheets were moistened with 100 mM sodium borate (pH 9.5) before electrophoresis (700 V for 20 min) in a cooled Bio-phoresis horizontal electrophoresis cell (Bio-Rad). Bands were visualized by spraying with 5 g l⁻¹ ninhydrin in methanol/acetic acid (20:1 (v/v)) and development at 80 °C.

SDS/PAGE was carried out on slab gels using the buffer system of Laemmli (Laemmli, 1970). The seperation gel contained 15% (m/v) acrylamide/bisacrylamide and 6 M urea. Protein test mixture 5 (Serva) was used as molecular-mass standard. Proteins were visualized by staining with Coomassie brilliant blue R250 (Sambrook et al., 1989).

Amino acid analysis

Amino acid analyses were carried out after acid hydrolysis (6 M HCl, 110 °C for 18 h) by the pre-column derivatization method with 4-(dimethylamino)azobenzene-4-sulfonly chloride as described (Kamp, 1997).

Derivatization of free carboxyl groups

Selective derivatization of free carboxyl groups of the polymer was achieved by esterification with methanolic HCl to the methyl esters and subsequent reduction with sodium borohydride. The procedure followed a protocol given by Simon and Weathers (1976). After chemical conversion the polymer was precipitated with ethanol as described above, washed with acetone and dried in vacuo. Amino acids and amino alcohols in the derivative polymer were analyzed as described under “amino acid analysis”. Arginine methyl ester (Bachem), argininol (prepared by chemical reduction of arginine methyl ester with sodium borohydride as in Simon and Weathers, 1976), and L-homoserine (Sigma) served as additional standards.

Mass spectroscopy

Electrospray ionization/MS and fragmentation analysis were performed with an Ion Trap ESQUIRE LC instrument (Bruker Daltonik). Samples were infused by a nanospray source in water/methanol/acetic acid (1:1:0.01 (v/v/v)). Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectra were recorded by AnagnosTec GmbH, Luckenwalde, Germany.

Results and Discussion

Identification of cyanophycin synthetase homologs in the genomes of non-cyanobacterial eubacteria

We have critically examined the preliminary identification of several bacterial ORFs as genes...
Fig. 1. Alignment of the deduced amino acid sequence of the ORF of *Desulfitobacterium hafniense* with homology to cyanophycin synthetase genes and the primary sequence of cyanophycin synthetase CphA of *Synechocystis* sp. PCC 6803. Scystis, *Stnicipsy*; Dhafnie, *Desulfitobacterium hafniense*. Overall sequence identity is 41% (369/876), overall similarity 59% (523/876). Positions with identical amino acid residues are shaded. Two ATP-binding sites have been postulated for cyanophycin synthetases, a binding site of the ATP-grasp fold type, characterized by a B-loop and a J-loop, and a second with a Rossmann fold and a P-loop motif. The positions of these loops are indicated. For *Desulfitobacterium hafniense* the amino acid sequence deduced from the sequence of the cloned fragment is given. It differs in amino acid residue Val313 (indicated by an asterisk) from the published sequence (http://www.jgi.doe.gov), where this amino acid residue is an alanine.
encoding cyanophycin synthetases, focussing mainly on the data base of “Experimental and Unfinished Genomic Sequences” published by the Munich Information Center for Protein Sequences (mips, http://pedant.gsf.de). Forty-six sequenced or partially sequenced genomes of prokaryotes other than cyanobacteria were inspected. Taken into account were the length of the deduced amino acid sequences, the overall similarity to cyanophycin synthetase, and the presence of amino acid residues fully conserved in the cyanobacterial enzymes. As highly probable cyanophycin synthetase genes, we finally regarded ORF1 in contig2227 of the genomic sequence of Bordetellabronchiseptica RB50, ORF29 in contig108 of Bordetella parapertussis 12822, and ORF25 in contig828 of Nitrosomonas europaea ATCC 25978 (annotations according to mips, date 09-23-2001). The similarity of the deduced amino acid sequences of these ORFs to the primary sequence of cyanophycin synthetase are surprisingly high. The calculated amino acid identities and similarities to the enzyme of Synechocystis sp. PCC6803 are 41% (360/874) and 55% (489/874) for B. parapertussis, 40% (352/874) and 56% (497/874) for N. europaea and 39% (325/826) and 56% (466/826), respectively, for B. bronchiseptica. Highest homology was found, however, for an ORF in the genome of Desulfitobacterium hafniense DCB-2, comprising bps 5589–8243 of contig3152 of the D. hafniense genome project (DOE Joint Genome Institute, http://www.jgi.doe.gov). An alignment of the putative D. hafniense protein with the amino acid sequence of cyanophycin synthetase of Synechocystis sp. PCC6803 is given in Fig. 1. Additional support for the preliminary identification of this D. hafniense ORF as a cyanophycin synthetase gene was the observation that it is immediately preceded by an ORF (bps 4648–5466 of contig3152) probably encoding a cyanophycinase homolog (amino acid identity and similarity to the cyanophycinase of Synechocystis sp. PCC6803 are 39% (102/258) and 59% (156/258), respectively). A very similar clustering of the genes for cyanophycinase and for cyanophycin synthetase has been found in all cyanophycin-forming cyanobacteria examined (Ziegler et al., 1998; Aboulmagd et al., 2000). Genes that may encode cyanophycinase were not found in the sequences of B. parapertussis, B. bronchiseptica, and N. europaea. They are either missing or have not been sequenced yet.

Expression of the CphA homolog of Desulfitobacterium hafniense in Escherichia coli and purification of its polymeric reaction product

In order to verify that it encodes a functional enzyme, we have expressed the D. hafniense ORF in E. coli and analyzed the transgenic cells for the formation of cyanophycin-like material. No such material was obtained when a standard isolation method (Simon, 1976) was used. This method is based on the insolubility of cyanophycin at neutral pH and its solubility in dilute acids. However, SDS-PAGE analysis of crude extracts of transgenic E. coli cells showed the presence of a polydispers, Coomassie-positive polymer that was absent from control cells. Further analysis showed that this material is water-soluble. A protocol was developed for the purification of the polymer from transgenic E. coli. The protocol comprises the following steps: (i) extraction of the soluble protein fraction, (ii) heating to 60°C and removal of denatured proteins, (iii) digestion with proteinase K to which the polymer is resistant, (iv) precipitation of the material with ethanol to remove the products of the preceding digestion, and (v) anion exchange chromatography. This last step did not alter the polypeptide pattern of the preparation but resulted in removal of unknown UV-absorbing materials. The purification steps are documented in Fig. 2A. The UV/VIS spectrum of the polymer dissolved in water exhibited a single absorption band peaking at 195 nm, indicative of peptide bonds (data not shown).

Amino acid analysis

The only amino acids present in substantial amounts in acid hydrolysates of the polymer were aspartic acid, arginine and lysine. A molar ratio of aspartate/arginine/lysine of approximately 1.0:0.9:0.1 was calculated. The absence of aromatic amino acids is corroborated by lack of absorption in the 260–280 nm region of the UV/VIS spectrum (not shown). The composition of the polymer is thus very similar to the composition of cyanophycin isolated from transgenic E. coli, expressing cphA.

Determination of molecular mass

In SDS-PAGE, the polymer formed a broad, intensively stained band with an apparent molecular
Fig. 2. A: Purification of the polymer synthesized in E. coli expressing the cyanophycin synthetase homolog of Desulfitobacterium hafniense. Analysis was by SDS-PAGE and Coomassie staining. Lane 1, crude extract of transgenic E. coli; lane 2, after heat treatment and centrifugation; lane 3, after treatment with proteinase K; lane 4, after precipitation with ethanol; lane 5, after anion exchange chromatography. The positions of standard proteins are indicated. B: SDS-PAGE of the degradation of the polymer by cyanophycinase CphB from Synechocystis sp. PCC6803. Lane 1, control; lane 2, after treatment with cyanophycinase. The conditions for the digestion are given in Section 2. The control was treated identically except that cyanophycinase was omitted.

The mass of 30–35 kDa (Fig. 2). The diffuse band in the range of 22–26 kDa might either be caused by an irregular migration of a minor amount of the polymer or may be an impurity. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectroscopy (data not shown) of the polydispers material gave an average value of approximately 29.5 kDa for the [M+H]+ ion, with a maximal intensity at m/z = 29361 and a broadness (full width at half-maximal intensity) of about 1100 m/z. Minor peaks around 59218 m/z and 14663 m/z could be ascribed to [2M+H]+ and [M+2H]2+ ions. The molecular mass obtained by this method is in good agreement with the estimate from SDS-PAGE analysis. Also detected by MALDI-TOF MS were distinct peaks in the range of 1100 to 3300 m/z spaced at intervals of 271 m/z (271 Da correspond to one Asp–Arg residue). The amino acid composition of these molecules, apparently short polymer chains or hydrolysis products, was deduced from the measured m/z values as Asp4Arg4, Asp3Arg5, Asp2Arg6, etc. The masses of a second group were consistent with the composition Asp2Arg1Lys, Asp5Arg2Lys, Asp4Arg2Lys etc.

Digestion with cyanophycinase and analysis of reaction products

Cyanophycinase depolymerizes cyanophycin by hydrolysis of the α-peptide bonds between the aspartate residues of the backbone to yield β-aspartyl-arginine (see Richter et al., 1999). The enzyme from Synechocystis sp. PCC6803 readily degraded the reaction product of the cyanophycin synthetase homolog from D. hafniense (Fig. 2B). By ESI-MS, the molecular mass of the principal product was determined as 290.2 m/z (Fig. 3). The molecular mass expected for a dipeptide consisting of arginine and aspartic acid (calculated mass of the [M+H]+ ion, 290.28 Da). Three of the additional, minor peaks observed in the spectrum (Fig. 3) could be assigned. The peak with 175.1 m/z was identified as arginine (calculated m/z of the [M+H]+ ion, 175.2 Da) which may be liberated by fragmentation within the mass spectrometer, the peak with 262.2 m/z as a dipeptide consisting of aspartate and lysine (calculated m/z of the [M+H]+ ion, 262.27 Da) and that with 281.2 m/z as a tetrapeptide consisting of two aspartate and two arginine residues (calculated m/z of the [M+2H]+ ion, 281.27 Da). Additional MS/MS analysis by collision-induced fragmentation (results not shown) unequivocally confirmed the identification of the main product and the presence of lysine in the component at 262.2 m/z. Because the MS analysis did not allow to distinguish between α- and β-bonded dipeptides, the reaction products were further analyzed by thin-layer electrophoresis (Fig. 3, insert). The dominant degradation product migrated like synthetic β-aspartyl-arginine. By contrast, α-aspartyl-arginine had a very different electrophoretic mobility. It should be noted that the colour developed with ninhydrin on the electrophorograms was yellowish-brown for the synthetic β-aspartyl-arginine as well as for the principal product of the cyanophycinase reaction, whereas α-aspartyl-arginine stained purple.

Derivatization of free carboxyl groups

According to the chemical structure of cyanophycin (see Introduction), the only free carboxyl groups are those of the arginine residues and, as a minor fraction, the α-carboxyl groups of the C-terminal aspartic acid residues (Simon and Weath-
Esterification of the carboxyl groups of the polymer to methyl esters and subsequent reduction should therefore result in the formation of the amino alcohol argininol from arginine, but should leave nearly all aspartyl residues unaffected. We have applied this method of selective esterification and reduction, followed by acid hydrolysis, to get further insight into the structure of the polymer. Amino acid analysis showed that the treatment changed the molar ratio of aspartate/arginine from approximately 1.0:0.9 in the native to about 1.0:0.4 ± 0.1 in the treated polymer. This efficiency of derivatization is similar to that reported for authentic cyanophycin (Simon and Weathers, 1976). In the reversed phase chromatography used to separate the 4-(dimethylamino)azo-benzene-4-sulfonyl derivatives of amino acids (Kamp, 1997), two new peaks appeared that co-eluted with argininol and arginine methyl ester. There was no evidence for the conversion of aspartic acid residues to methyl esters or their reduction to 3-amino-4-hydroxybutyric acid or 2-amino-4-hydroxybutyric acid (the products of the reduction of either the α- or β-carboxyl group of aspartic acid). This result is consistent with a chemical structure of the polymer in which both the α-carboxyl and β-carboxyl groups of the aspartate residues participate in covalent linkages, whereas either all or at least a large part of the carboxyl groups of the arginine residues are free.

Its degradation by cyanophycinase to β-aspartyl-arginine, as well as the chemical and mass spectroscopic analysis of the polymer formed by the recombinant synthetase from D. hafniense show that this material is very similar to cyanophycin, except for its solubility properties. Its water-solubility must be caused by rather subtle differences in structure. A hint to a possible structural difference was provided by a parallel experiment in which cyanophycin, formed by expression of the cyanophycin synthetase of the cyanobacterium Anabaena variabilis ATCC 29413 in E. coli was digested with cyanophycinase. The products were analyzed by ESI-MS as above. The principal difference of that mass spectrum to the one shown in Fig. 3 was the absence of the peak at 281.2, i.e. the \([\text{M}+2\text{H}]^{2+}\) ion of the tetrapeptide Asp$_2$Arg$_2$. An explanation for this difference would be that the soluble polymer formed by the synthetase from D. hafniense may contain branches in addition to the isopeptide bonds between the β-carboxyl groups of the poly-(α-aspartate) backbone and the...
amino groups of arginine residues. If the *D. hafniense* synthetase would occasionally form an α-peptide bond between the carboxyl group of such an arginine residue and the amino group of an aspartate, then a branch could start and a local structure would be generated that could not be hydrolyzed by cyanophycinase to β-aspartyl-arginine.

In summary, we have shown that genomes of eubacteria contain genes with rather strong homology to the cyanophycin synthetase of cyanobacteria. When such a gene from *Desulfitobacterium hafniense* was expressed in *E. coli*, the enzyme formed a polymer which is very similar to cyanophycin in composition and structure. In contrast to cyanophycin, however, this polymer is water-soluble, probably because of subtle differences in structure and/or composition. Very recently, Krehenbrink et al. (2002) have reported that expression in *Escherichia coli* of a gene of *Acinetobacter* with similarity to cphA led to the formation of a polymer with the chemical composition and the solubility properties like those of cyanobacterial cyanophycin.

Our results suggest that the distribution of cyanophycin-like reserve polymers amongst prokaryotes is more wide-spread than previously thought, and that the polymer must not necessarily accumulate in the form of microscopically visible granules.

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