FEMS Microbiology Letters 252 (2005) 273–278

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

A new soluble 10 kDa monoheme cytochrome *c*-552 from the anammox bacterium *Candidatus* "*Kuenenia stuttgartiensis*"

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Received 17 June 2005; received in revised form 29 August 2005; accepted 4 September 2005

First published online 22 September 2005

Edited by J. Simon

Abstract

The chemolithoautotrophic anammox bacterium *Candidatus* "*Kuenenia stuttgartiensis*" grows anaerobically using ammonium as electron donor for nitrite reduction. More than 10% of the proteins in cell extracts of "*K. stuttgartiensis*" consist of *c*-type heme proteins. A 10 kDa soluble cytochrome *c* was purified from cell extracts using ultracentrifugation and anion exchange chromatography. The UV/Vis spectrum of the reduced cytochrome showed the γ, β and α absorption maxima at 419, 522 and 552 nm, respectively. The N-terminal amino acid sequence and peptide fragments of the tryptic digest of the protein were used to identify the corresponding gene. Analysis of the gene product showed that the protein was preceded by a 30 amino acids long leader sequence and that it belonged to the low-spin class ID cytochrome *c*. The CXXCH motive was located at the N-terminal site of the protein. The gene organization of the cytochrome showed some resemblance to cytochrome *c* clusters of unknown function in the genome of *Nitrosomonas europaea* and *Geobacter sulfurreducens* PCA.

Keywords: Anammox; Anaerobic ammonium oxidation; *Kuenenia stuttgartiensis*; Cytochrome *c*; Electron transfer

1. Introduction

Anaerobic ammonium-oxidizing (anammox) bacteria are recognized as an ecologically and environmentally important group of microorganisms [1,2]. The anammox bacteria use ammonium as an electron donor for nitrite reduction under anoxic conditions [3]. Due to the extremely low growth rate (doubling time at best 11 days) the cultivation of the anammox bacteria requires very efficient biomass retention [4]. On the basis of 16S rRNA gene phylogeny the bacteria responsible for the anammox process have been identified as very deep branching Planctomycetes [5,6]. So far three groups of anammox bacteria have been described: the fresh water species *Candidatus* "*Brocadia anammoxidans*" [1,7], *Candidatus* "*Brocadia fulgida*" [8] and *Candidatus* "*Kuenenia stuttgartiensis*" [9] found in waste water treatment systems and the marine species *Candidatus* "*Scalindua sorokinii*" discovered in the Black Sea [2].

The metabolic pathway for anaerobic ammonium oxidation in "*B. anammoxidans*" has been elucidated via ^{15}N-labelling experiments. These experiments

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0378-1097/$22.00
doi:10.1016/j.femsle.2005.09.007

showed that ammonium and nitrite are combined to yield dinitrogen gas [10]. In batch experiments with excess hydroxylamine and ammonium, a transient accumulation of hydrazine was observed, indicating that hydrazine is an important intermediate of anammox bacteria [11]. The oxidation of hydrazine to dinitrogen gas is known to be mediated by the hydroxylamine oxidoreductase (HAO) of the aerobic ammonium-oxidizing bacterium *Nitrosomonas europaea* [12]. Likewise, a homotrimeric, 24 *c*-type heme containing anammox enzyme has been purified to homogeneity from "*B. anammoxidans*" [13]. The purified HAO-like enzyme was able to catalyze the oxidation of both hydroxylamine and hydrazine. The oxidation of hydrazine to dinitrogen gas yields 4 electrons which are used for the reduction of nitrite.

Like "*B. anammoxidans*", "*K. stuttgartiensis*" exhibits a high content of cytochromes *c*, which can constitute more than 10% of the total protein. Cytochromes are a heterogeneous group of proteins involved in electron transfer and are often associated with membrane-bound protein complexes. It is currently hypothesized that these *c*-type cytochromes in "*K. stuttgartiensis*" may play an important role in the electron transfer from hydrazine to nitrite. The present study therefore concentrated on the isolation and identification of a prominent soluble cytochrome *c* of "*K. stuttgartiensis*".

2. Material and methods

2.1. Reagents

Chemicals and biochemicals were of analytical or reagent grade and were obtained from Sigma, Aldrich or Fluka (www.sigmaaldrich.com) and Merck BV (Amsterdam, The Netherlands) unless mentioned otherwise. Solutions were prepared using water of MilliQ grade.

2.2. Growth of Candidatus "K. stuttgartiensis"

Cells of "*K. stuttgartiensis*" were grown anaerobically on bicarbonate medium containing ammonium, nitrite and mineral salts [14] in a continuous gaslift reactor with biomass retention at 37 °C. The medium was flushed with argon/carbon dioxide gas (95%/5%, v/v) at 7 ml min^{-1}. Mixing of cell aggregates and medium was achieved through gas recycling from the headspace. The cells were harvested from the effluent under oxic conditions.

2.3. Purification of the cytochrome

Cells of "*K. stuttgartiensis*" (30 g wet weight) were suspended in 120 ml of 10 mM potassium phosphate buffer, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 μg $μl^{-1}$ DNaseI, 2 mM phenylmethanesulfonyl fluoride (PMSF). The bacteria were broken using an ultrasonic disintegrator (MSE) for 20 min under constant cooling. Sonication was repeated three times with cooling periods. Cell debris was removed by centrifugation at 8600*g* at 4 °C. The cell extract was centrifuged for 15 h at 100,000*g* at 4 °C. Additionally, 0.1% Triton (w/v) was added to the supernatant to prevent protein aggregation. The supernatant was stored in 20% glycerol at −20 °C or directly applied to a Q-Sepharose Fast Flow anion exchange column (1.5 × 25 cm, Amersham Bioscience, Roosendaal, The Netherlands). Proteins were eluted in a linear gradient of 0–1 M NaCl in 10 mM potassium phosphate buffer, pH 7.0. The fractions containing cytochrome *c*, monitored by their absorption spectrum, were pooled and concentrated with Centriprep-10 Concentrators (Millipore BV, Amsterdam, The Netherlands). The concentrated protein fractions were desalted using a Sephadex G-25 gel filtration (1 × 10 cm, Amersham Bioscience, Roosendaal, The Netherlands) using 10 mM potassium phosphate buffer, pH 7.0. The cytochrome *c* containing fraction was loaded onto a pre-packed TSK DEAE-5PW anion exchange column (0.75 × 7.5 cm, Tosoh Bioscience GmbH, Stuttgart, Germany). A gradient of 0–0.5 M NaCl in potassium phosphate buffer, pH 7.0 was applied. After an additional concentration step the protein was stored at −20 °C in 20% glycerol until further use.

2.4. Protein determination, polyacrylamide gel electrophoresis, and UV/VIS spectrometry

Protein concentration was determined using the bicinchoninic acid method [15] with reagents from Pierce (Perbio Science, Etten-Leur, The Netherlands). Bovine serum albumine and horse heart cytochrome *c* were used as standards.

Denaturing Tris/Tricine polyacrylamide gel electrophoresis [16] was performed in a Mini Protean apparatus (BioRad, Veenendaal, The Netherlands). The protein samples were denatured overnight in loading buffer [17] containing 5% SDS and 10 mM DTT at room temperature. Broad range Precision Protein Standard (BioRad, Veenendaal, The Netherlands) was used as molecular mass marker. The gels were fixed in 10% glacial acetic acid, and stained with Coomassie Blue G-250 (Serva Electrophoresis GmbH, Heidelberg, Germany).

Heme peroxidase activity was detected and visualized in SDS- gels with 3,3′-diaminobenzidine [18].

The absorption spectra of the air-oxidized and dithionite-reduced protein were recorded with a Hewlett Packard 8453 spectrophotometer at room temperature.

2.5. MALDI-TOF mass spectroscopy

The cytochrome *c* was dialysed against 10 mM NH_4HCO_3. Trypsin digestion and matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of native and digested protein was carried out as described before [19]. Spectra were recorded on a Biflex III (Bruker Daltonic B.V., Wormer, The Netherlands).

2.6. N-terminal sequencing and sequence analysis

The N-terminus of 100 pmol of the purified protein was sequenced at the Sequence Centre Utrecht, University of Utrecht, The Netherlands. The amino acid sequencing was carried out via automated Edman degradation using a Perkin Elmer/Applied Biosystems 476A. The partial "*K. stuttgartiensis*" genome assembly available at Genoscope (Evry, France) was screened for the presence of the N-terminus. Similar sequences were located and aligned using BlastP [20] and ClustalW 1.8 [21]. The presence of a signal peptide was analysed with SignalP [22] and the prosthetical groups were predicted by InterProScan [23]. The DNA and protein sequence of the cytochrome *c* have been deposited in the GenBank/EMBL databases under accession number DQ023144.

3. Results and discussion

3.1. Purification of cytochrome c

A 10 kDa *c*-type cytochrome from the soluble fraction of *Candidatus* "*K. stuttgartiensis*" was purified to homogeneity by extended ultracentrifugation and anion exchange chromatography. After prolonged ultracentrifugation the small cytochrome *c* remained in the supernatant, mainly with other low molecular mass proteins. The supernatant was applied on a Q-Sepharose anion exchange column from which cytochrome *c* eluted at a NaCl concentration of 0.4 M as judged by the UV–Vis absorption spectrum. The desalted cytochrome fraction eluted from the TSK anion exchange column at 0.24 M NaCl. The chromatographic steps yielded a homogeneous protein preparation which exhibited one protein band with an apparent molecular mass of 10 kDa on SDS-PAGE (Fig. 1). The smear at a molecular mass above 250 kDa was probably caused by protein aggregation or precipitation. The protein band at 10 kDa was also stained by specific heme staining (Fig. 1).

3.2. Characterization of cytochrome c

Absorption spectra of the cytochrome *c* were recorded in its oxidized and reduced form. The reduced cytochrome had absorption maxima at 552 (α), 522 (β) and 419 nm (γ, Soret peak), and the oxidized form had a maximum at 410 nm. The protein has been designated as a cytochrome *c*-552 on basis of the wavelength maximum of the ferrous α-band. An absorption charge transfer band at 695 nm, indicative for His/Met coordination, was not observed. The spectral characteristics point to a low-spin six-coordinate heme iron which might be involved in electron transfer.

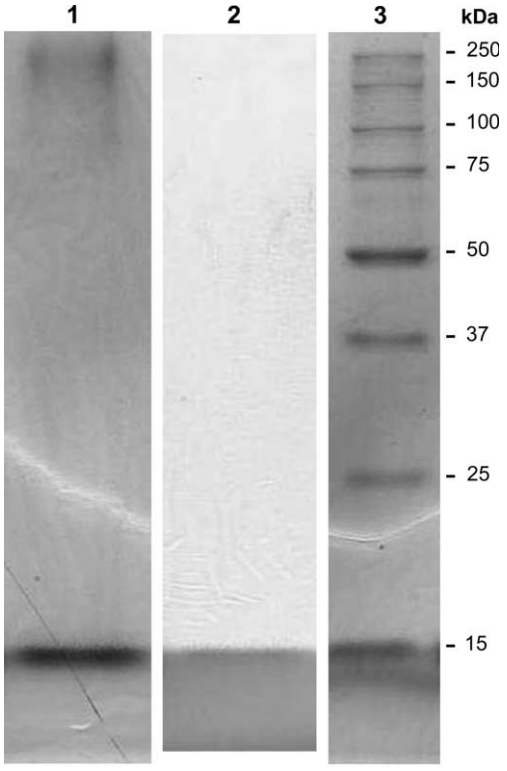

Fig. 1. SDS-PAGE of 2 μg purified cytochrome *c* from "*Kuenenia stuttgartiensis*". Lane 1, Coomassie Brilliant Blue G-250 staining. Lane 2, heme staining. Lane 3, protein markers.

The accurate molecular mass of the protein was determined by MALDI-TOF MS (Fig. 2). The spectrum shows the mass peaks representing the singly and doubly charged ions $[M]H^+$, $[M]2H^+$ and the dimeric singly charged ion, $[2M]H^+$. The mass of the purified cytochrome *c* protein appeared to be 10,118 ± 24 Da. No higher molecular mass peaks were observed in the MALDI-TOF MS spectrum.

3.3. Protein and gene sequence of cytochrome c

The N-terminal sequence of the purified cytochrome *c* was determined as XNIDGMKLYLQH. The N-terminus allowed the identification of the gene sequence for this cytochrome *c* in the partial genome data assembly of "*K. stuttgartiensis*". The deduced protein sequence of gene 4414 matched the N-terminus (Fig. 3). The protein was 115 aa in size and exhibited one heme *c*-binding signature CXXCH. The theoretical molecular mass was 12,639 Da and the calculated *pI* was 7.8. Analysis of the primary sequence with SignalP revealed the presence of a signal peptide of 30 amino acid residues, which was

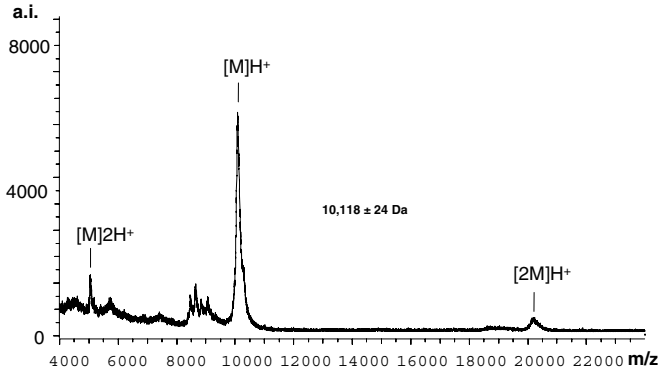

Fig. 2. MALDI-TOF MS spectrum of the purified cytochrome *c* from "*Kuenenia stuttgartiensis*". The spectrum shows peaks representing the singly and doubly charged monomeric mass ($[M]H^+$, $[M]2H^+$) and the singly charged dimeric mass of the protein ($[2M]H^+$).

absent in the mature protein. This was supported by the N-terminal fragment as determined by Edman degradation which corresponded to positions 31–42 of the cytochrome *c*. The signal peptide contained the typical sec features of charged/hydrophilic amino acids at the N-terminal end, followed by a hydrophobic amino acid stretch. The residues near the cleavage site, followed the (−1, −3) rule which states that these residues should be small and hydrophobic [22]. The calculated molecular mass for the mature apoprotein was 9505 Da. Together with the molecular mass of one heme ligand ($C_{34}H_{32}N_4O_4Fe = 616.5$ Da) this results in a calculated average mass of 10,123.4 Da [24,25]. The molecular mass determined by MALDI-TOF MS for the purified cytochrome *c* perfectly matches the calculated value, confirming the presence of a *c*-type heme covalently bound to the protein. Another proof that the deduced amino acid sequence of gene 4414 is identical to the purified protein came from MALDI-TOF MS analysis of a tryptic digest. The peptides identified were ALVPVVR (*m/z* 753.66), LTPEEVK (*m/z* 815.00), FADAEWQAK (*m/z* 1065.50), KFADAEWQAK (*m/z* 1193.55), FADAEWQAKTSDER (*m/z* 1653.00). The peak observed at *m/z* 1467.70 could correspond to LYLQHCK with an attached heme devoid of iron and apparently also the second cysteine was detached from the heme group. Possible aspecifically cleaved peptides were LVPVVRGGFKK (*m/z* 1142.44), MMMPF (*m/z* 656.00) and PEEVKA (*m/z* 762.0). These peptides covered 45% of the sequence.

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1   MKNKNTLTLA LFSVTIGASA FFFGTSVSKA SNIDGMKLYL  40
41  QHCKTCHGVD GNPTDLGEGL GARKFADAEW QAKTSDERII  80
81  EQINEGTPEM MMPFKEKLTP EEVKALVPVV RGFKK       115
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Fig. 3. Amino acid sequence of the cytochrome *c* from "*K. stuttgartiensis*". The sequenced N-terminus is underlined and the putative signal peptide is shown in italic. Cleavage occurs between positions 30 and 31. Sequence fragments detected after tryptic digestion and MALDI-TOF MS analysis are shown in bold.

3.4. Comparison of "K. stuttgartiensis" cytochrome c_{552}

BLAST searches for homologous proteins yielded class I *c*-type cytochromes. In these proteins the heme attachment signature CXXCH is located between position 43 and 47. Three methionine residues, Met-89, Met-90 and Met-91, surrounded by proline residues are present in the sequence stretch that commonly contains the sixth methionine ligand for the heme iron. Multiple alignments showed that Met-91 might be the most likely iron ligand (Fig. 4). These features are typical for low-spin cytochromes *c* of class ID with presumed functions in electron transport [26].

Comparison of the mature protein sequence to a variety of small soluble *c*-type cytochromes (Fig. 4) revealed about 23–26% identity to the cytochrome *c*-552 from the aerobic ammonium-oxidizing bacterium *N. europaea* [27] (AAB46987, 1A56), the cytochrome *c*-551 (NirM) from the denitrifying bacterium *Pseudomonas aeruginosa* (AAG03907), the cytochrome *c*-551/*c*-552 from aerobic methane-oxidizing bacterium *Methylococcus capsulatus* strain Bath [28] (AAU91528), and the cytochrome *c*-551/*c*-552 from *Thiobacillus denitrificans* ATCC25259 (ZP_00334625). The latter bacterium is capable of nitrate-dependent sulfide oxidation under anoxic conditions.

Analysis of the of "*K. stuttgartiensis*" cytochrome *c*-552 with PredictProtein (http://cubic.bioc.columbia.edu/predictprotein/) revealed a high α-helical content as was previously described for other members of the c-551/c-552 family [27]. The N-terminal α-helix involved in bending the thioether bonds (Cys^{13} and Cys^{16}) to orient His^{17} for ligation to the heme seems to be conserved (Fig. 4). The second α-helix observed in structural studies has a lower probability in protein 4414, while the third and fourth α-helixes are fully conserved.

Immediately downstream of the isolated cytochrome *c*-552 in the partial genome data assembly of "*K. stuttgartiensis*", a second small cytochrome (gene 4414) was found. The genes were separated by only 43 nucleotides which may indicate that they are co-transcribed. The

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P.aeruginosa      ----------EDPEVLFKNKGCVACHAIDTKM--VGPAY--KDVA-AKFAG  36
N.europaea        ----------DADLAKKNNCIACHQVETKV--VGPAL--KDIA-AKYAD  34
M.capsulatus      ----------SEDLAKAKNCVMCHSVDKKI--LGPAF--KDVA-QKYAG  34
T.denitrificans   ----------ADEMALAQKNACMSCHGVDKKI--VGPAF--KEVA-KKYAG  36
4414              GSVIPFLSRGEEKAIDARNLFEYH-CAKCHGLTGEANKRGKALKAPDLCDPGWQN  54
4415              -----------SNIDGMKLYLQH-CKTCHGVDGNPTDLGEGLGARKFADAEWQA  42
                                *   : *  **    :    *      .     :

P.aeruginosa      QAGAEAELAQRIKNGSQGVWGPIPMP-PN-AVSDDEAQTLAKWVLS--QK--    82
N.europaea        KDDAATYLAGKIKGGSSGVWGQIPMP-PNVNVSDADAKALADWILT--LK--    81
M.capsulatus      QQGADVKLAEKVMKG SGVWGTMVMP-PNPQVSEAEAKQLVQWILS--LK--    81
T.denitrificans   DKGAEDKLVAKVKAGGKGVWGQIPMP-PNPQVKDEDAHKIVAWVLS--LK--    83
4414              SK-TDKEILYSITNG------KNKMPAWNERLTPEEIEALARYVRKLSKKQR   99
4415              KT-SDERIIEQINEGTP----EMMMP-FKEKLTPEEVKALVPVVRGF-KK--    85
                     :   :   :  *          **  :  :.  :   :.  :     *
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Fig. 4. ClustalW multiple alignment of the small cytochrome *c* proteins from "*Kuenenia stuttgartiensis*" (genes 4414 and 4415) with some selected low molecular mass cytochromes *c*-551/552. The signal peptides of the proteins were not included in the alignment. Organisms and their accession numbers are: *Nitrosomonas europaea* (AAB46987, 1A56); *Pseudomonas aeruginosa* (AAG03907); *Methylococcus capsulatus* strain Bath (AAU91528) and *Thiobacillus denitrificans* ATCC25259 (ZP_00334625). The underlined parts in the *N. europaea* and "*K. stuttgartiensis*" sequences are the α-helixes obtained using the PredictProtein software.

neighbouring cytochrome *c* consisted of 121 aa and had a theoretical molecular mass of 13,751 Da and a *pI* of 9.7. The calculated values for the mature holo-cytochrome *c* after removal of the predicted signal peptide were 11,848 Da and *pI* 9.4. The mature proteins showed 38% identity (Fig. 4). A similar isolated arrangement of two small cytochromes *c* of unknown function was detected in the genome of *N. europaea* ATCC 19718 (gi_30248745 and gi_30248744) and the iron and nitrate reducing delta-proteobacterium *Geobacter sulfurreducens* PCA (gi_39997608 and gi_39997610), which showed 36% and 20% identity, respectively, towards each other. Also here the two cytochromes *c* were of similar molecular masses (8.9–12.2 kDa), but their *pI* differed 1.6–1.9 units. The reason for this apparent gene duplication is presently unknown.

The similarity of the "*K. stuttgartiensis*" cytochrome *c*-552 to class ID cytochromes indicates that it might be involved in electron transfer. Homologies with cytochromes *c* from organisms capable of nitrate reduction and ammonium oxidation may suggest the involvement of the cytochrome *c*-552 purified from "*K. stuttgartiensis*" in nitrogen conversion. Future studies will address the redox function of the cytochrome *c*-552 after sufficient protein is expressed in *E. coli*.

Acknowledgement

This work was funded by the Dutch Science Foundation for Earth and Life Sciences (ALW), grant 810.35.001.

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