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Resolution of two native monomeric 90 kDa nitrate reductase active proteins from *Shewanella gelidimarina* and the sequence of two *napA* genes

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ABSTRACT

The reduction of nitrate to nitrite in the bacterial periplasm occurs in the 90 kDa NapA subunit of the periplasmic nitrate reductase (NAP) system. Most *Shewanella* genomes contain two *nap* operons: *napEDABC* and *napDAGHB*, which is an unusual feature of this genus. Two native, monomeric, 90 kDa nitrate reductase active proteins were resolved by hydrophobic interaction chromatography from aerobic cultures of *Shewanella gelidimarina* replete with reduced nitrogen compounds. The 90 kDa protein obtained in higher yield was characterized as NapA by electronic absorption and electron paramagnetic resonance spectroscopies and was identified by LC/MS/MS and MALDI-TOF/TOF MS as NapA from the *napEDABC*-type operon. The other 90 kDa protein, which was unstable and produced in low yields, was posited as NapA from the *napDAGHB*-type operon. Two *napA* genes have been sequenced from the *napEDABC*-type and *napDAGHB*-type operons of *S. gelidimarina*. Native NAP from *S. putrefaciens* was resolved as one NapA monomer and one NapAB heterodimer. Two amino acid substitutions in NapA correlated with the isolation of NAP as a NapA monomer or a NapAB heterodimer. The resolution of native, redox-active NapA isoforms in *Shewanella* provides new insight into the respiratory versatility of this genus, which has implications in bioremediation and the assembly of microbial fuel cells.

1. Introduction

The catalytic subunit NapA of the periplasmic nitrate reductase (NAP) system belongs to the dimethylsulfoxide reductase family of mononuclear Mo enzymes, which catalyze two-electron reduction reactions of oxyanions (nitrate, selenate, arsenate) or marine osmoregulating agents in bacteria [1,2]. NAP has been isolated from *Desulfovibrio desulfuricans* [3,4] and from *Escherichia coli* [5] as a NapA subunit containing a *bis*-molybdopterin guanine dinucleotide (Mo-MGD) unit and one [4Fe–4S] iron–sulphur cluster, and from *Rhodobacter sphaeroides* as a tightly bound ($K_d \sim 0.5$ nM) NapAB protein heterodimer [6]. The di-haem cytochrome NapB transfers electrons to NapA. The NAP system displays variable arrangements and combinations of *napCDEFGHKLM* genes that accompany the *napAB* genes [7–9]. A recent systems biology study established that most *Shewanella* genomes encode two NAP isoforms, NapEDABC (NAP-α) and NapDAGHB (NAP-β) [8]. The physiological significance of this unusual feature has not been established and requires 'bottom-up' studies that explore the resolution of native NAP isoforms from culture and the resolution of *napA* genes from the *napEDABC*-type and *napDAGHB*-type operons.

Here, we report the resolution of two native monomeric, 90 kDa nitrate reductase active proteins from *Shewanella gelidimarina* and the sequence of two *napA* genes from the *napEDABC*-type and *napDAGHB*-type operons. Native NAP from *S. putrefaciens* was studied in concert. Our results contribute to a fuller understanding of the metabolic versatility of *Shewanella*, which is a genus at the forefront of environmental biotechnology [10–12].

2. Materials and methods

2.1. Culturing Shewanella

Shewanella gelidimarina ACAM 456^T [13] and *S. putrefaciens* ATCC 8071^T were kindly provided by Dr. D.S. Nichols (University of Tasmania, Australia). Base medium was 0.5% w/v bactopeptone, 0.2% w/v yeast extract (both Oxoid, Bacto), 3.5% w/v sea salts, 5 mM KNO_3, 0.5 μM Na_2MoO_4 and 0.5 μM $FeCl_3$ (all Sigma). Inoculated cultures in 2.5–3 L batches were shaken at 100 rpm at 4 °C (*S. gelidimarina*) or at ~20–25 °C (*S. putrefaciens*). Cells were harvested at mid-log phase (4000*g*, 4 °C, 30 min) at 8 days (*S. gelidimarina*) or 40 h (*S. putrefaciens*). Cells were washed in ice-cold 10 mM Tris–HCl, pH 8.0, and repelleted and processed by total cell lysis [14] or periplasmic extraction, coupled with assaying for malate dehydrogenase activity [15].

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2.2. Assaying nitrate reductase activity and cytochrome-containing proteins

Nitrate reductase activity was determined using the Griess reaction [14] or the anaerobic benzyl viologen depletion assay [16]. Native PAGE gels were stained for *in situ* nitrate reductase activity [17] and SDS–PAGE gels, run with non-heat-treated samples, were stained for cytochrome-containing proteins [18].

2.3. Native NAP from S. gelidimarina

Proteins were extracted from 34.0 g wet weight of *S. gelidimarina* cells by total cell lysis. Finely ground $(NH_4)_2SO_4$ was added to 60% saturation and the suspension was centrifuged (16,000*g*, 40 min, 4 °C) and the pellet discarded. The supernatant was brought to 90% $(NH_4)_2SO_4$ saturation and, after centrifugation, the pellet was resuspended in a minimum volume of working buffer (20 mM Tris–HCl, pH 7.8) and was loaded onto a column (internal diameter = 26 mm, column volume (CV) = 38 mL) containing butyl-sepharose resin and run with 1.0–0 M $(NH_4)_2SO_4$ in working buffer over six CV. The fraction at $[(NH_4)_2SO_4]$ = 0.20 M was purified on an Akta HPLC (Table S1) to yield 0.48 mg of protein that was identified from mass spectrometry as NapA from the NapEDABC-type isoform (NapA-α_{Sgel}). The fraction at $[(NH_4)_2SO_4]$ = 0.53 M was less stable and purified as a 90 kDa monomer in a yield of ~2.5 μg and was designated as NapA from the NapDAGHB-type isoform (NapA-β_{Sgel}). Specific nitrate reductase activity and activity yields (benzyl viologen) were 15 U mg^{-1} and 0.21 U g^{-1} (NapA-α_{Sgel}) and 170 U mg^{-1} and 0.01 U g^{-1} (NapA-β_{Sgel}).

2.4. Native NAP from S. putrefaciens

Proteins from 28.1 g wet weight of *S. putrefaciens* cells were extracted and processed as above on butyl-sepharose resin to give two nitrate reductase active peaks. The fraction at $[(NH_4)_2SO_4]$ = 0.72 M gave 0.57 mg of a red-brown protein of M_r = 106 kDa that identified within the limits of SDS–PAGE, native PAGE *in situ* nitrate reductase activity, staining for cytochrome-containing proteins and bioinformatics as NapAB from the NapEDABC-type isoform (NapAB-α_{Sput}). The fraction at $[(NH_4)_2SO_4]$ = 0.20 M gave ~0.23 mg of a 90 kDa monomer and was designated as NapA from the NapDAGHB-type isoform (NapA-β_{Sput}). Specific nitrate reductase activity and activity yields (benzyl viologen) were 3 U mg^{-1} and 0.06 U g^{-1} (NapAB-α_{Sput}) and 0.8 U mg^{-1} and 0.006 U g^{-1} (NapA-β_{Sput}).

2.5. Electronic absorption and electron paramagnetic resonance spectroscopies

The electronic absorption spectrum from NapA-α_{Sgel} (0.96 mg mL^{-1}) was recorded using a Cary 50 spectrophotometer. The X-band Mo(V) electron paramagnetic resonance (EPR) spectrum from NapA-α_{Sgel} (9.2 mg mL^{-1}) was acquired using a Bruker eleXsys spectrometer coupled to a Bruker microwave bridge (ER 049X): temperature, 163 K; microwave frequency, 9.358 GHz; microwave power, 1.978 mW; number of scans, 50; modulation frequency, 50 kHz; and modulation amplitude, 0.1 mT [19]. The spectrum was simulated using QPOW.

2.6. Mass spectrometry

Two different preparations of NapA-α_{Sgel} were analyzed by the Australian Proteome Analysis Facility (APAF) using LC/MS/MS or MALDI-TOF/TOF MS of trypsin-digested protein fragments and MASCOT peptide recognition software. Sequence alignments were performed with ClustalW.

2.7. Sequencing two napA genes from S. gelidimarina

Degenerate PCR oligonucleotides were designed based on a consensus alignment of 12 NapA proteins from the NapEDABC-type isoform (NapA-α) and the NapDAGHB-type isoform (NapA-β) from other *Shewanella* species [8] and synthesized by Sigma–Genosys. Genomic DNA was isolated from an aliquot of cells from a mid-log-phase culture of *S. gelidimarina* using a GenomicPrep™ Kit (GE Healthcare). PCR products were visualized on a 1% agarose 1× TAE gel containing 1:25,000 SYBR Safe DNA Gel Stain (Invitrogen) and were excised from the gel and purified with a Qiaprep PCR Purification Kit (Qiagen) prior to ligation into pCR-Blunt-II-Topo cloning vector (Invitrogen). Ligations were transformed into competent 5-α cells (Bioline) and plasmid DNA was isolated using a High Pure Plasmid Isolation Kit (Roche). DNA sequencing was performed by SUPAMAC (University of Sydney Prince Alfred Medical Analysis Centre). The GenBank accession numbers are HM037038 (NapA-α_{Sgel}) and HM037039 (NapA-β_{Sgel}).

3. Results

3.1. Native NAP from S. gelidimarina

Hydrophobic interaction chromatography of the total cell lysis extract from *S. gelidimarina* cells showed two well resolved nitrate reductase active peaks at $[(NH_4)_2SO_4]$ = 0.20 M and $[(NH_4)_2SO_4]$ = 0.53 M (Fig. 1A). Analysis of the fractions by S200 HPLC gel filtration chromatography (Fig. 1B) showed that: (i) the major peak in each trace corresponded to the nitrate reductase activity (Fig. 1B, marked with arrows); (ii) the co-eluting nitrate reductase active proteins were of a comparable globular size; and (iii) the fraction at $[(NH_4)_2SO_4]$ = 0.20 M (Fig. 1B, black) was purer than the fraction at $[(NH_4)_2SO_4]$ = 0.53 M (Fig. 1B, grey). The difference in purity was evident from the analysis of the individual fractions by SDS–PAGE (Fig. 1C) and native PAGE *in situ* nitrate reductase activity (Fig. 1D), which showed a single co-migrating activity band ($[(NH_4)_2SO_4]$ = 0.53 M and $[(NH_4)_2SO_4]$ = 0.20 M) that co-migrated with a major band at 90 kDa on the SDS–PAGE gel of the $[(NH_4)_2SO_4]$ = 0.20 M fraction (Fig. 1C). Therefore, these fractions contained nitrate reductase active proteins that were resolved by hydrophobic interaction chromatography (Fig. 1A), but which had similar molecular masses (90 kDa), such as would occur for two nitrate reductase isoforms. The fraction at $[(NH_4)_2SO_4]$ = 0.20 M was purified (Table S1) to furnish a protein that was subsequently identified by mass spectrometry as NapA of the NapEDABC-type isoform, and was designated as NapA-α_{Sgel}. As expected for a monomeric NapA [3,5,19], a concentrated solution of NapA-α_{Sgel} was green-brown in colour, purified as a single M_r = 90 kDa subunit (Fig. 2A, lane 2 and 2B, lane 6), and was active in the native PAGE *in situ* nitrate reductase activity gel (Fig. 2C, lane 11 and Fig. 1D). The fraction at $[(NH_4)_2SO_4]$ = 0.53 M was partially purified and showed minor contaminating bands in the SDS–PAGE gel and a major band at M_r = 90 kDa (Fig. 2B, lanes 7 and 8) that was positive in the native PAGE *in situ* nitrate reductase activity (Fig. 2C, lanes 9, 10 and Fig. 1D). This protein was designated as NapA of the NapDAGHB-type isoform (NapA-β_{Sgel}). Neither NapA-α_{Sgel} (Fig. 2F, lane 8) nor NapA-β_{Sgel} gave bands that stained positive for cytochrome-containing proteins, which supports that these proteins have purified as NapA monomers and not as NapAB heterodimers.

Although NAP is located in the periplasm, we found that nitrate reductase active proteins were not released from cells of *S. gelidimarina* using a periplasmic extraction procedure. The agglutination of *S. gelidimarina* cells appeared to impede the access of periplasmic extraction reagents to the cell surface. The possibility that cytoplasmic nitrate reductase (NAS) was the enzyme responsible

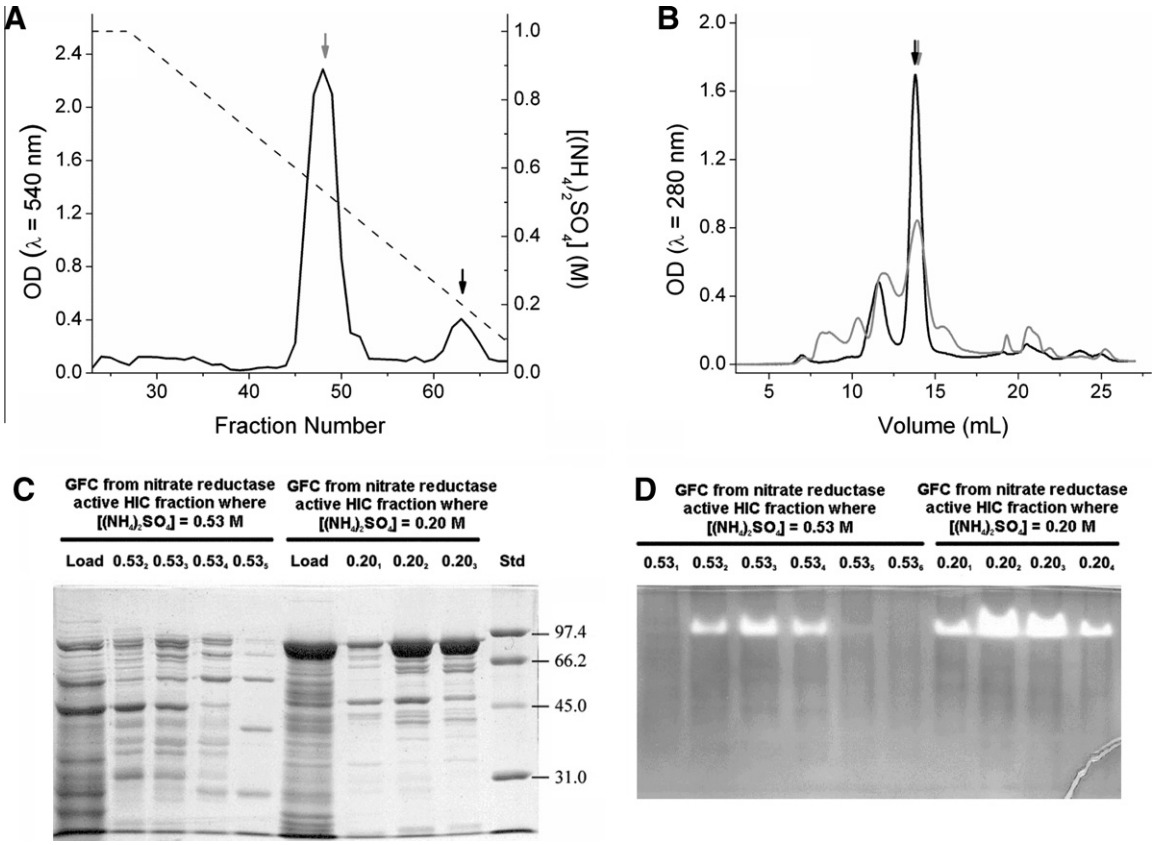

Fig. 1. (A) Nitrate reductase activity of *S. gelidimarina* cell extracts purified by hydrophobic interaction chromatography (HIC) and (B) OD280 and activity (marked with arrows) of the HIC fractions ($[(NH_4)_2SO_4]$ = 0.53 M (grey) and $[(NH_4)_2SO_4]$ = 0.20 M (black)) purified by gel filtration chromatography (GFC). (C) Protein-stained SDS–PAGE and (D) native PAGE *in situ* nitrate reductase activity of the GFC fractions. Load, loaded samples; Std, low-molecular-weight range standards.

for the nitrate reductase activity was discounted based upon the: (i) high concentration of reduced nitrogen compounds in the medium (20 μM ammonium and reduced nitrogen compounds in bactopeptone), which would suppress NAS production (if this gene is present on the *S. gelidimarina* genome) and (ii) the discrepancy in the M_r values between the two nitrate reductase proteins from *S. gelidimarina* (both 90 kDa) and a putative NAS [20] from *S. frigidimarina* NCIMB 400 (101 kDa).

3.2. NapA from S. gelidimarina: spectroscopy and mass spectrometry

The electronic absorption spectrum from a solution of NapA-α_{Sgel} showed features (A_{400}/A_{280} = 0.19) (Fig. 3A) similar to NapA from *D. desulfuricans* (A_{400}/A_{280} = 0.17), which contains a *bis*-Mo-MGD cofactor and one [4Fe–4S] iron–sulphur cluster [3]. No transitions characteristic of cytochromes were present in the spectrum. The X-band EPR spectrum of NapA-α_{Sgel} as isolated (Fig. 3B, expt), showed a Mo(V) signal typical of the high *g* resting state of NapA from *R. sphaeroides* [6] and *Paracoccus denitrificans* [19]. The EPR spectroscopic parameters of the simulated spectrum (Fig. 3B, sim; g_1 = 1.9985, A_1 = 6.4 G; g_2 = 1.9902, A_2 = 5.2; g_3 = 1.9806, A_3 = 5.5 G) correlated with the parameters for NapA from *P. denitrificans* [19], although the interaction with a second I = ½ nucleus (noted on the g_1 signal for NapA from *P. denitrificans*) was not resolved in the NapA-α_{Sgel} spectrum.

Two preparations of NapA-α_{Sgel} were analyzed by APAF. The MASCOT searches (LC/MS/MS) returned significant hits with NapA from *Vibrio fischeri* ES114 (NapA of the NapEDABC-type (NAP-α)) and *Photobacterium profundum* SS9 (NapA of the NapEDABC-type (NAP-α)). The matched fragments (MALDI-TOF/TOF) were aligned to the NapA of *S. oneidensis* MR-1 (NapA of the NapDAGHB-type (NAP-β)) (Figs. 3C and S1). Three residues returned from the MALDI-TOF/TOF analysis identify the higher-yielding protein from *S. gelidimarina* as NapA of the NapEDABC-type (NAP-α) isoform (NapA-α_{Sgel}). In NapA of the NapEDABC-type (NAP-α) isoform in *Shewanella*, the residues at general coordinates 54, 166 and 558 are conserved as T, M and E (or D in some species), respectively [8]. These residues (T, M and E) are present in native NapA-α_{Sgel} (residues marked with ‡ in Figs. 3C and S1). In NapA from the NapDAGHB-type isoform (NAP-β) in *Shewanella*, the residues at these same coordinates are absolutely conserved in 18 species as V, H and G, respectively.

3.3. Sequencing two napA genes from S. gelidimarina

If *S. gelidimarina* was producing two NapA isoforms, the genome must contain two *napA* genes. PCR amplification of genomic DNA from *S. gelidimarina* using primers *napA*-β_{fwd} and *napA*-β_{rev} (Table S2) generated a band of the expected size of *napA* of 2.5 kb. This product was cloned into the pCR-Blunt-II-Topo cloning vector and individual positive transformants were sequenced, yielding the 2481 nucleotide sequence of *napA*-β which translates to a protein of 827 amino acid residues of 92.7 kDa (Table S3). Amplification reactions using the *napA*-α_{fwd} and *napA*-α_{rev} primers also generated 2.5 kb bands, but this product was found by sequencing to be *napA*-β. Reactions using *napA*-α_{fwd} and a degenerate oligonucleotide designed to recognize both *napA*-α and *napA*-β, *napA*-α/*nap*-β_{rev}, also yielded only the *napA*-β isoform. The design of primers specific for the *napA*-α isoform was more complicated: a 0.7 kb product was generated from *napA*-α_{fwd} and *napA*-α_{revX}; no

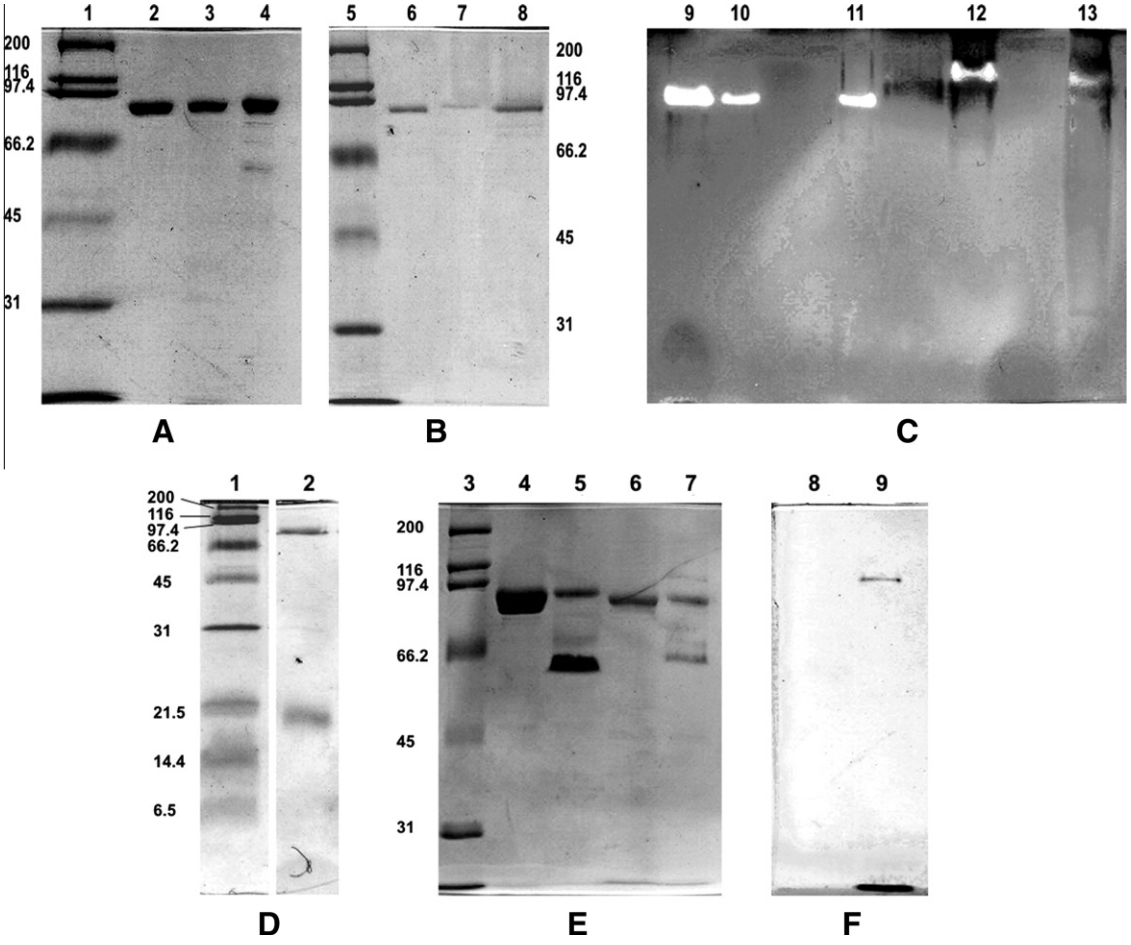

Fig. 2. SDS–PAGE (A and B) and native PAGE *in situ* nitrate reductase activity (C) of nitrate reductase active proteins from *S. gelidimarina* (NapA-α_{Sgel}, NapA-β_{Sgel}) or *S. putrefaciens* (NapAB-α_{Sput}, NapA-β_{Sput}). Gels (A), (B) and (C): lanes 1 and 5, broad-molecular-weight range standards; lanes 2 (1 μg), 6 (0.3 μg) and 11 (0.7 μg), NapA-α_{Sgel}; lanes 3 (0.8 μg) and 12 (6 μg), NapAB-α_{Sput}; lanes 4 (1 μg) and 13 (5 μg), NapA-β_{Sput}; lanes 7 (0.08 μg), 8 (0.4 μg), 9 (0.2 μg) and 10 (0.04 μg), NapA-β_{Sgel}. SDS–PAGE (15% gel) (D) and SDS–PAGE (10% gel with heat-treated and non-heat-treated samples) (E and F) of nitrate reductase active proteins from *S. gelidimarina* (NapA-α_{Sgel}) and *S. putrefaciens* (NapAB-α_{Sput}) stained *in situ* (F) for cytochrome-containing proteins. Gels (D), (E) and (F): lanes 1 and 3, broad-molecular-weight range standards; lanes 2, 6 (heat-treated), 7 (non-heat-treated) and 9 (non-heat-treated), NapAB-α_{Sput}; lanes 4 (heat-treated), 5 (non-heat-treated) and 8, NapA-α_{Sgel} (non-heat-treated).

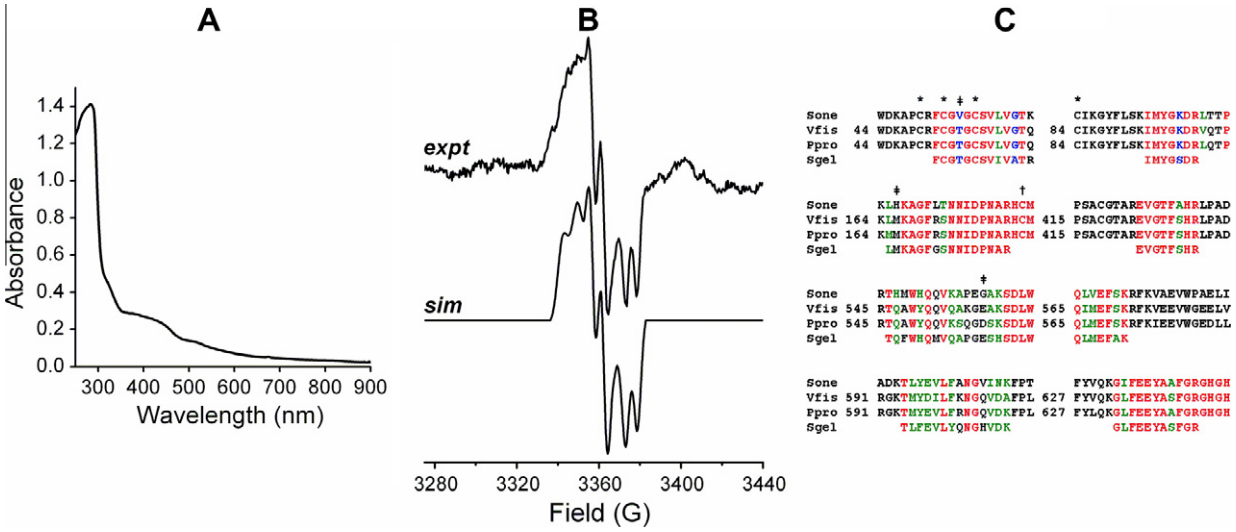

Fig. 3. (A) Electronic absorption spectrum of NapA-α_{Sgel} (0.96 mg mL^{-1}) and (B) Mo(V) EPR spectrum at 163 K of NapA-α_{Sgel} (9.2 mg mL^{-1}) as isolated (expt) and simulated (sim). (C) MALDI-TOF/TOF MS analysis of NapA-α_{Sgel} (Sgel) aligned with NapA from *Vibrio fischeri* ES114 (Vfis, NapA-α form), *Photobacterium profundum* SSP (Ppro, NapA-α form) or *Shewanella oneidensis* MR-1 (NapA-β form). The cysteine residues bound to the [4Fe–4S] iron–sulphur cluster or the Mo ion in NapA from *R. sphaeroides* [6] are denoted by ∗ or †, respectively. Residues that differentiate NapA-α from NapA-β are denoted by ‡.

product was generated from *napA*-α_{fwdX} and *napA*-α_{rev}; and a 1.8 kb fragment of *napA*-α was generated from *napA*-α_{fwdX} and *napA*-α/*napA*-β_{rev}. These products were cloned and sequenced to generate a contiguous 2439 nucleotide sequence of *napA*-α from *S. gelidimarina* (Table S3). The amino acid identity between NapA-α_{Sgel} and NapA-β_{Sgel} is 69%, which accords with previous data [8].

3.4. Native NAP from S. putrefaciens

Two nitrate reductase active peaks from *S. putrefaciens* ATCC 8071^T extract eluted from the butyl-sepharose column at $[(NH_4)_2SO_4]$ = 0.72 M and at $[(NH_4)_2SO_4]$ = 0.20 M. The fraction at $[(NH_4)_2SO_4]$ = 0.72 M contained more protein and was purified to give a nitrate reductase active protein that showed one band at M_r = 90 kDa and one band at M_r = 16 kDa (Fig. 2D, lane 2) on a 15% SDS–PAGE gel. Two lanes on the same SDS–PAGE gel, which were loaded with non-heat-treated samples, were stained separately for protein or for cytochrome-containing proteins. The feint band at M_r = 106 kDa that stained for protein (Fig. 2E, lane 7) also stained positive for cytochrome-containing proteins (Fig. 2F, lane 9), together with a second positive band that ran in the region of the unresolved M_r standards. These properties showed that this fraction isolated as a NapAB heterodimer. Based on bioinformatics data (detailed below), this protein was designated as NapAB of the NapEDABC-type isoform (NapAB-α_{Sput}). The isolation of NapAB-α_{Sput} as a NapAB heterodimer is further supported by the: (i) red-brown colour of the protein solution; (ii) slower running positive band in the native PAGE *in situ* nitrate reductase activity (Fig. 2C, lane 12) and (iii) elution under native HPLC gel filtration conditions of NapAB-α_{Sput} (M_r = 106 kDa) before NapA-α_{Sgel} (M_r = 90 kDa). The fraction that eluted at $[(NH_4)_2SO_4]$ = 0.20 M was partially purified and showed several bands in the SDS–PAGE gel and a major band at M_r = 90 kDa (Fig. 2A, lane 4) that was positive in the native PAGE *in situ* nitrate reductase activity (Fig. 2C, lane 13). This fraction did not give any bands that stained positive for cytochrome-containing proteins and eluted in the same window as NapA-α_{Sgel} and NapA-β_{Sgel} in HPLC gel filtration chromatography experiments. This NapA monomer was designated as NapA of the NapDAGHB-type isoform (NapA-β_{Sput}). The genome of *S. putrefaciens* CN-32 contains two *nap* operons (*napEDABC*-type and *napDAGHB*-type) [8], which supports the production of two nitrate reductase active proteins in *S. putrefaciens* ATCC 8071^T.

4. Discussion

Since most *Shewanella* genomes contain two *nap* operons (*napEDABC* and *napDAGHB*), we examined whether native NapA isoforms could be resolved from culture. The two nitrate reductase active proteins produced by each of *S. gelidimarina* and *S. putrefaciens* cultures were characterized at the level of SDS–PAGE, native PAGE *in situ* nitrate reductase activity, cytochrome-containing protein staining and benzyl viologen-based nitrate reductase activity assays. MALDI/TOF/TOF analyses positively identified the protein produced in higher yield from *S. gelidimarina* as NapA of the NapEDABC-type isoform (NapA-α_{Sgel}). Both NapA-α_{Sgel} and NapAB-α_{Sput} are likely to serve roles in redox balancing, akin to the aerobic NapA of the *napEDABC*-type operon of *P. denitrificans* [9,21]. Since the genome of the obligate respiratory nitrate ammonifier *S. oneidensis* contains only the *napDAGHB*-type operon [8], the NapDAGHB-type isoform (NAP-β) is functional in anaerobic respiratory nitrate ammonification [22]. The facultative anaerobic phenotype of *Shewanella* may manifest in the production of detectable amounts of NapA from the NapDAGHB-type isoform together with NapA from the NapEDABC-type isoform.

We resolved NAP from *S. gelidimarina* as two 90 kDa monomers and NAP from *S. putrefaciens* as one NapAB heterodimer and one NapA monomer. In *R. sphaeroides* [6], *P. denitrificans* [23], *Ralstonia eutrophus* H16 [24] and *S. putrefaciens* (one isoform: NapAB-α_{Sput}), NAP isolated as a NapAB heterodimer. In *Sinorhizobium meliloti*, NAP is predicted to isolate as a NapAB heterodimer [25]. In *E. coli* [5], *S. putrefaciens* (one isoform: NapA-β_{Sput}) and *S. gelidimarina* (two isoforms: NapA-α_{Sgel} and NapA-β_{Sgel}), NAP isolated as a NapA monomer. An alignment of NapA proteins showed two residues present at the NapA–NapB interface that are absolutely conserved in the four experimental NapAB-purifying systems and which differ from the residues in the NapA-purifying systems (Fig. 4A). As referenced to NapAB from *R. sphaeroides* [6], these residues are E47 and S772. In NapA from *R. sphaeroides*, E47 is in close proximity with R83 in NapB to enable intersubunit interactions via H-bonding and/or salt bridges [6] (Fig. 4B). In all four of the NapA-purifying systems, E47 is replaced with P (marked with † in Fig. 4A). The loss of this NapA(acid)–NapB(base) interaction could disrupt the integrity of the NapAB heterodimer during NAP purification. In NapA-purifying systems, S772 is replaced with R in the three monomeric NapAs from *Shewanella* (NapA-α_{Sgel}, NapA-β_{Sgel} and NapA-β_{Sput}) and with A in *E. coli* NapA. The NapA of the NapEDABC-type isoform (NapA-α) encoded on the *S. putrefaciens* CN-32

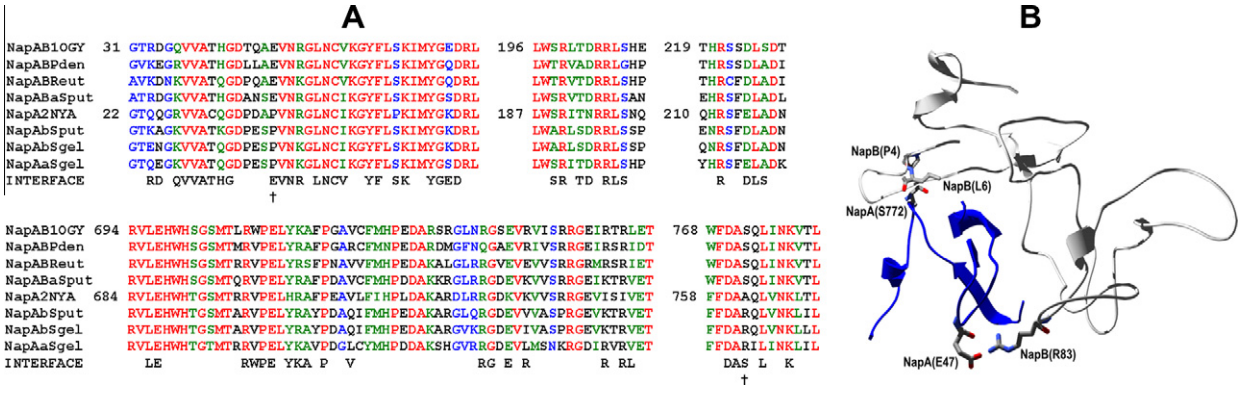

Fig. 4. (A) Alignment of NapA proteins from NAP systems that purify as NapA monomers: *S. gelidimarina* (NapA-α_{Sgel}, NapAaSgel; NapA-β_{Sgel}, NapAbSgel), *S. putrefaciens* (NapA-β_{Sput}, NapAbSput) and *Escherichia coli* (NapA2NYA); and as NapAB heterodimers: *S. putrefaciens* (NapAB-α_{Sput}, NapABaSput), *Rhodobacter sphaeroides* (NapAB1OGY), *Paracoccus denitrificans* (NapABPden) and *Ralstonia eutrophus* H16 (NapABReut). NAP from *Sinorhizobium meliloti* 1021 is predicted as a NapAB heterodimer [25]. Residues in NapA within 4 Å of the NapA-NapB interface [6] are shown in INTERFACE. Residues at the NapAB INTERFACE that are absolutely conserved in NapAB-purifying systems and that are different from the residues in the NapA-purifying systems are marked with †. (B) The NapAB interface in *Rhodobacter sphaeroides* [6] (NapA, blue; NapA, grey). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genome contains the two residues (E and S) that predict that this NAP would purify as a NapAB heterodimer. This is consistent with the isolation of one of the two native nitrate reductase active proteins from *S. putrefaciens* ATCC 8071^T as a NapAB heterodimer (M_r = 106 kDa, NapAB-α_{Sput}). The other monomeric NapA from *S. putrefaciens* was designated as NapA-β_{Sput}. This designation is consistent with the E47P and S772R substitutions in the NapA of the NapDAGHB-type isoform (NapA-β) encoded on the *S. putrefaciens* CN-32 genome, which predicts the isolation of monomeric NapA. These NapA amino acid substitutions (E47P and S772R(A)) may predict whether NAP isolates as a NapA monomer (P, R(A)) or a NapAB heterodimer (E, S).

This work has provided: (i) experimental evidence of the production of two native NAP isoforms in two species of *Shewanella*; (ii) positive identification of one of the two monomeric, 90 kDa nitrate reductase active proteins from *S. gelidimarina* as NapA of the NapEDABC-type isoform; (iii) primers that are specific for *napA*-α and *napA*-β in *Shewanella* and (iv) a tool that predicts whether native NAP isolates as a NapA monomer or as a NapAB heterodimer. The first resolution of NAP isoforms sheds new light on the metabolic versatility of *Shewanella*, which has implications in fully realizing the applications in environmental biotechnology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.002.

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