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A cytochrome *c* encoded by the *nar* operon is required for the synthesis of active respiratory nitrate reductase in *Thermus thermophilus*

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Abstract A cytochrome *c* (NarC) is encoded as the first gene of the operon for nitrate respiration in *Thermus thermophilus*. NarC is required for anaerobic growth and for the synthesis of active nitrate reductase (NR). The α and δ subunits (NarG, NarJ) of the NR were constitutively expressed in *narC::kat* mutants, but NarG appeared in the soluble fraction instead of associated with the membranes. Our data demonstrate for NarC an essential role in the synthesis of active enzyme and for the attachment to the membrane of the respiratory NR from *T. thermophilus*. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Nitrate respiration; Thermophilic; Cytochrome *c*; *Thermus*

1. Introduction

Respiratory nitrate reductases (NRs) are widely distributed enzymes that allow the use of nitrate as electron acceptor by many facultative anaerobes. One of the best studied enzymes is the NRA from *Escherichia coli*, expressed from a four gene operon (*narGHJI*), which encodes the catalytic molybdoprotein or α subunit (NarG), an iron–sulfur protein or β subunit (NarH), a specific chaperone (δ subunit, or NarJ), and a diheme cytochrome *b* or γ subunit (NarI) [1,2]. During nitrate respiration different donors transfer their electrons to naphtoquinones [3,4], which donate them to the acceptor site of NarI at the outer face of the cytoplasmic membrane [2,5]. Electrons are then transferred to NarH and finally to the active site of NarG, where they are used to reduce nitrate.

A large nitrate respiration gene cluster (*narCGHJIK1K2*) is present in *Thermus thermophilus* HB8 [6], as part of a conjugative plasmid integrated into its chromosome [7]. No nitrite reductase activity is found in this bacterium [6], and nitrite is finally excreted through two nitrate/nitrite transporters (NarK1 and NarK2) [8]. As in other organisms, the transcription of the *nar* operon from *T. thermophilus* HB8 requires nitrate and low oxygen concentrations [6]. The promoter region of this operon has been identified immediately upstream of the *narC* gene, and used for the controlled expression of an eukaryotic ribozyme in this thermophile [9].

In this article we describe the sequence of *narC*, the first gene of the *narCGHJIK1K2* operon. We present evidence that it encodes a cytochrome *c* which is required for the attachment to the membrane and activation of the respiratory NR from *T. thermophilus*.

2. Materials and methods

2.1. Bacterial strains

T. thermophilus HB8 (ATCC 27634) [10] was obtained from the American Type Culture Collection (Rockville, MD, USA). *T. thermophilus* HB27::*nar* was a derivative of the aerobic HB27 strain that contains the *nar* cluster [7]. The *E. coli* strains DH5αF′ (Bethesda Research Laboratories, Gaithersburg, MD, USA) and GM2929 (*E. coli* Genetic Stock Center) were used for genetic constructions and for the isolation of unmethylated DNA, respectively.

2.2. Cell growth and nar induction

Aerobic and anaerobic growth of *T. thermophilus* was developed in rich medium (TB) as described in [6]. For the induction of nitrate-anoxia dependent promoters two alternative procedures were followed. For 'progressive' inductions the cells were grown at 70°C under shaking (150 rpm) in a medium containing KNO_3 (40 mM), and induction was concomitant with the oxygen consumption that parallels the increase in cell mass during growth. For 'sudden' inductions cells were grown under shaking as above in the absence of nitrate up to an OD_{550} of ~0.5, and the induction was activated by the addition of KNO_3 (40 mM) and the simultaneous arresting of the shaker.

2.3. Isolation of narC::kat mutants

For *narC* inactivation, we inserted the *kat* cassette, encoding a thermostable resistance to kanamycin, at position 125 from its ATG start codon. For gene replacement, we included upstream and downstream sequences from positions −719 to +1049 as recombination arms. Gene replacement was done as described in [8] on *T. thermophilus* HB27::*nar*. The mutation was then transferred to the HB8 strain by chromosomal transformation of competent cells. Two *narC::kat* derivatives (CKN1 and CKN2) were selected for further analysis.

2.4. Nucleic acids analysis

Methods for DNA purification, restriction analysis, and Southern blot were developed as described in [11,12]. Automatic methods were used for sequencing. Computer predictions were carried out through PROSITE [13], MAXHOM alignment [14], PHD [15] and TMHMM1.0 [16] programs, in the EXPASY molecular biology server.

Total RNA from *T. thermophilus* was purified with the Fast RNA kit-blue from BIO101 (CA, USA) and analyzed by Northern blot [12]. For detection, specific oligonucleotides were labeled with UTP–fluorescein and visualized with the ECL detection kit (Amersham Phar-

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macia). Oligonucleotides O-citC (5′-CAGTGGGTTCCGTAGA-3′) and O-narG (5′-TAGAACTCCTCCCACTT-3′) were used for detection of *narC* and *narG* mRNAs, respectively.

2.5. Protein analysis

The NR activity was measured at 80°C [6] with methyl-viologen as the electron donor and potassium nitrate (40 mM) as the electron acceptor [17]. NarG and NarJ were detected by Western blot with specific rabbit antisera [6] and visualized with the ECL detection kit. To localize NarG, cell samples equivalent to three OD_{550} units were harvested from the cultures, re-suspended in 1/10 volume of TS buffer (50 mM Tris–HCl, 50 mM NaCl, pH 7.5), and broken by sonication. After discarding unbroken cells, particulate and soluble fractions were separated by two consecutive centrifugation steps (22 000×*g*, 15 min) and equivalent amounts of cell fractions were subjected to Western blot. For heme c detection, soluble or particulate fractions were incubated for 5 min at 37°C in SB buffer (Tris–HCl 50 mM, 100 mM NaCl, 1% SDS, pH 7.5), and solubilized proteins were immediately subjected to SDS–PAGE and stained for heme c as described in [18].

3. Results

3.1. NarC is the first gene of the narCGHJIK1K2 operon

The sequence of the *narC* gene is available under the accession number AJ409166. It encodes a 262 amino acid long protein, for which the ATG start codon is preceded by a putative ribosome binding site (RBS) sequence identical to that of *narG* (GGAGGTGA). There is no other open reading frame encoded immediately upstream of *narC* in the same DNA strand. The last two codons of *narC* overlap the RBS of *narG*, and no transcription terminator sequences are apparently located between these two genes. In fact, Northern blots of samples from progressively anoxic cultures of *T. thermophilus* HB8 (Section 2) resulted in labeled bands of identical sizes (∼10 kbp) and increasing intensities (Fig. 1A) when probes against *narC* (O-citC) or *narG* (O-narG) were used. As the distance between the start and stop codons of *narC* and *narK2*, respectively, is 9792 bp, it seems clear that *narC* constitutes the first gene of the *narCGHIJK1K2* operon.

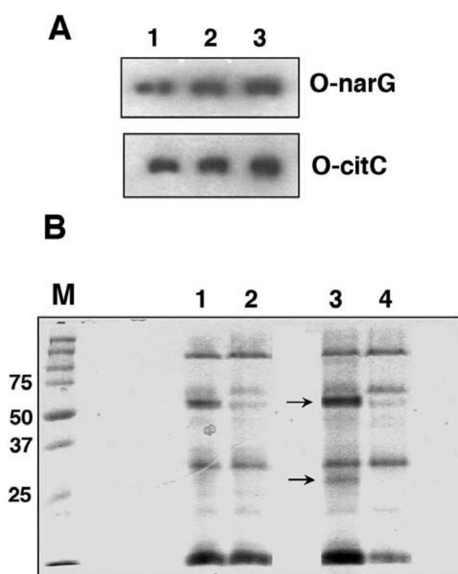

Fig. 1. NarC is a cytochrome *c* encoded by the *nar* operon. A: Northern blot on progressively induced samples of *T. thermophilus* HB8 hybridized with the indicated probes. Samples correspond to OD_{550} of 0.27 (1), 0.79 (2) and 1 (3). B: Heme c staining on total proteins of the wild-type (1, 3) and the CKN1 mutant (2, 4) cells of *T. thermophilus* HB8. Cells were grown aerobically (1, 2) or subjected to 'sudden' induction for 16 h (3, 4). The size (kDa) of the markers is indicated. Arrows label the proteins specifically induced in the wild-type strain which are absent in the mutant.

3.2. NarC is a membrane cytochrome c

The first ∼100 amino acid domain of NarC shows high sequence similarity to different cytochrome *c*'s, like the periplasmic cytochrome c_{552} (38% identical) and the cytochrome *c*-aa3 oxidase subunit IIc (32% identical) from *T. thermophilus*, the cytochrome oxidase subunit III from *Campylobacter jejuni* (31% identical), the cytochrome *c*-554 from *Paracoccus* sp. (30% identical), etc. In fact, this N-terminal domain contains a sequence signature for the binding of a heme c group ($\underline{C}_{30}AS\underline{C}HG_{35}$). By contrast, the sequence of the last ∼160 amino acids of NarC does not show significant homology with any other protein in the gene banks, despite having an additional sequence signature for the binding of a second heme c group ($\underline{C}_{141}QA\underline{C}HT_{146}$).

A 19 amino acid long signal peptide is located at the N-terminus of NarC. Such a peptide ends at a putative processing site (LA-Q) identical in sequence to that of the periplasmic cytochrome c_{552} precursor from *T. thermophilus* HB8 [19]. The sizes expected for the precursor and for a putatively processed product of NarC are 27.1 and 25.1 kDa, respectively. An additional ∼1 kDa had to be added if two heme c groups were bound to the protein. A putative transmembrane segment (positions 226–243), followed by a positively charged sequence (six arginines in a 19 amino acid long sequence), is found at the C-terminus of NarC. The presence of the signal peptide and this transmembrane segment support NarC being a periplasmic protein attached to the cytoplasmic membrane.

The presence of at least one heme c group associated with NarC is supported by the results shown in Fig. 1B. In this figure, a heme c-carrying protein of around 27 kDa is synthesized in the parental strain (arrow) when subjected to nitrate-anoxia 'sudden' induction. This heme c protein is not detected in a *narC::kat* mutant (CKN1). As expected from the predictions on NarC, this ∼27 kDa cytochrome *c* was located in the particulate fraction of the cells (not shown). Based on these experiments we concluded that *narC* actually encodes a 27 kDa membrane cytochrome *c*. An additional heme c-containing protein of ∼54 kDa was also expressed under these conditions in the wild-type (arrow), but was absent in the *narC::kat* mutant, suggesting that NarC could dimerize.

3.3. NarC is required for nitrate respiration and NR synthesis

In the insertion mutants, the *kat* gene was cloned in the same transcription sense as the *nar* operon to allow the expression of downstream genes. Nevertheless, *narC::kat* mutants were unable to grow anaerobically with nitrate (not shown). In order to test if this was due to a defect in NR synthesis, a series of induction experiments were developed in which the synthesis of NarG and NarJ was followed by Western blot and the NR activity assayed in parallel with methyl-viologen as electron donor.

As shown in Fig. 2A, NarG and NarJ were constitutively expressed in a *narC::kat* mutant, an effect expected from the transcriptional activity of the *kat* cassette, which does not wear a transcription terminator. Due to this, the amounts of both proteins were not far below that of induced cultures of the wild-type strain (compare lanes 3 and 4–6 of Fig. 2A).

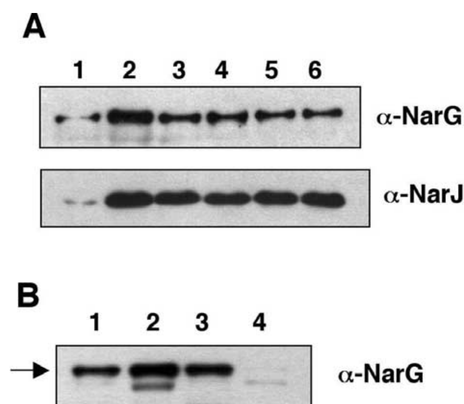

Fig. 2. NarG is expressed as a soluble and inactive form in *narC::kat* mutants. A: Total proteins from equivalent cell amounts from progressively induced cultures of the wild-type strain (1–3) and the CKN1 mutant (4–6) were subjected to Western blot for the detection of NarG and NarJ. Samples correspond to OD_{550} of 0.32 (1), 0.68 (2), 1.2 (3), 0.25 (4), 0.75 (5), 1.3 (6). B: Western blot for the detection of NarG in soluble (1, 3) and particulate (2, 4) cell fractions of the wild-type (1, 2) and the CKN1 (3, 4) strains, subjected to 'sudden' induction for 4 h.

However, whereas no NR activity was detected in the mutants, activities of ~700 and ~1000 units (nmol of nitrite per ml, min, and OD_{550} unit) were associated with the wild-type samples corresponding to lanes 2 and 3, respectively. These data demonstrate that NarC is required not solely for anaerobic respiration, but also for the synthesis of a NR active in nitrate reduction with methyl-viologen as electron donor.

3.4. *NarG is expressed as a soluble protein in narC::kat mutants*

A cell fractionation procedure was followed to localize NarG in a *narC::kat* mutant. In agreement with previous results [6], most of the NarG protein was associated with the particulate fraction of the wild-type strain (Fig. 2B, lane 2), whereas in the mutant this protein appeared in the soluble fraction (lane 3). Thus, NarG is unable to bind to the cytoplasmic membrane in the absence of NarC.

4. Discussion

We have shown that *narC* constitutes the first gene of the largest *nar* operon so far described (*narCGHJIK1K2*). The nature of NarC as a cytochrome *c* is based both on its sequence and on the nitrate-anoxia dependent induction in wild-type cells of a heme c-containing protein of the size expected for NarC; the latter did not appear in *narC::kat* mutants. Interestingly, a second heme c-containing protein of around twice this size (~54 kDa) was also induced in the wild-type but not in the mutants, suggesting that NarC could form dimers that withstand the mild extraction conditions required for this protocol. A confirmation of this hypothesis would require the use of specific antisera, which are unavailable at present.

A second point deals with the location of NarC. We have evidence to support that NarC is associated with the particulate cell fraction (membranes), a fact that agrees with the predictions for a transmembrane helix followed by a positively charged domain at the C-terminus of the protein. In addition, the presence of a putative signal peptide with a potential processing site identical (LA-Q) to that of the periplasmic cytochrome c_{552} from *T. thermophilus* HB8 strongly suggests that NarC is a secreted protein. Consequently, the most likely nature for NarC is that of a periplasmic diheme cytochrome *c* anchored to the membrane through its C-terminus. Such a location makes its requirement for the synthesis of an active NR even more intriguing.

To discuss the putative role of NarC in NR biosynthesis, two kinds of data are relevant. First, the NR subunits are actually synthesized, but they are unable to couple nitrate reduction to the oxidation of methyl-viologen (Fig. 2A), an artificial electron donor which apparently donates the electrons directly to the molybdopterin guanine dinucleotide (MGD) cofactor [20]. Such a result supports that either the MGD is not present in the α subunit of *narC::kat* mutants, or that a wrong conformation of the enzyme blocks the electron transfer to the nitrate. Purification of the αβ complex from the *narC::kat* mutants would be required to determine if the MGD is actually present in the enzyme.

The second type of data indicates that NarC is required for the attachment of the α subunit to the membrane. At present, it is not possible to know if it is the wrong conformation of the αβ complex that impedes its attachment to the membrane, or if it is the inability to bind to the membrane that is responsible for the wrong conformation. Clues from the behavior of mutants in the γ subunit favor the second possibility. In *E. coli*, mutants in NarI (γ subunit) produce soluble forms of the αβ complex, which can reduce nitrate with viologens as electron donors [1]. Thus, membrane attachment seems to be not required for the 'activation' of the NRA in this mesophile. By contrast, *narI::kat* mutants of *T. thermophilus* produce soluble but inactive forms of the NR ([21] and unpublished data), a phenotype which is indistinguishable from that of the *narC::kat* mutants described here. Consequently, we propose that in *T. thermophilus* a membrane binding step is required for a yet unknown 'activation' process of the NR. Unfortunately, limitations in the genetic tools available for this extreme thermophile make it impossible at present to check if it is the short cytoplasmic domain of NarC that functions directly as a binding site for NarG, or if it is the absence of NarC that produces an incorrect folding of the γ subunit, making it unable to bind NarG.

Whatever the mechanism implicated, it remains clear from our data that *narC* is required for membrane attachment and activation of the respiratory NR. The nature of NarC as a cytochrome *c* and its probable stoichiometric synthesis with the NR subunits suggest a role for this protein as a component of the electron transport chain toward the NR. In this sense, the presence of a 16 kDa diheme cytochrome *c* (NapB) as part of the periplasmic NR from *Paracoccus pantotrophus* could support such a potential role for NarC [1]. Having in mind that *Thermus* belongs to one of the oldest evolutionary branches of the bacteria domain, it is tempting to speculate that its NR represents an evolutionary precursor of both membrane and periplasmic respiratory NRs. Future work on the biochemistry of NarC will define these and other aspects of the essential role of NarC in the nitrate respiration of *T. thermophilus*.

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