Transcriptional regulation of nitric oxide reduction in \textit{Ralstonia eutropha} H16

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Abstract

Nitric oxide reduction in \textit{Ralstonia eutropha} H16 is catalysed by the quinol-dependent N0 reductase NorB. \textit{norB} and the adjacent \textit{norA} form an operon that is controlled by the $\sigma^{54}$-dependent transcriptional activator NorR in response to NO. A NorR derivative containing MalE in place of the N-terminal domain binds to a 73 bp region upstream of \textit{norA} that includes three copies of the putative upstream activator sequence GGT-(N7)-ACC. Mutations altering individual bases of this sequence resulted in an 80–90% decrease in transcriptional activation by wild-type NorR. Similar motifs are present in several proteobacteria upstream of genes encoding proteins of NO metabolism. The N-terminal domain of NorR contains a GAF module and is hypothesized to interact with a signal molecule. A NorR derivative lacking this domain activates the \textit{norAB} promoter constitutively. Amino acid exchanges within the GAF module identified a cysteine residue that is essential for promoter activation by NorR. Signal sensing by NorR is negatively modulated by the iron-containing protein NorA.

Introduction

NO and derived RNS (reactive nitrogen species) are versatile signal molecules in biological systems. In prokaryotes, various transcriptional regulators have been shown to respond to NO and RNS. In most of the denitrifying bacteria, expression of nitrite reductase and NO reductase is controlled by Fnr-like regulators on induction with NO [1]. An NO-responsive two-component system (ResDE) is present in \textit{Bacillus subtilis} [2]. In the denitrifying $\beta$-proteobacterium \textit{Ralstonia eutropha} H16, NO reduction depends on the NO-responding transcriptional regulator NorR [3]. NorR orthologues present in \textit{Escherichia coli} K12 [4,5] and \textit{Pseudomonas aeruginosa} PAO1 (H. Arai, personal communication) activate expression of the NO-detoxifying enzymes flavorubredoxin (NorV) and NO dioxygenase (Hmp) respectively.

In \textit{R. eutropha} H16, three genes for NO reduction (\textit{norRAB}) are located on the conjugative megaplasmid pHG1. A chromosomally encoded paralogue cluster termed \textit{norR2AB2} has not been extensively studied, but mutational analyses indicate that either of the two \textit{nor} clusters is sufficient for denitrification.

The \textit{norB} gene encodes a single-subunit NO reductase of the qNor type [6]. The precise function of the \textit{norA} gene product has not been established. NorR belongs to the class of $\sigma^{54}$-dependent transcriptional activators and shows the typical domain structure including a putative helix–turn–helix motif for DNA binding and a central AAA+ domain.

The N-terminal domain contains a GAF module, a widely distributed unit for binding of small molecules [7].

NorR binds to conserved DNA motifs

In \textit{R. eutropha}, both \textit{norA} and \textit{norB} are transcribed from a promoter upstream of \textit{norA}. Since attempts to use purified NorR in DNA-binding studies were not successful, a NorR derivative was constructed that contains MalE in place of the N-terminal domain (MalE-NorR$'$. A DNase I footprint with purified MalE-NorR$'$, a 73 bp DNA region upstream of \textit{norA} was protected from cleavage. This region contains three copies of an inverted repeat GGT(N7)ACC, designated NorR-box. Gel retardation assays indicate that two NorR-boxes are sufficient for DNA binding of MalE-NorR$'$ \textit{in vitro}. However, promoter studies in cells containing wild-type NorR demonstrated that the alteration of conserved bases within NorR-boxes 1, 2 or 3 lowers \textit{norA} promoter activity by 80–90%. Thus DNA-binding of MalE-NorR$'$ \textit{in vitro} does not completely reflect the situation \textit{in vivo}, probably since the fusion protein differs from wild-type NorR with respect to DNA affinity and co-operativity.

A database survey indicates that genes for NorR orthologues are present in several proteobacteria from the $\beta$- and $\gamma$-group. An alignment of DNA segments containing \textit{norR} genes from several organisms showed that, in most cases, three consecutive NorR-boxes are located adjacent to \textit{norR}. In these boxes, the first (G) and the last (C) bases of the \textit{R. eutropha} consensus are frequently replaced by T and A respectively. The conservation of the GT(N7)AC core sequence suggests that NorR proteins also contain conserved residues for DNA binding. In fact, a comparison of several NorR orthologues revealed the presence of an almost perfectly conserved KLAKRL sequence at the end of the putative recognition helix of the HTH (helix–turn–helix)

Key words: denitrification, DNA-binding motif, nitric oxide, \textit{Ralstonia eutropha} H16, $\sigma^{54}$, transcriptional activator.

Abbreviations used: RNS, reactive nitrogen species; SNP, sodium nitroprusside.

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motif. Therefore these residues are probable candidates for the recognition of conserved DNA sequences.

The N-terminal domain of NorR is essential for RNS sensing

Activation of the norA promoter by NorR is induced by NO and the NO\(^+\) donor SNP (sodium nitroprusside) [3]. A NorR derivative lacking the GAF module (NorR\(^{‘}\)) activates the norA promoter constitutively, suggesting that NorR is a one-component system that senses RNS by the GAF module. Several proteins known to sense RNS contain metal cofactors for that purpose. Prominent examples are haem in soluble guanylate cyclase [8] of eukaryotes and iron–sulphur clusters in, e.g. SoxR [9] and FNR [10] of E. coli. In contrast, R. eutropha NorR, as purified from E. coli, did not contain iron and had no significant features in the UV/Vis redox difference spectrum.

Alternatively, NorR may sense RNS by direct modification of amino acid residues. Both the nitration of tyrosine residues and nitros(yl)ation of cysteine residues have been implicated in signal transduction [11,12]. Sequence alignments of several NorR orthologues with GAF domains from distantly related proteins indicate the presence of NorR-specific residues conserved in the GAF domains of NorR proteins, including a tyrosine and a cysteine residue (Tyr\(^{95}\) and Cys\(^{112}\) in R. eutropha NorR).

The isolated N-terminal domain of R. eutropha NorR is susceptible to modification by RNS in vitro. Tyr\(^{95}\) can be nitrated by peroxynitrite. S-nitrosation can be detected after the addition of cysteine-NO and SNP. Nonetheless, NorR carrying a Tyr\(^{95}\)Leu exchange is still capable of activating the norA promoter in response to SNP in vivo. In contrast, a Cys\(^{112}\)Ser exchange dropped promoter activation to background levels. Therefore we suggest that Cys\(^{112}\) plays an important role in signal sensing by NorR.

NorA is a negative modulator of signal sensing by NorR

NorA of R. eutropha is highly similar to ScdA from Staphylococcus aureus, NipC from Salmonella enterica and DmrN from Pseudomonas stutzeri. Furthermore, genomes of several proteobacteria and some firmicutes contain orthogonal genes for NorA. A ScdA mutant is affected in cell division [13], whereas a NipC mutant increased the pathogenicity of S. enterica in infected mice [14]. Both nipC and norA are up-regulated in response to RNS in S. enterica [14] and R. eutropha respectively suggesting a role for the gene product in NO metabolism. A NorA mutant of R. eutropha is neither affected in aerobic growth nor in growth by denitrification. However, norA promoter activation by SNP is increased 3-fold in the absence of NorA. This effect does not occur with a constitutively activating NorR’ lacking the N-terminal domain. These data indicate that NorA acts as a negative modulator of signal sensing by NorR, possibly by interaction with RNS. In fact, NorA contains one non-haem iron atom per molecule and can reduce nitrite to NO. This activity ceases after a few turnover cycles, suggesting that either the protein is not designed for turnover of nitrite or nitrite is not the in vivo substrate.

References
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