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The primary structure of cytochrome P460 of *Nitrosomonas europaea*: presence of a *c*-heme binding motif

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Abstract Cytochrome P460 and hydroxyamine oxidoreductase of *Nitrosomonas europaea* both catalyze the oxidation of hydroxylamine and contain a 460 nm-absorbing chromophore. The gene (*cyp*) encoding cytochrome P460 was cloned and sequenced. The predicted amino acid sequence contains a single *c*-heme binding motif (CXXCH) near the carboxy-terminus. Cytochrome P460 shows little sequence homology to other *c*-cytochromes including hydroxyamine oxidoreductase. The presence of a signal peptide and a possible *c*-heme binding site suggest that the cytochrome P460 of *N. europaea* is periplasmic.

Key words: Ammonia oxidation; *cyp*; Cytochrome P460; Hydroxylamine oxidation; *Nitrosomonas europaea*

1. Introduction

Nitrosomonas europaea, an autotrophic ammonia-oxidizing bacterium, contains two cytochromes which catalyze the oxidation of hydroxylamine to nitrite; the abundant hydroxylamine oxidoreductase (HAO) [10] which is periplasmic [4], and the much less abundant enzyme, cytochrome P460 [5]. Both cytochromes contain an unusual chromophore, heme P460 [5,10,19], with a ferrous absorbancy maximum at 463 nm.

HAO is an oligomer of 63 kDa subunits, each containing seven *c*-hemes and heme P460 [3]. The heme P460, which occupies the active site of HAO [8], resembles a *c*-heme in that a protoheme IX is bound by thioether linkages to two cysteine residues of the polypeptide, but has an additional novel covalent linkage between a tyrosyl residue of the polypeptide and a meso carbon of the heme [3]. Cys^{229} and Cys^{232} and Tyr^{467} are involved [21].

Cytochrome P460, a trimer of identical 18 kDa subunits, contains the P460 chromophore but no *c*-hemes [15, 17]. The structure of the P460 of cytochrome P460 is not known but may be similar to that of the P460 of HAO. They have similar optical spectra in the reduced state and the pyridine ferrohemochrome [5] and similar resonance Raman [1] spectra. Mossbauer spectra of the two chromophores are unique and, by analogy with model compounds, suggest a strongly anionic axial ligand [2,16]. Like P460 of HAO, the chromophore of cytochrome P460 is not extracted by acid acetone [5], Ag_2SO_4 or *o*-nitrophenylsulfenyl chloride [17], suggesting the existence of a covalent bond between the heme and the polypeptide in addition to any possible thioether linkages.

The genes for cytochrome P460 and HAO of *N. europaea* are separate [14], hence the former is not a proteolytic fragment of the latter. The genes for P460 and HAO are present in one and three copies, respectively. It is not known whether the two proteins share significant sequence, a reasonable possibility in light of their spectral and catalytic similarities.

A protein similar to P460 of *N. europaea* has recently been found in the methane oxidizing bacterium, *Methylococcus capsulatus* Bath, where it is thought to oxidize hydroxylamine produced from ammonia by methane monoxygenase [22]. Although it has spectral and enzymatic properties similar to cytochrome P460 of *N. europaea*, only three of the 10 amino-terminal amino acid residues are the same.

Here we report that the amino acid sequence of cytochrome P460 of *N. europaea* contains a *c*-heme binding motif, but otherwise shares no similarities with the sequence of HAO of *N. europaea*.

2. Materials and methods

A degenerate oligonucleotide, 5′-GGI-GTI-GCI-GA(A/G)-TT(T/C)-AA(C/T)-GA(A/G)-AA(A/G)-GA(C/T)-CT-3′, was synthesized based on the N-terminal amino acid sequence of cytochrome P-460 reported by McTavish et al. [13]. Restriction endonucleases were used as directed by the manufacturers (Life Technologies, Gaithersburg, Maryland USA and Promega Corporation, Madison, Wisconsin, USA). The oligonucleotide probe was 5′ end labeled with [γ-^{32}P]ATP with T-4 polynucleotide kinase [20] and longer probes were made using the Prme-A-Gene kit for random hexamer priming method (Promega Corporation). Agarose gel electrophoresis, bacterial colony lifts, and Southern blots were performed using standard procedures [20]. Hybridization and washing of Southern blots were done as described previously [13]. Double-stranded plasmid DNA was sequenced by the dideoxy-chain termination method with the Sequenase Version 2.0 kit (United States Biochemicals, Cleaveland, OH, USA).

3. Results and discussion

3.1. *Cloning* cyp, *the gene for cytochrome P460*

In Southern blots of genomic DNA of *N. europaea*, 3.1 kb *Kpn*I and 7.8 kb *Bam*HI fragments hybrized to the degenerate oligonucleotide probe [14]. A size-fractionated genomic library of *Kpn*I-digested fragments was created in the plasmid vector pUC119. Twenty-seven of the approximately 2000 colonies containing the library (*E. coli* strain DH5α MCR, Life Technologies, Inc. Gaithersburg, Maryland, USA) hybridized to the probe. Sequencing plasmid DNA from one of the clones revealed a 3.1 kb *Kpn*I fragment coding for the 5′ part of the gene

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The nucleotide sequence reported in this paper has been deposited in the Genbank database under Accession number U15305.

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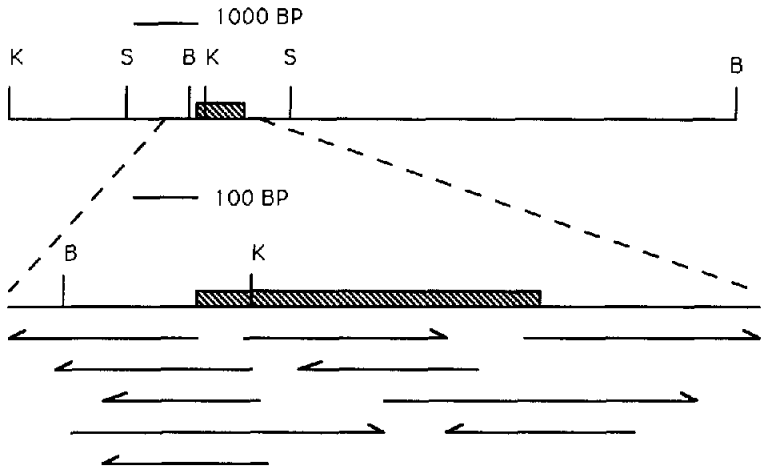

Fig. 1. Diagram showing the regions of the *N. europaea* chromosome cloned and sequenced. The *cyp* gene is indicated by crosshatches. *Bam*HI (B), *Kpn*I (K), and *Sma*I (S) restriction sites are indicated, and regions sequenced are shown by arrows.

for cytochrome P460 (which we designate as *cyp*) including a region encoding the 18 amino-terminal residues of the mature cytochrome (Figs. 1 and 2).

A 286 bp *Kpn*I–*Bam*HI fragment of this clone was then used as a probe to screen a size-fractionated library of *Bam*HI-digested genomic fragments in pUC119. Five of the 1,100 colonies screened hybridized with the probe. One clone contained the entire cytochrome P460 coding region (Figs. 1 and 2).

3.2. *Nucleotide sequence of* cyp

The open reading frame (ORF) encoding cytochrome P460 begins at base 131 and ends at a stop codon (TAA) at base 725 (Fig. 1). A probable ribosome binding site (AGGAG) is at bases 120–124. The first 26 amino acids of the predicted polypeptide probably comprise a signal peptide for export, suggesting that this soluble protein is periplasmic [18]. The amino-terminus of the mature cytochrome [14] is alanine 27.

The mature polypeptide is predicted to have a molecular mass of 18,850 (without heme) and a pI of 5.1. In keeping with the soluble nature of cytochrome P460, a hydrophobicity plot [11] (not shown) indicated that the mature polypeptide contains no extensive hydrophobic regions.

A sequence, CAACH, containing the motif (CXXCH) common to all *c*-heme binding sites, is present near the carboxy-terminus of the polypeptide (Fig. 1). Hence the heme may be attached by two thioether linkages to substituents of the pyrroles and by one histidine axial ligation to the iron. Since the heme remains attached to the protein following treatments which would have cleaved the putative thioether linkages [17] an additional covalent attachment is probably present, possibly between one of the eight tyrosine residues and the heme of cytochrome P460 as seen with P460 of HAO [3]. This putative linkage may account for the similar spectral properties of the P460 chromophores of cytochrome P460 and HAO. The presence of the two putative cysteine thioether heme linkages suggests a periplasmic location since all known soluble bacterial *c*-cytochromes are periplasmic [6].

3.3. *Similarity to other cytochromes*

Search of the Swissprot and combined GenEMBL data bases

*Bam*HI

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  1 GGATCCGGGCGCCGCACTGCAAGCCTGTTTACGACAAACCTGACGTAAGTGAGCTGCACCATCAGTCGGGTAACTATCTCCCCGACCACGATATAACAAT

101 AATGCTTCAATCAACCAAAAGGAGACCTTTATGAACTTCAGGAAACAACTGACAGGCGGTTTGAGCAGCCTGATTCTTTCAGCAGTCATGTCCGGCAGCC
                              M  N  F  R  K  Q  L  T  G  G  L  S  S  L  I  L  S  A  V  M  S  G  S  L
                                                                                          KpnI

201 TGCTGGCAGCAGGTGTGGCAGAGTTTAACGACAAGGGAGAACTGCTGCTGCCGAAAAATTACCGTGAATGGGTGATGGTCGGTACCCAGGTAACACCTAA
      L  A  A  G  V  A  E  F  N  D  K  G  E  L  L  L  P  K  N  Y  R  E  W  V  M  V  G  T  Q  V  T  P  N

301 CGAATTGAACGATGGCAAAGCACCCTTCACCGAAATCAGAACAGTCTATGTCGACCCGGAAAGCTATGCTCACTGGAAAAAAACCGGTGAATTCAGAGAT
      E  L  N  D  G  K  A  P  F  T  E  I  R  T  V  Y  V  D  P  E  S  Y  A  H  W  K  K  T  G  E  F  R  D

401 GGGACGGTAACTGTAAAAGAACTGGTCAGCGTCGGTGACCGTAAAGGCCCAGGCAGTGGAAACGGCTACTTCATGGGTGACTATATCGGGCTCGAGGCAT
    G  T  V  T  V  K  E  L  V  S  V  G  D  R  K  G  P  G  S  G  N  G  Y  F  M  G  D  Y  I  G  L  E  A  S

501 CGGTCAAGGATTCACAACGCTTTGCAAACGAGCCGGGAAACTGGGCTTTTTATATTTTCTATGTTCCTGACACGCCGCTGGTTGCTGCAGCCAAAAATCT
      V  K  D  S  Q  R  F  A  N  E  P  G  N  W  A  F  Y  I  F  Y  V  P  D  T  P  L  V  A  A  A  K  N  L

601 GCCCACAGCGGAATGTGCTGCCTGCCACAAGGAAAATGCGAAGACAGACATGGTCTTCACACAATTTTATCCTGTGTTACGTGCCGCCAAAGCAACCGGT
      P  T  A  E  C  A  A  C  H  K  E  N  A  K  T  D  M  V  F  T  Q  F  Y  P  V  L  R  A  A  K  A  T  G

701 GAATCCGGTGTAGTCGCGCCCAAGTAAATAACAGATACAAATTTGGCAAGCAGCAGGAGTATTGCGGTAACCCGATAGTTGCGTTACCGCAGCGAAATAC
    E  S  G  V  V  A  P  K  Stop

801 TCCTGTTTTTTCTCCTCGTTTTCCCCGCAGCTACGGCCAGTTACAGCCTGGTGATAAAATGCTGGCACTATGGCCAACATTACCATCCGCGAATTTCCTG
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Fig. 2. The nucleotide sequence of the *cyp* gene of *Nitrosomonas europaea*. *Bam*HI and *Kpn*I restriction sites are indicated.

with the FastA and TFastA programs, respectively [6] revealed no significant homology between the cytochrome P460 of *N. europaea* and other proteins. Significantly, although cytochrome P460 and HAO of *N. europaea* have similar chromophores and catalyze the unique oxidation of hydroxylamine, no sizeable regions of local homology were detected in the amino acid sequences [21] using the Bestfit algorithm [6].

The presence of the P460 chromophore and unique primary structure indicate that cytochrome P460 of *N. europaea* represents a new class of cytochromes, which may also include the cytochrome P460 of *M. capsulatus* [22] .

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