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Research Letters

The structural gene for cytochrome c_{551} from *Pseudomonas aeruginosa*

The nucleotide sequence shows a location downstream of the nitrite reductase gene

Margareta Nordling*, Simon Young*, B. Göran Karlsson* and Lennart G. Lundberg*,°

*Department of Biochemistry and Biophysics, University of Göteborg and Chalmers University of Technology, S-412 96 Göteborg and °Department of Biochemistry, Cardiovascular Research, AB Hässle, S-431 83 Mölndal, Sweden

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The gene coding for *Pseudomonas aeruginosa* cytochrome c_{551} has been cloned and its nucleotide sequence determined. Cytochrome c_{551} is expressed as a 104 amino acid pre-protein from which a signal peptide of 22 amino acids is cleaved off during the translocation across the cytoplasmic membrane. The gene is located just downstream of the gene coding for nitrite reductase on the *Pseudomonas aeruginosa* chromosome, suggesting that these genes form an operon.

Cytochrome c_{551}; Nitrite reductase; Operon; (*Pseudomonas aeruginosa*)

1. INTRODUCTION

Cytochrome c_{551} is a small protein with a molecular mass of 8685 Da (unmodified chain). It contains a type *c* heme covalently attached to the protein via Cys-12 and Cys-15, whereas His-16 together with Met-61 are the axial ligands of the heme iron [1]. Cytochrome c_{551} is located in the periplasmic space of *Pseudomonas aeruginosa* [2], and delivers electrons to nitrite reductase (EC 1.9.3.2) either directly or via azurin [2,3]. These 3 proteins in the *Pseudomonas* respiratory chain are located in the periplasmic space [2]; this is also confirmed by the nucleotide sequence in the 3 genes, showing the existence of a signal peptide [4,5].

The proteins in this electron transfer process are well characterized [6–9]. The 3-dimensional structure of azurin [10] and cytochrome c_{551} [1] from *Pseudomonas aeruginosa* have been determined by X-ray crystallography. For electron transfer studies these 3 proteins are often used as a model system. The reaction between cytochrome c_{551} and azurin has been thoroughly studied [11,12] and recently site directed mutagenesis of the azurin gene has been used to further elucidate the electron transfer pathway [13]. To fully understand the electron transport in the system, it will be necessary to obtain mutant proteins of cytochrome c_{551} as well. For that purpose we have cloned and sequenced the structural gene for cytochrome c_{551}.

Correspondence address: L.G. Lundberg, Department of Biochemistry, Cardiovascular Research, AB Hässle, S-431 83 Mölndal, Sweden

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

Pseudomonas aeruginosa strain ATCC 10145 was obtained from the Deutsche Sammlung von Mikroorganismen. *Escherichia coli* K 12 strain TG1 [14] was used as a host for construction of the library. Plasmid pUC19 [15] was obtained from Boehringer Mannheim GmbH, FRG.

2.2. *Oligonucleotides*

All oligonucleotides used are listed in table 1. They were purchased from Symbicom, Umeå, Sweden.

2.3. *DNA techniques*

Pseudomonas aeruginosa chromosomal DNA was isolated from the cell paste of a 100 ml culture (LB-medium) according to Marmur [16].

A *Pseudomonas aeruginosa* DNA library from *Sal*I digested chromosomal DNA was constructed in plasmid pUC19 according to Arvidsson et al. [4].

The library was screened by colony hybridization using oligonucleotide no. 1 labeled with ^{32}P. Hybridization conditions were 6 × SSC, 65°C [17]. The positive insert was subcloned into pUC19. DNA sequencing was carried out according to Sanger et al. [18] with M13 universal primer and oligonucleotides nos. 1, 2 and 3 (table 1). Due to the very high GC contents (70%) of *Pseudomonas* DNA, dITP was sometimes used to resolve compressions.

3. RESULTS

Oligonucleotide no. 1 was derived from the known amino acid sequence complementary to amino acids nos. 12–20. It was used for Southern blotting analysis and for screening of the *Pseudomonas aeruginosa* DNA library. Southern hybridization performed on *Sal*I digested chromosomal DNA with this oligonucleotide

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Table 1

Oligonucleotides used in screening the *Pseudomonas* DNA library and for nucleotide sequencing of the cloned insert

No. 1	**ACC ATC TTG GTG TCG ATG GCG TGG CA**
No. 2	**CGT ACG CAC TGC TTT CGC TGC TCC G**
No. 3	**CTG AAG CTC AAG GCC GTG GTC AAG**
No. 4	**CTT GAC CAC GGC CTT GAG CTT CAG**

Oligonucleotide no. 1 was used to screen the library and to select for the positive clone in the subcloning experiments. The codon usage chosen for this was based on the codon usage frequency of azurin from *Pseudomonas aeruginosa* [4]. All the oligonucleotides were used for sequencing the insert

showed a positive 1400 bp fragment. This fragment was cloned into plasmid pUC19. One positive clone was found out of 700 colonies. The positive 1400 bp fragment was further subcloned into bacteriophage M13mp18/19 for sequencing.

The nucleotide sequence shows two open reading frames (fig.1). The first is the C-terminal of nitrite reductase [5] and the second is cytochrome c_{551}. Analysis of the nucleotide sequence shows that cytochrome c_{551} is expressed as a 104 amino acid pre-protein. The mature protein, however, consists of only

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   GTCGACACCACCTTCAACCCCGACGCCAGGATCAGCCAGAGCGTCGCGGTGTTCGACCTG
 1 ---------+---------+---------+---------+---------+---------+ 60
   ValAspThrThrPheAsnProAspAlaArgIleSerGlnSerValAlaValPheAspLeu

   AAGAACCTCGACGCCAAGTACCAGGTGCTGCCGATCGCCGAATGGGCCGATCTCGGCGAA
61 ---------+---------+---------+---------+---------+---------+ 120
   LysAsnLeuAspAlaLysTyrGlnValLeuProIleAlaGluTrpAlaAspLeuGlyGlu

   GGCGCCAAGCGGGTGGTGCAGCCCGAGTACAACAAGCGCGGCGATGAAGTCTGGTTCTCG
121 ---------+---------+---------+---------+---------+---------+ 180
   GlyAlaLysArgValValGlnProGluTyrAsnLysArgGlyAspGluValTrpPheSer

   GTGTGGAACGGCAAGAACGACAGCTCCGCGCTGGTGGTGGTGGACGACAAGACCCTGAAG
181 ---------+---------+---------+---------+---------+---------+ 240
   ValTrpAsnGlyLysAsnAspSerSerAlaLeuValValValAspAspLysThrLeuLys

   CTCAAGGCCGTGGTCAAGGACCCGCGGCTGATCACCCCGACCGGTAAGTTCAACGTCTAC
241 ---------+---------+---------+---------+---------+---------+ 300
   LeuLysAlaValValLysAspProArgLeuIleThrProThrGlyLysPheAsnValTyr

   AACACCCAGCACGACGTGTACTGAGACCCGCGTGCGGGGCACGCCCCGCACGCTCCCCCC
301 ---------+---------+---------+---------+---------+---------+ 360
   AsnThrGlnHisAspValTyrEND

   TACGAGGAACCGTGATGAAACCGTACGCACTGCTTTCGCTGCTCGCCACCGGCACCCTGC
361 ---------+---------+---------+---------+---------+---------+ 420
                  MetLysProTyrAlaLeuLeuSerLeuLeuAlaThrGlyThrLeuLeu
                 -22
   TCGCCCAGGGCGCCTGGGCCGAAGACCCCGAAGTGCTGTTCAAGAACAAGGGCTGTGTGG
421 ---------+---------+---------+---------+---------+---------+ 480
     AlaGlnGlyAlaTrpAlaGluAspProGluValLeuPheLysAsnLysGlyCysValAla
                      +1
   CCTGCCATGCCATCGACACCAAGATGGTCGGCCCGGCCTACAAGGACGTCGCCGCCAAGT
481 ---------+---------+---------+---------+---------+---------+ 540
     CysHisAlaIleAspThrLysMetValGlyProAlaTyrLysAspValAlaAlaLysPhe

   TCGCCGGCCAGGCCGGCGCGGAAGCGGAACTCGCGCAGCGGATCAAGAACGGCAGCCAGG
541 ---------+---------+---------+---------+---------+---------+ 600
     AlaGlyGlnAlaGlyAlaGluAlaGluLeuAlaGlnArgIleLysAsnGlySerGlnGly

   GCGTCTGGGGCCCGATCCCGATGCCGCCGAACGCGGTCAGCGACGACGAGGCGCAGACCC
601 ---------+---------+---------+---------+---------+---------+ 660
     ValTrpGlyProIleProMetProProAsnAlaValSerAspAspGluAlaGlnThrLeu

   TGGCGAAGTGGGTCCTGTCGCAGAAATGAACGCCCTCCGGACTTCCGGCGCGCCGCAGCC
661 ---------+---------+---------+---------+---------+---------+ 720
     AlaLysTrpValLeuSerGlnLysEND
```

Fig.1. Nucleotide sequence and the amino acid sequence of the two open reading frames found in the cloned insert. The sequence is written in the 5′ to 3′ direction of the coding strand. The first open reading frame corresponds to the C-terminal part of the *Pseudomonas aeruginosa* nitrite reductase [5]. The second open reading frame corresponds to the cytochrome c_{551} structural gene. The putative ribosome binding site is underlined by a double line, the heme binding amino acids are underlined by a continuous line and the ligands of the heme iron are underlined by a dashed line.

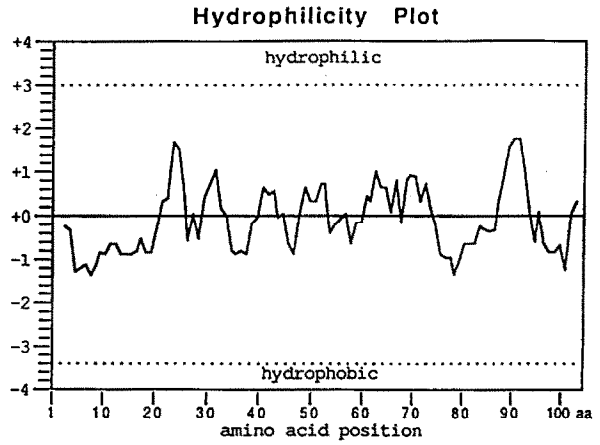

Fig.2. Hydrophilicity plot of *Pseudomonas aeruginosa* cytochrome c_{551} pre-protein. Calculations were made by the method described by Hopp and Woods [22], using the DNA analysis package; DNA Inspector IIe from Textco, NH.

82 amino acids [19] and is found in the periplasmic space. The N-terminal region of the pre-protein shows the significant features of a signal peptide [20,21]: (i) a positive net N-terminal charge (Lys at position −21); (ii) a hydrophobic core (the region −6 to −18, fig.2); (iii) a secondary structure disrupting residue at position −4 (Gly); (iv) an Ala-X-Ala sequence preceding the cleavage site. This results in a mature protein starting with Glu, which is in agreement with the result of Ambler [19].

4. DISCUSSION

The translational start of cytochrome c_{551} begins only 49 nucleotides downstream of the stop codon of nitrite reductase (fig.1). The genes are separated by a structure suggested to be a transcriptional terminator. Since no promoter region can be defined in this region, the proteins are probably transcribed on the same mRNA. This is in accordance with the findings of Wood [2] who reports an almost 1:1 stoichiometry between cytochrome c_{551} and the nitrite reductase monomer in *Pseudomonas aeruginosa* grown under anaerobic conditions. The transcriptional terminator suggested by Silvestrini [5] may then act as a transcriptional pause signal. Cloning the promoter region of the operon will give valuable information about the regulation.

Horio [3] reports that nitrite reductase cannot be found in *Pseudomonas aeruginosa* grown under aerobic conditions. He further suggests that both azurin and cytochrome c_{551} receive electrons from cytochrome b_{560} and different type *c* cytochromes before delivering them to nitrite reductase. It has also been suggested that azurin and cytochrome c_{551} may substitute for each other [2] in receiving electrons from cytochrome c_1 and delivering electrons to nitrite reductase. On the other hand, Martinkus et al. [23], referring to kinetic data

and cellular concentration measurements of azurin from *Pseudomonas denitrificans*, conclude that azurin cannot be the major physiological electron donor to nitrite reductase.

Azurin is not transcribed on the same messenger as cytochrome c_{551} and nitrite reductase, but on a monocistronic mRNA [4], with two promoter regions, one constitutive and one inducible under denitrifying conditions. This gives rise to a question concerning the true biological role of azurin.

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