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A fourth subunit is present in cytochrome *c* oxidase from the thermophilic bacterium PS3

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A new putative subunit was found in cytochrome *c* oxidase (aa_3-type) of the thermophilic bacterium PS3. The N-terminal amino acid sequence of this ~12 kDa protein coincides with the deduced sequence of an open reading frame found downstream from the gene encoding subunit I of the PS3 cytochrome oxidase [(1988) J. Biochem. 103, 606–610]. This small hydrophobic protein, composed of 109 amino acid residues after the initial methionine residue has been processed, shows homology with the subunit IV (*cyoD* product) of cytochrome *bo*-type quinol oxidase of *Escherichia coli*.

Cytochrome aa_3; Cytochrome *c* oxidase; DNA sequence; Subunit structure; (Thermophilic bacterium PS3)

1. INTRODUCTION

Bacterial cytochrome aa_3-type terminal oxidase is structurally and functionally similar to the mitochondrial enzyme, although its subunit structure is much simpler. For example, the enzyme from the thermophilic *Bacillus* PS3 contains cytochrome aa_3 and Cu_A and Cu_B as prosthetic groups and shows proton pumping activity in addition to transmembrane electron transfer from cytochrome *c* to O_2 [1,2]. The enzyme was reported to have 3 subunits that correspond to the 3 largest subunits of its mitochondrial counterpart [1–3]. Similar aa_3-type oxidases with 3 subunits have been purified from *Bacillus subtilis* [4]. *Bacillus stearothermophilus* [5], *Bacillus firmus* [6] and *Paracoccus denitrificans* [7]. As the original preparation of the *Paracoccus enzyme* consists of two subunits [8,9], the reported two-subunit aa_3-type oxidases of bacteria (see [10,11] for reviews) may have lost the third subunit.

Instead of aa_3-type oxidase, *Escherichia coli* contains a cytochrome *bo*-type oxidase, in which the chromophores are two protohemes. This enzyme is reported to have 2 [12] or 4 subunits [13]. Recently Au and Gennis [14] cloned of *cyo* operon encoding the subunits of cytochrome *bo*. The deduced amino acid sequences show striking similarity to the 3 largest subunits of the mitochondrial [15,16] and the *P. denitrificans* cytochrome aa_3s [17] and subunit I of the PS3 enzyme [18] (see [19]).

It seemed necessary to examine whether PS3 cytochrome oxidase has additional subunits, before concluding that bacterial aa_3-type oxidases are always composed of 3 subunits homologous to the 3 core subunits of the mitochondrial enzyme.

Here we report the presence of a fourth putative subunit in the preparations of the PS3 aa_3-type oxidase. The gene for this subunit is downstream from the gene encoding subunit I in the same operon.

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

Cytochrome *c* oxidase (aa_3-type) was prepared from the thermophilic bacterium PS3 cultured with vigorous aeration as described previously [1,20]. Peptide sequences were determined by Edman degradation using an Applied Biosystems' model 470A gas-phase sequencer.

The methods for preparation of a DNA library of PS3, cloning of the gene with synthetic oligonucleotide probes and sequencing DNA were the same as described previously [18]. Clone 2-1, which contains PS3 genes encoding both subunits I and II (to be published elsewhere), was used for DNA sequencing. A small *Eco*RI fragment (235 bp) and the adjacent *Eco*RI-*Hae*III fragments (cf. fig.3) were used for sequencing after subcloning into M13 mp18 and mp19. The sequence data were analyzed with a software program (Genentyx, Tokyo) adapted for an NEC PC9801 computer.

SDS-PAGE was carried out essentially by the method of Laemmli. Polyacrylamide concentration was 13% and 6 M urea was included in

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the gels [21], unless otherwise described. Durapore filter (GVHP) for blotting and peptide sequencing was purchased from Nippon Millipore Co. (Yamagata).

A G-3000SW column (0.75 × 60 cm) from Toso Co. (Tokyo) for high-performance gel-permeation chromatography was used in a Waters apparatus consisting of a pump 510 and a detector 490 for separation of the subunits of PS3 cytochrome oxidase.

3. RESULTS AND DISCUSSION

3.1. *Presence of a fourth subunit*

Fig.1 shows the typical SDS-PAGE pattern of PS3 cytochrome oxidase. Besides 3 larger subunits (I,II and III), a small polypeptide was seen slightly behind the dye front close to a marker protein, lysozyme, when the gel concentration was raised to 13% (fig.1). In previous experiments with 10% or 12% acrylamide gel this polypeptide seemed to move at the dye front (e.g. fig.3A, lane 5 in [22]). In the Weber-Osborn system with 7.5% acrylamide gel and 8 M urea this band was detected in the same position as equine cytochrome *c*, corresponding to about 12 kDa (fig.1B); the band is, however, faint and diffuse (see also fig.4, in [1].

Fig.2 shows the elution pattern on gel permeation chromatography in the presence of SDS. The shoulder in a region of very high molecular weight is due to undissociated or partially-dissociated PS3 oxidase, and the next four peaks (I–IV) are those of subunits I–IV, respectively. The material in the fifth peak in the region of low molecular weight shows absorption at about 420 nm and does not react with Folin reagent [24]. Thus the fifth peak is likely to be due to free heme a. The second peak shows absorption at 408 nm, indicating that it is subunit II which also has heme c [1,18].

These data suggest that PS3 cytochrome aa_3-type oxidase is probably composed of 4 subunits, not 3 as we formerly thought [1–3].

3.2. *N-Terminal peptide sequence of subunit IV*

The material in the peak fractions of subunits IV was pooled (bar in fig.2), and its amino acid sequence was determined from the N-terminus. The sequence was found to be NH_2-Ala-Asn-Gln-Thr-Asn-Ser-Gly-Asn-Glu-Arg-Val-Asp-Leu-Ala-Tyr-Arg-. This sequence was confirmed by direct sequencing of the spot blotted to Durapore paper after SDS-PAGE.

3.3. *DNA sequence*

The nucleotide sequence of the gene for subunit I of the PS3 cytochrome oxidase has been reported [18]. Analysis of the 3′-downstream region of this gene indicates the presence of genes for subunit III and subunit IV. Fig.3 shows the restriction map of this genomic region and DNA sequence of the gene coding for subunit IV and its deduced amino acid sequence. The open reading frame is preceded by a putative Shine-Dalgarno box (underlined). It can encode a small hydrophobic protein composed of 110 amino acid residues. Since the first residue of the subunit IV protein is alanine, the first methionine has been processed to form a polypeptide with 109 amino acid residues. It is also noteworthy that a terminator-like structure is present in the 3′-downstream region close to the stop

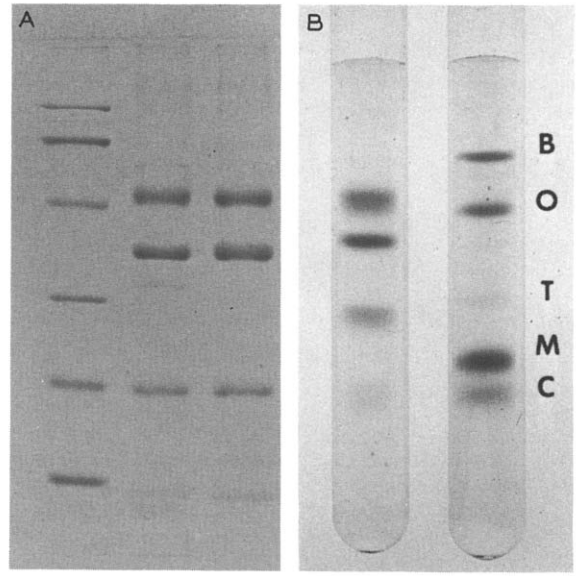

Fig.1. SDS-PAGE patterns of PS3 cytochrome oxidase. (A) Electrophoresis of two samples of the PS3 enzyme (10 μg each) in 13% acrylamide gel containing 6 M urea with the marker proteins phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme (left-hand side lane). (B) Electrophoresis of the PS3 enzyme (12 μg) using the method of Weber-Osborn [23] in 7.5% acrylamide gel containing 8 M urea with marker proteins. B, bovine serum albumin; O, ovalbumin; T, trypsin; M, myoglobin; C, equine cytochrome *c* (right-hand side lane).

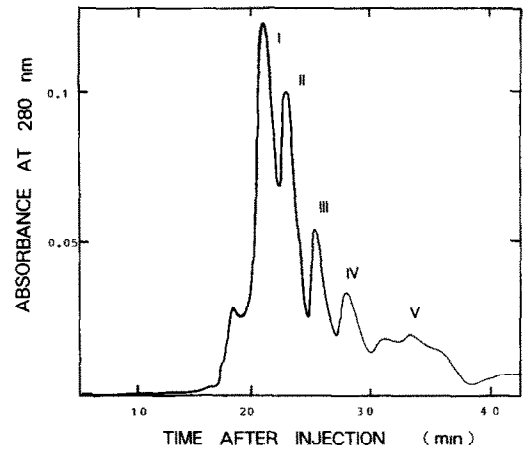

Fig.2. High-performance gel permeation chromatography of the PS3 enzyme in the presence of SDS. PS3 cytochrome oxidase (0.4 mg protein) denatured in 0.05 ml of 6% SDS was applied on a column of Toso G3000SW equilibrated with 0.1% SDS containing 0.1 M Na_2SO_4, 1 mM EDTA, 10 mM NaPi buffer (pH 7.0). The flow rate was 0.6 ml/min.

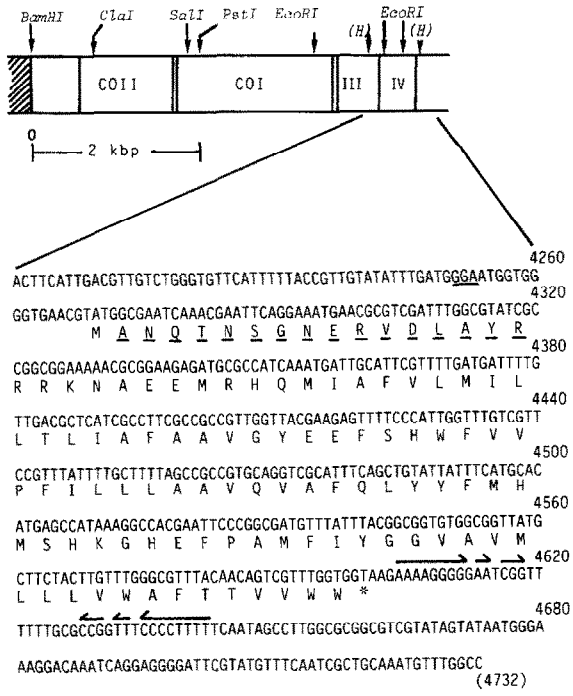

Fig.3. Map of the PS3 chromosome in the cytochrome oxidase locus, and the DNA and deduced amino acid sequence of the fourth subunit. A putative Shine-Dalgarno box (underlining) and terminator structure (arrows) are marked. Several restriction sites including the adjacent *Hae*III sites (H) are also shown.

codon as marked with arrows. Thus all the genes for subunits of the PS3 enzyme are in the same locus forming an operon. This is in contrast to the genomic organisation in *P. denitrificans*, in which the gene for subunit I and the genes for subunits II and III are separated in different operons [17].

3.4. *Comparison of the sequence*

Of the various bacterial cytochrome aa_3-type and *bo*-type oxidases hitherto studied, only *E. coli* cytochrome *bo*-type oxidase is known to have a fourth subunit [13]. Fig.4 shows Harr plots that compare subunit IV of the PS3 cytochrome aa_3-type oxidase with subunit IV of the *E. coli bo*-type oxidase (fig.4A) and the mitochondrial subunit IV (fig.4B). Clear homologous alignments can be seen with the *E. coli* subunit IV, but not with the mitochondrial subunit IV. Moreover the amphipathic mitochondrial subunit IV has a single hydrophobic segment, whereas the subunits IV of PS3 and *E. coli* have 3 hydrophobic segments (data not shown). Nevertheless, very recently we found that an antiserum against subunit IV of beef heart cytochrome cross-reacted with the PS3 subunit IV (S. Chan et al., to be published). No homology between the minor subunits of the mitochondrial cytochrome oxidase and subunit IV reported here has been found. Thus subunit IV of the PS3 aa_3-type oxidase seems most similar to the corresponding subunit in the *E. coli bo*-type oxidase. The characteristics of the PS3 cytochrome oxidase may be common to gram-positive bacteria, because usual preparations of the enzymes contain at least 3 subunits including a small subunit III [1,4–6], while the enzymes isolated from gram-negative purple bacteria and their relatives have usually been found to have two subunits [10,11].

The present finding of a fourth subunit in the PS3 cytochrome aa_3-type oxidase that is homologous to the subunit IV (*cyoD* product) of the *bo*-type oxidase provides a new clue to the origin and evolution of respiratory complexes in aerobic bacteria. The role of subunit IV in bacterial cytochrome oxidases (both aa_3-type and *bo*-type) requires further study.

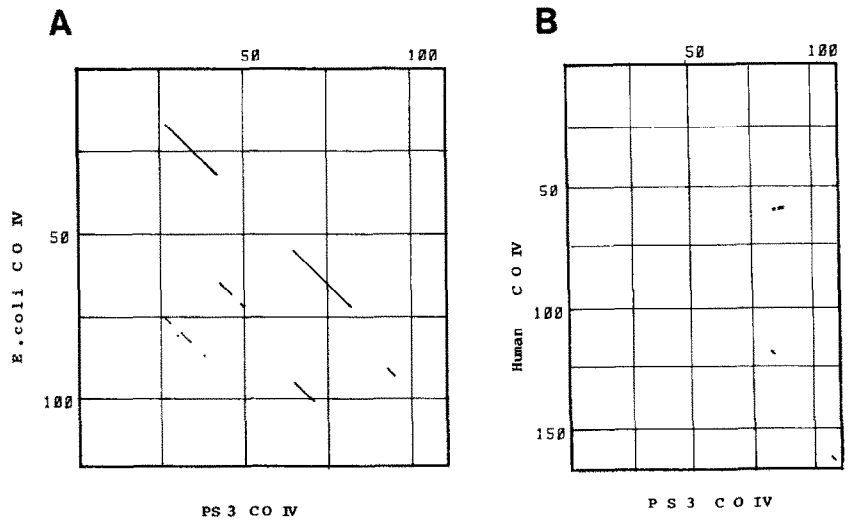

Fig.4. Comparisons of the PS3 cytochrome oxidase subunit IV with subunit IV of the *E. coli* cytochrome *bo*-type oxidase (A) and the human mitochondrial subunit IV (B). The latter sequences are taken from Chepuri et al. (submitted) and Zeviani et al. [25]. The plots show points with a score above 1.4 in a window of 22 residues.

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