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Molecular Cloning of the Cytochrome aa_3 Gene from the Archaeon (Archaebacterium) *Halobacterium halobium*

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SUMMARY: A novel aa_3-type cytochrome oxidase from the extremely halophilic archaeon, *Halobacterium halobium*, differs significantly from those of other prokaryotic and eukaryotic cytochrome oxidases (Fujiwara,T., Fukumori,Y., and Yamanaka,T. (1989) *J. Biochem.* **105**, 287-292). In the present study, we cloned and sequenced the gene which encodes the cytochrome aa_3 by using the polymerase chain reaction methods. The deduced amino acid sequence of subunit I of *H. halobium* cytochrome aa_3 was more similar to that of subunit I of the eukaryotic cytochrome (44%, maize mitochondria) than that of the cytochrome from other bacteria (36%, *Paracoccus denitrificans*). The consensus sequence in putative metal binding residues is well-conserved also in *H. halobium* cytochrome aa_3.

Cytochrome *c* oxidase (E.C. 1.9.3.1), also called cytochrome aa_3, plays a central role in the terminal process of the electron transport chain, which catalyzes the reduction of O_2 to water and proton translocation across mitochondrial inner or cytoplasmic membrane. In general,it contains four redox metal centers; two copper atoms, Cu_A and Cu_B, and two heme *a* groups, cytochrome *a* and cytochrome a_3 (1-7). The respiratory enzymes occur as integral membrane components. In eukaryote, cytochrome aa_3 is localized in the mitochondrial inner membrane, and comprises of 13 kinds of subunits; the major three subunits (I, II, and III) are encoded by miochondrial DNA, others by nuclear DNA. On the other hand, prokaryotic cytochrome aa_3 has much simpler subunit composition; its subunits correspond to the largest three subunits of the mitochondrial counterpart. These

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Abbreviations: PCR, polymerase chain reaction; PS3, thermophilic bacterium PS3.

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results suggest that prokaryotic cytochrome aa_3 is the minimal functional core of the mitochondrial enzyme .

Prokaryotic respiratory chains are usually branched and contain several terminal oxidases; under air-limited conditions, *o*-type cytochrome functions as an alternative terminal oxidase. Recent progress of DNA sequence analysis of the cytochrome oxidase genes from various organisms has revealed that the amino acid sequences of subunit I is well-conserved through cytochrome aa_3 and cytochrome *o*, they constitute one genetically-related family (8-17).

Recently, novel cytochrome aa_3 has been purified from the extremely halophile, *Halobacterium halobium* (18-20). *H. halobium* belongs to archaea (archaebacteria) and its evolutional status is very intriguing (21-23). The purified cytochrome aa_3 consists of only a single subunit whose *Mr* value has been estimated to be 40,000, and lacks Cu atoms. In this communication, we presented cloning of the gene which encodes *H. halobium* cytochrome aa_3 in order to compare directly its primary structure with those of the other oxidases. The deduced amino acid sequence of subunit I of this archaeal cytochrome aa_3 was more similar to the sequence of the eukaryotic mitochondrial oxidase than to those of the eubacterial oxidase. The consensus sequences in the metal-binding part of the oxidase are well-conserved also in *H. halobium* cytochrome aa_3, suggesting that the *H. halobium*, mitochondrial, and eubacterial oxidases have possibly been derived from a common ancestor.

Materials and Methods

Isolation of cytochrome aa_3 and peptide sequencing ---- Cytochrome aa_3 was purified from *H. halobium* as described in the previous paper (19). In order to determine the internal sequences, the purified cytochrome was digested with trypsin, and the peptides obtained analyzed by a gas phase protein sequencer (Applied Biosystems 470A, U.S.A.).

Cloning and sequencing of coxI gene ---- The genomic DNA was extracted from *H. halobium* cells (24). A polymerase chain reaction (PCR) was carried out to prepare a DNA fragment for screening a genomic DNA library of *H. halobium*. The 5' primer (TCTGGTTCT(T/A)CGG(C/G)CACCC) and the 3' primer (ACATGTGGTG(C/G)(C/G)CCCA(C/G)AC) were synthesized by a DNA synthesizer (Applied Biosystems 381A, U.S.A.) according to the amino acid sequence, FWFFGHP and VWAHHMF, respectively, which are conserved regions found in cytochrome oxidases from various organisms. The distance between the two primers was predicted to be around 36 amino acids. PCR reactions were carried out in 95°C, 1 min; 56°C, 2 min; 72°C, 1.5 min; 35 cycles. The amplified 120 base pair DNA fragment was purified, cloned into pUC118, and confirmed by sequencing. Radioactive 120bp fragment was used as a probe for screening the *H. halobium* gene library. Construction of the genomic library and screening by colony hybridization were carried out as describrd in (25). Nucleotide sequence analysis was conducted according to the dideoxy chain termination method. All

DNA sequences were confirmed by sequencing both strands.Computer analysis was performed with Genetyx (Software Development Co.).

Results and Discussion

The DNA probe for cloning the gene encoding cytochrome aa_3 of *H. halobium* was prepared by the PCR method, and a single DNA fragment coding for a part of subunit I of the cytochrome was obtained. After screening the *H. halobium* genomic library, a 9 kb-DNA fragment containing full-length of the subunit I gene was isolated.

The nucleotide sequence of subunit I of *H. halobium* cytochrome aa_3 and its deduced amino acid sequence are presented in Fig. 1. The gene encoding subunit I of cytochrome aa_3, deginated as *coxI*, contained 1,782 nucleotides corresponding to a protein with *Mr* of 65,220 comprising of 593 amino acid residues. This value is higher than *Mr* of the cytochrome estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. This

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                                                                  CGCCGACATTTCCGTGGACAATCCTAAGCTTTCTTTACCGTCGAATCCTGACTAGGAGGC
   1 ATGGCTACAGCAGCGTCATCGATTACACTCACTGTGCTGATGGGCGTGTTGCTCGTCGGCGTGGTCGCTGTCCTCGCGCGACTCGAGGACTGGCGGTCGTACACGCCCCTCAGCGACGTG
     M  A  T  A  A  S  S  I  T  L  T  V  L  M  G  V  L  L  V  G  V  V  A  V  L  A  R  L  E  D  W  R  S  Y  T  P  L  S  D  V

 121 GGCGGTGGGCTCGGGGAGCGCACCGGCTACACGCACGAGGAGAAGCCTGGGGGCATTATCCGCTGGTTCACGACGGTCGATCACAAGGACATCGGGATCCTGTACGGCGTCTACGGCACC
     G  G  G  L  G  E  R  T  G  Y  T  H  E  E  K  P  G  G  I  I  R  W  F  T  T  V  D  H  K  D  I  G  I  L  Y  G  V  Y  G  T

 241 ATCGCGTTCGCATGGGGCGGGGTGTCGGTGCTCCTGATGCGGACTGAGCTTGCGACGAGCAGTGAGACGCTGATCAGCCCGTCGCTGTACAACGGGCTGTTGACGAGTCACGGGATCACG
     I  A  F  A  W  G  G  V  S  V  L  L  M  R  T  E  L  A  T  S  S  E  T  L  I  S  P  S  L  Y  N  G  L  L  T  S  H  G  I  T   1

 361 ATGTTGTTCCTGTTCGGGACGCCGATGATCGCGGCCTTCGGGAACTACTTCATCCCGCTGTTGATCGACGCCGACGACATGGCGTTCCCGCGCATCAACGCGATCGCGTTCTGGCTGCTG
     M  L  F  L  F  G  T  P  M  I  A  A  F  G  N  Y  F  I  P  L  L  I  D  A  D  D  M  A  F  P  R  I  N  A  I  A  F  W  L  L   1

 481 CCGCCGGGCGCGATCCTCATCTGGTCCGGGTTCCTGATCCCGGGGATCGCCACCGCACAGACGTCGTGGACGATGTACACGCCGCTGTCGCTCCAGATGTCGAGTCCCGCGGTGGACATG
     P  P  G  A  I  L  I  W  S  G  F  L  I  P  G  I  A  T  A  Q  T  S  W  T  M  Y  T  P  L  S  L  Q  M  S  S  P  A  V  D  M   2

 601 ATGATGCTGGGGCTGCACCTCACCGGCGTGTCCGCGACGATGGGTGCGATCAACTTCATCGCGACAATCTTCACCGAGCGCGGTGAGGACGTCGGATGGCCGGATCTGGACATCTTCTCG
     M  M  L  G  L  H  L  T  G  V  S  A  T  M  G  A  I  N  F  I  A  T  I  F  T  E  R  G  E  D  V  G  W  P  D  L  D  I  F  S   2

 721 TGGACGATGCTCACCCAGTCCGGACTGATCCTGTTCGCGTTCCCGCTGTTCGGCAGCGCCCTCATCATGCTGCTGCTCGACCGGAACTTCGGAACGACGTTCTTCACGGTCGCCGGCGGG
     W  T  M  L  T  Q  S  G  L  I  L  F  A  F  P  L  F  G  S  A  L  I  M  L  L  L  D  R  N  F  G  T  T  F  F  T  V  A  G  G   2

 841 GATCCGATCTTCTGGCAGCACCTCTTCTGGTTCTTCGGCCATCCCGAAGTGTACGTGCTCGTGTTGCCGCCGATGGGGATCGTGTCGCTGATCCTGCCGAAGTTCTCGGGGCGGAAGCTG
     D  P  I  F  W  Q  H  L  F  W  F  F  G  H  P  E  V  Y  V  L  V  L  P  P  M  G  I  V  S  L  I  L  P  K  F  S  G  R  K  L   3

 961 TTCGGGTTCAAGTTCGTCGTCTACTCGACGCTGGCGATCGGCGTGCTGAGTTTCGGGGGTGTGGGCCCACCACATGTTCACGACGGGCATCGACCCGCGGATTCGGTCGAGTTTCATGGCG
     F  G  F  K  F  V  V  Y  S  T  L  A  I  G  V  L  S  F  G  V  W  A  H  H  M  F  T  T  G  I  D  P  R  I  R  S  S  F  M  A   3

1081 GTGTCGCTGGCGATTTCGATCCCGTCGGCGGTGAAGGTGTTCAACTGGATCACCACGATGTGGAACGGGAAACTGCGCCTGACCGCGCCGATGCTGTTCTGCATCGGGTTCGTGCAGAAC
     V  S  L  A  I  S  I  P  S  A  V  K  V  F  N  W  I  T  T  M  W  N  G  K  L  R  L  T  A  P  M  L  F  C  I  G  F  V  Q  N   4

1201 TTCATCATCGGCGGTGTGACCGGCGTGTTCCTCGCGGTCATTCCGATCGACCTCATCCTGCACGACACCTACTACGTGGTCGGTCACTTCCACTTCATCGTCTACGGTGCGATCGGGTTC
     F  I  I  G  G  V  T  G  V  F  L  A  V  I  P  I  D  L  I  L  H  D  T  Y  Y  V  V  G  H  F  H  F  I  V  Y  G  A  I  G  F   4

1321 GCGCTGTTCGCGGCGTCGTACTACTGGTTCCCGATGGTCACGGGCCGGATGTACCAGAAGCGCCTGGCGCACGCGCACTTCTGGACGGCGCTCGTCGGCTCCAACGCGACGTTCCTGGCG
     A  L  F  A  A  S  Y  Y  W  F  P  M  V  T  G  R  M  Y  Q  K  R  L  A  H  A  H  F  W  T  A  L  V  G  S  N  A  T  F  L  A   4

1441 ATGTTGTGGCTCGGCTACGGCGGGATGCCACGGCGGTACGCGACGTACATCCCGCAGTTCGCGACCGCACACCGGCTCGCAACAGTGGGGGCGTTCCTGATCGGGGTGTCGACGCTGATC
     M  L  W  L  G  Y  G  G  M  P  R  R  Y  A  T  Y  I  P  Q  F  A  T  A  H  R  L  A  T  V  G  A  F  L  I  G  V  S  T  L  I   5

1561 TGGCTGTTCAACATGGCGACCTCGTGGCGTGAGGGGCCACGCGTCGACTCCACTGACCCATGGGACCTAGAGGAGACCGACCAGTTCACCAACGACTGGGCGTGGTTCCGTGCGAAAGAG
     W  L  F  N  M  A  T  S  W  R  E  G  P  R  V  D  S  T  D  P  W  D  L  E  E  T  D  Q  F  T  N  D  W  A  W  F  R  A  K  E   5

1681 GAGACGACCGTGCTCCCCGACGGCGGTGACGAAGCGCAATCCGAGGCGGACGCGGTGACTGACGGCGGGCAGCCGGCCGCGGACAGCGACACCGAGTCGTAGCCGCCGTCAGCGACGTTT
     E  T  T  V  L  P  D  G  G  D  E  A  Q  S  E  A  D  A  V  T  D  G  G  Q  P  A  A  D  S  D  T  E  S  End                5
```

Fig.1. Nucleotide sequence of the gene encoding subunit I of *H. halobium* cytochrome aa_3 and deduced amino acid sequence. Independently determined peptide sequences by a gas-phase protein sequencer are *underlined*. Potential ribosome-binding sites are both *over-* and *underlined*. Amino acids are numbered on the *right* and nucleotides on the *left*.

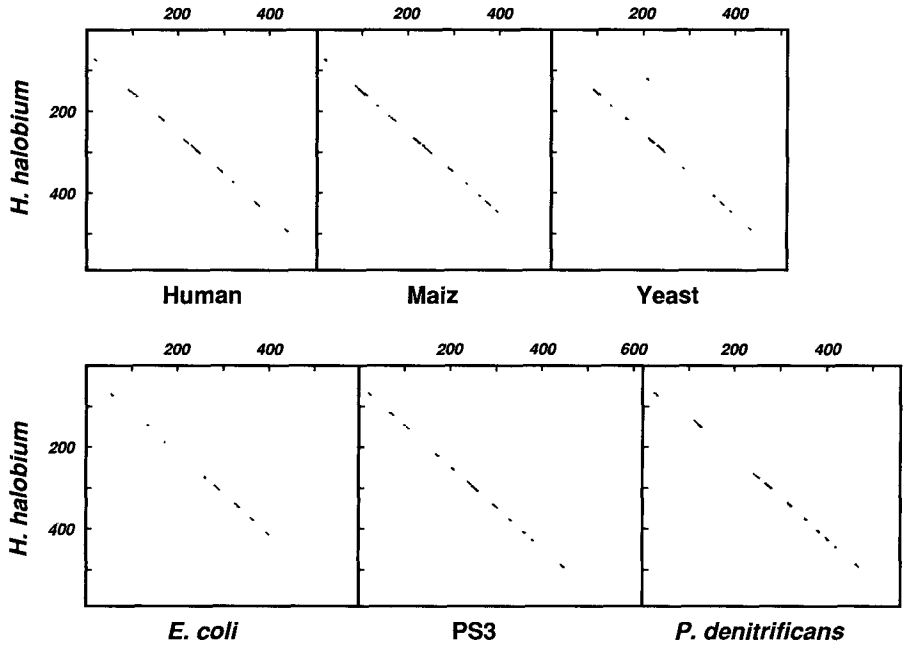

Fig.2. Comparison of the amino acid sequence of subunit I of *H. halobium* cytochrome aa_3 with the sequences of the cytochromes from several organisms using a Diagon plot (26). The comparison used an 11-residue segment, and a dot was generated if the number of identical amino acid residues extended for more than 7. Sequences for cytochrome aa_3 from human, maize, yeast, *Escherichia coli,* thermophilic bacterium PS3, and *Paracoccus denitrificans* were obtained from Anderson *et al.* (34), Isaac *et al.* (35), Bonitz *et al.* (36), Chepuri *et al.* (13), Ishizuka *et al.* (14), and Raitio *et al.* (15), respectively.

discrepancy may be caused by the hydrophobic property of this polypeptide. The identity of this coding frame to the gene for *H. halobium* cytochrome aa_3 was confirmed by comparing the deduced amino acid sequence with the partial amino acid sequence of the cytochrome (Fig. 1, underlined).The AGGAGG sequence located one base upstream of ATG, is complementary to that near the 3' terminus of 16 S rRNA of this organism (5' GAUCACCUCCU 3'). It may thus possibly be a ribosome binding site.The GC content of the *coxI* gene is 65 %, which is consistent with those reported for other *H. halobium* genes.

Fig.2 shows the amino acid sequence similarities of subunit I between *H. halobium* and various organisms by a Diagon plot (26). It is generally accepted that the primary structure of subunit I of cytochrome aa_3 has been highly conserved in eukaryotes and prokaryotes (1, 4, 5, 7). The sequence of subunit I of the *H. halobium* cytochrome is also homologous to those of the human (42%), maize (44%), yeast (41%) *Paracoccus denitrificans* (36%), (thermophilic bacterium) PS3

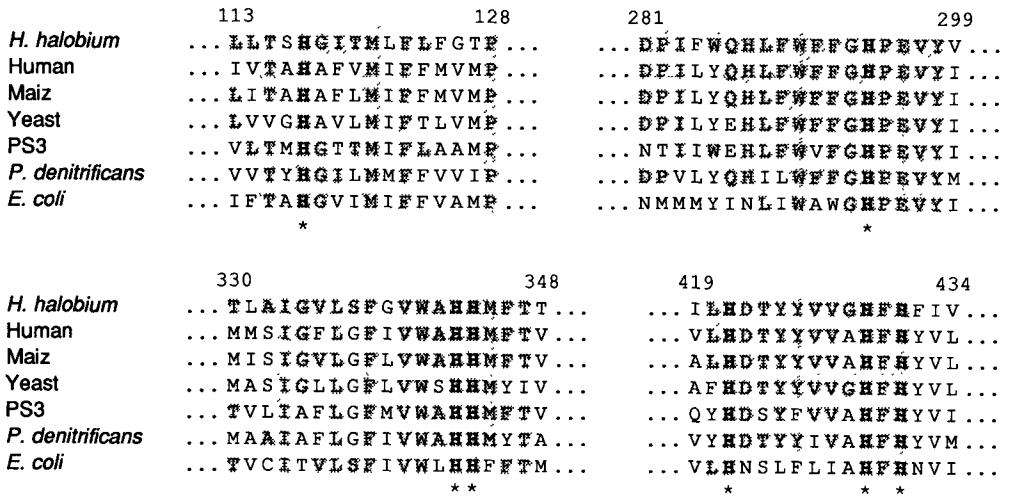

Fig.3. Alignment of sequences containing putative metal-binding residues. *Shadowed sequences* are identical. The asterisk (*) indicates a conserved histidine residue. The numbers refer to subunit I of *H. halobium* cytochrome aa_3 sequence.

(41%), and *Escherichia coli* (36%) cytochromes. Moreover, it is worth notice that the primary structure of subunit I of *H. halobium* cytochrome aa_3 is more similar to those of the cytochromes from higher plants than to those of cytochromes from other organisms. Such relationships of archaea with higher plants are also reported for other biochemical data of archaea (27, 28).

It has been shown that consensus sequences concerning the metal binding part seems to occur in many proteins of diverse origins. For the four redox centers in the cytochrome aa_3 molecule, 8 His and 2 Cys residues are required. Holm *et al.* have reported that 6 His residues in subunit I of cytochrome aa_3 are binding sites of heme *a*, heme a_3, and Cu_B (29). As shown in Fig. 3, subunit I of *H. halobium* cytochrome aa_3 possesses these consensus sequences; subunit I of *H. halobium* cytochrome aa_3 thus possibly functions as putative cytochrome *a*, cytochrome a_3, and Cu_B-binding subunit. Cu_B has not been found in our purified cytochrome aa_3. It might have been lost from the cytochrome preparation during purification. Sequence alignments of cytochrome aa_3 from various organisms show that subunit I of cytochrome oxidase contains 7 conserved His residues (Fig. 3), while subunit II of the oxidase, which is thought to have the cytochrome *c* binding site where Cu_A binding site is located, has 2 conserved His and 2 conserved Cys residues. Since attempts to isolate subunit II of *H. halobium* cytochrome aa_3 was unsuccessful,

imformation of the extensive sequence analysis of this archaeal *cox* operon should be required.

Recently, attention was focused on the prokaryotic cytochrome oxidases because of their simpler subunit structures and functional similarities to the more complexed mitochondrial enzymes. Studies on archaeal respiratory chains also lead us to questions about their adaptation to the extreme environments and their phylogenetic status, although, at present, the cytochromes other than our presentations have been reported only from a few archaea, including *H. halobium* (30), *Sulfolobus acidocaldarius* (31, 32), and *Thermoplasma acidophilum* (33). In the present study, we presented the deduced amino acid sequence of subunit I of *H. halobium* cytochrome aa_3. Extensive structural analysis of this archaeal *cox* operon is now in progress and will be published elsewhere. Accumulation of the sequence data of archaeal cytochrome oxidase will clarify its evolutional relationships with other known cytochrome oxidases.

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