Vol. 181, No. 1, 1991
November 27, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 437-442

THE CYTOCHROME *C* OXIDASE GENES IN BLUE-GREEN ALGAE AND CHARACTERISTICS OF THE DEDUCED PROTEIN SEQUENCE FOR SUBUNIT II OF THE THERMOPHILIC CYANOBACTERIUM *SYNECHOCOCCUS VULCANUS*

Hiroyuki Tano and Morio Ishizuka

Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, Kasuga, Bunkyo-ku, Tokyo, 112, Japan

Nobuhito Sone

Department of Biochemical Engineering and Science, Kyushu Institute of Technology, 680-4, Kawazu, Iizuka, Fukuoka-ken, 820, Japan

Received October 23, 1991

SUMMARY: Blue-green algae (cyanobacteria) contain both primitive photosynthetic and respiratory systems in their membranes. The controversial genes coding for an aa_3-type cytochrome oxidase in cyanobacteria were examined. The DNA probe coding for the most conserved part of subunit I hybridized with DNA fragments from four cyanobacterial species. We have cloned the genes coding for subunits I and II from the genomic library of the thermophilic cyanobacterium *Synechococcus vulcanus* and determined the nucleotide sequence of the subunit II gene. The deduced protein sequence (327 amino acid residues) indicates that there are two hydrophobic segments near the N-terminus and a hydrophilic intermembrane domain containing ligands for Cu_A (the ESR-active Copper) similar to other subunit IIs. The *S.vulcanus* subunit II does not contain the cytochrome *c* moiety that is present in bacilli and thermophiles.

To elucidate the origin and evolution of respiratory systems, it is important to identify and characterize the corresponding terminal oxidases in cyanobacteria. Cyanobacteria are interesting because they are evolutionarily primitive, and both photosynthetic and oxidative phosphorylation systems coexist in their biomembranes. In oxygen-producing cyanobacteria, removal of poisonous oxygen might have been a primitive process for respiration. There have been several reports supporting the presence of cytochrome aa_3-type oxidases in cyanobacteria (for example, [1-4]), although identification of the terminal oxidases is rather difficult. Bacterial cytochrome aa_3-type oxidases are structurally and functionally similar to the corresponding enzyme in mitochondria, although they have fewer subunits [5-7]. Genes coding for these bacterial oxidases have been cloned from *Paracoccus denitrificans* [8], the thermophilic bacterium PS3 [9,10], *Bacillus subtilis* [11], and *Thermus thermophilus* [12]. The deduced sequences for subunit I (COI) are very similar to each other as well as to those of mitochondrial COI's. These subunits contain three chromophores (haem *a*, haem a_3 and Cu_B) out of four redox active metal centers. The sequences of COII are also similar to each other, especially around the putative Cu_A-binding domain. The bacterial COII genes of PS3, *B. subtilis* and *T. thermophilus* encode proteins with a cytochrome *c* domain at the C-termini, whereas the sequence of *Paracoccus* COII does not [8].

Abbreviations: COI, COII, COIII, cytochrome *c* oxidase subunits I, II, III, respectively; ORF, open reading frame; PCR, polymerase chain reaction; bp, base pair.

0006-291X/91 $1.50

Here we report the cloning and sequencing of the genes coding for cytochrome aa_3-type oxidase in the thermophilic cyanobacterium *Synechococcus vulcanus*.

MATERIALS AND METHODS

T4-DNA ligase, DNA polymerase of *Thermus aquaticus*, restriction enzymes, plasmid vector pUC119, and M13 bacteriophages mp18 and mp19 were obtained from Takara Shuzo Co. (Kyoto, Japan). Hybond N^+ (nylon membranes for DNA blotting) and [α-^{32}P]dCTP were purchased from Amersham Corp.

Cyanobacterial cells were kindly donated to us as follows: *S. vulcanus* [13], by Dr. Y. Inoue of the Institute of Physical and Chemical Research, Wako-shi; *Synechococcus elongatus* [14], from Prof. S. Katoh of the University Tokyo; *Plectonema boryanum* and *Anabaena variabilis* [15], from Dr.T. Katoh of Kyoto University.

The library of genomic DNA of *S. vulcanus* was kindly given to us by Dr. Y. Inoue. This library was constructed by ligating DNA (13-18-kbp) with *Sau*3AI partially digested to the *Bam*HI-site of the lambda DASH vector. The ^{32}P-labeled probe was prepared by the PCR method using 5'-AGCTTGGCGGAAATACG (sense primer for *B. subtilis* COI at positions 3362-3378 in [11]) and 5'-GCAAAAACGTATAAACC (antisense primer at positions 4021-4005 in [11]) and DNA (20ng) of *B. subtilis* W168 as a template. PCR with a thermal cycler (Perkin-Elmer Cetus) was carried out 20 times with a cycle of 94° C(1 min)- 48° C (2 min)- 75° C(2 min). The oligonucleotides used for PCR were synthesized by an Applied Biosystems model 380B DNA synthesizer.

DNA sequencing was performed by the dideoxy chain terminator procedure [16] using [α-^{32}P]dCTP. Other methods used for molecular cloning were based on those of Maniatis et al. [17].

The sequence data were analyzed with a software program (Genetyx,Tokyo) adapted for an NEC PC9801 computer.

RESULTS AND DISCUSSION

Analysis by hybridization

To clone the genes coding for cytochrome oxidase in cyanobacteria, a conserved region in COI of *B. subtilis* cytochrome caa_3-type oxidase was used as the probe. For this, a set of oligonucleotides, a sense primer and anti-sense primer (see Materials and Methods) were synthesized chemically, and chain reactions were carried out with *B.subtilis* DNA as the template to prepare the DNA of the most conserved part of COI containing hydrophobic segments VI-X [7,10,11]. The product, mainly composed of 660-bp DNA as judged by agarose gel electrophoresis (not shown), was labeled with [α-^{32}P]dCTP. Figure 1 shows the result of hybridization to Southern blotted genomic DNA from four species of cyanobacteria. The probe hybridized well to fragments of *S. vulcanus* and of *S. elongatus* DNA at 63° C and at 58° C, respectively. The patterns were almost the same. In contrast, the probe did not hybridize to the DNA of *P. boryanum* or that of *A. variabilis* at 63° C (not shown), but hybridized rather well both DNAs at 58° C.

Cloning of the genes coding for cyanobacterial oxidase

Out of about 5000 recombinants, 20 clones were selected by plaque hybridization of the genomic library of *S.vulcanus*. Three clones contained the 2.4-kbp *Xho*I-fragment which hybridized to the probe well (see Fig. 1). Further sequence analysis showed that at least one of these clones (clone 4) indeed contained the genes for cytochrome oxidase subunits.

DNA sequence

Figure 2 shows a restriction map and the order of genes encoding for the three subunits (COII, COI and COIII). Nucleotide sequencing of the subcloned *Hind* III/*Xho*I fragment in M13 revealed that it was homologous to the sequences of other COI's including the sense priming site used for PCR (underlined). The nucleotide sequence of the *Eco*RI/*Hind* III fragment (1178 bp) contained the genes for COII.

Figure 3 shows the nucleotide sequence of the genes coding for COII and COI (partial) and the deduced amino acid sequence for COII. The open reading frame for *S. vulcanus* COII starts from an ATG at position 1954 (numbered from the *Eco*RI site in Fig. 2) and terminates at TAG at 2935, coding for a 327 amino acid residue protein.

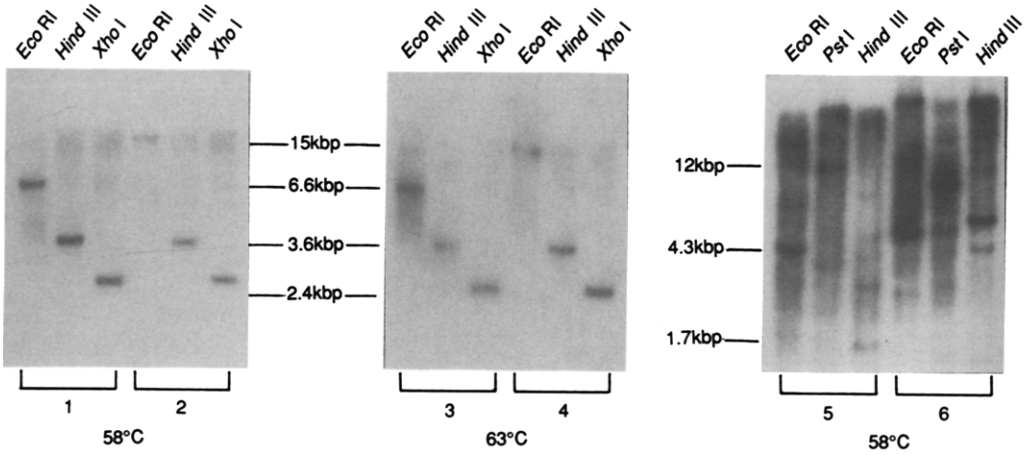

Fig. 1. Southern blot analyses of cyanobacterial DNA with the *B. subtilis* COII gene (*ctaC*) as a probe. Genomic DNAs from *S. vulcanus*, *S. elongatus*, *P. boryanum* and *A. variabilis* were hydrolyzed with *Eco*RI, *Hind*III or *Xho*I, electrophoresed, and blotted to a Hibond N^+ filter. Hybridization and washing were carried out at the indicated temperature. 1 and 3: *S. vulcanus*, 2 and 4: *S. elongatus*, 5: *A. variabilis*, 6: *P. boryanum*.

Characteristics of the deduced protein sequence for COII

Figure 4 shows the alignment of COII sequences. *S.vulcanus* COII is closely related to other COII's. There is a signal sequence adjacent to the N-terminal (underlined), which may be be processed as in the PS3 COII. The first hydrophobic transmembrane segment (B) is found at positions 76-98. In these parts, respective residues are not conserved, but as a whole, the characteristics of a signal peptide sequence (A) and a transmembrane helix (B) are conserved. The second hydrophobic segment is present at positions 117-135 (C) as in other COII's. Close to and inside of this segment, E-112, W-115, T-116 and P-119 are strictly conserved. This hydrophobic segment may interact to another hydrophobic segment in COI as pointed out in Ref. [7]. This N-terminal third domain is likely to form a membrane anchor domain composed of two hydrophobic hairpins.

The rest, the C-terminal two thirds, is a functionally important part that probably contains ligands to Cu_A and binds cytochrome *c* as a substrate. Three portions are highly conserved: an

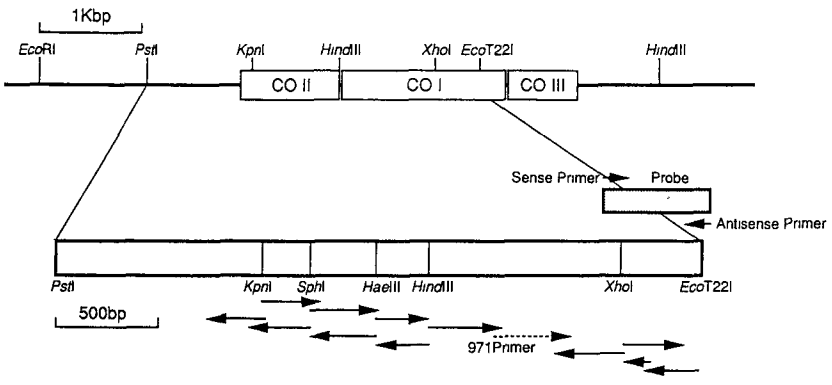

Fig.2. A map of *S. vulcanus* genes coding for subunits of cytochrome oxidase. The restriction sites used for subcloning and sequencing are also shown. The sequence of the dashed portion was obtained by using an oligonucleotideprimer.

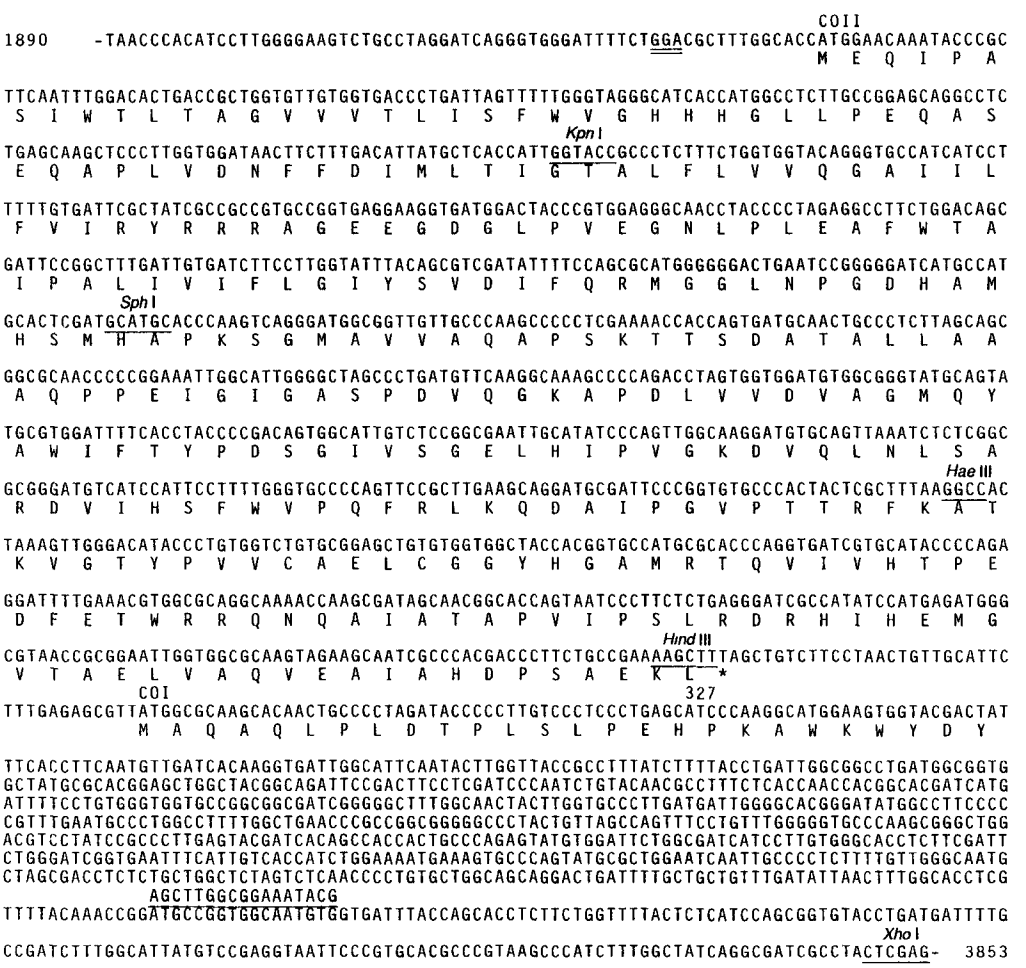

Fig. 3. Nucleotide and deduced amino acid sequences of *S.vulcanus* gene coding for COII and COI (partial). Putative ribosome-binding sites (double underlined), the restriction sites shown in Figure 1, and the comparative sequence of the sense primer (underlined) are marked.

aromatic cluster at positions 165-171, the residues around H-207, and -CXEVCGXXHXXM- at position 248-259. According to a model for the structure of Cu_A, two histidine and two cysteine residues may directly ligate the Cu atom [18,19]. H-207, C-248, C-252, and H-256 in *S. vulcanus* COII are all conserved as those in other proteins. The vicinity of H-221 is the second conserved portion; in addition to V-205 and P-212, two carboxylic acids (#), D-204 and D-219, are conserved in the cyanobacterial COII. The portion of the aromatic cluster is also highly conserved, but in the *S. vulcanus* sequence -YAWIFTYPD-, the second A-166 is unique, since in other COII sequences, tyrosine or tryptophane is found in this position. Many proline and glycine residues are conserved in *S. vulcanus* COII as in others, which may indicate that the basic folding of COII is the same.

In mitochondria and *Paracoccus*, cytochrome *c* is a protein detached from the oxidase which transfers electrons from the cytochrome bc_1 complex to cytochrome oxidase. Cytochrome *c* is covalently bound to COII by gene fusion in PS3 [9,10], *B. subtilis* [11] and *T. thermophilus* [12]. However, the *S. vulcanus* COII has no cytochrome *c* moiety. It is advantageous to Gram-positive bacteria to bind cytochrome *c* covalently, because they have no periplasmic space. It is not evident why the gram negative *T.thermophilus* contains fused cytochrome *c* in COII.

Fig. 4. An alignment of COII sequences. One mitochondrial (human, Hu) and five bacterial proteins are shown. The signal sequences of *S.vulcanus* (Sv) and PS3 (PS) are underlined. Parts of C-terminus sequences are omitted in *T.thermophilus* (Tt), PS3 and B.subtilis (Bs). The long insertion in Sv alignment (++) is -KSGMAVVAQAPSKTTSDATALLAAAQPPEIGIGASPDVQGKAPD -. Those in Hu (!!) and Pd ($$) are -LIFNSYMLPPLFLEPGDLR- and -AFDALMLEKEALADAGYSE-DEYL-, respectively. Two transmembrane segments (===) and the conserved carboxy groups (#) are also marked.

Comparison of COII's and conclusion

Table 1 summarizes numbers of identical amino acid residues in the alignment shown in Fig. 4. The highest identity of *S. vulcanus* COII is observed with *T. thermophilus* COII, followed by PS3 COII and *B. subtilis* COII. Since the *T. thermophilus* COII shows the highest identity with PS3 COII, as well as those in Gram-positive bacteria, *T. thermophilus* seems to be closely related to cyanobacteria [cf. 20]. *S. vulcanus* COII is less homologous to human mitochondrial COII or *P.denitrificans* COII. The sequence of *S.vulcanus* COII is rather close to those fused with cytochrome *c* such as bacilli and *Thermus* COII's, but different from those without cytochrome *c* such as mitochondorial and purple bacterial COII's. This fact suggests that the genes coding for cytochrome *c* and cytochrome *c* oxidase were independent in the primitive form; and later, a both the genes fused in a group including *Bacillus* and *Thermus*. The sequence of the COII of *S.vulcanus* is moderately different from those of other COII's of different mitochondria and bacteria listed, and may thus be useful for elucidating the structure-function relationship and evolution of cytochrome oxidases.

In conclusion, *S. vulcanus* possesses the genes for the subunits of cytochrome *c* oxidase. The genes seem to be expressed and functioning, since the deduced protein sequence of *S.vulcanus* COII, at least, has consensus amino acid residues at every important portion of the molecule.

Table 1. Comparative matrix for COII from different sources

Source	1	2	3	4	5	6	7
1. Human	227						
2. *P.denitrificans*	69	277					
3. *E. coli (bo)*	43	41	260				
4. *T. thermophilus*	57	56	58	245			
5. PS3	43	58	61	78	271		
6. *B. subtilis*	36	57	55	66	165	269	
7. *S. vulcanus*	47	59	36	77	72	71	283

Alignments of the cytochrome *c* portion in the C-terminal region were not included
Number of identical residues.

Acknowledgments

We thank Dr. M. Saraste for his kindly providing us with information on the sequence data of *ctaB-F* genes for *B. subtilis* cytochrome *c* oxidase before publication and critical reading of the manuscript; Dr.Y.Inoue for providing the gene library and cells of *S.vulcanus*; Prof. S. Kato for providing cells of *S. elongatus*; and Dr. T. Katoh for providing cells of *P. boryanum* and *A. variabilis*. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Lockau, W. (1981) Arch. Microbiol., 128, 336-340.
2. Omata, T. and Murata, N. (1985) Biochim. Biophys. Acta, 810, 354-361.
3. Hafele, U., Scherer, S., Boger, P. (1988) Biochim Biophys. Acta 934, 186-190.
4. Peschek, G.A., Wastyn, M., Trnka,M., Molitor, V., Fry, I.V., Packer, L. (1989) Biochemistry, 28, 3057-3063.
5. Poole, R.K. (1988) in Bacterial Energy Transdusduction (C. Anthony ed) pp. 231-316, Academic Press, London.
6. Sone, N. (1990) in The Bacteria (vol. 12), Bacterial Energetics (T.A. Krulwich ed) pp.1-32, Academic Press, New York.
7. Saraste, M. (1991) Quart. Rev. Biophys. 23, 331-366.
8. Raitio, M., Jalli, T., and Saraste, M. (1987) EMBO J. 6, 2825-2833.
9. Sone, N., Yokoi,F., Fu, T., Ohta, S.,Metso, T., Raitio,M. and Saraste, M. (1988) J.Biochem. 103, 606-610.
10. Ishizuka, M., Machida, K., Shimada, S., Mogi, A., Tsuchiya, T., Ohmori, T., Souma, Y., Gonda, M. and Sone, N. (1990) J. Biochem. 108, 866-873.
11. Saraste, M., Metso, T., Nakari, T., Jalli, T., Lauraeue, M., van der Oost, J. (1991) Eur. J. Biochem., 196, 517-525.
12. Mather, M.W., Springer, P., and Fee, J.A. (1991) J. Biol. Chem. 266, 5025-5035.
13. Koike, H., and Inoue, Y. (1983) in The Oxygen Evolving System of Photosynthesis (Y.Inoue, A.R. Croft, Govindjee, N. Murata, G. Renga and K.Satoh eds) pp.257-263, Academic Press,Tokyo.
14. Yamaoka, T., Satoh, K. and Katoh, S. (1978) Plant Cell Physiol. 19, 943-954.
15. Ohki, K., Katoh, T. (1975) Plant Cell Physiol. 16, 53-64.
16. Sanger, F., Nicklen, S. and Coulson, A. E. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467.
17. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) in A Laboratory Manual, Cold Spring Harbor, NY.
18. Stevens, T.H., Martin, C.T., Wang, H., Brudvig, G.W. Scholes, C.P. and Chan, S. I. (1982) J.Biol. Chem. 257, 12106-12113.
19. Holm, L., Saraste, M. and Wikstrom, M. (1987) EMBO J. 6, 2819-2823.
20. Woese, C.R. (1987) Microbiol. Rev., 51, 221-271.