

LEGIBILITY NOTICE

A major purpose of the Technical Information Center is to provide the broadest dissemination possible of information contained in DOE's Research and Development Reports to business, industry, the academic community, and federal, state and local governments.

Although a small portion of this report is not reproducible, it is being made available to expedite the availability of information on the research discussed herein.

LA-UR 90-2733

LA-UR--90-2733

DE90 016512

SEP 07 1990

Los Alamos National Laboratory is operated by the University of California for the United States Department of Energy under Contract W 7405-ENG-56

TITLE Relationship of Cytochrome *caa3* from *Thermus thermophilus* to other Heme- and Copper-Containing Terminal Oxidases

AUTHOR(S) Michael W. Mather, Penelope Springer, and James A. Fee

SUBMITTED TO Proceedings of the 41st Mosbach Colloquium published by Springer Verlag, Heidelberg, FRG

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

By acceptance of this article, the publisher recognizes that the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or to allow others to do so, for U.S. Government purposes.

The Los Alamos National Laboratory requires that the publisher identify this article as work performed under the auspices of the U.S. Department of Energy.

LOS ALAMOS

Los Alamos National Laboratory
Los Alamos, New Mexico 87545

MASTER

Form No. 816 RA
5/79 No. 2629 5/81

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

Relationship of Cytochrome *caa₃* from *Thermus thermophilus* to other Heme- and Copper-Containing Terminal Oxidases

M. W. Mather, P. Springer, and J. A. Fee

Stable Isotope Resource, Isotope and Structural Chemistry Group, Los Alamos
National Laboratory, Los Alamos, New Mexico, USA 87545

1 Introduction

Cytochrome oxidases are a key component of the energy metabolism of most aerobic organisms from mammals to bacteria. They are the final enzyme of the membrane associated respiratory chain responsible for converting the chemical energy of reduced substrates to a transmembrane electrochemical potential, which is used by the cell for a wide variety of energy-requiring processes. The most widely studied oxidase is the cytochrome *c* oxidase (cytochrome *aa₃* oxidase) of the mammalian mitochondrion. This complex, integral membrane protein contains 13 subunits and four canonical metal centers: heme centers *a* and *a₃*; copper centers *Cu_A* and *Cu_B*. It is responsible for electron transfer from reduced cytochrome *c* to dioxygen with the concomitant reduction of dioxygen to water and the coupled vectorial transfer of protons across the mitochondrial membrane. (see Chan and Li 1990 and Palmer 1987 for recent reviews.)

Bacterial membranes have been shown, in many cases, to contain respiratory components very similar to those of the mitochondrion. In particular, the bacterial cytochrome *aa₃* oxidases were found to possess similar spectroscopic and functional properties to the mitochondrial enzyme, but a

much simpler subunit structure. Purified, active bacterial oxidases contain the four canonical metal centers, but consist of only one to three subunits (reviewed in Anraku 1988). In the case of *Paracoccus denitrificans* (Raitio et al. 1987) these three subunits were shown to be homologous to the three largest subunits of the eukaryotic oxidase, which are synthesized in the mitochondrion (the remaining subunits being imported from the cytoplasm). From recent results, it is now apparent that there are at least three types of heme- and copper-containing terminal oxidases: the classical *aa₃* type discussed above; the recently discovered *ba₃* type, in which the heme A of cytochrome *a* is replaced by protoporphyrin IX (heme B) (Zimmermann et al. 1988); and the bacterial cytochrome *bo* oxidase (a quinol oxidase), containing two B hemes in place of the two A hemes of the *aa₃* type and at least one copper with properties resembling *Cu_B* (Kita et al. 1984, Hata et al. 1985, Salerno et al. 1989). The cytochrome *bo* oxidase from *E. coli*, like the *aa₃* oxidase from *P. denitrificans*, has now been shown to contain subunits homologous to the three largest subunits of the mitochondrial oxidase (Chepuri et al. 1989). The sequence of cytochrome *ba₃* is not yet available, but, because the metal centers reside in similar environments (Zimmermann et al. 1988), it seems likely that it too will contain homologies to one or more of the mitochondrial subunits. The recently isolated heme A- and copper-containing oxidase from thermophilic archaeobacteria (Annemüller and Schäfer 1989), which appears to have only one subunit, may prove to be yet another sub-type of this superfamily of oxidases.

The thermophilic eubacterium *Thermus thermophilus* has proven to be a good source of stable, comparatively simple respiratory enzymes (reviewed in Fee, et al. 1986). Under appropriate growth conditions, the membrane fraction contains significant amounts of two terminal oxidases, cytochrome *caa₃* and

cytochrome *ba₃*. The *ba₃* oxidase has been purified as a single subunit enzyme of 35 kDa and shown to contain cytochrome *a₃*, *Cu_A* and *Cu_B* centers similar to the mitochondrial *aa₃* oxidase, but to contain cytochrome *b* in place of cytochrome *a* (Zimmermann et al. 1988). Cytochrome *ba₃* is the subject of ongoing and future studies in our laboratory. This communication will principally be concerned with recent results from our studies of cytochrome *caa₃* oxidase. Cytochrome *caa₃* has been isolated as a two subunit (55 kDa and 33 kDa) enzyme containing the four canonical metal centers and an additional C heme (Hon-nami and Oshima 1983, Yoshida, et al. 1984). The enzyme has been extensively characterized and shown to be essentially identical spectroscopically to a combination of cytochrome *aa₃* oxidase plus cytochrome *c* (see Fee, et al. 1986 and references cited therein). The *caa₃* oxidase has been shown to function as a redox-coupled proton pump when reconstituted in proteoliposomes (Sone et al. 1983, Yoshida and Fee 1984). The finding that the 33 kDa subunit, when resolved from the *caa₃* complex and separately purified, bound the C heme but apparently contained no A heme or copper was a principal factor leading to the hypothesis that the 55 kDa subunit (presumably homologous to subunit I of the mitochondrial oxidases) contains the four canonical metal centers and constitutes the catalytic core of the oxidase (Yoshida et al. 1984; see also Fee et al. 1986). This hypothesis conflicts with the more generally accepted hypothesis that subunit I contains the two A hemes and *Cu_B*, but that *Cu_A* is bound to subunit II. The latter hypothesis is consistent with a large number of spectroscopic, copper depletion, and other experiments (see Chan et al. 1990, Holm et al. 1987, Palmer 1987), but may be inconsistent with the isolation of the single subunit cytochrome *ba₃* oxidase and the preparation of an enzymatically digested cytochrome *aa₃* oxidase from *P. denitrificans* which consists of the major portion of subunit I only, yet retains

activity and the optical spectrum of the native enzyme (Müller et al. 1988a and 1988b). In this communication we will describe preliminary results of DNA sequencing experiments with the cytochrome *caa₃* oxidase, initially undertaken to determine the nature of the subunits of this oxidase and shed light on the distribution of the metal centers. We will speculate on oxidase gene and protein structures and evolutionary relationships in the light of these results and recent sequencing results from other groups.

2 Experimental Procedures

The procedures used to clone the oxidase genes from *T. thermophilus* have been described (Fee et al. 1988 and Mather 1988). DNA sequencing was performed using the dideoxy method (Sanger et al. 1977). Several methods were used to overcome polymerase terminations, gel compressions, and other problems caused by the high guanine/cytosine content of *Thermus* DNA, including the use of 7-deaza-guanine and inosine in the sequencing reactions; details will be published elsewhere (Mather, et al, 1990). Computer sequence analyses were performed using the University of Wisconsin GCG programs (Devereux et al. 1984) and additional software written by one of the authors (MWM).

3 Results and Discussion

3.1 Summary of Results

We have cloned and sequenced a ~4kb region of the *Thermus thermophilus* chromosome containing genes encoding subunits of the cytochrome *caa₃* oxidase. Fig. 1 is a schematic representation of this portion of the chromosome. A few small regions of uncertainty in the DNA sequence remain to be clarified

by additional sequencing experiments, most notably, the region following the second *Hind*III site and presumably containing the end of subunit I and the start of subunit III. Comparative analyses of the deduced amino acid sequences of these subunits together with those of other available oxidase sequences lead to a number of conclusions:

- *T. thermophilus* contains genes for three oxidase subunits: I, IIc, and III. These genes are contiguous on the *T. thermophilus* chromosome.
- The sequences of the *T. thermophilus* oxidase subunits are very similar to the corresponding sequences of all other oxidase subunits I, II, and III.
- Hydrophobicity analyses show that all oxidase subunits I, II, and III which have been sequenced have hydrophobic regions (putative transmembrane helices) at corresponding positions. This, together with the sequence and spectroscopic similarities, suggests a basic structural similarity for all the heme/copper oxidases from mitochondrial and eubacterial sources¹.
- Subunit III of the *T. thermophilus* oxidase is apparently lost during purification, as in the case of *P. denitrificans* (Saraste et al. 1986).
- An acidic cytochrome *c* domain is fused to the carboxy-terminus of the *T. thermophilus* subunit II. This may represent a fusion of substrate (cytochrome *c*) to enzyme (cytochrome *c* oxidase).
- The 2 cysteine and 2 histidine residues proposed to coordinate Cu_A in subunit II (Holm et al. 1987) are retained in *T. thermophilus* subunit IIc, as in all other cytochrome *aa₃* oxidases. The sequences near the putative ligands are highly conserved. *E. coli* *bo* oxidase subunit II, in contrast, specifically lacks the proposed Cu_A liganding residues.
- Subunit I is the most highly conserved subunit. Comparison of all subunit I sequences indicates that only six histidines are conserved. According to

the results of spectroscopic experiments, these must constitute a minimal or near minimal set of ligands for the metal cofactors found in subunit I : cytochrome *a* (2 His), cytochrome *a₃* (1 His), *Cu_B* (2-3 His).

- Comparison of the order and extent of the bacterial oxidase genes among several species suggests that genomic rearrangements involving the subunit III gene have occurred.

In the remainder of the communication we discuss some specific results regarding three interesting points: (1) The overall similarity of subunits I, II, and III among all the oxidases, (2) some possible genomic variations which may result in variations in the position of the amino terminal portion of subunit III among the bacterial oxidases, and (3) the conservation of sequence near the proposed *Cu_A* coordinating residues in subunit II.

3.2 Similarity of Oxidase Sequences and Structures

We have prepared alignments of all available sequences of oxidase subunits I, II, and III using the multiple alignment algorithm of Gribskov et al. (1987) with some minor adjustments made upon consideration of conserved amino acid residues and relative locations of hydrophobic regions (which tend to be less well conserved and thus more difficult to align). The alignments will be published elsewhere. Here we present a compilation of the degree of identity of subunits I, II, and III from some selected species (Table I). The general rule of thumb is that a percent identity of 25% or more is good evidence of a significant match between two sequences, while scores below 15% provide no evidence of relatedness (Doolittle 1981 and 1986). The strong sequence conservation of the oxidase subunits, especially of subunit I, among these evolutionarily distant organisms is readily apparent. In the case of subunits II

and III of the *E. coli* *bo* oxidase, comparison to eukaryotic subunits II leaves some doubt with regard to the significance of the postulated relationship, e.g. note the relatively low percent identities in the bovine/*E. coli* comparison of Table 1. Comparison to the subunits from *T. thermophilus* (Table 1) or *Bacillus* PS3 (not shown), however, clearly establishes the relationship of the cytochrome *bo* oxidase subunits to the corresponding cytochrome *aa₃* oxidase subunits. It is interesting to note that the *T. thermophilus* subunits have the greatest similarity to those from the gram positive thermophile *Bacillus* PS3 (except in the case of subunit III); otherwise they are not appreciably more similar to the other bacterial oxidase subunits than to subunits from mitochondrial oxidases.

A separate tabulation for the hydrophilic portion of subunit II is presented in Table 1 to illustrate the higher degree of conservation of this portion of the subunit. The hydrophobic segments in the amino terminal portion of the subunit may be less conserved because they function as a membrane anchor for the peptide (Bisson et al. 1982a), which apparently requires only that the overall hydrophobicity of the segments be maintained.

As noted above, hydrophobicity plots of aligned oxidase subunits show that these subunits sequenced from a wide variety of species contain hydrophobic regions in essentially identical positions in the sequence alignments. For example, compare the top two traces in Fig. 2 (see, for example, Wikström et al. 1985 for additional comparative hydrophobicity plots of oxidase subunits). It is likely that many of these hydrophobic segments are transmembrane helices, as was found for the reaction centers from photosynthetic bacteria (Michel et al. 1986). Further, this alignment of the hydrophobic regions, and alternating hydrophilic regions, predicts a basic structural similarity¹ among the individual subunits (I, II, and III) from all sources examined so far. It is very likely, then, that

general conclusions drawn from studies of the bacterial oxidases are valid for all the heme/copper oxidases.

3.3 Organization of Bacterial Genes Encoding Oxidase Subunits

The oxidase genes of *T. thermophilus* are contiguous on the chromosome in the order IIc, I, and III² (Fig. 1 and Fig. 3). The same gene order is found in the case of *Bacillus* PS3 and *E. coli* (see Fig. 3) (Chepuri et al. 1989, Ishizuka et al. 1989). However, the genes for subunits III from *Bacillus* PS3 and *E. coli* are shorter than those of the other species. The translated peptide sequences do not have a region which corresponds to the amino terminal portion of subunit III from other sources, including the first two (of seven) hydrophobic segments. As shown in Fig. 2 and Fig. 3, however, there is an extra sequence element at the end of the *E. coli* subunit I gene which translates to a peptide sequence containing two hydrophobic segments, and this peptide sequence can be aligned to the beginning of the subunit III sequence alignment. A similar sequence element occurs in the case of *Bacillus* PS3; it is found as a separate open reading frame (orf) between the genes encoding subunits I and III (Fig. 3) (Ishizuka et al. 1989). This suggests that transfer of the initial portion of the gene for subunit III to a separate gene or to the end of the subunit I gene has occurred without disrupting the basic structure of the oxidase protein complex. Since the subunit III gene follows the subunit I gene on the chromosome of these bacteria, it is easy to envision how this could be accomplished by mutations producing a stop codon at the appropriate place in the gene encoding subunit III, and, in the case of *E. coli*, eliminating a stop codon at the end of an originally shorter subunit I gene. We note that even more drastic genomic rearrangements were found in the case of bacterial operons encoding sugar phosphotransferase systems, which are also membrane-associated

(reviewed in Fobillard and Lolkema 1988). The nature of the junction region for the subunit I-subunit III genes in *T. thermophilus* is still under investigation².

3.4 Sequence Conservation In the Proposed Cu_A Binding Region of Subunit II

The most highly conserved region among subunits II of the cytochrome aa_3 oxidases is the region near the carboxy terminus which contains the 2 conserved histidines (161 and 204 of the bovine sequence) and 2 conserved cysteines (positions 196 and 200 of the bovine sequence) proposed to coordinate Cu_A (see Holm et al. 1987 and Martin et al. 1988). It also contains a conserved methionine which was initially proposed to be a ligand to Cu_A (Steffens and Buse 1979) and conserved aspartate and glutamate residues thought to be involved in cytochrome *c* binding (Bisson et al. 1982b, Millet et al. 1983). The positions of these residues can be seen in Fig. 4. Since the *E. coli* cytochrome *bo* oxidase has been found to lack a Cu_A center, while retaining two hemes and a Cu_B , it is interesting to note that the quinol oxidase subunit II does not retain any of the conserved residues proposed to coordinate Cu_A or to participate in cytochrome *c* binding, yet has a high degree of overall similarity in this region to subunit IIc from the *T. thermophilus* caa_3 oxidase (37% identity; Fig. 4)³. The *E. coli* cytochrome *bo* oxidase, then, has a homologous subunit II, but the proposed Cu_A ligands are absent from this subunit; this result is consistent with the proposal that the conserved cysteines and histidines in subunit II of the cytochrome aa_3 oxidases are ligands to Cu_A . In light of the hypothesis that Cu_A is a site of proton pumping in cytochrome aa_3 oxidase (Gelles, et al. 1986), it is also interesting to note that cytochrome *bo* oxidase has recently been found to translocate protons (Puustinen et al. 1989).

Acknowledgments

We thank Drs. Sieghard Hensel and Gerhard Buse for providing peptide sequence data and discussions; Drs. Robert B. Gennis and Nobuhito Sone for providing DNA sequences prior to publication; and Dr. Mark Wilder and the Life Sciences Division of LANL for assisting us with the use of their computer facilities. This work was supported by USPHS Grant GM35342 and performed under the auspices of the U. S. Department of Energy.

References

- Anderson, S., de Bruijn, M. H. L., Coulson, A. R., Eperon, I. C., Sanger, F., & Young, I. G. (1982) Complete sequence of bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. *J. Mol. Biol.* **156**:683-717.
- Annemüller, S. & Schäfer, G. (1989) Cytochrome *aa₃* from the thermuacidophilic archaebacterium *Sulfolobus acidocaldarius*. *FEBS Lett.* **244**:451-455.
- Anraku, Y. (1988) Bacterial electron transport chains. *Annu. Rev. Biochem.* **57**:101-132.
- Bisson, R., Steffens, G. C. M., & Buse, G. (1982a) Localization of lipid binding domain(s) on subunit II of beef heart cytochrome *c* oxidase. *J. Biol. Chem.* **257**:6716-6720.
- Bisson, R., Steffens, G. C. M., Capaldi, R. A., & Buse, G. (1982b) Mapping of the cytochrome *c* binding site on cytochrome *c* oxidase. *FEBS Lett.* **144**:359-363.
- Bonitz, S. G., Coruzzi, G., Thalenfeld, B. E., & Tzagoloff, A. (1980) Assembly of the mitochondrial membrane system. Structure and nucleotide sequence of the gene coding for subunit I of yeast cytochrome oxidase. *J. Biol. Chem.* **24**:11927-11941.
- Chan, S. I. & Li, P. M. (1990) Cytochrome *c* oxidase: Understanding nature's design of a proton pump. *Biochemistry* **29**:1-12.
- Chepuri, V., Lemieux, L., Au, D. C.-T., & Gennis, R. B. (1989) manuscript submitted.

- Clary, D. O. & Wolstenholme, D. R. (1983a) Nucleotide sequence of a segment of *Drosophila* mitochondrial DNA that contains the genes for cytochrome *c* oxidase subunits II and III and ATPase subunit 6. *Nucleic Acids Res.* **11**: 4211-4227.
- Clary, D. O. & Wolstenholme, D. R. (1983b) Genes for cytochrome *c* oxidase subunit I, *urf2*, and 3 tRNA's in *Drosophila* mitochondrial DNA. *Nucleic Acids Res.* **11**: 6859-6872.
- Coruzzi, G. & Tzagoloff, A. (1979) Assembly of mitochondrial membrane systems. DNA sequence of subunit 2 of yeast cytochrome oxidase. *J. Biol. Chem.* **254**:9324-9330.
- Dayhoff, M. O., Schwartz, R. M., & Orcutt, B. C. (1978) A model of evolutionary change in proteins. In: Dayhoff, M. O. (ed.) *Atlas of protein sequence and structure*, vol. 5, suppl. 3. National Biomedical Research Foundation, Washington, D. C., pp 345-352.
- Degli Esposti, M., Ghelli, A., Luchetti, R., Crimi, M., & Lenaz, G. (1989) New approaches to the prediction of the folding of membrane proteins with redox function. *Ital. J. Biochem.* **38**:1-22.
- Doolittle, R. F. (1981) Similar amino acid sequences: Chance or common ancestry? *Science* **214**:149-159.
- Doolittle, R. F. (1986) *Of urfs and orfs. A primer on how to analyze derived amino acid sequences.* University Science Books, Mill Valley, California.
- Fee, J. A., Kuila, D., Mather, M. W., & Yoshida, T. (1986) Respiratory proteins from extremely thermophilic, aerobic bacteria. *Biochim. Biophys. Acta* **853**:153-185.
- Fee, J. A., Mather, M. W., Springer, P., Hensel, S., and Buse, G. (1988) Isolation and partial sequence of the A-protein gene of *Thermus thermophilus* cytochrome *c*_{1aa3}. *Ann. New York Acad. Sci.* **550**:33-38.

- Gelles, J., Blair, D. F., & Chan, S. I. (1986) The proton-pumping site of cytochrome *c* oxidase: A model of its structure and mechanism. *Biochim. Biophys. Acta* **853**:205-236.
- Gribskov, M., McLachlan, A. D., & Eisenberg, D. (1987) Profile analysis: detection of distantly related proteins. *Proc. Natl. Acad. Sci. USA.* **84**:4355-4358.
- Hata, A., Kirino, Y., Matsuura, K., Itoh, S., Hiyama, T., Konishi, K., Kita, K., & Anraku, Y. (1985) Assignment of ESR signals of *Escherichia coli* terminal oxidase complexes. *Biochim. Biophys. Acta* **810**:62-72.
- Holm, L., Saraste, M., & Wikström, M. (1987) Structural models of the redox centres in cytochrome oxidase. *EMBO J.* **6**:2819-2823.
- Hon-nami, K. & Oshima, T. (1984) Purification and Characterization of cytochrome *c* oxidase from *Thermus thermophilus* HB8. *Biochemistry* **23**:454-460.
- Ishizuka, M., Machida, K., Shimada, I., and Sone, N. (1989) manuscript in preparation.
- Kita, K., Konishi, K., & Anraku, Y. (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. *J. Biol. Chem.* **259**:3368-3374.
- Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein.
- Mather, M. W. (1988) Base composition-independent hybridization in dried agarose gels: Screening and recovery for cloning of genomic DNA fragments. *BioTechniques* **6**:444-447.
- Mather, M. W., Springer, P., & Fee, J. A., (1990) manuscript in preparation.
- Martin, C. T., Scholes, C. P., & Chan, S. I. (1988) On the nature of cysteine coordination to *CU_A* in cytochrome *c* oxidase. *J. Biol. Chem.* **263**:8420-8429.

- Michel, H., Weyer, K. A., Gruenberg, H., Dunger, I., Oesterhelt, D., & Lottspeich, F. (1986) The 'light' and 'medium' subunits of the photosynthetic reaction centre from *Rhodospseudomonas viridis*: isolation of the genes, nucleotide and amino acid sequence. *EMBO J.* **5**:1149-1158.
- Millet, F., de Jong, C., Paulson, L., & Capaldi, R. A. (1983) Identification of specific carboxylate groups on cytochrome *c* oxidase that are involved in binding cytochrome *c*. *Biochemistry* **22**:546-552.
- Müller, M., Schläpfer, B., & Azzi, A. (1988a) Preparation of a one-subunit cytochrome oxidase from *Paracoccus denitrificans*: Spectral analysis and enzymatic activity. *Biochemistry* **27**:7546-7551.
- Müller, M., Schläpfer, B., & Azzi, A. (1988b) Cytochrome *c* oxidase from *Paracoccus denitrificans*: Both hemes are located in subunit I. *Proc. Natl. Acad. Sci. USA.* **85**:6647-6651.
- Palmer, G. (1987) Cytochrome oxidase: a perspective. *Pure and Appl. Chem.* **59**:749-758.
- Puustinen, A., Finel, M., Virkki, M., & Wikström, M. (1989) Cytochrome *o* (*bo*) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli*. *FEBS Lett.* **249**:163-167.
- Raitio, M., Tuulikki, J., & Saraste, M. (1987) Isolation and analysis of the genes for cytochrome *c* oxidase in *Paracoccus denitrificans*. *EMBO J.* **6**:2825-2833.
- Salerno, J. C., Bolgiano, B., & Ingledew, W. J. (1989) Potentiometric titration of cytochrome-*bo* type quinol oxidase of *Escherichia coli*: Evidence for heme-heme and copper-heme interaction. *FEBS Lett.* **247**:101-105.

- Saraste, M., Raitio, M., Tuulikki, J., & Perämaa, A. (1986) A gene in *Paracoccus* for subunit III of cytochrome oxidase. *FEBS Lett.* **206**:154-156.
- Sone, N., Yokoi, F., Fu, T., Ohta, S., Metso, T., Raitio, M., & Saraste, M. (1988) Nucleotide sequence of the gene coding for cytochrome oxidase subunit I from the thermophilic bacterium PS3. *J. Biochem. (Tokyo)* **103**:606-610.
- Sone, N., Yanagita, Y., Hon-Nami, K., Fukumori, Y., & Yamanaka, T. (1983) Proton-pump activity of *Nitrobacter agilis* and *Thermus thermophilus* cytochrome *c* oxidases. *FEBS Lett.* **155**:150-154.
- Steffens, G. J., & Buse, G. (1979) Studies on cytochrome *c* oxidase, IV. Primary structure and function of subunit II. *Hoppe-Seyler's Z. Physiol. Chem.* **360**:613-619.
- Steinrücke, P., Steffens, G. C. M., Pankus, G., Buse, G., & Ludwig, B. (1987) Subunit II of cytochrome *c* oxidase from *Paracoccus denitrificans*. DNA sequence, gene expression and the protein. *Eur. J. Biochem.* **167**:431-439.
- Sweet, R. M. & Eisenberg, D., (1983) Correlation of sequence hydrophobicities measures similarity in three-dimensional protein structures. *J. Mol. Biol.* **171**:479-488.
- Thalenfeld, B. E. & Tzagoloff, A. (1980) Assembly of the mitochondrial membrane system. Sequence of the *Oxi 2* gene of yeast mitochondrial DNA. *J. Biol. Chem.* **255**:6173-6180.
- Wikström, M., Saraste, M., & Penttillä, T. (1985) Relationships between structure and function in cytochrome oxidase. In: Martonosi, A. M., ed. *The enzymes of biological membranes*, vol. 4. Plenum, New York.

- Yoshida, T., Lorence, R. M., Choc, M. G., Tarr, G. E., Findling, K. L., & Fee, J. A. (1984) Respiratory proteins from the extremely thermophilic aerobic bacterium, *Thermus thermophilus*. *J. Biol Chem.* **259**:112-123.
- Yoshida, T. & Fee, J. A. (1984) Studies on cytochrome *c* oxidase activity of the cytochrome *c₁aa₃* complex from *Thermus thermophilus*. *J. Biol. Chem.* **529**:1031-1036.
- Zimmermann, B. H., Nitsche, C. I., Fee, J. A., Rusnak, F., & Münck, E. (1988) Properties of a copper-containing cytochrome *ba₃*: A second terminal oxidase from the extreme thermophile *Thermus thermophilus*. *Proc. Natl. Acad. Sci. U. S. A.* **85**: 5779-5783.

FOOTNOTES

¹ Sweet and Eisenberg (1983) have pointed out the basic correlation between structural elements and hydrophobicity patterns. Thus, the probability of structural similarity does not depend solely on any specific structural interpretation of the hydrophobicity plot, such as the existence of transmembrane helices formed from extended segments of hydrophobic amino acids.

² The exact end of the subunit I gene and beginning of the subunit III gene in *T. thermophilus* is uncertain because we have not yet obtained high quality sequencing gels for a small region which apparently includes the end of subunit I and the start of subunit III (Fig. 1); so far we have not identified a stop codon following subunit I. This should be clarified shortly; we are attempting to prepare additional subclones for this region and are preparing a specific oligonucleotide primer for this region.

³ The replacements of the conserved residues which are found in the *E. coli* subunit II are, however, in most cases evolutionarily conservative: Cys→Ser is the most frequent cysteine replacement; His→Asn, Asp→Ser and Glu→Ser are also common (Dayhoff et al. 1978).

Table 1

Per cent of identical amino acid residues^a in cytochrome or quinol oxidase subunits I, II, and III from six species compared to the corresponding subunits from the Bovine mitochondrial oxidase or from the *T. thermophilus* oxidase

Comparison to the Bovine oxidase			
Source ^b	Subunit		
	I	II / IIs ^c	III
<i>D. yakuba</i>	75.5	59.8 / 65.3	65.0
<i>S. cerevisiae</i>	59.6	44.7 / 50.0	44.2
<i>P. denitrificans</i>	54.3	36.1 / 43.1	51.0
<i>Bacillus</i> PS3	44.0	24.9 / 29.4	24.9 ^d
<i>T. thermophilus</i>	39.8	26.8 / 35.6	29.5
<i>E. coli</i>	40.1	17.7 / 22.5	23.4 ^d

Comparison to the <i>T. thermophilus</i> oxidase			
Source ^b	Subunit		
	I	II / IIs ^c	III
Bovine	39.8	26.8 / 35.6	29.5
<i>D. yakuba</i>	40.8	24.6 / 33.7	28.2
<i>S. cerevisiae</i>	39.4	27.3 / 33.3	27.8
<i>P. denitrificans</i>	42.6	28.9 / 36.9	30.1
<i>Bacillus</i> PS3	47.1	33.3 / 39.4	29.0 ^d
<i>E. coli</i>	38.2	31.0 / 33.7	29.2 ^d

^aper cent identity was calculated from an alignment of all published sequences for each subunit (and prepublication sequences for *T. thermophilus*, *Bacillus* PS3, and *E. coli*) over the extent of the shortest sequence in the alignment for each subunit and not including the initial methionine (this corresponds to residues 3-512, 10-214, and 2-259 of Bovine subunits I, II, and III, respectively). The alignments will be published elsewhere. Sequences from plant or protozoan mitochondrial oxidases which have not been corrected for RNA editing were not included in the alignments. The formula used was: %ID = 100 X (number of identical residues) / (length of sequence in common).

^bReferences for the sequences used: Bovine (Anderson et al. 1982, Steffens and Buse 1979); *Drosophila yakuba* (Clary and Wolstenholme 1983a and 1983b); *Saccharomyces cerevisiae* (Coruzzi and Tzagoloff 1979, Bonitz et al. 1980, Thalenfeld and Tzagoloff 1980.); *Paracoccus denitrificans* (Raitio et al. 1987, Steinrücke et al. 1987); *Bacillus* PS3 (Sone et al. 1988, Ishizuka et al. 1989); *Thermus thermophilus* (Mather et al. 1990); and *Escherichia coli* (Chepuri et al. 1989).

^cIIs refers to the hydrophilic portion of subunit II which constitutes approximately the carboxy terminal three fifths of the subunit (residues 91-214 in the bovine oxidase subunit II for purposes of calculating the per cent identity).

^dSubunits III from *Bacillus* PS3 and from *E. coli* are missing sequences corresponding to an amino terminal segment present in the other oxidase subunits III (however, see the text for possible alternate locations of this sequence). The per cent identity for these two subunits III is calculated for a shorter sequence, corresponding to residues 66-259 of bovine subunit III.

FIGURE CAPTIONS

Fig. 1. Map of the region of the *T. thermophilus* chromosome containing the genes encoding the subunits of the cytochrome *caa₃* oxidase. The solid line indicates the region for which DNA sequence has been obtained. Arrows, labelled underneath as IIc, I, or III, indicate the positions of the genes for the corresponding subunits of the oxidase. Vertical marks indicate the positions of restriction endonuclease sites: B, *Bam*HI; Bc, *Bcl*I; H, *Hind*III; P, *Pst*I; and Pv, *Pvu*II. The question mark indicates the region of current ambiguity in the DNA sequence near the end of the subunit I gene/beginning of the subunit III gene.

Fig. 2. Hydrophobicity plot of *T. thermophilus* (upper dashed trace) and bovine (middle solid trace) cytochrome oxidase subunits III together with the carboxy-terminal portion of *E. coli* cytochrome *bo* oxidase subunit I (lower dashed trace). The hydrophobicities were calculated using the MPH scale of (Degli Esposti, et al. 1989), and moving averages calculated with a window of 17 residues (Kyte and Doolittle, 1982); the abscissa of the plot is taken from the position of the central residue of the window with respect to an alignment of subunit III sequences from 20 species (to be published elsewhere after finalization of the *T. thermophilus* sequence). Gaps in the plot occur where one or more sequences have inserts relative to the sequence of the plotted species (e.g., the gap between the first two peaks of hydrophobicity occurring at about position 43 to 51 is due to additional residues present in the sequence of *P. denitrificans* subunit III which do not align with the other sequences). This type of hydrophobicity plot provides an assessment of whether the various hydrophobic

and hydrophilic regions of each sequence occur in essentially the "same place". The bovine sequence was taken from: Anderson et al. (1982), the *T. thermophilus* sequence was data from our laboratory (Mather et al. 1990), and the *E. coli* sequence was prepublication data graciously provided by Dr. R. B. Gennis (Chepuri et al. 1989). The similarity of the sequences in the region of the first two hydrophobic segments (positions 1 thru 80) is relatively low; in particular the alignment of the *E. coli* subunit I carboxy-terminal sequence is speculative (not significant by statistical tests).

Fig. 3. Organization of the genes encoding oxidase subunits in several species. The relative positions of the oxidase genes are depicted for bovine mitochondrial (Bovine), *Paracoccus denitrificans* (Pd), *Bacillus* PS3 (PS3), *Thermus thermophilus* (Tt), and *Escherichia coli* (*E. coli*) chromosomes. The gene encoding subunit I of the *P. denitrificans* oxidase is found in a separate location on the bacterial chromosome; this is indicated by two slashes in the figure. Vertical bars indicate the approximate positions of DNA coding sequence segments which translate to hydrophobic amino acid segments (putative membrane spanning helices); the unfilled bars denote the first two such hydrophobic segments present in all eukaryotic subunit III genes and peptides and their putative homologs in the bacterial oxidase genes. Genes labeled I, II, III, and IV in the various species appear to encode homologous protein subunits. Orf1 (Orf = Open reading frame) from *P. denitrificans* and from *E. coli* and possibly from PS3, also appear to encode homologous peptides. "?" indicates regions where the DNA sequence was unknown or as yet uncertain. The PS3 and *E. coli* organization is based on DNA sequence data graciously provided prior to publication by Dr. N. Sone (Ishizuka et al. 1989)

and Dr. R. B. Gennis (Chepuri et al. 1989). Data for bovine mitochondria was taken from Anderson, et al (1982) and for *P. denitrificans* from Raitio, et al (1987).

Fig. 4. Comparison of the the amino acid sequences of subunits II from *Thermus thermophilus* and *Escherichia coli* in the region containing the proposed ligands of **Cu_A** (residues 130 through 195 in the *T. thermophilus* subunit II sequence, corresponding to residues 145 through 209 in the bovine subunit II sequence). Residues identical in the two sequences are shown as **OUTLINE** characters. Residues totally conserved among all cytochrome **aa₃** oxidases are overprinted by *; conserved residues proposed to function as ligands to **Cu_A** or to be involved in cytochrome **c** docking, and which are absent from the *E. coli bo* oxidase, are underprinted by x. (Taken from an alignment of bacterial oxidases to be published elsewhere.)

FIG. 1

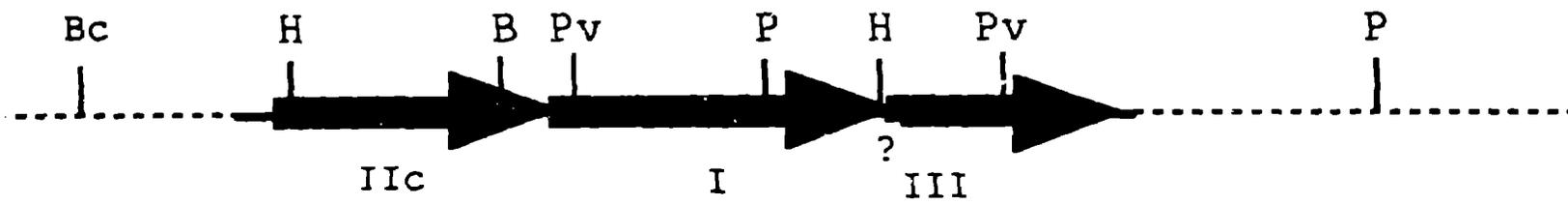


FIG. 2

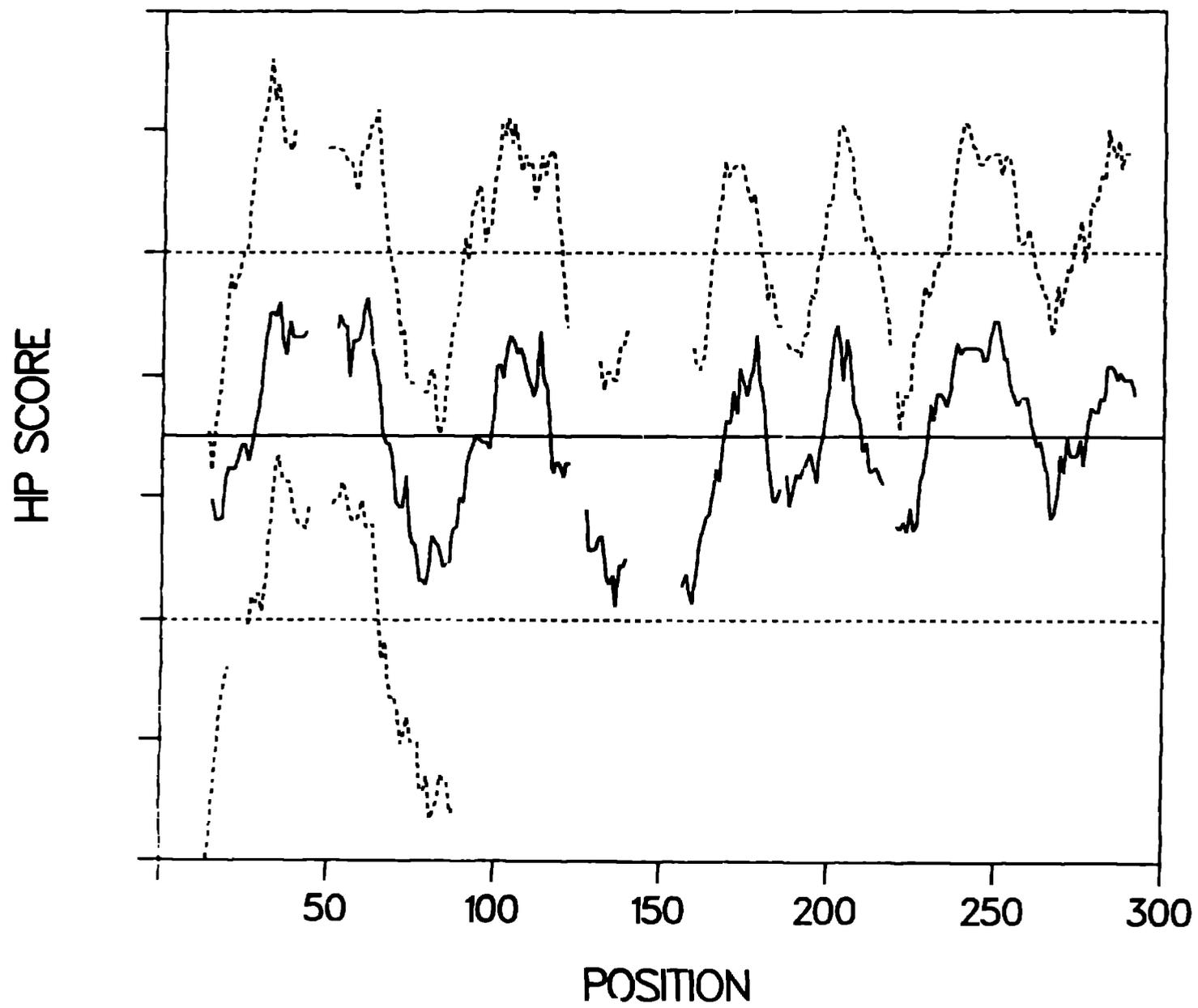


FIG. 3

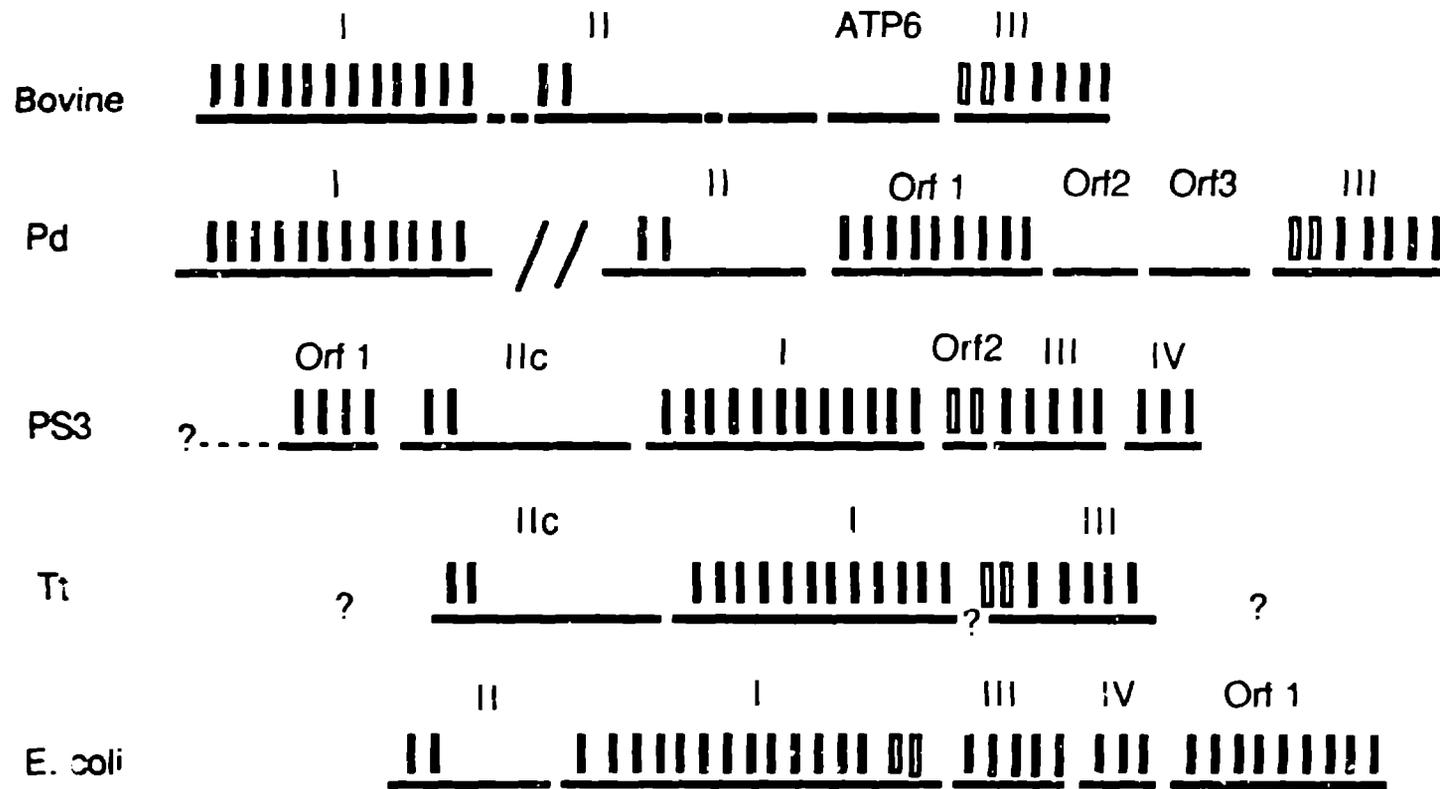


FIG. 4

<i>Tt</i>	PAGVPVELEITSKDVIHSFWVPGLAGKRDAIPG
<i>Ec</i>	PANTPVYFKVTSNSVMNSFFIPRLGSQIYAMAG
	* * * * *
	x x

<i>Tt</i>	QTTRISFEPKEPGLYYGFCAELCGASHARMLFR
<i>Ec</i>	MQTRLHLIANEPGTYDGISASYSGPGFSGMKFK
	* * * * *
	x x x x