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Cytochrome caa_3 from the Thermophilic Bacterium *Thermus thermophilus*: A Member of the Heme-Copper Oxidase Superfamily

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The subject of this short review is the cytochrome *c* oxidase (caa_3) from the thermophilic bacterium *Thermus thermophilus*. First, some of the extensive physical and enzymological results obtained with this enzyme are reviewed, and two experiments are described, involving isotope substitutions in combination with Mössbauer and ENDOR spectroscopies, which have provided novel insight into the active sites of the enzyme. Second, we summarize recent molecular genetic work showing that *Thermus* cytochrome caa_3 is a *bona fide* member of the superfamily of heme-copper oxidases. Finally, we present a rough three-dimensional model and speculate about certain features of the metal-binding sites.

KEY WORDS: Thermus cytochrome caa_3 protein sequences; metal coordination; cyanide binding; structural model.

INTRODUCTION

Evidence for the presence of a cytochrome aa_3-like enzyme in the plasma membranes of *Thermus thermophilus* was first presented in 1978.[1] This consisted of UV-visible electronic spectra showing cytochromes *a* whose spectral features were altered by reduction with NADH and by subsequent treatment with CO. Low-temperature EPR revealed the characteristic signal of a Cu_A site which disappeared upon addition of dithionite. Extraction of the membranes with detergents and fractionation with ion-exchange resins yielded a two-subunit protein having properties remarkably similar to those of eukaryotic cytochrome aa_3 but which contained a heme C associated with the smaller subunit.[2] This communication reviews some of the extensive physical and enzymological results obtained with this enzyme, summarizes data from recent molecular genetic work showing that *Thermus* cytochrome caa_3 is a *bona fide* member of the superfamily of heme-copper oxidases, and presents a rough three-dimensional model for its cytochromes *a* and a_3, and Cu_B active sites.

RATIONALE FOR WORKING WITH *Thermus thermophilus*

T. thermophilus is a nonmotile, nonsporulating, thermophilic eubacterium.[3] It has long been known that proteins from thermophilic organisms are generally more tolerant toward the rigors of isolation and characterization than those from mesophilic organisms, and the increased stability of *Thermus* enzymes (growth at $\sim 70°C$) has contributed greatly to the success of our effort, notably work with the Rieske protein.[4] It was also assumed that enzymes of prokaryotes having similar function would be hom-

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ologous to the more extensively studied eukaryotic enzymes. Additionally, study of a bacterial enzyme would permit application of powerful physical tools through isotope substitution, deductions from classical and molecular genetic manipulation, and ultimately insight from site-directed mutagenesis.

In the early stages of the project, we examined the electronic and EPR spectra of plasma membranes from several thermophilic bacteria: *Bacillus* PS3, *Bacillus caldolyticus*, and *Thermus thermopohilus* HB8.[1] All showed optical evidence for an aa_3-type oxidase, but only membranes from *T. thermophilus* HB8 yielded a high signal-to-noise Cu_A EPR spectrum that was unobscured by Mn^{2+}. In addition, essentially all the cytochrome *a* in a detergent extract of *Thermus* membranes was reversibly adsorbed to an anion exchange column, effectively removing the large amount of carotenoid in the membranes. These observations led us to undertake purification of the cytochrome aa_3 from this organism.

PURIFICATION PROCEDURES

Purification of bacterial terminal oxidases is, in general, substantially more time-consuming than cytochrome aa_3 from bovine heart tissue. This is true for enzymes from *T. thermophilus, P. denitrificans*,[5] *Bacillus* PS3,[6] and others, all of which involve detergent extraction followed by fairly standard protein purification methods. Yoshida *et al.*[7] and Hon-nami *et al.*[8] described detailed procedures for obtaining purified cytochrome caa_3 from *T. thermophilus* HB8. In our laboratory, 2 kg of wet cell paste yields ~70 mg of highly purified protein and requires ~2 months work time. The protein is stable, well-behaved, and suitable for use in a wide variety of physical and chemical experimentation.

PROPERTIES OF THE PURIFIED PROTEIN

The purified enzyme exhibits two bands in SDS gel electrohoresis experiments.[2,7,8] In our hands, the larger one runs with an apparent M_r of ~52 ± 2 kDa while the smaller one exhibits an M_r of ~33 kDa. Pyridine hemochrome analyses revealed a composition of 21.3 ± 1.1 nmol heme A/mg of protein and 8–11 nmol of heme C/mg of protein. The tentative molecular weight of this complex from protein assays is 94 kDa (somewhat larger than the sum of the two M_r values obtained from SDS-PAGE). Elemental analyses revealed the presence of ~1.1 g-atom of phosphorous, probably as a tightly bound phospholipid; 3.37 g-atom iron; and 1.85 g-atom copper. These data, along with a wide variety of physical measurements (see below), suggested a minimal enzyme unit containing 1 heme C, 2 Cu, and 2 heme A in 2 protein subunits.

Assay of heme-dependent peroxidase activity[10] revealed that the heme C was associated with the smaller subunit. Accordingly, we named this the C-protein. We presumed that the other cofactors were associated with the large subunit and named it the A-protein. Recent molecular genetic studies (see below) have greatly clarified the protein chemistry of this enzyme, revealing that the C-protein is actually a fusion protein consisting of an N-terminal subunit II and a C-terminal cytochrome *c*,[11] while the A-protein is really an N-terminal subunit I fused at its C-terminus to a subunit.[5]

CYTOCHROME caa_3 AS AN ENZYME

Early work with the bovine enzyme showed that 4 mol of cytochrome *c* were oxidized by 1 mol of O_2 with formation of 2 mol of water.[12] Later, it was discovered that purified bovine cytochrome aa_3 also "pumped" protons across a membrane,[13] and the now generally accepted "oxidase" reaction is

$$4c^{2+}_{out} + O_2 + 8H^+_{in} \rightarrow 4c^{3+}_{out} + 2H_2O_{in} + 4H^+_{out}$$

Yoshida and Fee[9] demonstrated that the *Thermus* caa_3 also reduced O_2 to H_2O, finding stoichiometries of 3.7 ± 0.2 mol c^{2+} and 1.8 ± 0.15 mol ascorbate oxidized per mole of O_2 reduced. Proton pumping experiments revealed that 0.9 ± 0.2 H^+ were extruded from vesicles for each *Thermus* cytochrome c_{552} that was oxidized; as confirmed by Sone *et al.*[14] These data demonstrate that caa_3 is a catalyst of the "oxidase" reaction.

Steady-state kinetic studies were carried out using TMPD, horse heart, and *C. crusei* cytochromes *c*, and cytochrome c_{552} isolated from the periplasmic space of

[5] Mather, M. W., Springer, P., Hensel, S., Buse, G., and Fee, J. A. Submitted. This manuscript reports the sequence of *caaB* and provides an in-depth analysis of oxidase subunit I and III amino acid sequences. In unpublished work (P. Springer and J. A. Fee) we have demonstrated that DCCD binds exclusively to the A-protein of caa_3 under the same experimental conditions that it binds to subunit III of bovine aa_3.

T. thermophilus.[9] All mediated caa_3-catalyzed oxidation of ascorbate and showed simple Michaelis–Menten kinetics with the largest V_{max} of ~130 electrons per second per mole enzyme being comparable to other oxidases.[15,16] The oxidation of organic dyes and cytochromes *c* by caa_3 is independent and additive, suggesting there are two substrate binding sites on the enzyme. We speculated that dyes transferred electrons to the *c*- of caa_3 while the cytochromes *c* transferred electrons directly to the aa_3 portion of the enzyme. Why covalent attachment of cytochrome *c*? The answer is, of course, not known, but several possibilities can be mentioned: to permit direct interaction with the bc_1 complex, to guard against loss of the cytochrome *c* at increased thermal energies or in the absence of an outer membrane as in the *Bacilli*, or to interact with periplasmic dehydrogenases.

We also examined the redox behavior of *Thermus* cytochrome caa_3.[17] The 549 nm chromophore, corresponding to the cytochrome *c*, behaves as an independent electron transfer center with $n = 1$ and $E_m = \sim 200$ mV. The 603 nm band, with contributions from both *a* and a_3, appears to report for an $n = 1$, $E_m \sim 270$ mV center and an $n = 2$, $E_m \sim 360$ mV center. The absence of new EPR signals during reductive titration (cf. Ref. 18) suggests that the a_3/Cu_B pair acts as a two-electron acceptor in this enzyme. This is to be contrasted with the behavior of the a_3/Cu_B pair in bovine enzyme which undergoes sequential one-electron transfers. The observed potentials fall within the range reported for bovine aa_3 and appear to contain contributions from cooperative interactions.

In summary, caa_3 is a cytochrome *c* oxidase which exhibits characteristically complicated redox behavior, discriminates among different electron donors, reduces O_2 to H_2O, and pumps protons.

PHYSICAL CHARACTERISTICS

Thermus cytochrome caa_3 has been subjected to a wide variety of spectroscopic examinations. The first and commonest comparison of cytochromes is made with their electronic spectra. We have previously compared an equimolar mixture of bovine aa_3 and horse cytochrome *c* with one of *Thermus* caa_3.[18] The near identity of the spectra provided evidence that the environments of the hemes in these proteins are similar. In the near-infrared spectrum, both aa_3 and caa_3 have an absorption band at ~800 nm that is thought to arise from charge transfer transitions in Cu_A^{2+}.[18] Quantitative and qualitative features of the EPR spectrum of caa_3 have been compared with those of bovine aa_3. Excepting a resonance at $g \sim 3.3$ due to the low-spin ferric heme of cytochrome *c*,[6] the spectra are essentially indistinguishable, in each case revealing one copper, Cu_A, and one low-spin ferric heme, cytochrome *a*; the potential signals from cytochrome a_3 and Cu_B are eliminated through spin coupling between these centers.[18] EXAFS studies by B. Chance and his colleagues revealed the X-ray absorption properties of the Cu and Fe centers in caa_3 to be indistinguishable from those observed with the bovine enzyme.[19] Ogura *et al.*[20] examined the resonance Raman properties of *Thermus* caa_3, noting their similarity to those of the bovine enzyme, and substituted ^{54}Fe for the naturally occurring ^{56}Fe to demonstrate that the resonance Raman band at 210 cm^{-1}, observed in fully reduced oxidases, is due to an Fe–N(His) vibrational mode (see also Ref. 18). Fourier-transform infrared studies analogous to those reported by Alben and co-workers[21] with bovine aa_3 showed that upon photolysis of the fully reduced, CO-liganded enzyme the CO leaves the Fe and binds to the Cu_B.[22] This result provides strong qualitative evidence for functional and structural similarity of the a_3/Cu_B site in the two enzymes. A comparative ENDOR study of Cu_A in caa_3 and bovine aa_3 revealed the presence of two coordinated ^{14}N atoms in both enzymes (Gurbiel *et al.*, submitted)[7] (see below). Enriching the protein with ^{57}Fe permitted the first high signal-to-noise Mössbauer studies of a cytochrome *c* oxidase. This work confirmed previously made assignments of low-spin ferric cytochromes *a* and *c* and high-spin ferric cytochrome a_3 spin coupled to an $S = 1/2$ center (Cu_B^{2+}) in the oxidized protein, and low-spin ferrous cytochromes *a* and *c* and high-spin ferrous cytochrome a_3 in the fully reduced protein, with a_3 being converted to the low-spin configuration upon binding CO. This body of spectral data demonstrates unequivocally that

[6] The EPR signal at $g = 3.37$ combined with the size of the C-protein led us to suggest that this protein may be a cytochrome c_1 [2]. However, the sequence of this protein shows no relation to that of bovine cytochrome c_1 [11], and we have discontinued the use of c_1 aa_3 to specify this enzyme.

[7] Gurbiel, R. J., Werst, M. M., Surerus, K. K., Fee, J. A., and Hoffman, B. M. Submitted. This manuscript describes a 35 GHz ENDOR study of ^{14}N and $^{63,65}Cu$ resonances in cytochromes aa_3, ba_3, and caa_3. We were able to obtain the latter from cells grown in the presence of labeled histidine.

aa_3-type enzymes from these widely divergent species retain fundamentally similar active site environments.

Two examples follow which demonstrate how isotope substitution permits spectroscopic studies not otherwise possible, often yielding powerful insight on active site structure.

Mössbauer Studies of the Cyano Complex of Fully Oxidized Cytochromes (c)aa_3. The structure of the cyano complex of aa_3 remains controversial. When oxidized aa_3 is exposed to HCN, the optical spectrum changes, suggesting that high-spin ferric a_3 is converted to a low-spin ferric compound;[23] no new EPR signals appear, but there is a decrease in magnetic susceptibility.[24,25] Caughey and co-workers[26] have studied this system with infrared spectroscopy, concluding that CN^- is bound to Cu_B but not to Fe and suggesting a schematic structure:

$$Fe^{3+}_{LS} - X - Cu^{2+}_B - CN^-, \qquad X \neq CN^-$$

Thompson and Greenwood and their co-workers[27] have examined the magnetic field and temperature dependence of the MCD spectrum of this cyano complex, concluding that the electronic ground state is an $m_s = \pm 1$ doublet, i.e., a ferromagnetic coupling occurs between Fe^{3+}_{LS} and Cu^{2+}_B. They propose a schematic structure in which the four atoms lie on a line:

$$Fe^{3+}_{LS} - CN^- - Cu^{2+}_B$$

Münck and his co-workers[28] found that the 4 K Mössbauer spectrum of ^{57}Fe-enriched cyano-caa_3 from *Thermus* consisted of a doublet of very narrow lines superimposed on broad spectral features due to low-spin ferric cytochromes *a* and *c*. The $\Delta E_q = 1.13$ mm/s and $\delta = 0.22$ mm/s Mössbauer parameters are typical of low-spin heme-cyano complexes, thereby providing compelling evidence for an Fe–CN bond. Moreover, when a very weak magnetic field (60 mT) is applied to the sample, the sharp doublet broadens dramatically. This results from a mixing of the $m_s \pm 1$ states and indicates they are split by $D < 0.3\,cm^{-1}$, a result fully consistent with the conclusions of Thomson *et al.*[27] The experiment is shown in Fig. 1A and B.

The extreme sharpness of the lines in the a_3-CN compound of caa_3 suggested to Palmer and his co-workers[29] that a similar signal might be observed in bovine aa_3 without ^{57}Fe enrichment. As shown in Fig. 1C and D, this proved to be the case. Again the Mössbauer parameters are typical of a low-spin heme-cyano complex, and the broadening effect of the applied magnetic field indicates ferromagnetic coupling

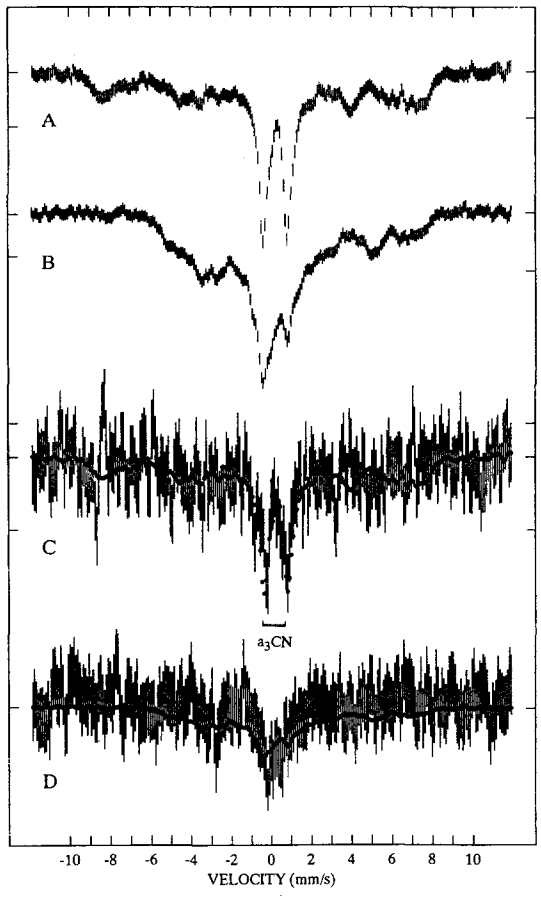


Fig. 1. Mössbauer spectra of the cyano derivatives of oxidized *Thermus* caa_3 (A and B) and bovine aa_3 (C and D). The temperature is 4 K, and a magnetic field of 60 mT was applied during the recording of B and D. Other details are given in Refs. [28] and [29].

of the Fe to Cu^{2+}_B yielding $S = 1$ with $m_s \pm 1$ ground state. The combined IR, MCD, and Mössbauer data suggest a schematic structure

$$Fe^{3+}_{LS} - CN^- - Cu^{2+}_B - CN^-$$

in which the Fe, CN, and Cu_B are colinear, but the CN^- bound to Cu_B is not necessarily in line with the other atoms. The structure of the oxidized cyano complex is nominally identical in both enzymes.

High-field ENDOR of the Cu_A site in caa_3. Cu_A posseses unusual spectroscopic properties.[30] It has an intense (ε = 2,000–3,000 $M^{-1}cm^{-1}$) optical absorption band located at ~800 nm and an EPR signature that has puzzled biophysicists for many years. Peisach and Blumberg[31] set the tone for research on this center in 1966 with their suggestion that the unpaired electron of the system resided predominantly on a

sulfur atom, thereby showing some of the properties of an RS$^{\cdot}$ radical. Steffens *et al.*[32] recognized a unique sequence in subunit II of bovine oxidase that could serve as the Cu_A site, the so-called "Cu_A motif" which contains two conserved histidines and two conserved cysteines (see below). Chan and co-workers, among others, combined these ideas and have championed $\{(His)_2(Cys)_2\}$ ligation;[30] Covello and Gray[33] further noted the conserved methionine in the "Cu_A motif" and suggested that this residue may also coordinate Cu_A, as noted in some "blue" copper proteins.

High-field (35 GHz) ENDOR spectroscopy provides a frequency window in which only ^{14}N (or ^{15}N) resonances appear (cf. Ref. 34), allowing them to be examined without interference from ^{1}H signals. However, when multiple resonances are overlapping, it may remain impossible to determine the number of contributing resonances. Substitution with ^{15}N (which lacks a quadrupole moment) greatly facilitates counting equivalent resonances and obtaining their coupling constants (see, for example, Ref. 34). Further, if only the coordinating amino acid is labeled with ^{15}N, one also demonstrates the nature of that amino acid. Once the coupling constants are known, the field dependence of the ^{14}N signal can provide detailed three-dimensional structural information about a paramagnetic center.[34] The ENDOR spectra of [U-^{14}N]caa_3 and [U-^{14}N; δ, ε-$^{15}N_2$-histidine]caa_3, shown in Fig. 2, demonstrate that two histidines coordinate Cu_A in this protein. Hoffman and his co-workers have examined the 35 GHz ENDOR spectra of bovine aa_3 and the *Thermus* enzymes caa_3 and ba_3 in great detail (see footnote 7), and the results demonstrate that the Cu_A site is nominally identical in the three proteins. Additional isotope substitution studies now underway in this laboratory with *Thermus* caa_3 should provide further tests of the above structural hypotheses and provide new information on spin distribution and electronic structure.

These examples illustrate how the homologous bacterial oxidases, subject to facile isotopic manipulation, can provide information about all the heme-copper oxidases.

MOLECULAR GENETIC STUDIES

It is now known that *Thermus* cytochrome caa_3 contains the common structural motifs of all heme-copper oxidases including protein subunits I, II, and III. However, the code for this information and Nature's embellishments to it are novel and interesting in their own right. Scheme I shows the arrangement of the two *caa* genes on the *Thermus* chromosome.

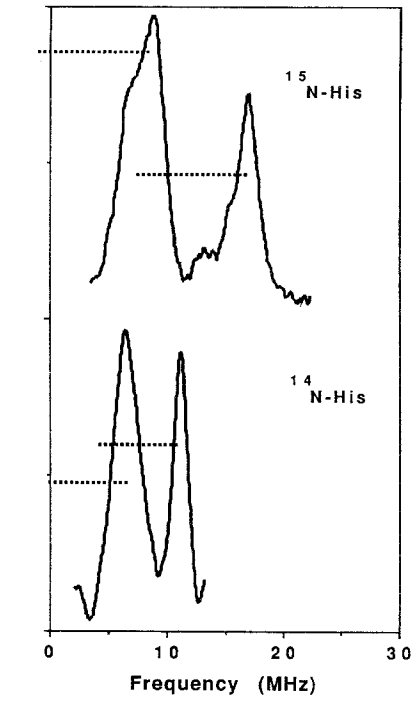


Fig. 2. ^{14}N and ^{15}N ENDOR spectra of Cu_A in *Thermus* cytochrome caa_3. The upper spectrum was obtained from enzyme grown under normal conditions [7], and the lower spectrum was obtained from cells of a His$^-$ strain grown in the presence of [δ,ε-$^{15}N_2$] histidine. Details are given in Gurbiel *et al.* (Footnote 7).

Scheme I

The structural genes of caa_3 appear to be on a single operon with the promoter somewhere upstream of *orfI* and a termination palindrome immediately downstream from *caaB*[11] (see footnote 5). *orfI*, which terminates only a few nucleotides upstream from the first codon of *caaA*, bears good resemblance to the predicted *P. denitrificans orfI* gene product[35] and the *B. subtilis* CtaB protein (Ref. 36; Saraste, personal communication), the products of which are thought to be essential for proper assembly of aa_3 in those organisms.

Translations of *caaA* and *caaB* have been made[11]

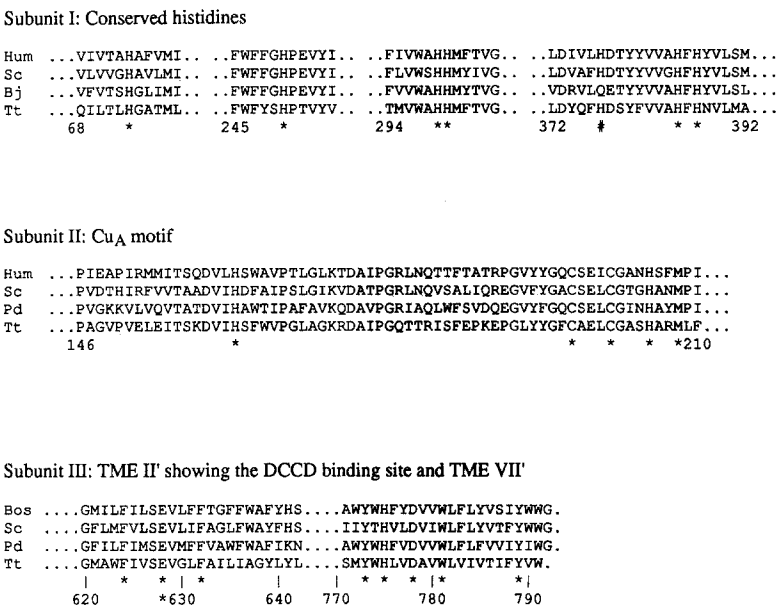


Fig. 3. Partial sequence comparisons of *Thermus* caa_3 subunits with other oxidase sequences. Hum, *Homo sapiens*; Sc *Sacharomyces cerevisiae*; Bj, *Bradyrhizobium japonicum*; Tt, *Thermus thermophilus*; Pd, *Paracoccus denitrificans*.

(see footnote 5) and selected portions are compared with other oxidase sequences in Fig. 3. As noted above, *caaA* encodes a fusion protein consisting of a typical subunit II amino acid sequence whose C-terminus is fused to the N-terminus of a typical cytochrome *c* amino acid sequence (see footnote 5). Similarly, *caaB* encodes a fusion protein consisting of a typical subunit I amino acid sequence whose C-terminus is fused to the N-terminus of a typical subunit III amino acid sequence. In the following, we treat these as units, although it is possible that the I/III protein has been cleaved somewhere downstream to transmembrane helix III′ in subunit III (see footnote 5 below). Extensive statistical comparisons of these translated amino acid sequences with other, relevant amino acid sequences have been made, and we are convinced that *Thermus*' subunits I, II, and III are homologous to all known subunits I, II, and III[11] (see footnote 5). Some complications in deciding on homology are discussed.

Homology, among phylogeneticists, means derived through evolution from a common ancestor.[37] The farther apart evolution has taken two species, the more difficult it may be to recognize this relationship between their respective proteins. In sequence comparisons, this is most evident by amino acid substitutions which can occur to a very significant extent. For example, subunit I of *Thermus* caa_3 is 38.8% identical to that of the bovine enzyme but only 18.6% identical with subunit I of *S. acidocaldarius* (see footnote 5). Additional genetic mechanisms can obscure homology.[38] We previously noted that the N-terminal sequence of subunit III could reside either on an extended subunit I in combination with an attenuated subunit III or remain as the originally observed ("holo" or mitochrondrial type) subunit III.[39] The occurrence of fused genes, in *Thermus* as well as other microorganisms,[40] suggests that collateral functionalities can be "captured" by a gene in this fashion, thereby obscuring the relation of the resulting enzyme to its homologs. Additional variations on this theme will likely be discovered as more bacterial enzymes are studied. Therefore, in deciding on homology, we have considered several factors including similarity of biological function, active-site structures, hydropathy profiles, and placement of conserved amino acids within 1°/2° models (see below). We have already discussed the first two of these.

Hydropathy Profiles and Secondary Protein Structure. The calculated hydropathy plots of all subunit I, II, and III oxidase sequences have short regions where apolar residues predominate and which are generally thought to form helical structures that traverse the membrane. There are 12 such regions in subunits I,

two in subunits II, and seven in subunits III. Figure 4 shows alternating 1° and 2° dimensional models for the *Thermus* caa_3 sequences. All known oxidase sequences display this pattern of alternating hydrophilic–hydrophobic regions of sequence, and expression of fusion genes supports the alternating pattern shown in Fig. 4.[41,42] Conserved residues occur at very nearly the same positions within this pattern. Notable among many examples of this are the DCCD binding site of subunit III[43] and the conserved histidine residues in subunits I and II (see Fig. 3). The topological models contain a variety of other information.

Charge and Mass Distribution. The 1°/2° models of Fig. 4 permit one to calculate some features of the molecule as a whole, and these are presented in Fig. 5. Each component has been drawn with an area roughly proportional to the number of amino acids represented in the models of Fig. 4. Some very interesting observations can be made from these numbers. First, the overall charge on the molecule is only 1^-, yet the number of charges on the *out* side of the complex is 13^-, while on the *in* side it is 11^+; potentially there are two + charges and one − charge within the membrane. Subunit I is net positively charged (9^+), subunit II is net negatively charged (7^-), and subunit III is weakly negative (3^-). More of the mass is on the *out* side of the membrane, where subunit II is the major contributor, than on the *in* side; the largest fraction of the mass is actually integral to the membrane. The strong electric dipole across the membrane may have some functional significance in assembly and/or vectorial transport of protons. In this regard, it would be very interesting to compare similar diagrams obtained from other oxidase sequences.

Distribution of the Metals. The question of where the metals are bound is an important one that has been approached in a variety of ways for some time (cf. Ref. 14). By analogy to other heme and copper proteins, histidine residues are likely to be important in metal coordination, and considerable physical evidence now indicates two histidines are bound to Cu_A (see above), two to cytochrome *a*, one to cytochrome a_3, and three or four to Cu_B. This adds up to eight or nine histidines involved in metal coordination. There are two histidines conserved in subunit III (see footnote 5), but these are not involved in coordinating the cannonical metals because subunit II can be selectively removed from the bovine enzyme without loss of function,[45,46] and it is often lost from bacterial enzymes. Within subunit II there are only two conserved histidine residues, and these are found in the "Cu_A motif" (see Fig. 3). In subunit I there are six conserved histidines and an additional one that is present in all sequences except that from *B. japonicum*[47,48] where it is replaced with a glutamine (see Fig. 3). This "seventh" histidine (or glutamine) could be a ligand to Cu_B in some states of the enzyme, but present information does not permit definite statements. The other conserved histidines are all reasonably accounted for as metal ligands.

Comparisons between members of the heme-copper oxidase family now permit reasonable assignments of metal binding sites. Cytochrome *bo* from *E. coli* is a two-heme, one-copper enzyme that lacks Cu_A but is composed of subunits, including I and II, that are homologous to those of cytochrome aa_3.[49] The 6(7) conserved histidine residues of subunit I reasonably support the low-spin cytochrome *b* (analogous to cytochrome *a*), the high-spin cytochrome *o* (analogous to cytochrome a_3), and a Cu_B site. While the subunit II of *E. coli* cytochrome *bo* is homologous to other subunits II, it differs by having mutations in the "Cu_A motif" that would prevent this stretch of sequence from being a Cu-binding site (see Ref. 11 for discussion). Accepting the functional similarity[50] of cytochromes *bo* and aa_3 and the homology of their proteins, places two hemes and one Cu in subunit I of both proteins and requires all the conserved histidines to serve as metal ligands. The direct demonstration of two histidines bound to Cu_A in *Thermus* caa_3 by ^{15}N-histidine substitution leads to the conclusion that Cu_A is associated with the "Cu_A motif" of subunit II (see Fig. 3) because this region of sequence contains the only conserved histidine residues which remain unassigned.

Location of the Cu_A site. Some idea of the location of Cu_A with respect to the membrane can be obtained by perusal of Figs. 4 and 5. Subunit II is anchored to the membrane by two membrane-spanning helices and has a relatively large hydrophilic portion facing the periplasmic (intermembrane) space. The "Cu_A motif" is well into the hydrophilic portion of the sequence, and it is difficult to imagine a close approach (< 10 Å) of Cu_A to cytochrome *a* (see below). However, previous estimates of this distance in bovine aa_3 suggest a distance between 8 and 13 Å.[51,52]

Location of the a, a_3, and Cu_B sites. As shown in Fig. 3, the conserved histidine residues lie within otherwise hydrophobic stretches of amino acid sequence that are visualized as α-helices in Fig. 4, where the conserved histidines are found near the

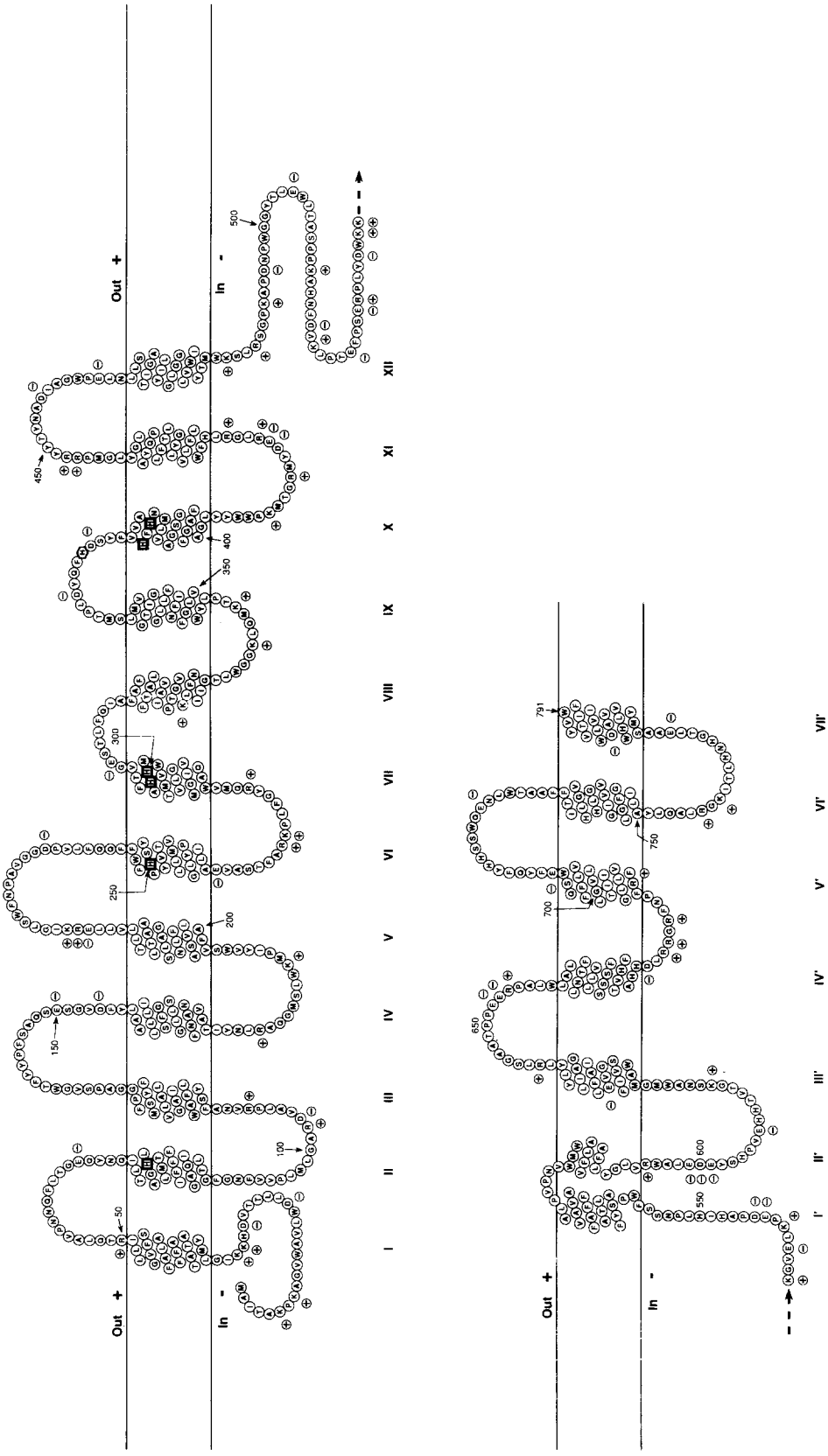


Fig. 4. Primary and secondary strucral models of the subunits of *T. thermophilus* cytochrome caa_3. (A) Subunit I/III. Conserved histidines are denoted by squares in helices II, VI, VII, and X, while the "seventh" histidine is denoted by a hexagon in the external loop between helices IX and X. (B) Subunit II. Possible metal ligands in the "CuA motif" are indicated by an underlying caret. Sequence information for subunits I/III is taken from Mather *et al.* (Footnote 5) and that of IIc is taken from [11].

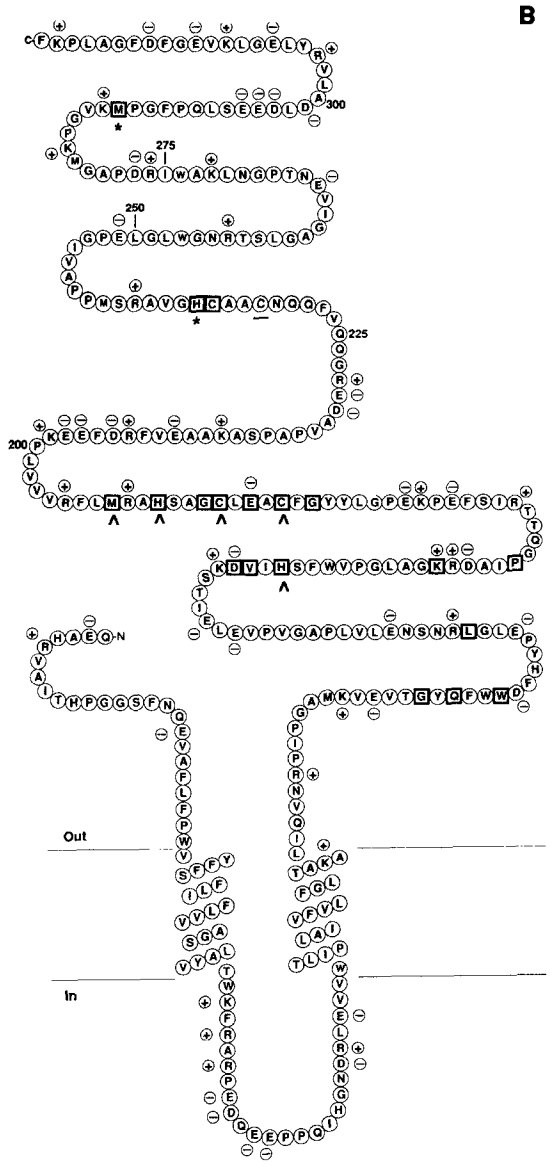


Fig. 4. Continued.

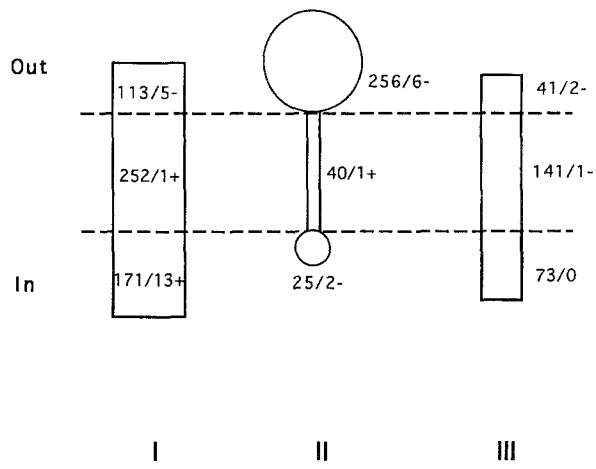
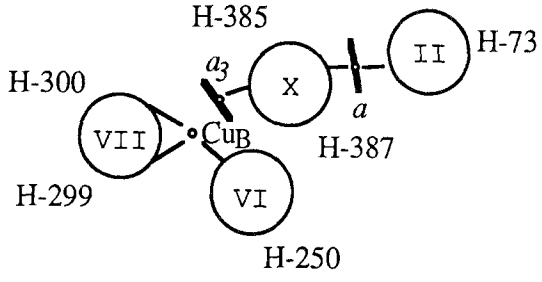


Fig. 5. Cartoon presentation of charge/mass relations of the *Thermus* cytochrome caa_3 derived from the 1°/2° models of Fig. 4.



Scheme II

outer side of the membrane. This suggests that cytochrome *a* and the cytochrome a_3/Cu_B pair may lie on a plane roughly parallel with that of the membrane. The site-directed mutagenesis work of Gennis, Anraku, and Ferguson-Miller and their co-workers with cytochromes *bo* and aa_3 have implicated the six conserved histidines in subunit I with specific cofactors.[53-55] A current interpretation of these data is shown in Scheme II using *Thermus* numbering:

Toward a Molecular Model of caa_3. We have constructed a three-dimensional model of this region of the molecule using a procedure suggested by Dr. David Richardson of Duke University. The helices are constructed by sliding 11/16″ Tygon tubing over metal rods attached via a movable connector to a 3-cm-diameter magnetic base; the latter allows facile location on a steel base plate. The size of the tubing defines a scale of 0.51 cm/Å. The helices are visualized by wrapping helical nets, printed to the correct scale, around the tubes and taping them in place. Points marked on the paper denote the position of the α-carbons. The β-carbons can be visualized by inserting a push pin into the tubing at the appropriate angle and correspond roughly to the portion of the pin against the tubing; the head of the pin must be ignored. Side chains are easily constructed from soft aluminium (welding) wire and inserted into a small hole drilled into the base of the push pin. Hemes were similarly constructed. The metals were made by wrapping fine copper wire about itself and were suspended in the model with the same. A scale model of Scheme II was

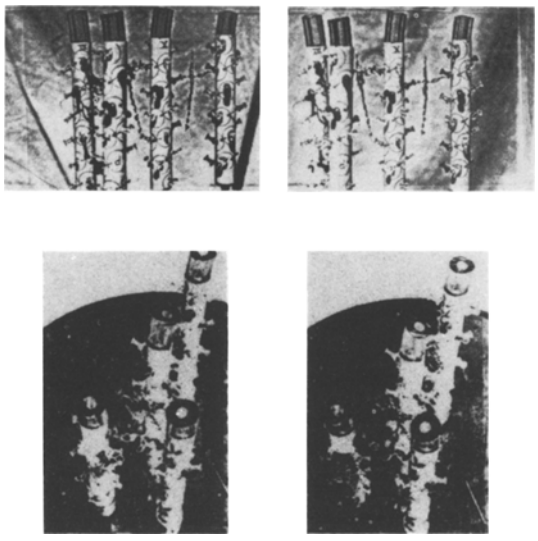


Fig. 6. Stereo presentation of photographs showing a rough three-dimensional model for the arrangement of cytochrome *a* and the cytochrome a_3/Cu_B pair of cytochromes aa_3. Details become apparent with the use of a standard stereoscope.

thus constructed and stereo photos of two views are shown in Fig. 6.

Cytochrome *a* fits nicely between two helices where it is coordinated to His-73 from helix II and His-387 from helix X. The propionyl side chains reach the outer plane defined by the membrane but are surrounded by apolar side chains from both helices II and X. If ionized, their charges could be compensated by complementary charges in the external loops. On the opposite side of helix X from His-387 lies His-385 which coordinates cytochrome a_3. The propionate carboxyl groups of this heme also lie near the projected outer plane of the membrane but are surrounded by fewer apolar side chains than the *a*-heme (see below). The polyisoprenoid side chain from each heme extends to approximately halfway between the planes of the membrane within the hydrophobic interior of the molecule. The two iron atoms lie just below the plane defined by the *out* side of the membrane, and the distance between them, in this model, is ~15 Å, a value that falls within the range predicted from spin relaxation studies.[51,56,57] The approximate distance between helices II and X is 18 Å, which is somewhat large as helices tend to pack with a center-to-center distance of ~10 Å (D. C. Richardson, personal communication). His-250 on helix VI and His-299 and His-300 on helix VII can be brought into close proximity to form a binding site for Cu_B which lies ~2 Å below the line connecting *a* and a_3. As photographed in Fig. 6, the interhelix distances are all about 16–18 Å, which brings the copper atom to within 5–6 Å of the a_3 Fe atom. This is too great a distance between these two centers, which is predicted from EXAFS work to be only ~3.8 Å.[19] In order to achieve this distance in the present model, however, helices VI and VII must approach much closer to helix X than shown here.

Manipulation of the model indicates that forcing the metals to approach more closely than this is likely to cause severe steric crowding between helices VI/VII and X. One mechanism to avoid this is to unwind helix VII beyond position 297 or 298 such that Cu-coordinating atoms of H-299 and H-300 along with that of H-250 can form a plane roughly parallel with that of the a_3-heme. Such an arrangement might allow the "seventh" histidine, H-377 (on the loop between helices X and XI), to have access to Cu_B.[8] We are currently examining this region of the model with computer graphics.

We have argued elsewhere (see footnote 5) that helix VIII might be in proximity to helices VII and X. Helix VIII has three rather hydrophilic residues that are conserved and present themselves along one face. When thus brought into close proximity to a_3, it is apparent that the a_3-heme binding pocket contains quite a large number of conserved residues and is more polar than that of the *a*-heme in this model. If real, this could contribute to differences in the apparent redox potentials of the hemes.

While imprecise, the model is of considerable value in orienting the observer to relative sizes and distances, and angular relations between components; it promises to be a good starting point for pursuing computer graphics studies of selected regions of the model.

CONCLUSIONS

Cytochrome caa_3 from *Thermus thermophilus* was one of the first heme-copper oxidases to be isolated

[8] A recent study of the cyano compound of *Thermus* cytochrome ba_3 shows that four ^{14}N atoms bind to Cu_B, forming a tetragonal complex [58]. Because there appear to be no changes in coordination at the other centers, at least seven histidines would be required to form such a complex. From the sequence of this portion of the ba_3 protein (J. A. Keightley, M. W. Mather, and J. A. Fee, unpublished results), it is reasonable that the "seventh" histidine participates in this compound as a ligand to Cu_B.

from bacterial plasma membranes (1980). Its composition, enzymatic and redox behavior, the detailed structural features of its metal centers, and its protein sequences all indicate its homologous relation to other cytochromes aa_3. Deductions from experimental observations made with this protein are thus generally transferable to other aa_3-type oxidases.

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