A Novel Heme a Insertion Factor Gene Cotranscribes with the *Thermus thermophilus* Cytochrome *ba*3 Oxidase Locus

Carolin Werner, Oliver-Matthias H. Richter, and Bernd Ludwig*

Molecular Genetics, Institute of Biochemistry, Goethe University, Max-von-Laue-Strasse 9, D-60438 Frankfurt am Main, Germany

Received 12 May 2010/Accepted 29 June 2010

Terminal oxidases catalyze the reduction of molecular oxygen to water, coupling the available redox energy to proton translocation across the cytoplasmic (or mitochondrial inner) membrane. Most oxidases belong to the heme/copper superfamily, which is quite diverse in terms of electron donors, subunit compositions, and heme types (30). Two different terminal cytochrome *c* oxidase complexes have been described for the extremely thermophilic eubacterium *Thermus thermophilus*, both members of this superfamily. In contrast to the *caa*3 oxidase, the *ba*3 oxidase is expressed under limited oxygen supply (20) and is composed of three protein subunits, I, II, and III. The most prominent features are associated with subunit I, the core element of the complex, where a protein scaffold consisting of 13 membrane helices provides the ligands for hemes *b* and *a*3 (23). Heme *a*3 together with Cu₃p forms the active site, and in *Thermus* is characterized by the presence of a hydrophobic hydroxyethyl geranyleranyl moiety instead of the hydroxyethyl farnesyl side chain found in conventional *a*-type hemes. All of the *N* termini of the three subunits are located on the cytoplasmic (negative) side of the membrane, whereas the hydrophilic domain of subunit II containing the primary electron acceptor, a mixed-valence binuclear Cu₄A center, faces the periplasmic space (32).

The biogenesis of cytochrome *c* oxidases involves numerous steps in which specific chaperones assist the translation of subunits, their insertion or translocation across the membrane, the integration of essential cofactors, and the assembly and final maturation of the enzyme complex. This assembly process, and in particular the exact order of events, is still not very well understood, and most effort has been directed to the mitochondrial enzyme (14, 17, 21, 36)

In this work, we show that the *ba*3 locus comprising the three structural genes for the complex (*cbaDBA*) also encodes two additional putative proteins (*CbaX* and *CbaY*) downstream from the subunit I gene on the same transcript. One of them, *CbaX*, is obviously required for proper maturation of the enzyme, because its deletion results in a severe deficit in the heme *a* content and consequently a drastic decrease in *ba*3 oxidase activity. From the analysis of the cytochrome *ba*3 transcription unit and from complementation studies, we conclude that *CbaX* is critically involved in heme *a* incorporation into the high-spin site of subunit I of the *ba*3 cytochrome *c* oxidase. Even though it lacks any sequence homology, it shares essential functional similarities with the previously identified Surf1 protein (5, 6, 25, 31, 35), which is required for the biogenesis of the mitochondrial oxidase and many bacterial oxidases. Furthermore, we demonstrate that an unbalanced expression of *CbaX* and the oxidase subunit genes leads to incorporation of heme *a* even into the low-spin site of the enzyme.

**MATERIALS AND METHODS**

Construction of the *ba*3 deletion strains. All deletion strains (Fig. 1) were obtained by double homologous recombination using suicide plasmids, each a derivative of pUC18. The structural genes of the *ba*3 oxidase alone (see i below), as well as together with a 56-bp fragment downstream of subunit I (see ii below), were replaced by the bleomycin resistance gene under the control of the surface layer protein *A* gene promoter (sl*Ap*) (13). The bleomycin resistance gene, including sl*Ap*, was amplified via PCR using the plasmid pWUR-bleo (4) and the primer pair 17/18 (primer sequences are listed in Table 1). The forward primer (primer 17) introduced an XbaI site, and the reverse primer (primer 18) introduced a PstI site. (i) Upstream and downstream regions flanking the *ba*3 locus (flank A and flank B) were amplified via PCR using *T. thermophilus* genomic DNA. For flank A (genomic positions 747813 to 748315) the forward primer (primer 1) introduced an EcoRI site, and the reverse primer (primer 2) introduced an Xbal site. For flank B (genomic positions 750807 to 751361), the forward primer (primer 3) gave rise to a Psrl site, and the reverse primer (primer 4) introduced a HindIII site. The resulting products (506 bp for flank A and 558 bp for flank B) were digested and cloned via a four-claw ligation, together with the digested bleomycin resistance gene, into pUC18, leading to pWE8 (Fig. 1C). (ii) Flank A was produced as described above; for flank B (genomic positions 750861 to 751361), the forward primer (primer 5) gave rise to a Psrl site...
TABLE 1. Sequences of primers used in this work

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCAGGAATTCGGATCCTACAGCAAGGTGCCC</td>
</tr>
<tr>
<td>2</td>
<td>GCCTCAGATTAAGAACCAGCCGCCAC</td>
</tr>
<tr>
<td>3</td>
<td>AAACCATCGCAAGAGCTCTTCGCGCCG</td>
</tr>
<tr>
<td>4</td>
<td>CCACAGCTTGGCAGAGGAGGTAGAGCAG</td>
</tr>
<tr>
<td>5</td>
<td>GTAACTCTGCGCCTAGGCCCTTTGACC</td>
</tr>
<tr>
<td>6</td>
<td>ATATCGACGATATGGAAGAAAGCCAAAGGGCC</td>
</tr>
<tr>
<td>7</td>
<td>GTGATGTTGATGTTGATGTTGATGCTGATCCGCCGGGAGGTTCGCCCGCTTCCAGC</td>
</tr>
<tr>
<td>8</td>
<td>CTCATACCATACATACATACATACATACATACATTACGAGGAGGTAGAGCAG</td>
</tr>
<tr>
<td>9</td>
<td>GTATTATCGCAAGCTCAGACAGCCGGCCACCCGGC</td>
</tr>
<tr>
<td>10</td>
<td>CTTTGGTCCATGCCCTTCAACTCC</td>
</tr>
<tr>
<td>11</td>
<td>GAGCTATCTGAGCTTACCCAGGATAGG</td>
</tr>
<tr>
<td>12</td>
<td>GCAGCCCGGGGGATCCACTAGAGG</td>
</tr>
<tr>
<td>13</td>
<td>TCAACGCCGGGGCCTTCCAGAG</td>
</tr>
<tr>
<td>14</td>
<td>ATCTCCGACGCGCAAAG</td>
</tr>
<tr>
<td>15</td>
<td>TTAGAATGTGCTGATCATCGACAGGCGGAGG</td>
</tr>
<tr>
<td>16</td>
<td>ATGGCCAATTTGACCAGTGCCGTT</td>
</tr>
<tr>
<td>17</td>
<td>CGCATCAGAGGCGGCGCATCATTCCAG</td>
</tr>
<tr>
<td>18</td>
<td>ATAGCTCGACGCTTCCGCGTCAGATCCTTGTGAG</td>
</tr>
</tbody>
</table>

*See Materials and Methods.*
difference spectra, samples were diluted in 20% (vol/vol) pyridine, 0.1 M NaOH. Native (see i below) and pyridine hemochromogen (see ii below) redox spectra were recorded, and the heme/protein concentrations were determined using the Lowry determination (see above) and the following extinction coefficients: (i) \( \Delta A_{500-600} \) 26 cm\(^{-1}\) M\(^{-1}\) for heme \( b \) (8), and \( \Delta A_{515-528} \) nm 6.3 cm\(^{-1}\) M\(^{-1}\) for heme \( a \) (37); (ii) \( \Delta A_{557-540} \) nm 22.1 cm\(^{-1}\) M\(^{-1}\) for heme \( b \) (33), and \( \Delta A_{563-620} \) nm 21.7 cm\(^{-1}\) M\(^{-1}\) for heme \( a \) (33). For cyanide binding spectra, samples were reduced with dithionite, and potassium cyanide (neutralized) was added to the samples to a final concentration of 100 nM.

Enzymatic-activity measurements. Activity measurements were recorded at room temperature on a Hitachi U-3000 spectrophotometer using 20 \( \mu \)M reduced \( T. \) thermophilus cytochrome \( c_{552} \) (50 mM Tris-HCl, 0.02% [wt/vol] DDM, pH 8.0; \( \Delta A_{562} \) nm = 21 cm\(^{-1}\) M\(^{-1}\)).

RESULTS

The cytochrome \( ba_3 \) oxidase locus contains additional ORFs in its transcription unit. The aim of the project presented here was to establish the organization of the cytochrome \( ba_3 \) (\( cba \)) locus, since the genomic sequence identifies two additional open reading frames (ORFs) only 56 bp downstream of the three structural genes of the \( ba_3 \) oxidase itself (Fig. 1A). A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of ORF 1 (here termed \( cbaX \)) did not reveal any specific motif for the putative protein (CbaX) and indicated similar sequences only in \( T. \) aquaticus (67% sequence identity) and two \( M. \) thermus strains (\( M. \) silvanus, 34% sequence identity; \( M. \) thermus \( ruber, 35% \) sequence identity). A topology search with TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) identified the gene product as a membrane protein of 156 amino acids (16.8 kDa) and predicted three transmembrane helices; with respect to its functional role, we note that the protein lacks any histidine residues as potential heme ligands (see Discussion). The gene product of ORF 2 (\( cbaY \), overlapping with \( cbaX \) by 4 nucleotides) aligned with a number of sequences, mostly annotated as permeases, with a conserved major facilitator superfamily (MFS) domain.

The intergenic distance between \( cbaA \) (encoding subunit I [Fig. 1A]) and \( cbaX \) is 56 bp and therefore long enough to potentially accommodate its own promoter element. To address this issue, total RNA was isolated from \( T. \) thermophilus HB27 wild-type cells, and cDNA was synthesized using a gene-specific primer that binds at the 3′ end of \( cbaX \) (see Materials and Methods). The primers used for PCR with this synthesized cDNA (Fig. 2) give rise to a product only if \( cbaX \) is part of the same transcription unit as the preceding \( ba_3 \) structural genes. The PCR product obtained had a size identical to that of the 1,030-bp positive control. This clearly shows that the \( ba_3 \) transcription unit includes at least \( cbaX \), so that the 56 bp of intervening DNA most likely contains a ribosome binding site (RBS) but no separate promoter acting on \( cbaX \). Due to the low degree of consensus between different RBSs in \( T. \) thermophilus (11), its exact position remains unclear. The \( cbaX \) and \( cbaY \) reading frames overlap by 4 nucleotides, strongly suggesting that the downstream \( cbaY \) reading frame is part of the \( cbaX \) transcript, as well.

Deletion of \( cbaX \) leads to severe loss of heme \( a \) in the purified \( ba_3 \) oxidase. To assign the \( cbaX \) gene product a functional role, a genomic deletion of the structural genes (as well as the promoter region) of the \( ba_3 \) oxidase, including the downstream stretch of 56 bp, was generated (strain WE6) (Fig. 1 B), preserving, on the other hand, as much of the ORFs in question as possible. Assuming an RBS was present within the 56 bp downstream of \( cbaA \), this deletion strain should no longer have been able to express the CbaX protein. Strain WE6 was then complemented by a plasmid encoding only the \( ba_3 \) subunits (pWE4) (Fig. 1 D). For ease of purification, subunit I was provided with an internal 12-mer His tag located on a cytoplasmically oriented loop (Fig. 3) between transmembrane helices IV and V, thus avoiding any interference with cytochrome \( c_{552} \) binding on the periplasmic side of the enzyme complex. A C-terminal tag position resulted in a nonfunctional enzyme.

FIG. 2. Transcriptional analysis of \( cbaX \). Total RNA was isolated from \( T. \) thermophilus HB27, and cDNA was produced using the gene-specific primer 15. After cDNA synthesis, PCR was performed using the primer pair 14 and 15 on different templates. Lane 1, cDNA as a template; lane 2, RNA as a template (negative control); lane 3, plasmid DNA as a template (positive control; pWE7) (see Materials and Methods); lane 4, λ DNA marker (restricted with EcoRI and HindIII; relevant fragment sizes, from bottom: 0.83, 0.95, 1.38, and 1.58 kb).

FIG. 3. Schematic representation of the \( T. \) thermophilus \( ba_3 \) oxidase. Subunits II (harboring the \( Cu_b \) center) and IIa are shown as grey transparent outlines, while subunit I is displayed mainly with its 13 transmembrane helices, partly presented as transparent segments to increase cofactor visibility. The low-spin heme \( b \) and the high-spin heme \( a \)/\( Cu_a \) site of subunit I are denoted by \( ls \) and \( hs \), respectively. The boxed amino acid sequence corresponds to the internal His tag in subunit I, inserted into the cytoplasmic loop connecting transmembrane helices IV and V between residues N174 and P175, embedded by three additional linker amino acids on either side, as indicated (Protein Data Bank [PDB] code, 1EHK; graphics generated by VMD [http://www.ksu.uiuc.edu/Research/vmd/]).
Possibly due to steric hindrance, while tag lengths beyond 7 residues on the N terminus (8) apparently are also not tolerated (C. Werner, unpublished data). The internally tagged enzyme, when expressed and purified from HB27 wild-type cells, allowed a single-step purification leading to an oxidase preparation (estimated to have 95% purity) that was fully competent in its enzymatic and spectral properties (Fig. 4, pWE4/HB27) and provided a convenient tool to quickly assess the putative effects of a cbaX deletion.

The loss of the putative cbaX gene product drastically lowered the ba3 activity to about 7% of that of the wild type (Fig. 4A), and less than 10% of the expected heme a content was deduced from the typical peak position at 613 nm in the native redox spectrum of the purified oxidase (Fig. 4B). However, we note that the trace of the redox spectrum appeared rather “noisy,” with small spectral features present on either side of this peak. In contrast to the native spectrum with its severe loss of the heme a peak at 613 nm, the pyridine hemochromogen spectrum revealed that around 60% of the wild-type heme a level was detectable in the enzyme complex (see Discussion). Heme b was not affected by the deletion of cbaX, as the specific heme b content, based on native and denaturing spectra (Fig. 4C), matched the wild-type value.

To prove that the diminished oxidase activity, as well as the loss of heme a, was indeed caused by the deletion of cbaX, a second complementation plasmid was generated, comprising the ba3 structural genes, the 56-bp intergenic region, and cbaX (pWE7-2) (Fig. 1E). After complementation, the resulting ba3 enzyme was again affinity purified and examined (Fig. 4): both the activity and the heme a content of the purified ba3 oxidase were fully restored in this strain.

Expression of cbaX under the control of a strong promoter leads to partial incorporation of heme a into the low-spin site of the ba3 cytochrome c oxidase. To separate the expression of the three structural genes of the cytochrome ba3 oxidase from that of the cbaX gene, we constructed a second deletion strain in which the structural genes of the ba3 oxidase were deleted...
while the intergenic stretch of 56 bp was still present (WE8) (Fig. 1C, vertical arrow). Here, the structural genes of the \( ba_3 \) oxidase were exchanged for the bleomycin resistance gene placed under the control of the \( slpA \) promoter. To confirm a transcriptional read-through into the \( cbaX \) gene, thereby mimicking the wild-type situation, we ascertained that no transcriptional terminator was present at the 3' end of the bleomycin resistance gene. For experimental confirmation, total RNA was isolated from this deletion strain, and again, cDNA was produced with the same gene-specific primer binding at the 3' end of \( cbaX \). The primer pair used for this PCR with the synthesized cDNA as a template can result in a product only if \( cbaX \) is cotranscribed, this time with the bleomycin resistance gene. Figure 5 shows that indeed a PCR product with a length identical to 1 kb of the positive control was obtained, clearly demonstrating cotranscription of both genes.

To check the phenotype of the \( ba_3 \) oxidase, strain WE8 was complemented with a plasmid carrying only the \( ba_3 \) subunit genes with the internal His tag in subunit I (pWE4) (Fig. 1D). The resulting enzyme was purified and assayed for its enzymatic activity as well as spectral properties. The native spectrum (Fig. 6A) showed the typical heme \( b \) (560-nm) and heme \( a \) (613-nm) peaks, but unexpectedly, one additional peak at 582 nm arose. To reveal its identity, heme extraction and separation (data not shown) were performed and a pyridine hemochromogen spectrum (Fig. 6B) was recorded, both confirming the presence of no heme type other than \( a \) and \( b \). The expressed oxidase in this strain showed an increase of the heme \( a/b \) ratio to 2:1 and a decrease in the turnover number to about 65% of that of the wild-type \( ba_3 \) enzyme. Determination of the specific heme content (Fig. 6C) verified that the general heme \( (a + b) \)-to-protein ratio was not influenced, i.e., the purified enzyme did not carry any surplus heme.

Our finding that the complementation of WE8 leads to a distorted heme \( a/b \) ratio raised the question of how the high- and low-spin sites of the enzyme are populated by both heme types. To determine this, a spectrum of the isolated, reduced, and cyanide-ligated enzyme was recorded (Fig. 7). In contrast to the oxidized form of the \( ba_3 \) oxidase (32), the reduced form readily reacts with cyanide, and a shift of the heme \( a \) peak in the \( \alpha \)-region from 613 nm to 592 nm has been reported for the five-coordinate \( a_\beta \) site (29), while hemes present in the six-coordinate low-spin site were not affected. Accordingly, the recorded wild-type spectrum (Fig. 7, solid line) showed two peaks at 560 nm (low-spin heme \( b \)) and at 592 nm (high-spin heme \( a_\beta \)). When the enzyme isolated from strain pWE4/WE8 was analyzed (Fig. 7, dotted line), an additional shoulder at around 580 nm was observed. The difference spectrum (Fig. 7, gray line) revealed a loss of signal for the heme \( b \) peak at 560 nm and a distinct extra peak at 582 nm. A comparison of Fig. 6 and 7 clearly shows that neither the position of the 560-nm heme \( b \) peak nor that of the extra 582-nm heme \( a \) peak was shifted by cyanide treatment. Due to the observed inaccessibility for external ligands, we therefore conclude that both heme types, \( a \) and \( b \), are found in the low-spin site in contrast to the wild-type situation: heme \( a \) populating this low-spin site replaces one-third of the regular heme \( b \) moiety (Fig. 7).

**DISCUSSION**

The mitochondrial cytochrome \( c \) oxidase complex is composed of up to 13 subunits encoded by both the mitochondrial and the nuclear genomes. Defects in the assembly pathways for its subunits and redox centers are frequently associated with severe respiratory deficiencies. From extensive work, mostly with yeast and mammalian oxidases, a considerable body of information on various chaperones and biogenesis factors has been established (for reviews, see, e.g., references 7, 21, and 36).

The cytochrome \( ba_3 \) oxidase from the extremely thermophilic eubacterium *T. thermophilus*, on the other hand, consists of only three subunits. This organism not only provides a much simpler biogenesis system, but, given its evolutionary location in the deepest branch of the phylogenetic tree (34), is also an interesting object for the study of assembly events of an evolutionarily ancient and thermophilic oxidase.

Only three types of specific auxiliary proteins associated with cytochrome \( c \) oxidase assembly have been identified so far in *T. thermophilus*: a prenyltransferase and a heme \( a \) synthase, catalyzing the last two steps of heme \( a \) biosynthesis, and Sco1, previously linked to the formation of the \( Cu_\alpha \) center in subunit II (2, 9; see also reference 1, which presents different results). Two further assembly factors of critical importance found almost ubiquitously in all aerobic organisms have not yet been identified in *T. thermophilus*: CtaG, thought to be responsible for the formation of the \( Cu_\beta \) center (2, 9, 15), and Surf1, necessary for heme \( a \) insertion into subunit I (5, 6, 25, 35).

Here, we show by transcriptional analysis that a novel protein, CbaX, is encoded in the same operon with the structural genes of the \( ba_3 \) oxidase. Our present data suggest that the 56-bp sequence preceding the \( cbaX \) gene (Fig. 1A) harbors an RBS. While the \( cbaX \) gene was shown to be cotranscribed (Fig. 2), loss of its presumed translational start signal within the 56-bp stretch (deleted in strain WE6) (Fig. 1B) is responsible for the observed phenotype. Absence of CbaX caused a drastic decrease in the activity of the \( ba_3 \) oxidase complex, due to a...
dramatically lowered content of correctly inserted heme a. Even though there is no sequence homology to any known oxidase assembly protein, the reported phenotype suggests this protein is a prime candidate in the biogenesis pathway of the \( ba_3 \) oxidase, possibly responsible for the correct insertion of heme a into subunit I.

As shown in Fig. 4, the most notable effect of the \( cbaX \) deletion on the cytochrome \( ba_3 \) oxidase consists of a decrease in its enzymatic activity to 7% of that of the wild type, and this observation is paralleled by an apparent loss of heme a of about 90%, as judged from the canonical 613-nm peak in the native spectrum (Fig. 4B, strain pWE4/WE6). However, when the total amount of heme a was assessed for this oxidase preparation under denaturing conditions (Fig. 4C), this loss amounted to only 40% of the heme a of the wild type. This obvious discrepancy may be explained by only a small fraction of correctly assembled heme a contributing to the 613-nm spectral peak, indicative of a normal ligand environment in the high-spin \( a_3 \) site of subunit I; the majority of heme a present in the oxidase from this assembly mutant strain may be liganded in a nonnative environment, giving rise to spectral “noise” observed on either side of the 613-nm redox spectrum peak (Fig. 4B).

Whereas the deletion of \( cbaX \) caused a loss of correctly

---

FIG. 6. Redox spectra and specific heme content of the affinity-purified oxidase from strain pWE4/WE8. (A) Besides the typical heme b peak at 560 nm and the heme a peak at 613 nm, an additional peak at 582 nm was visible in the native spectrum. (B) The pyridine spectrum, however, revealed only heme b at 557 nm and heme a at 587 nm. (C) While the absolute value for the specific heme content of the \( ba_3 \) oxidase purified from pWE4/WE8 was 20.4 nmol/mg (hemes a and b), close to the value for the wild type (23.6 nmol/mg), the heme a/b ratio had been shifted to 2:1 in the \( ba_3 \) oxidase from the complemented strain relative to the wild type.

FIG. 7. Cyanide spectra of the affinity-purified cytochrome \( ba_3 \) oxidase from the wild type and from strain WE8 complemented with pWE4 (Fig. 1). The high-spin heme a peak (pWE4/HB27) shifted from 613 nm to 592 nm on cyanide incubation, while the low-spin heme b peak remained at 560 nm. In the case of pWE4/WE8, the spectrum displayed a shoulder near 582 nm (dotted line), clearly visualized in the difference spectrum (pWE4/WE8 minus pWE4/HB27). The spectra were normalized for heme a at 592 nm.
assembled heme \(a\) in the cytochrome \(ba_{3}\) oxidase and dramatically diminished its turnover number, the use of a strong promoter for the expression of CbaX, detached from that of the subunit genes in WE8, led to a distorted heme \(a/b\) ratio of about 2:1 (Fig. 6C). Compared to the wild-type situation, where the heme \(a/b\) ratio is 1:1, a disturbed ratio of heme \(a\) was reflected in pWE4/WE8 by an additional peak at 582 nm (Fig. 6A). As shown in Fig. 6C, the specific heme content (the sum of heme \(a\) and heme \(b\)) was not changed in this strain; therefore, the presence of any surplus heme sticking to the enzyme is excluded.

The unusual spectral position of the 582-nm redox peak may be explained by fortuitous side chains surrounding the low-spin site, now liganding the heme \(a\) artificially. This assumption is supported by the cyanide spectrum (Fig. 7): as expected for the wild type, the high-spin heme \(a\) peak was shifted to 592 nm by the cyanide binding, while the position of the low-spin heme \(b\) peak remained unchanged (Fig. 7, solid line). In pWE4/WE8 (dotted line), the 592-nm heme \(a\) peak displayed an unexpected shoulder with a maximum at 582 nm; this spectral assignment became more obvious in the difference spectrum (gray line). Thus, this wavelength was identical to that of the additional peak in the native redox spectrum (Fig. 6A) and therefore represented heme \(a\) occupying the low-spin site. The measured enzymatic activity, two-thirds that of the wild type, supports this notion, where one out of three oxidases carries a heme \(a\) moiety instead of heme \(b\) in its low-spin site. Because of the bulky geranylgeranyl side chain of heme \(a\), it is highly unlikely that this heme \(a\) group would fit into the low-spin site in an orientation identical to that of heme \(b\) and be properly positioned between the two histidine ligands, as found in the native structure (32). We conclude that these severe oxidase defects are caused by overexpression of CbaX due to the use of the strong \(slpA\) promoter; since S-layer proteins represent up to 15% of the total cellular protein of an S-layer-carrying cell, the promoters of surface layer genes are among the strongest found in prokaryotes (3, 19). We hypothesize that in the wild type, where the transcription of \(cbaX\) is linked to that of the subunit genes, a balanced protein level, not necessarily stoichiometric with subunit I, is assured; such a regulated expression may be necessary for proper \(ba_{3}\) assembly and function. Whether the role of CbaX is a truly catalytic one or rather that of a generic chaperone maintaining subunit I in a state competent to receive the heme \(a\) moiety remains unclear. However, the low yield for CbaX, even when purified from overexpressing strains, may rather argue for a catalytic function. We note that the CbaX protein is devoid of any histidine residues typically involved in liganding heme groups, thus not favoring direct heme transport.

The lowered activity, the distorted heme \(a\) content, and the location of the \(cbaX\) gene within the \(ba_{3}\) operon (6, 31) clearly suggest a Surf1-like function for CbaX. This assumption, on the other hand, is not substantiated on the sequence level, where CbaX and Surf1 share no meaningful sequence identity in either of the two \(T.\) thermophilus genome sequences (16).

Data on a \(surfI\) deletion in \(P.\) denitrificans or \(R.\) sphaeroides show that the activities of the resulting oxidases are much less affected (6, 31) than in the results presented here. Also at variance in the case of \(P.\) denitrificans, where two heme \(a\)-carrying terminal oxidases require two independent \(surfI\) gene variants with noncomplementary functions encoded in each particular operon (5, 6), no further operon-specific homology was found within the \(caa_{3}\) oxidase operon (12) or elsewhere in the genome of \(T.\) thermophilus. At present, we consider it very unlikely that assembly of the cytochrome \(caa_{3}\) oxidase is also dependent on proper CbaX function, as \(T.\) thermophilus HB27 completely relies on oxidative phosphorylation and lacks fermentative pathways (16). Therefore, a severe defect in the assembly of the cytochrome \(caa_{3}\) oxidase in the CbaX deletion strain would result in serious growth rate retardation, which was not observed here. The role of the second gene, \(cby\), which is most likely also part of the \(cbabra\) transduction unit, remains unclear, because its deletion had no effect upon proper function of the cytochrome \(ba_{3}\) oxidase (data not shown), apart from a sharp drop in the yield of the enzyme.

A BLAST search found homologues of CbaX only in members of the \(Thermaceae\) group: in the few cases with genomic information available, this gene was located in a position either next to the \(ba_{3}\) structural genes (in \(T.\) thermophilus, as well as in \(T.\) aquaticus) or next to \(sco1\) (as in either of the two \(M.\) thermus strains). However, in all the above-mentioned bacteria with a CbaX protein present, a Surf1 homologue was missing. The existence of an alternative heme \(a\) insertion factor may well reflect an adaption of this biogenesis pathway to hyperthermophilic conditions.

In conclusion, we identified a novel membrane protein, CbaX, that is vital for proper maturation of a thermophilic enzyme, the \(ba_{3}\) cytochrome \(c\) oxidase of \(T.\) thermophilus. It is encoded, together with the structural genes of the enzyme, in one transcription unit, and its absence causes a severe loss of correctly assembled heme \(a\) and therefore of enzymatic activity. We conclude that this protein, devoid of homologues outside the \(Thermaceae\) group, plays a specific role in heme \(a\) insertion into the high-spin site of subunit I of the \(ba_{3}\) oxidase by an as-yet-unknown mechanism. It may act as a chaperone, keeping the transmembrane helices of newly synthesized subunit I polyepitides in a state competent to sterically position the heme \(a\) group, thus facilitating proper formation of the active site. Furthermore, balanced expression seems to be important, as overexpression of CbaX leads to misincorporation of heme \(a\) into the low-spin site of the \(ba_{3}\) enzyme, thereby once again emphasizing the specificity of CbaX in heme \(a\) incorporation.

ACKNOWLEDGMENTS

The Collaborative Research Center “Molecular Bioenergetics” (SFB 472), the Center for Membrane Proteomics (CMP), and the Cluster of Excellence Frankfurt (Macromolecular Complexes, DFG Project EXC 115) are acknowledged for financial support.

We thank B. Averhoff for kindly providing the pWUR-bleo vector and H.-W. Müller for excellent technical assistance.

REFERENCES


