

Primary structure of a novel subunit in *ba*₃-cytochrome oxidase from *Thermus thermophilus*

TEWFIK SOULIMANE,¹ MANUEL E. THAN,² MANFRED DEWOR,¹ ROBERT HUBER,²
AND GERHARD BUSE¹

¹Rheinisch-Westfälische Technische Hochschule Aachen, Institut für Biochemie, Pauwelsstraße 30, D-52057 Aachen, Germany

²Max-Planck Institut für Biochemie, Am Klopferspitz 18a, D-82152 Planegg-Martinsried, Germany

(RECEIVED April 12, 2000; FINAL REVISION July 5, 2000; ACCEPTED August 18, 2000)

Abstract

The *ba*₃-type cytochrome *c* oxidase from *Thermus thermophilus* is known as a two subunit enzyme. Deduced from the crystal structure of this enzyme, we discovered the presence of an additional transmembrane helix “subunit IIa” spanning the membrane. The hydrophobic N-terminally blocked protein was isolated in high yield using high-performance liquid chromatography. Its complete amino acid sequence was determined by a combination of automated Edman degradation of both the deformylated and the cyanogen bromide cleaved protein and automated C-terminal sequencing of the native protein.

The molecular mass of 3,794 Da as determined by MALDI-MS and by ESI requires the N-terminal methionine to be formylated and is in good agreement with the value calculated from the formylmethionine containing sequence (3,766.5 Da + 28 Da = 3,794.5 Da). This subunit consists of 34 residues forming one helix across the membrane (Lys5–Ala34), which corresponds in space to the first transmembrane helix of subunit II of the cytochrome *c* oxidases from *Paracoccus denitrificans* and *bovine heart*, however, with opposite polarity. It is 35% identical to subunit IV of the *ba*₃-cytochrome oxidase from *Natronobacterium pharaonis*.

The open reading frame encoding this new subunit IIa (*cbaD*) is located upstream of *cbaB* in the same operon as the genes for subunit I (*cbaA*) and subunit II (*cbaB*).

Keywords: *ba*₃-cytochrome *c* oxidase; gene location; sequence analysis; subunit IIa; *Thermus thermophilus*

Thermus thermophilus HB8 (ATCC 27634) is an extremely thermophilic gram-negative eubacterium. Depending on the fermentation conditions, this organism expresses several *c*- and *b*-type cytochromes (T. Soulimane, unpubl. results) as well as two terminal cytochrome *c* oxidases that belong to the *caa*₃- and *ba*₃-type (Fee et al., 1980; Zimmermann et al., 1988). This is in accordance with the fact that respiring bacterial cells usually express simultaneously several terminal oxidases (Garcia-Horsman et al., 1994). Both *T. thermophilus* oxidases are presently studied in our laboratories with proteinchemical, crystallographic, and functional methods (Soulimane et al., 1995, 1997, 2000; Than et al., 1997; Buse et al., 1999). The *ba*₃-oxidase was originally described as a single subunit enzyme (Zimmermann et al., 1988). More recent studies, however, have shown that this enzyme is a two subunit complex (Bresser & Buse, 1993; Keightley et al., 1995; Soulimane et al., 1995).

The amino acid sequence of its subunit I shows a clear, but distant sequence homology to other members of the heme-copper oxidases superfamily. This has been suggested to be the result of an early gene duplication preceding the separation of bacteria and archaea (Castresana & Saraste, 1995). The archaeal oxidases, the *ba*₃-oxidase from *T. thermophilus* and the *bo*₃-oxidase from *Bacillus stearothermophilus* belong to the *SoxB*-type, while most other eubacterial oxidases are of the *SoxM*-type (Lübben et al., 1992; Mattar & Engelhard, 1997).

The primary structure of subunit II was obtained by both proteinchemical methods (Bresser, 1995) and sequencing of the corresponding gene (Keightley et al., 1995). Subunit II binds the electron-donating substrate and contains the homodinuclear copper centre in cytochrome *c* oxidases, which is missing in quinol oxidases. Homology searches show that the subunits II of the archaeal *Sulfolobus acidocaldarius* quinol oxidase, *soxA*, (Lübben et al., 1992, 1994), the *Natronobacterium pharaonis* *ba*₃-cytochrome *c* oxidase, *cbaB* (Mattar & Engelhard, 1997), the gram-positive *B. stearothermophilus* *bo*₃-cytochrome *c* oxidase (Nikaido et al., 1998), *cbaB*, and the *T. thermophilus* *ba*₃-oxidase lack the first transmembrane helix, a typical characteristic of *SoxB*-type oxidases. All *SoxB* cytochrome *c* oxidases do not contain the classical subunit III

Reprint requests to: Tewfik Soulimane, Rheinisch-Westfälische Technische Hochschule Aachen, Institut für Biochemie, Pauwelsstraße 30, D-52057 Aachen, Germany; e-mail: tsoulimane@post.klinikum.rwth-aachen.de.

with seven transmembrane helices. Both subunits I and II of the *ba*₃-oxidase show a relatively high sequence identity (38–43%) to their counterparts in the *SoxB*-type *ba*₃-oxidase from *N. pharaonis* (Mattar & Engelhard, 1997).

The analysis of the hemes present in the *T. thermophilus ba*₃-oxidase and in the other two archaeal *SoxB* oxidases has shown that the A-type hemes are different from the hemes A, which are normally present in eubacterial and eucaryotic terminal oxidases: the farnesyl chain is replaced by a geranylgeranyl-group in these enzymes (Lübben & Morand, 1994).

In contrast to the *T. thermophilus ba*₃- and *B. stearothermophilus bo*₃-enzyme, which are known as two subunit enzymes, the above cited archaea oxidases *SoxABCD* from *S. acidocaldarius* and *cbaDBAC* from *N. pharaonis* contain four subunits. However, *SoxCD* and *cbaCD* show no sequence similarity to each other or to the known subunits of other oxidases. Keightley et al. (1995) describe the existence of a further ORF for the *T. thermophilus ba*₃-oxidase, downstream of the *cbaA*-gene (subunit I). Its relative location in the gene locus would resemble that of *cbaC* from *N. pharaonis* (Mattar & Engelhard, 1997).

In addition to the known structures of the homologous 4 and 2 subunit cytochrome *c* oxidases from *Paracoccus denitrificans* (Iwata et al., 1995; Ostermeier et al., 1997) and the 13 subunit oxidase from bovine heart mitochondria (Tsukihara et al., 1995, 1996; Yoshikawa et al., 1998), we have solved the crystal structure of the *ba*₃-cytochrome *c* oxidase from *T. thermophilus* (Soulimane et al., 2000). Surprisingly, after placing all of the visible residues of the known subunits I and II into the experimental electron density, one additional transmembrane spanning helix was found.

The present paper describes the isolation, characterization, and sequence analysis of the novel subunit of the *ba*₃-oxidase from *T. thermophilus* as well as the location of the corresponding gene. The sequence similarity to other subunits of known oxidases, especially to subunit IV from the *N. pharaonis ba*₃-oxidase, is discussed.

Results and discussion

Purification and amino acid sequencing

The *ba*₃-type cytochrome *c* oxidase from *T. thermophilus* has been isolated from cells fermented under low aeration. The cells were disrupted and membranes prepared essentially as described by Soulimane et al. (1997). The membrane proteins have been solubilized using TX-100. The *ba*₃-oxidase was purified with a DEAE-Biogel, DEAE-Biogel CL-6B, and Fractogel TMAE-650 (S) anion-exchange column as well as gel filtration on a fast protein liquid chromatography Superdex 200 column.

SDS-PAGE of the purified enzyme shows after denaturation in SDS and staining with Coomassie Blue the two known subunits with the apparent molecular weights 46 and 18 kD. The additional subunit described in this work cannot be detected by this method.

N-terminal sequence analysis of the native oxidase shows only the N-terminal residues of subunit II. Both subunit I and the newly identified subunit IIa are N-terminally blocked. However, the crystal structure of the native enzyme clearly shows the existence of an additional transmembrane helix, which cannot be interpreted using the known amino acid sequences of subunits I and II (Soulimane et al., 2000).

We have used high-performance liquid chromatography (HPLC) to separate the subunits of (1) the purified enzyme and (2) *ba*₃-oxidase crystals dissolved in 0.05% dodecyl- β -D-maltoside (10 mM Tris/HCl; pH 7.4). Figure 1 shows the HPLC chromatogram for the purified enzyme. Subunit II, consisting mainly of a hydrophilic domain that contains the Cu_A binding site, elutes first with a retention time of 17 min. The novel subunit elutes at 32 min and shows the lowest absorption. The very hydrophobic subunit I containing 13 transmembrane helices elutes at 41 min. This elution gradient has been optimised for hydrophobic proteins. The HPLC of *ba*₃-oxidase crystals shows a similar chromatogram.

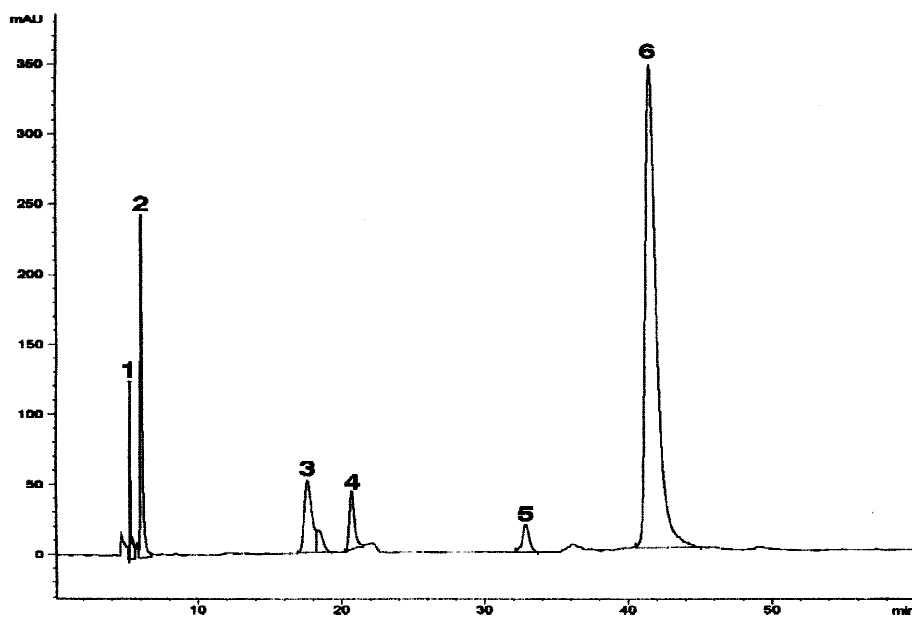


Fig. 1. Separation of the *ba*₃-type cytochrome *c* oxidase subunits by RP-HPLC. Peaks 1–6: 1, injection peak; 2, nonseparated enzyme; 3, subunit II; 4, heme; 5, subunit IIa; 6, subunit I.

Amino acid analysis of the novel subunit (Table 1) shows the presence of a high amount of hydrophobic amino acids Leu6, Ile2, Val5, Ala4, Gly3, Phe3, and Trp1. It also contains five acidic residues (Glu2, Tyr1, Thr2) and three positively charged groups (Lys2, Arg1) leading to a calculated pI of 9.5. Furthermore, it contains one methionine that allows cleavage with cyanogen bromide.

To start the sequence analysis of this subunit, the protein has been cleaved with cyanogen bromide as described in Materials and methods. Sequence analysis of the mixture (500 pmol) shows only one PTH amino acid in each cycle confirming the presence of one methionine in the primary structure of the novel subunit. The following sequence has been obtained:

EEKPKGALAV ILVLTTLTILV FWLGVYAVFF ARG.

It is common that proteins expressed in bacteria are N-terminally blocked with formylmethionine. To determine the first amino acids, the HPLC purified SuIIa was deformedylated as described in Materials and methods. Automated Edman degradation of the resulting preparation (500 pmol) gave the following sequence.

MEEKPKGALA VILVLTTLTIL VFWLGVYAV

indicating that the cleaved methionine is the N-terminal residue of the novel subunit.

Table 1. Amino acid composition of subunit IIa of the *ba*₃-type cytochrome *c* oxidase from *T. thermophilus*^a

Amino acid	Residues per molecule subunit IIa	
	Amino acid analysis	N-terminal sequence analysis
Asp	0	0
Thr	1.8	2
Ser	0.2	0
Glu	2.2	2
Pro	n.d. ^b	1
Gly	3.3	3
Ala	4.3	4
Val	4.9	5
Met	1.1	1
Ile	1.8	2
Leu	6.0	6
Tyr	0.9	1
Phe	2.9	3
His	0.1	0
Lys	1.9	2
Arg	1.2	1
Cys	0.1 ^c	0
Trp	0.9 ^d	1

^aThe hydrolysis values are derived from multiple analyses under different conditions. The sequence values are taken from the sequence shown in Figure 2.

^bn.d., not determined.

^cPerformic acid oxidation.

^d0.4% thioglycolic acid.

For the determination of the C-terminal residues 100 pmol of the N-terminal, blocked protein have been sequenced C-terminally. The following six residues were obtained:

N-term ... VFFARG.

These data show that the cyanogen bromide cleaved protein was sequenced to its C-terminus using Edman degradation.

Molecular mass determination

The molecular mass of the novel subunit was calculated from the complete sequence to 3,766.5 Da. Since its molecular mass as determined by MALDI-MS (data not shown) is 3,794.6 Da, the N-terminal methionine must be formylated. Considering formyl-methionine as the N-terminal residue, the calculated molecular mass of the novel subunit is in good agreement with the MALDI data (3,766.5 Da + 28 Da = 3,794.5 Da). The existence of this N-terminal formylation is further supported by the requirement for a deformylation step prior to successful sequencing by Edman degradation (see above).

Comparison with other proteins

Homology searches with the sequence of the novel subunit, considering mainly other small subunits of cytochrome *c* oxidases and quinol oxidases, were performed with the Search and Alignment Program Fasta3_t as described in Materials and methods. The only hit with significant sequence similarity (35% identity; calculated for the 34 residues of the *ba*₃-oxidase subunit IIa) was found to subunit IV from the *N. pharaonis* cytochrome *c* oxidase (Accession No. Y10500, GenBank/EMBL Data Bank), which has extended N- and C-termini of 11 and 9 residues, respectively (Fig. 2). Interestingly, no sequence similarity was found to either the small hydrophobic peptide *SoxD* from the quinol oxidase from *S. acidocaldarius* (*SoxABCD*; Lübben et al., 1992) or the subunits IV of the structurally known cytochrome *c* oxidases from *P. denitrificans* (Iwata et al., 1995) and bovine heart (Tsukihara et al., 1996). A corresponding small subunit has not been described for the gram-positive *B. stearothermophilus* *bo*₃-cytochrome *c* oxidase.

The crystal structure analysis of the *ba*₃-oxidase from *T. thermophilus* (Soulimane et al., 2000) shows that the novel subunit superimposes with the first transmembrane helix of subunit II of the two structurally known cytochrome *c* oxidases, however, with opposite polarity (Fig. 3). Therefore, we introduce the name "subunit IIa" for this novel peptide. The presence of this transmembrane helix seems to be important for the function of cytochrome *c* oxidases. In addition to subunit IV of the *ba*₃-cytochrome *c* oxidases from *N. pharaonis* (Mattar & Engelhard, 1997), which shows the above mentioned high sequence similarity to this new subunit IIa, the *aa*₃-quinol oxidase from *S. acidocaldarius* contains also such a small domain, which was termed *SoxD* (Lübben et al., 1992). Since all four *SoxB*-type oxidases lack the first transmembrane helix of subunit II, its replacement by such an additional small subunit may be of a more general character. However, its existence has not been shown in *B. stearothermophilus* yet. Interestingly, the *ba*₃-oxidase does not contain a fourth subunit that might be related to either, apocytochrome *b* (*SoxC*) of the archaea *S. acidocaldarius* quinol oxidase or subunit III (*cbaC*) of *N. pharaonis* cytochrome oxidase.

```

MEEKPKGALAVILVLTTLTILVFWLGVYAVFFARG
      :  :  :  :  :  :  :  :  :  :  :
MGTDVEREIGHDEYDPKGTLLALIAIYFLLIAGLWIFTYFVEFLGNEMTVVGVVL

```

Fig. 2. Alignment of the sequences of subunit IIa from the *T. thermophilus* *ba*₃-oxidase and subunit IV from the *N. pharaonis* *ba*₃-oxidase. Identical residues are in red, and residues forming the transmembrane helix in the *T. thermophilus* structure are depicted in bold.

Genomic location of subunit IIa

The genomic nucleotide sequences of the *ba*₃-oxidase subunits I and II are available from the GenBank™/EMBL Data Bank with accession number L09121 (Keightley et al., 1995). Subunits II (*cbaB*) and I (*cbaA*) are located in one operon.

The comparison of the deposited nucleotide sequence of this operon with the amino acid sequence of subunit IIa shows that its gene is located directly upstream of the gene encoding subunit II (*cbaB*) (Fig. 4). Its ORF is, however, not in frame with the genes for the other two subunits (*cabA* and *cabB*). The same organization of the genes encoding subunits I, II, and the small subunit corresponding to subunit IIa described herein is observed for the *ba*₃-cytochrome *c* oxidase from *N. pharaonis* and the quinol oxidase from *S. acidocaldarius*. The genes encoding these three subunits are in different reading frames and even do slightly overlap. In line with the high sequence similarity to subunit IV of the *ba*₃-cytochrome *c* oxidase from *N. pharaonis* and the similar organization of the operon, we introduce the name *cbaD* for the gene encoding subunit IIa of the *ba*₃-oxidase from *T. thermophilus*. The putative ORF described for the *ba*₃-oxidase by Keightley et al. (1995) is located downstream of the *cbaA* gene and does not correspond to the *cbaD* gene described in this work.

Interestingly, the gene encoding subunit IIa (*cbaD*) seems to have an N-terminal extension of 25 residues if compared to the experimentally determined amino acid sequence (Fig. 4). Homology searches with the sequence of the first 25 residues were performed with the Search and Alignment Program Fasta3_t as described in Materials and methods. No sequence similarity to leader sequences (signal sequence) has been detected. To our knowledge, there is also no specific cleavage of the RRGME motif described in the literature. This and the fact that subunits I and II do not possess leader sequences lead to the conclusion that these 25 residues do not represent a signal sequence for the subunit IIa.

The nucleotide sequence of the entire putative ORF of the *ba*₃-oxidase subunit IIa shows the presence of a purine rich region upstream of the first ATG-codon (Met). However, this sequence does not correspond to the Shine-Dalgarno sequence described for *T. thermophilus* (ggagg; Gold & Stormo, 1990; Keightley et al., 1995) and the distance to the ATG-codon consists of only three nucleotides. However, a putative Shine-Dalgarno sequence could be identified at a distance of six nucleotides upstream of the second ATG-codon (encoding the formylated methionine of subunit IIa). These data suggest that the translation of the gene encoding the formylmethionine of subunit IIa (as described in this work) starts at the second methionine of the putative ORF. This conclu-

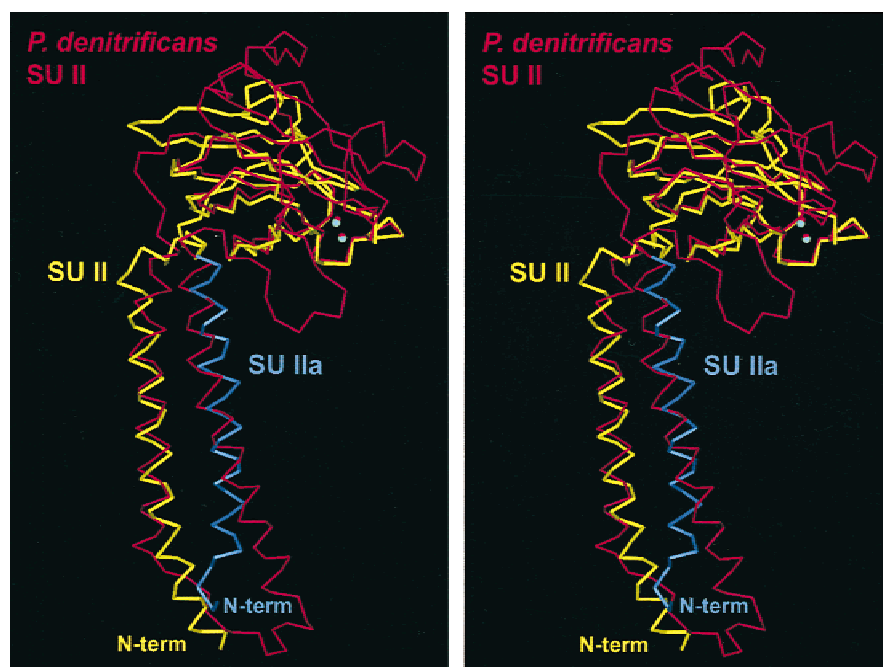


Fig. 3. Stereo plot of the superimposed C^α-traces of subunits II (yellow) and IIa (blue) from the *T. thermophilus* *ba*₃-cytochrome *c* oxidase and subunit II (red) from the *P. denitrificans* *aa*₃-cytochrome *c* oxidase (Iwata et al., 1995). The structures were aligned by a RMS method employing the five metal atoms common in the entire structures. This figure was prepared using MAIN (Turk, 1992).

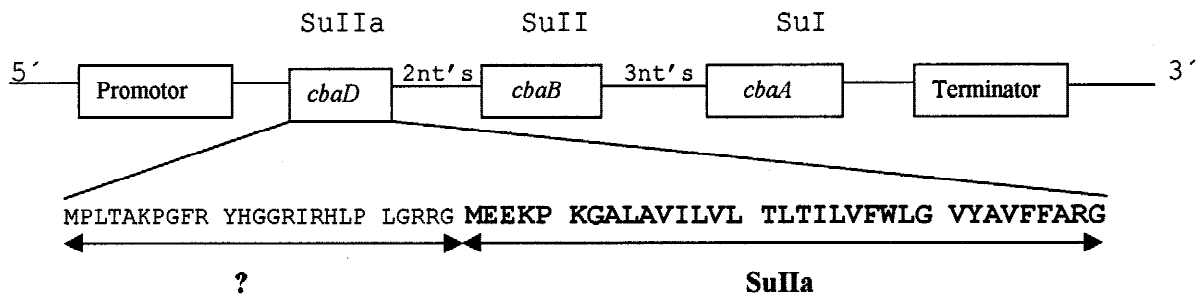


Fig. 4. Physical map of the gene locus of the *ba₃*-cytochrome *c* oxidases from *T. thermophilus*. The gene encoding subunit IiA (*cbaD*) is not in frame with the other two genes and located two nucleotides upstream of subunit Ii (*cbaB*). The enlargement shows the amino acid sequence of the ORF encoding for subunit IiA (bold type). The putative N-terminal extension as deduced from the nucleotide sequence is labeled with a question mark. For details refer to the text.

sion is further supported by the fact that the codon usage in the first part of the apparent reading frame MPL...RPG (Fig. 4) includes a very high percentage of rare codons—usually a sign of not being part of a true open reading frame.

Materials and methods

Chemicals

Detergents and Tris-(hydroxymethyl)-amino methane were purchased from Biomol (Hamburg, Germany), Fractogel EMD TMAE-650 (S) from Merck (Darmstadt, Germany), Superdex 200 and DEAE-CL6B-Sepharose from Pharmacia (Heidelberg, Germany), and DEAE-Biogel from Biorad (Hercules, California). All other chemicals were of analytical grade.

Purification

Fermentation of *T. thermophilus* HB8 (ATCC 27634) has been performed at the Gesellschaft für Biotechnologische Forschung (Braunschweig, Germany). The purification of the *ba₃*-oxidase is described in Giuffré et al. (1999). Further details and a protein chemical description of the enzyme and its crystallization will be given elsewhere.

Analytical procedures

Determination of cytochrome *c* oxidase concentration: Absorption spectra were recorded with a Perkin Elmer Lambda 5 UV/VIS spectrophotometer. Heme A concentrations were obtained from the dithionite reduced-minus-oxidised spectrum at 612 nm using a molar absorption coefficient of 6,300 M⁻¹ cm⁻¹ (Zimmermann et al., 1988).

Amino acid analysis

Quantitative amino acid determination was performed on a Biotronik amino acid analyzer LC 5001 (Eppendorf-Nethleler-Hinz, Maintal, Germany) equipped with a fluorescence detection of o-phthalaldehyde derivatives. The protein sample was hydrolyzed in 5.7 N HCl containing 0.2% thioglycolic acid for 90 min at 150 °C. Tryptophan was determined after hydrolysis in 5.7 N HCl containing 5% thioglycolic acid for 90 min at 150 °C. The cysteine content was determined after oxidation of the entire pro-

tein with performic acid (Henschen, 1986) to cysteinsulfonic acid before hydrolysis.

SDS-PAGE

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was performed according to Lämmler (1970) in the presence of 2-mercaptoethanol, 8M urea and other modifications according to Marx (1996).

Peptide separation by HPLC

The subunits of the *T. thermophilus ba₃*-oxidase were purified on a reversed-phase Synchropak C₄ column (250 × 4.6 mm) using a Hewlett-Packard 1050 HPLC System with multiple wavelength detector and the following solvents: (1) 5% formic acid, (2) 95% formic acid, (3) n-propanol, and (4) acetonitril. Gradients were formed with a microprocessor-controlled quaternary pump (HP) by low-pressure mixing of the four solvents in 60 min: (1) 14 to 0%, (2) 70% = constant, (3) 6 to 30%, and (4) 10 to 0% (by vol.).

Cleavage with cyanogen bromide

Methionyl bonds were cleaved with cyanogen bromide by the method of Gross and Morell (1974). Twenty nmol protein was dissolved in formic acid and mixed with 300 molar excess of cyanogen bromide and the reaction proceeded in the dark for 3 h. The reaction mixture was then evaporated under reduced pressure.

Deformylation

The n-formylmethionine blocked protein was deformylated with 0.5 M acetylchloride in the presence of methanol at 50 °C. After 20 min, the solution was evaporated. The pellet was dissolved in 75% formic acid and used for N-terminal sequencing.

Amino acid sequencing

Automated Edman degradation (Edman & Begg, 1967) was carried out in a Knauer 910 gas/liquid-phase protein sequencer on polybrene coated polyvinylidene fluoride membranes with autoconversion and on-line HPLC identification of the phenylthiohydantoin amino acids.

C-terminal sequencing

Two hundred forty pmol of the novel subunit, obtained after separation of all subunits of the enzyme by HPLC, were treated with carboxypeptidase P in 50 mM sodium acetate, pH 5.5, using

an E/S ration of 1:4. Analysis of the released amino acids was carried out on a 420 A Derivatizer with an on-line 130A Separation System (Applied Biosystems, Foster City, California).

Matrix-assisted laser desorption/ionization mass spectra (MALDI-MS)

The mass spectra were recorded on a time-of-flight Bruker REFLEX II spectrometer (Bruker-Franzen-Analytik GmbH, Bremen, Germany) equipped with a 337 nm nitrogen laser (pulse duration 4 and 3 ns). The acceleration voltage was 20 kV. The spectra were obtained by summing over 50 laser pulses. A 50 mM solution of α -cyano-4-hydroxycinnamic acid in aqueous 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) was used as the matrix. One and a half microliters of the matrix and 1.0 μ L of the purified subunit IIa sample were mixed on the target and air dried. The resulting deposit was washed in 0.1% TFA at 4 °C by immersing for about 10 s. This washing step is required for the removal of soluble ionic contaminants from the protein/matrix deposit, without removing the proteins or matrix. All measurements were performed in the linear mode of the spectrometer. Mainly singly and doubly positively charged molecular ions were detected. External mass calibration was accomplished by using bovine insulin ($M_r = 5,733.6$ Da) as protein standard obtained from Sigma Chemical Co. (St. Louis, Missouri). The masses of the proteins were determined by using the singly charged molecular ion only.

Homology search

Homology searches with the sequence of the novel subunit was performed with the Search and Alignment Program Fasta3_t. The protein sequence database Swall was used in combination with the mutation database matrix blosum50 and the Ktup 2, GapPenOpen -12 and GapPenExtend -2 specifications.

Structural analysis

The structure of the ba_3 -oxidase from *T. thermophilus* (Soulimane et al., 2000; Protein Data Bank (PDB) accession number 1EHK) was compared to the bacterial aa_3 -oxidase from *P. denitrificans* (Ostermeier et al., 1997; PDB accession number 1AR1) and to the mitochondrial aa_3 -oxidase from bovine heart (Tsukihara et al., 1996; PDB accession number 2OCC) by a root-mean-square (RMS) alignment of the five metal atoms.

Sequence data have been deposited in the SWISS-PROT protein sequence database "accession number P82543."

Acknowledgments

The authors are indebted to M. Tatarek-Nossol and H. Didden for excellent technical assistance. We thank Bart Samyn for C-terminal sequence analysis and Martin Hanna for MS spectra. This work was supported by the Deutsche Forschungs-gemeinschaft (Bu463/3-2) and the "START"-program (Klinikum-RWTH-Aachen) to T.S.

References

Bresser AH. 1995. Proteinchemische Charakterisierung von Endoxidasen aus *Thermus thermophilus* [PhD thesis]. RWTH-Aachen.
 Bresser AH, Buse G. 1993. Terminal oxidases from *Thermus thermophilus*. *Biol Chem Hoppe-Seyler* 374:736.
 Buse G, Soulimane T, Dewor M, Meyer HE, Blüggel M. 1999. Evidence for a copper coordinated histidine-tyrosine crosslink in the active site of cytochrome oxidase. *Protein Sci* 8:985-990.

Castresana J, Saraste M. 1995. Evolution of energetic metabolism: The respiration-early hypothesis. *Trends Biochem Sci* 20:443-448.
 Edman P, Begg G. 1967. A protein sequenator. *Eur J Biochem* 1:80-91.
 Fee JA, Choc MG, Findling KL, Lorence R, Yoshida T. 1980. Properties of a copper-containing cytochrome " c_1aa_3 " complex: A terminal oxidase of the extreme thermophile *Thermus thermophilus* HB8. *Proc Natl Acad Sci USA* 77:147-151.
 Garcia-Horsman JA, Barquera B, Rumbley J, Ma J, Gennis RB. 1994. The superfamily of heme-copper respiratory oxidases. *J Bacteriol* 176:5587-5600.
 Giuffré A, Forte E, Antonini G, D'Itri E, Brunori M, Soulimane T, Buse G. 1999. Kinetic properties of the ba_3 -oxidase from *Thermus thermophilus*: Effect of temperature. *Biochemistry* 38:1057-1065.
 Gold L, Stormo GD. 1990. High-level translation initiation. *Methods Enzymol* 185:89-93.
 Gross E, Morell JL. 1974. The reaction of cyanogen bromide with S-methyl-cysteine: Fragmentation of the peptide 14-29 of bovine pancreatic ribonuclease A. *Biochem Biophys Res Commun* 59:1145-1150.
 Henschen A. 1986. Advanced methods in protein micro sequence analysis. In: Wittman-Liebold B, Salnikow J, Erdmann VA, eds. *Advanced methods in protein micro-sequence analysis*. Berlin: Springer Verlag, pp 244-247.
 Iwata S, Ostermeier C, Ludwig B, Michel H. 1995. Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* 376:660-669.
 Keightley JA, Zimmermann BH, Mather MW, Springer P, Pastuszyn A, Lawrence DM, Fee JA. 1995. Molecular genetic and protein chemical characterization of the cytochrome ba_3 from *Thermus thermophilus* HB8. *J Biol Chem* 270:20345-20358.
 Lämmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
 Lübber M, Kolmerer B, Saraste M. 1992. An archaeobacterial terminal oxidase combines core structures of two mitochondrial respiratory complexes. *EMBO J* 11:805-812.
 Lübber M, Morand K. 1994. Novel prenylated hemes as cofactors of cytochrome oxidases. Archaea have modified hemes A and O. *J Biol Chem* 269:21473-21479.
 Lübber M, Warne A, Albracht SPJ, Saraste M. 1994. The purified SoxABCD quinol oxidase complex of *Sulfolobus acidocaldarius* contains a novel haem. *Mol Microbiol* 13:327-335.
 Marx M. 1996. Isoformen der cytochrom-c oxidase [PhD thesis]. RWTH-Aachen.
 Mattar S, Engelhard M. 1997. Cytochrome ba_3 from *Natronobacterium pharaonis*. An archaeal four-subunit cytochrome-c-type oxidase. *Eur J Biochem* 250:332-341.
 Nikaido K, Noguchi S, Sakamoto J, Sone N. 1998. The $cbaAB$ genes for ba_3 -type cytochrome *c* oxidase in *Bacillus stearothermophilus*. *Biochim Biophys Acta* 1397:262-267.
 Ostermeier C, Harrenga A, Ermler U, Michel H. 1997. Structure at 2.7 Å resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody Fv fragment. *Proc Natl Acad Sci USA* 94:10547-10553.
 Soulimane T, Buse G, Bourenkov GP, Bartunik HD, Huber R, Than ME. 2000. Structure and mechanism of ba_3 -cytochrome *c* oxidase from *Thermus thermophilus*. *EMBO J* 19:1766-1776.
 Soulimane T, Gohlke U, Huber R, Buse G. 1995. Three-dimensional crystals of cytochrome *c* oxidase from *Thermus thermophilus* diffracting to 3.8 Å resolution. *FEBS Lett* 368:132-134.
 Soulimane T, von Walter M, Hof P, Than ME, Huber R, Buse G. 1997. Cytochrome- c_{552} from *Thermus thermophilus*: A functional and crystallographic investigation. *Biochem Biophys Res Commun* 237:572-576.
 Than ME, Hof P, Huber R, Bourenkov GP, Bartunik HD, Buse G, Soulimane T. 1997. *Thermus thermophilus* cytochrome- c_{552} : A new highly thermostable cytochrome *c* structure obtained by MAD phasing. *J Mol Biol* 271:629-644.
 Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S. 1995. Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å. *Science* 269:1069-1074.
 Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S. 1996. The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. *Science* 272:1136-1144.
 Turk D. 1992. Weiterentwicklung eines Programms für Molekugraphik und Elektrodichte-Manipulation und seine Anwendung auf verschiedene Protein-Strukturaufklärungen [PhD thesis]. TU München, Germany.
 Yoshikawa S, Shinzawa-Itoh K, Nakashima R, Yaono R, Yamashita E, Inoue N, Yao M, Fel MJ, Libeu CP, Mizushima T, et al. 1998. The structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.3 Å. *Science* 280:1723-1730.
 Zimmermann BH, Nitsche CI, Fee JA, Rusnak F, Munck E. 1988. Properties of a copper-containing cytochrome ba_3 : A second terminal oxidase from the extreme thermophile *Thermus thermophilus*. *Proc Natl Acad Sci USA* 85:5779-5783.