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A four-subunit cytochrome bc_1 complex complements the respiratory chain of *Thermus thermophilus*

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Abstract

Several components of the respiratory chain of the eubacterium *Thermus thermophilus* have previously been characterized to various extent, while no conclusive evidence for a cytochrome bc_1 complex has been obtained. Here, we show that four consecutive genes encoding cytochrome bc_1 subunits are organized in an operon-like structure termed *fbc*CXFB. The four gene products are identified as genuine subunits of a cytochrome bc_1 complex isolated from membranes of *T. thermophilus*. While both the cytochrome *b* and the FeS subunit show typical features of canonical subunits of this respiratory complex, a further membrane-integral component (FbcX) of so far unknown function copurifies as a subunit of this complex. The cytochrome c_1 carries an extensive N-terminal hydrophilic domain, followed by a hydrophobic, presumably membrane-embedded helical region and a typical heme *c* binding domain. This latter sequence has been expressed in *Escherichia coli*, and in vitro shown to be a kinetically competent electron donor to cytochrome c_{552}, mediating electron transfer to the ba_3 oxidase. Identification of this cytochrome bc_1 complex bridges the gap between the previously reported NADH oxidation activities and terminal oxidases, thus, defining all components of a minimal, mitochondrial-type electron transfer chain in this evolutionary ancient thermophile.

Keywords: *Thermus thermophilus*; Respiratory chain; Cytochrome bc_1; Complex III; Electron transfer; Stopped flow kinetics

1. Introduction

Thermus thermophilus is an extremely thermophilic, Gram-negative eubacterium growing optimally in the range of 75–80 °C [1]. Representatives of this genus isolated from throughout the world were classified according to 16S rRNA analysis as a phylogenetically old lineage [2]. Nevertheless, several typical components of an aerobic electron transfer chain have been identified so far: a proton-translocating NADH:quinone oxidoreductase (NDH-1) composed of 14 subunits [3] with menaquinone-8 as the endogenous electron acceptor [4], a soluble cytochrome c_{552} [5] and two terminal oxidases: a ba_3- and a caa_3-type heme-copper oxidase [6,7]. In addition, menaquinone-8 can be reduced by succinate dehydrogenase (SDH) [8] or by a soluble two-subunit NDH-2 [9]. The crystal structure of both the ba_3 oxidase and its electron donor cytochrome c_{552} has been determined at high

Abbreviations: ET, electron transfer; aa, amino acid; ORF, open reading frame; I, ionic strength; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; MS, mass spectrometry; AP, atmospheric pressure; TMPD, tetramethyl-p-phenylendiamine; IPTG, isopropyl β-D-thiogalactopyranoside; NDH-1, NADH:quinone oxidoreductase-1 (energy transducing); NDH-2, NADH:quinone oxidoreductase-2; PSD, post source decay, spectrometer; MK-8, menaquinone-8

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resolution [10,11]. While no bc_1 complex has been described so far, a Rieske protein was identified as a component of a putative complex III [12], and the crystal structure of its soluble domain solved [13].

Quinol:cytochrome *c* oxidoreductase is a multisubunit enzyme complex operating in a wide variety of organisms. Members of the *bc* superfamily apparently have developed very early in evolution as they represent the only redox enzyme common to almost all respiratory (aerobic as well as anaerobic) and photosynthetic electron transfer chains [2]. On oxidation of a quinol, the bc_1 complex transfers electrons to reduce cytochrome *c* according to the Q-cycle mechanism; the free energy available from this reaction is thus used to establish a proton gradient across the membrane [14–16].

With only a limited number of thermophilic organisms studied in detail so far, it was of interest to characterize respiratory components of a thermostable and 'ancestral' bacterium. More specifically, we intended to elucidate the gap in the ET chain between the NADH:menaquinone-oxidoreductase (complex I) and the terminal oxidases of the ba_3- and caa_3-type.

Electron transfer reactions generally are fast and thereby involve only short-lived complexes [17], although specificity between reaction partners has to be maintained. For electron transfer of proteins from mesophilic organisms, a three-step mechanism has been described involving an electrostatically driven pre-orientation, followed by a rearrangement mediated mostly by hydrophobic amino acid residues, resulting in a productive electron transfer complex [18]. At elevated temperatures, ionic interactions are less favorable, and electrostatic forces only play a minor role for the ET reactions of thermophilic complexes and their partner proteins. For the interaction between cytochrome c_{552} and the ba_3 cytochrome *c* oxidase (complex IV) from *T. thermophilus* [19,20], hydrophobic interactions for ET reactions were suggested, in line with the electrostatic surface potentials of the predicted interaction sites deduced from the crystal structures of both proteins [10,11]. The electron donor for cytochrome c_{552} has not been known so far, but was suspected to be an as yet unidentified bc_1 complex.

On the basis of early data available from the *T. thermophilus* genome sequencing project (Genomics Laboratory, Göttingen), we analyzed a locus encoding a putative bc_1 complex in *T. thermophilus*. Three of the four open reading frames in an operon-like structure can be readily assigned to typical membrane-spanning components of bc_1 complexes: cytochrome c_1, the Rieske iron-sulfur protein and cytochrome *b*, while no relevant counterpart for the additional fourth gene product has been found so far. A four-subunit bc_1 complex was isolated from native membranes of *T. thermophilus* and by affinity chromatography after homologous expression of a His-tagged complex. The identification of all subunits as genuine gene products of the *fbc* operon was achieved by N-terminal sequencing and MALDI analysis, including the fourth subunit of yet unknown function. In addition, we report the cloning, heterologous expression and preliminary characterization of cytochrome c_1 as the full-size membrane-spanning protein, and its heme-containing soluble fragment. This soluble domain was used to study the kinetic behavior and ionic strength dependency of the electron transfer between cytochrome c_1 and its presumed acceptor protein cytochrome c_{552}.

2. Materials and methods

2.1. Growth conditions

T. thermophilus strains HB8 (ATCC 27634) and HB27 (ATCC BAA-163) were grown at 70 °C on LB-plates in a water-saturated atmosphere. Before inoculation of a liquid culture colonies were resuspended in LB-medium; for the plasmid- containing strain DM6 kanamycin was added at a concentration of 25 μg/ml. Fermentation was done at 70 °C overnight in a 10-l New Brunswick fermenter under aerobic conditions. Cells were harvested, resuspended in 50 mM KP_i buffer pH 8 (0.1 mg/ml lysozyme and 100 μM Pefabloc) and stored at −80 °C.

E. coli strain BL21(DE3) (Merck, Darmstadt) carrying the plasmid pMA37 or pDM3 (see below) in the presence of pEC86 (providing the heme maturation genes [21]) was grown aerobically at 32 °C in LB medium supplemented with 100 μg/ml ampicillin and 60 μg/ml chloramphenicol; induction by IPTG was not obligatory.

Cells expressing the soluble cytochrome c_1 fragment were harvested, followed by an immediate preparation of the periplasm performed essentially as described by Witholt et al. [22]. Cells producing the full-size cytochrome c_1 were resuspended in Tris–HCl-buffer pH 8 and stored in the presence of lysozyme and pefabloc at −80 °C.

2.2. Cloning and homologous expression of the bc_1 complex

For the expression of the entire bc_1 locus in *T. thermophilus* strain HB27 (ATCC BAA-163), the *Thermus/E. coli* shuttle vector pNTsp2 (NEB, Beverly, MA) [23] was optimized for growth temperatures of ~70 °C. To this end, the heat-stable kanamycin resistance gene (*HTK*) supplied by Hiroyuki Kagamiyama (Osaka, Japan) on vector pUC18-JHK3-1 [24] was amplified using primers A and B (Table 1, Supplementary material), providing restriction sites for *Kpn*I and *Bam*HI to clone the PCR product into the expression vector linearized with *Kpn*I and *Bgl*II yielding plasmid pMA34.

The bc_1 operon including the putative upstream promotor region was amplified from genomic DNA of HB8 (ATCC 27634) as two separate fragments, using primers C and D as well as E and F, thus allowing ligation of the two fragments after *Xho*I digestion. Encoding a His_6-tag at the C-terminus of cytochrome *b* subunit, this sequence also introduces a 5′*Kpn*I and a 3′*Pst*I restriction site for insertion into plasmid

pMA34. The construct was then introduced into the expression strain HB27 by electroporation [25], and clone DM6 isolated after kanamycin selection. Its identity was verified by PCR amplification of the *HTK* gene and part of the bc_1 operon via the primer pair E and B (see above) followed by sequencing.

2.3. Cloning and heterologous expression of the soluble and full-size cytochrome c_1

The gene sequence coding for the C-terminal 84 amino acids (aa) of cytochrome c_1 was amplified from *T. thermophilus* HB8 genomic DNA, using primers G and H (Table 1, Supplementary material); they introduce an upstream *Nco*I and a downstream *Hin*dIII restriction site for cloning into pET22b, an expression vector providing the *pel*B leader sequence (Merck, Darmstadt) directing export of the expressed protein into the periplasm, yielding pMA37. Expression of the cytochrome was achieved in *E. coli* BL21 cotransformed with the plasmid pEC86 [21] coding for the heme maturation complex.

The same cloning and expression strategy was applied for the membrane-spanning full-size cytochrome c_1 using primers I and J (Table 1, Supplementary material). The PCR product was cloned into pET22b linearized with *Nco*I and *Xho*I thereby fusing the vector-encoded sequence for a C-terminal His_6-tag for metal affinity purification (pDM3).

2.4. Purification of the bc_1 complex

Frozen *T. thermophilus* cells were thawed at 4 °C and broken by three passages through a Manton–Gaulin press (400 bar). After centrifugation at 130,000 × *g* for 45 min at 4 °C the membrane fraction was collected and resuspended in 50 mM KP_i-buffer pH 8, 150 mM NaCl and 1 mM EDTA. Membranes were diluted to 10 mg/ml (determined by Lowry protein assay [26]) with 50 mM KP_i pH 8, 300 mM NaCl, 30% glycerol, 1 mM EDTA. After addition of n-decyl-β-D-maltoside (3 mg detergent/mg protein; Biomol, Hamburg) the suspension was stirred for 3 h at 4 °C and then centrifuged at 130,000 × *g* for 1 h at 4 °C. The resulting reddish supernatant was loaded on a Ni-NTA column (Amersham Bioscience, Freiburg) equilibrated with 50 mM KP_i pH 8, 300 mM NaCl, 30% glycerol and 0.16% decylmaltoside. The column was washed with the same buffer adjusted to pH 7, followed by a second washing step at pH 6.5. Elution of the bc_1 complex was achieved at pH 5.5 (same buffer components). Red fractions were immediately adjusted to pH 8 with 1 M KP_i-buffer, concentrated and subjected to gel filtration on an Ultrogel AcA 34 column (Serva, Heidelberg).

2.5. Purification of the soluble and full-size cytochrome c_1

The soluble cytochrome c_1 fragment was purified in a two-step chromatographic protocol on a Q-Sepharose-Fast-Flow (Amersham Bioscience) in 20 mM Tris–HCl pH 8.0, 1 mM EDTA buffer and eluted with a salt gradient from 0 to 400 mM NaCl in the same buffer. This was followed by gel filtration on a Sephacryl S-100 column (Amersham Bioscience) in 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA.

E. coli membranes expressing the full-size cytochrome c_1 were prepared as described above for *T. thermophilus*, solubilized in 50 mM Tris–HCl-buffer pH 8 with Triton X-100 (3:1 excess over protein, w/w) and stirred for 2 h at 4 °C. After centrifugation the supernatant was applied on a Ni-NTA column equilibrated with 20 mM KP_i buffer pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 50 mM imidazole. After washing, the cytochrome c_1 was eluted with an imidazole gradient (50–150 mM) at 120 mM. The protein was collected, concentrated and applied to a Sephacryl S-100 gel filtration column (Amersham Biosciences).

2.6. In-gel digestion and mass spectrometry

For peptide mass fingerprinting and subsequent analyses, gels were sliced and subjected to an in-gel digestion protocol [27] adapted for the use on a Microlab® Star digestion robot (Hamilton, Bonaduz, Switzerland). Delayed extraction (DE) MALDI-TOF mass spectra were recorded on a Voyager-DE STR instrument (Applied Biosystems) as described in [28]. All AP-MALDI MS/MS experiments were performed using an LC 1100 quadrupole ion trap mass spectrometer (Agilent, Waldbronn, Germany) retro-fitted with an AP-MALDI source from Mass Technologies (Burtonsville, MD, USA) equipped with a 20-Hz, 337 nm N_2-Laser. All spectra were analyzed for MS/MS database search using Data Analysis V (Bruker Daltonic, Bremen).

For MALDI-PSD experiments either an Applied Biosystems 4700 ProteomicsAnalyzer (Applied Biosystems, Framingham, MA, USA) or a Bruker Ultraflex TOF/TOF (Bruker Daltonic) mass spectrometer was used. The 4700 ProteomicsAnalyzer TOF/TOF instrument is equipped with an Nd:YAG laser with 355-nm wavelength of <500 ps pulse and 200 Hz repetition rate in both MS and MS/MS modes. In MS mode, 1200 shots were accumulated and in MS/MS mode 3200 or 4800 were accumulated. LIFT mass spectra were acquired on a Bruker Ultraflex TOF/TOF mass spectrometer operated in the positive ion mode. Metastable fragmentation was induced by a nitrogen laser (337 nm; 25 Hz repetition rate) without the further use of collision gas. Precursor ions were accelerated and selected in a timed ion gate. In the LIFT-cell, the fragments were further accelerated to 19 kV; the reflector potential was 29 kV. In MS mode, again 1200 shots, and in MS/MS mode, 3200 or 4800 shots were accumulated.

2.7. N-terminal sequencing

Automated N-terminal sequence analysis was carried out in a Knauer 910 gas/liquid-phase protein sequencer on polyvinylidenfluorid (PVDF)-membranes with auto-conver-

sion and on-line HPLC identification of the phenylthiohydantoin amino acids.

2.7.1. Peptide separation

The subunits of the *Thermus* bc_1 complex were purified on a reversed phase Synchropak C_4 column (250 × 4.6 mm) using HPLC-equipment. A Hewlett-Packard 1050 System with a multiple wavelength detector in a mix-gradient of: (A) 5% formic acid, (B) 95% formic acid, (C) n-propanol, and (D) acetonitrile. Gradients were formed with the aid of a microprocessor-controlled quaternary pump (HP) and by low-pressure mixing of four solvents in two 30 min steps. (i) (A) 14% to 0%, (B) 70% = constant, (C) 6% to 11%, (D) 10% to 19%. (ii) (A) 0%, (B) 70% = constant, (C) 11% to 30%, (D) 19% to 0% (by vol.).

2.7.2. BrCN-cleavage

Methionyl bonds were cleaved with cyanogen bromide by the method of Gross and Morell [29]. 50 nmol protein was dissolved in formic acid and mixed with a 200-molar excess of cyanogen bromide and the reaction proceeded in the dark for 4 h. The reaction mixture was then evaporated under reduced pressure. The fragments were purified by HPLC on reversed phase Synchropak C_{18} column and used for N-terminal sequencing.

2.7.3. Deformylation

The N-formylmethionine blocked proteins were deformylated with 0.5 M acetyl chloride 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.

2.8. Spectroscopic procedures

Visible spectra were obtained on a U-3000 Hitachi spectrophotometer. Pyridine-hemochromogen spectra were recorded as previously described [30]. $K_3Fe(CN)_6$ was used to oxidize samples, ascorbate or sodium dithionite for reduction.

Concentrations of cytochrome c_{552} were determined using an extinction coefficient $\Delta\varepsilon_{\text{red-ox, 552nm}} = 21.0\ \text{mM}^{-1}\ \text{cm}^{-1}$ [31].

2.9. Western blotting

An antibody directed against the soluble cytochrome c_1 fragment was raised in a rabbit (Eurogentec, Belgium) after immunization with 250 μg protein, followed by three boosts of 80 μg each. Western blotting was performed essentially as described [32].

2.10. Co-immunoprecipitation

Initial evidence for a bc_1 complex in membranes of *T. thermophilus* was obtained by co-immunoprecipitation. To determine the optimal precipitation ratio the cytochrome c_1 antiserum was titrated against solubilized HB8 membranes (Triton X-100 3:1, w/w). After mixing and incubation at 4 °C for several days, the resulting precipitate was pelleted by low speed centrifugation and washed twice with 50 mM KP_i-buffer pH 8 containing detergent (0.5% Triton X-100). The pellet was then resuspended in SDS-sample buffer and analyzed by SDS-PAGE.

2.11. Stopped-flow spectroscopy and experimental protocol

Kinetic experiments were carried out using an Applied Photophysics stopped-flow apparatus (Leatherhead, UK, dead time of 1.3 ms) with a 1-cm observation chamber. The ET kinetics between the soluble cytochrome c_1 domain and cytochrome c_{552} were studied in both the forward (physiological) and reverse direction, by pre-reducing anaerobically one of the components with 1 mM ascorbate, following a protocol described previously [20].

After complete reduction, the solution was mixed in the stopped-flow apparatus with an anaerobic solution containing the oxidized ET acceptor protein. The time-dependent extinction changes were followed at 557 nm, the optimal wavelength previously determined by difference spectroscopy in double-sector cuvette mixing experiments. All steps were performed at 8 °C.

3. Results

3.1. Genomic sequence data reveal a common gene locus encoding T. thermophilus cytochrome bc_1 subunits

A tBLASTn search [33] of genomic sequence data using the consensus motif of the Rieske protein [34] resulted in two hits. One of them turned out identical to the sequence of the FeS soluble fragment, and was used to identify further genes encoding a putative bc_1 complex of *T. thermophilus*. This 3.1 kb locus *fbc*CXFB, with a GC-content of 69%, comprises four consecutive open reading frames. 45 bp upstream of the ATG codon of the first gene a typical procaryotic promotor region is found with a TATA-box (TATAGT) and a closely spaced −35 region (TTGGCC). Moreover a distinct termination loop (CCCCCACCGCGG CCGCGGTGGGG TTTT) is identified 16 bp downstream of the stop TAG of the last ORF. These features are indicative of an operon structure with one single polycistronic transcript.

In an identity search, three of the four gene products were immediately identified as bona fide cytochrome bc_1 subunits. The genetic organization of the four genes (encoding cytochrome c_1, FbcX, the Rieske subunit and cytochrome *b*) is given in Fig. 1, and the presence of the FbcX product as a genuine subunit of the purified *T. thermophilus* bc_1 complex confirmed, see below.

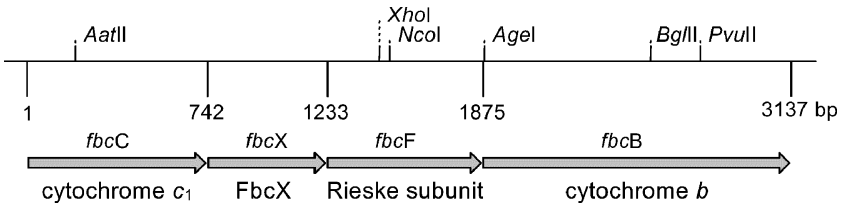

Fig. 1. Coding region of the *T. thermophilus fbc*CXFB locus specifying the four subunits of the bc_1 complex. Numbers denote first nucleotide of each start codon and the stop codon at the end of the cytochrome *b* gene.

3.2. The putative bc_1 operon *fbcCXFB* encodes four proteins

3.2.1. Cytochrome c_1

The first gene encodes a *c*-type cytochrome of 243 aa and 26 kDa (Table 1) with the typical heme-binding motif CxxCH (pos. 171–175). One putative central transmembrane helix of at least 12 aa starting at position 106 is suggested by the Dense Alignment Surface (DAS) transmembrane prediction algorithm [35] (Fig. 2A). Thus, in contrast to cytochrome c_1 components of the mitochondrial or other bacterial bc_1 complexes described so far (see Introduction), this cytochrome c_1 subunit of *T. thermophilus* (for a definition, see Discussion) exhibits an unusual structure consisting of two hydrophilic domains separated by a single transmembrane stretch: an N-terminal sequence of around 100 aa, presumably cytoplasmically oriented, and a C-terminal region of 125 aa, most likely facing outward, based on the fact that this domain contains the heme-binding motif and interacts with cytochrome c_{552} (see below). Moreover, there is extensive sequence identity only with the cytochrome *c* component of *Deinoccocus*.

3.2.2. FbcX

The second open reading frame encodes a putative protein FbcX of 17.6 kDa (Table 1) with so far unknown function. While no cofactor binding motif is revealed, nor any sequence identity to any of the other bc_1 subunits of mitochondria or bacteria, this highly hydrophobic sequence with its predicted 4 transmembrane helices (Fig. 2B) aligns well to the C-terminal part of cytochrome *c* from *D. radiodurans*. It is a genuine subunit of the isolated *T. thermophilus* cytochrome bc_1 complex, as shown below.

The Rieske protein is encoded by the third gene, resulting in a 22.4-kDa protein (Table 1). In the N-terminal part a twin-arginine motif within a distinctive TAT-export signal (SRRRLFLK) is detected (pos. 11–18, see Table 1) [36], followed by a predicted transmembrane helix of about 15 aa (starting at pos. 20) suggesting the Rieske protein to be a typical substrate of the TAT export pathway. Its C-terminal domain liganding the [Fe_2 S_2] cluster has already been expressed as a soluble protein in *E. coli*, and its crystal structure determined at high resolution [13]. The Rieske protein from *T. thermophilus* has been classified as a low potential (E'_0 = +150 mV) center involved in menaquinol-8 oxidation [37,38]. While there is only moderate sequence identity to other Rieske proteins, the overall structure of the *T. thermophilus* Rieske fragment is similar, especially in the cluster binding domain [13].

The cytochrome *b* subunit is encoded by the last structural gene of the *fbc* operon. The 47.5-kDa protein is composed of 420 aa (Table 1) and its eight presumed transmembrane helices suggest an unsplit full-size subunit structure (Fig. 2D). The heme-binding motifs may be attributed to helices two and four (pos. 91–105 and 193–208) in agreement with known structures of this subunit [2]. In an alignment, the highest degree of sequence identity (45%) is observed for the cytochrome *b* subunit of *D. radiodurans*, and only 25% for the homologous yeast protein.

3.3. Sequence differences of *fbcCXFB* in *T. thermophilus* strains HB8 and HB27

Sequencing the construct pDM6 (before publication of the complete HB8 genome sequence) revealed a total identity of 98% between the *T. thermophilus* strains HB8 (NC 006461) and HB27 (NC 005835). On the protein level,

Table 1
Subunit composition and characterization of the bc_1 complex from the two *T. thermophilus* strains examined

Subunit	Number of amino acids[a]	M_r/kDa[a]	$M_r$app/kDa[b]	N-terminal sequence[c]	Amino acids differing between strain HB8 and starin HB27
cytochrome *b*	420	47.5	36	f-MYR**WLDERLDLRG**	T133A, G200A
cytochrome c_1	243	26	28	MIVDRL**EVYLDQGTE**	D203N
Rieske subunit	210	22.4	24	MDEREVRLRQSRRRLFLK**TVIGTGIGLS**	K68Q, K76N, L103V, E111A, E146K, V157M, Y206C
FbcX	160	17.8	17	f-MYR**NDPILPTFAL**	Q39E, A111S

[a] Deduced from DNA sequence.
[b] Apparent mass deduced from Tricine-SDS-PAGE (see Materials and methods).
[c] Deduced from DNA sequence. The N-terminal residues have been obtained by N-terminal sequencing (underlined) and confirmed with peptide fragments identified by MALDI-MS (bold).

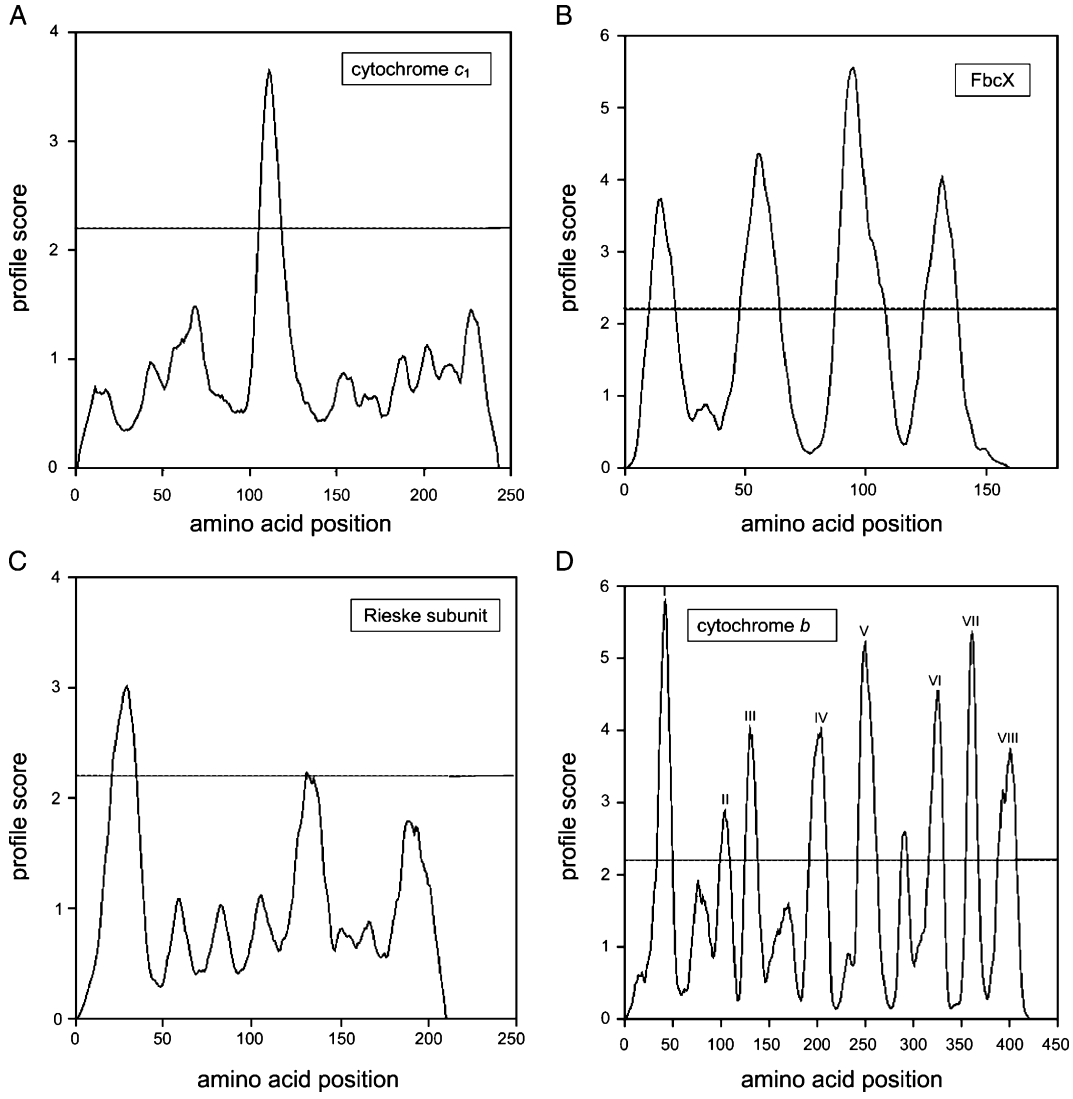

Fig. 2. (A-D) Hydropathy plots of the *T. thermophilus* bc_1 subunits as calculated by the DAS method [35]. Values above threshold (strict cut off; dotted line) denote presumed transmembrane helices.

both the translated sequences for the subunits cytochrome c_1 and cytochrome *b* show 99% identity between the two strains, corresponding to only one amino acid exchanged for cytochrome c_1 and two replacements each for cytochrome *b* and the FbcX subunit (see Table 1). Only for the Rieske protein a conspicuously lower identity of 96% is seen. Most of the seven exchanges (Table 1) are non-conservative, but exclude the consensus motif. We particularly note a shift in the charge pattern but no clear tendency between the two strains is seen.

3.4. The isolated T. thermophilus cytochrome bc_1 complex consists of four subunits encoded by the fbcCXFB operon

3.4.1. Co-immunoprecipitation

In an initial attempt to identify a hypothetical complex, the antibody directed against cytochrome c_1 was used for direct co-immunoprecipitation of the bc_1 complex from Triton X-100 solubilized membranes of *T. thermophilus* HB8. The resulting pellet was separated by SDS-PAGE, stained bands were excised, digested with trypsin and the resulting peptides subsequently analyzed using MALDI MS as well as MS/MS (see Figs. 1 and 2, Supplement): The two main protein bands proved the *T. thermophilus* cytochrome c_1 and cytochrome *b* subunits: for cytochrome c_1 60% of the protein sequence was covered and the precursor ion at m/z 1151.6 (NLPDGEHTLR) was additionally fragmented; the digest of cytochrome *b* resulted in a 15% sequence coverage, so that we further investigated the precursor ion at m/z 1357.7 (YMELPSEHPVR) to confirm the identity (Fig. 1, Supplement). No additional subunits of the bc_1 complex were detected under these detergent conditions.

3.5. Chromatographic purification of a four-subunit bc_1 complex

A DNA fragment comprising the entire *fbc*CXFB operon coding region and an additional 130 bp of the

upstream presumed promotor region was cloned into the *Thermus/E. coli* shuttle-vector pMA34 (see Material and Methods). After electroporation, this plasmid pDM6 was expressed in *T. thermophilus* HB27 and purified via the His-tag at the C-terminus of subunit cytochrome *b*. A two step purification procedure from membranes solubilized with n-decyl-β-D-maltoside was employed using a Ni-NTA affinity column followed by a gel filtration step at a yield of 2.5 mg per liter culture (see Material and methods).

The purified bc_1 complex from *T. thermophilus* is composed of four subunits as judged from SDS gel electrophoresis: cytochrome *b*, cytochrome c_1, Rieske protein, and FbcX, as shown in Fig. 3A (lane 1). All four subunits were isolated by reversed phase HPLC and used for N-terminal sequencing.

3.6. Characterization of the four-subunit complex

All four subunits were analyzed by MALDI MS and MS/MS (Bruker ultraflex TOF/TOF; see Figs. 1 and 2, Supplement) as well as by N-terminal sequencing (Table 1); subunits FbcX and cytochrome *b* are N-terminally blocked and were identified after deformylation as well as by internal peptide sequencing of the corresponding BrCN-fragments. The five N-terminal residues of the Rieske protein and the 103 residues of the heme *c* containing C-terminus of cytochrome c_1 were also obtained by Edman degradation. Moreover, the protein bands for cytochrome *b* (carrying the His_6-tag) and for cytochrome c_1 were identified by Western blotting with an anti-His antibody (data not shown) or with the cytochrome c_1 antiserum, respectively. Fig. 3B confirms identical migration behavior of the *c*-cytochrome for the isolated complex (lane 1), for *Thermus* membranes (lane M) as well as for the full-size cytochrome c_1 (carrying an additional His_6-tag) expressed in *E. coli* (lane 2, and see below).

When analyzed by SDS PAGE (Fig. 3A, lane 1), the cytochrome *b* subunit migrates with an apparent mass of around 36 kDa, deviating considerably from the expected size of 47.5 kDa, a fact observed for many strongly hydrophobic membrane proteins, and in particular for cytochromes *b*. In contrast, the cytochrome c_1 subunit shows an apparent molecular weight of about 28 kDa, slightly higher than the calculated 26 kDa, while the Rieske protein as well as the FbcX subunit appear at gel positions corresponding to their expected molecular mass (see Fig. 3A and Table 1).

Redox centers of the isolated complex are predominantly in their oxidized state (not shown). After ascorbate reduction, the spectrum only reveals absorption properties of the *c*-type heme in cytochrome c_1 with a maximum at 555 nm and a shoulder at 548.5 nm (Fig. 4C), in line with the spectral data for the cytochrome c_1 proteins expressed in *E. coli* (not shown). The dithionite-reduced sample shows a maximum at 561 nm corresponding to the two *b*-type hemes, in addition to the cytochrome c_1 absorption (Fig. 4A). Molar absorption coefficients were calculated based on pyridine-hemochrome spectra for cytochrome c_1: $\varepsilon_{555-620}$= 15.3 mM^{-1} cm^{-1} (in the ascorbate reduced state, Fig. 4) and heme *b*: $\varepsilon_{561-620}$=23.7 mM^{-1} cm^{-1}. Based on these values, a stoichiometry of 1.7 (cytochrome c_1 to heme *b*) is observed in the isolated bc_1 complex.

The presence of the Fe-S cluster of the Rieske subunit is demonstrated by EPR spectroscopy. Spectral properties and calculated g-values (Sevdalina Lyubenova, Fraser MacMillan, Frankfurt, to be published elsewhere) are closely related to those previously published for the soluble fragment of the Rieske protein [12].

3.7. Heterologous expression of cytochrome c_1

To analyze the composition of a hypothetical bc_1 complex in membranes of *T. thermophilus*, a polyclonal antibody directed against the cytochrome c_1 protein was

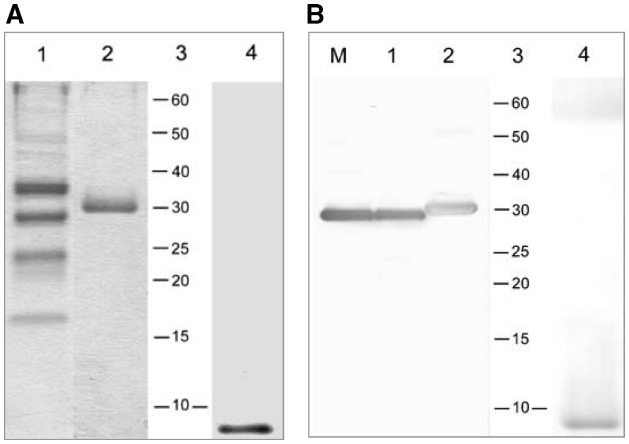

Fig. 3. The isolated *T. thermophilus* cytochrome bc_1 complex consists of four subunits. (A) 12% Tricine-SDS-PAG (lane 4: 15%) stained with Coomassie blue. (B) Western blot using a cytochrome c_1-specific antiserum. 1: purified bc_1 complex. 2: His_6-tagged full-size cytochrome c_1 expressed in *E. coli*. 3: molecular weight marker (kDa). 4: 84 aa C-terminal cytochrome c_1 fragment expressed in *E. coli*. M: solubilized *T. thermophilus* membranes.

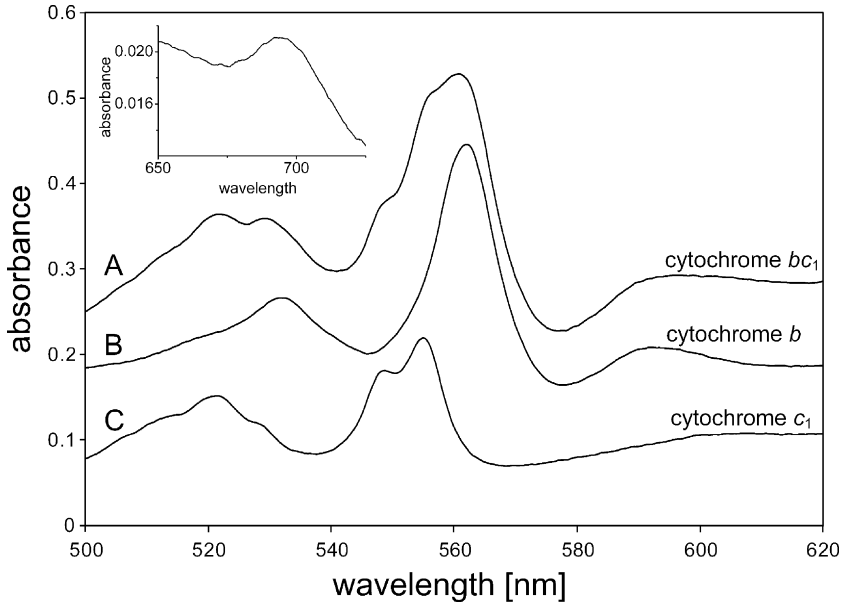

Fig. 4. Absorbance difference spectra of the isolated bc_1 complex. (A) Dithionite-reduced minus oxidized. (B) Calculated double difference spectrum (A–C). (C) Ascorbate-reduced minus oxidized. Charge transfer band of the ferricytochrome C_1.

generated. To this end, the C-terminal domain of the cytochrome c_1 subunit carrying the heme-binding motif was cloned (pMA37) and expressed as a soluble fragment in *E. coli* BL21(DE3) in the presence of plasmid pEC86 which specifies the heme maturation complex [21]. The purified fragment (8.7 kDa derived from DNA sequence) shows an apparent mass of <10 kDa on SDS gels (Fig. 3A, lane 4). Using nano-ESI on a Q-Trap the protein yields, when deconvoluted, a molecular mass of 9414 kDa; this value matches the last 84 aa of cytochrome c_1 including the heme cofactor (data not shown). The identity of the purified fragment was further confirmed by heme staining after SDS PAGE, and by redox spectroscopy, yielding a split á-band with a maximum at 555 nm and a shoulder at 548 nm in the reduced state (see Fig. 4 for the spectrum of the full-size c_1 subunit) as previously described [39].

Applying the same expression system, the full-size cytochrome c_1 was cloned and expressed. This membrane-integral protein was purified *via* its His_6-tag (see Fig. 3A, lane 2) and shows absorption properties identical to the fragment (Fig. 4). Western blotting using the c_1 antiserum confirms the expected full size of this cytochrome c_1 subunit. The apparent mass of 30 kDa for the heterologously expressed protein is in line with that of the subunit present in the purified complex as well as in solubilized *Thermus* membranes, with the expected difference in mass accounting for the His_6-tag (Fig. 3B). This further confirms that no major N-terminal proteolytic modification occurs in vivo.

3.8. The *T. thermophilus* cytochrome bc_1 complex reduces cytochrome c_{552}

The purified soluble cytochrome c_1 fragment was used to examine the ET route from the bc_1 complex towards the oxidases. Stopped-flow experiments were performed to study the interaction with the isolated soluble cytochrome c_{552} from *T. thermophilus*, expressed and purified basically as described previously [40]. The optimal wavelength to follow the reaction in the stopped-flow apparatus, 557 nm, was determined by double-sector cuvette experiments (for the difference spectrum, see Supplementary Fig. 3).

To study the electron transfer reaction, one of the two reaction partners was pre-reduced with ascorbate, which was present in the syringe to prevent autoxidation. The applicability of this approach was checked by determination of the bimolecular rate constant (k_{BM}) for the reaction of oxidized *Thermus* cytochrome c_1 with ascorbate at the given pH. By varying the ascorbate concentration, k_{BM} for the cytochrome c_1 reduction was calculated to be 2830 M^{-1} s^{-1} (data not shown), sufficiently slow not to interfere with the much faster electron transfer reaction between the two cytochromes. Previously, it had already been confirmed that under the same conditions also the *Thermus* cytochrome c_{552} reacts with ascorbate on a time scale much slower than the interprotein electron transfer rate [20]. Apparent bimolecular reaction rate constants were determined from the slopes of the pseudo-first order plots obtained by varying the cytochrome c_1 concentration.

In the forward (physiological) direction, with the pre-reduced c_1 fragment, the second-order rate constants at 8 °C range from ~2×10^7 M^{-1} s^{-1} at low ionic strength (10 mM KCl) to 0.7×10^7 M^{-1} s^{-1} at higher salt concentration (200 mM KCl) as shown in Fig. 5. The reverse reaction was analyzed by pre-reducing cytochrome c_{552}, and a bimolecular rate constant of 1.8×10^7 M^{-1} s^{-1} was determined for the reaction at 10 mM KCl. By calculating the forward rate constant ($k_{forward}$) over the reverse ($k_{reverse}$), a value for the

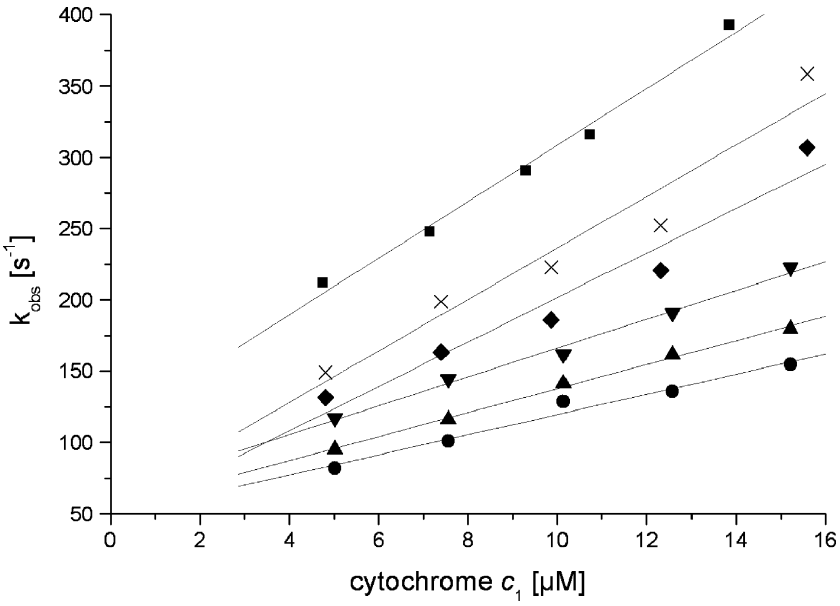

Fig. 5. The forward physiological electron transfer reaction between *T. thermophilus* cytochrome c_1 and cytochrome c_{552}. The fitted rate constants of the ET reaction between ferrous cytochrome c_1 (with concentrations ranging from 4.8 to 15.2 μM after mixing) and cytochrome c_{552} (5 μM after mixing) at various ionic strength conditions (10 mM: ■; 25 mM: x; 35 mM: ♦, 50 mM: ▼, 100 mM: ▲, 200 mM: ●) are plotted against the initial ferrous cytochrome c_1 concentration. Ascorbate concentration is 0.5 mM after mixing. Solid lines were calculated by linear regression of the data points.

equilibrium constant K_{eq} of 1.1 was obtained, indicating that the reaction is isoergonic. These very high bimolecular rate constants, determined for temperatures even far below optimum, suggest that the reaction approaches the limit imposed by diffusion and conclude that the two cytochromes indeed are physiological partners.

When the ionic strength dependency of bimolecular rates are analyzed, the Brønstedt Eq. (1) allows the quantitative determination of charges involved [41]. This function relates the observed bimolecular rate constant at ionic strength I (k_0 is the bimolecular rate at $I = 0$, B a term with a value of ~0.5 at 8 °C derived from the Debye–Hückel equation, and z_A and z_B representing the ET-sensitive charges on each of the protein surfaces). The bimolecular rate decreases as the ionic strength is increased, with a slope (the $z_A z_B$ product) of about −1.4 for this electron transfer couple (Fig. 6).

$$\log k = \log k_0 + 2Bz_A z_B\sqrt{I} \quad (1)$$

Thus, only around one effective charge of opposite sign on each interaction partner is involved in the electron transfer reaction, supporting the concept that hydrophobic contacts play a dominant role in the interaction of thermophilic proteins.

4. Discussion

Interest in the thermophile *T. thermophilus* has grown recently both for its phylogenetically old lineage [2] and its growing potential in biotechnological applications. With the genome sequences of the two strains HB8 (NC 006461) and HB27 (NC005835) made available in 2004 [42], our understanding of cellular strategies for survival at elevated temperatures, and for corresponding protein thermostability, are set to increase greatly.

We were mainly interested in the ET chain of this bacterium, where several of the respiratory components of *T. thermophilus* had already been described and partly determined to high structural resolution, but no evidence for a contiguous ET chain was available so far for redox reactions between NADH and oxygen (see Introduction), essentially lacking a quinone:cytochrome *c* activity. Only the soluble fragment of a FeS Rieske-type protein had been described so far, providing an early clue for the existence of a putative bc_1 complex (Fig. 7).

Based on the information of the *T. thermophilus* genome sequencing project (Genomics Laboratory, Göttingen, Germany), a consecutive chromosomal locus, termed *fbc*CXFB, was identified which may represent an operon structure: a typical promotor region (45 bp upstream of the start codon of the first ORF) and the presence of a single termination loop (16 bp downstream of the stop codon of the last gene) indicate a polycistronic transcription unit for the four coding genes.

4.1. The central component of the T. thermophilus ET chain is a four subunit bc_1 complex

Three of the four gene products (cytochrome c_1, Rieske protein, cytochrome *b*) share obvious sequence identities with bona fide bc_1 complex subunits, while the second open reading frame encodes a protein (FbcX) with no sequence identity to any known bc_1 complex subunit.

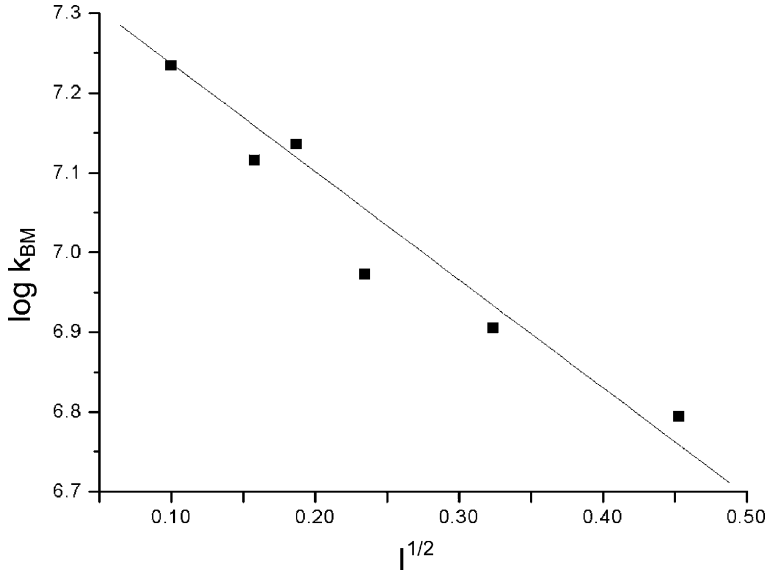

Fig. 6. Brønsted plot for the kinetic reaction between the *T. thermophilus* cytochrome c_1 fragment and cytochrome c_{552}. The logarithm of the bimolecular rate constant is plotted versus the square root of ionic strength. The slope yields the product $z_A \times z_B = -1.4$; for further details, see text.

On a protein basis, we give clear evidence that all four gene products assemble into a stable protein complex which therefore represents the *T. thermophilus* bc_1 complex. Identification of these four subunits, including the FbcX subunit, is provided by several protein chemical methods including N-terminal sequencing and MALDI mass spectrometry.

Initial attempts to obtain a native complex by co-immunoprecipitation after solubilization with Triton X-100 or dodecyl maltoside partly failed. Only the use of decyl maltoside yielded a stable complex association of all four subunits throughout several chromatographic steps, with an experimentally determined heme stoichiometry of 1.7 (*b* to *c*), only slightly short of the expected stoichiometry of 2:1.

In a preliminary attempt (data not shown) to quantify enzymatic activity of the purified, detergent solubilized complex, we obtained ubiquinol:cytochrome *c* (acceptor: horse heart *c*) electron transfer activity, specifically inhibited by menaquinone analoga (kindly supplied by Bernard Trumpower, Dartmouth). Using MK-8 analoga and the endogenous *T. thermophilus* c_{552}, strong autoxidation precluded further experiments; therefore, in-depth optimization of the assay conditions in terms of substrates and temperature will be required.

4.2. Cytochrome b and the Rieske subunit are largely similar to other known sequences

The *T. thermophilus* cytochrome *b* subunit is found as an unsplit version [2], especially the first half of the subunit (predicted helix I–IV, Fig. 2D) including the conserved heme binding motifs shows a high degree of identity to known cytochrome *b* sequences.

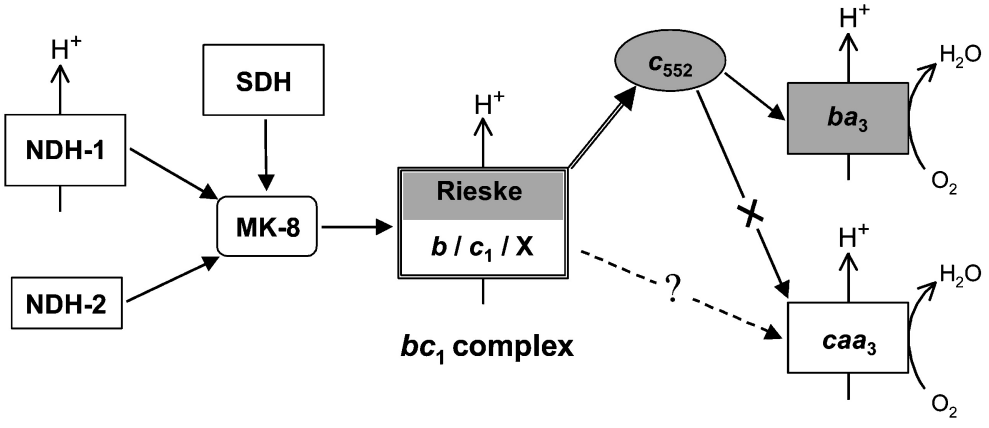

Fig. 7. The *T. thermophilus* cytochrome bc_1 complex and its interactions with previously described components of the respiratory chain. Double boxed elements: experimental evidence from this work, shaded: 3-D structures determined (see Introduction), dotted line: experimental evidence lacking, vertical upward arrows: presumed or confirmed energy transducing complexes. MK-8: menaquinone-8, NDH: NADH:quinone oxidoreductase, SDH: succinate reductase.

The soluble part of the Rieske protein has been described earlier [12,13]. By MALDI analysis, we identified the major part of the predicted transmembrane helix (Table 1) that exhibits the distinct TAT-export sequence motif. This hydrophobic region of about 15 aa (Fig. 2C), therefore, serves the dual purpose of a (unprocessed) signal sequence and a transmembrane anchor at the same time and confirms the full-length Rieske protein as a true membrane-integral subunit.

4.3. The cytochrome c_1 component shows an unusual topology

Hydropathy analysis of the cytochrome c_1 amino acid sequence suggests a topology at variance with other cytochromes c_1, with two soluble domains of about 100 aa and 125 aa separated by a 13 aa putative transmembrane helix (Fig. 2A). Using an antibody raised against the soluble fragment, we were able to assess the apparent mass of this subunit in native *T. thermophilus* membranes, after heterologous expression in *E. coli*, and in the purified complex (Fig. 3A). Thus, we can definitely confirm that the large N-terminal hydrophilic domain is indeed present in the mature subunit under all conditions of expression, and is not a transient one, such as an extensive signal sequence cleaved after secretion across the membrane. As shown in Fig. 3, the full length of the cytochrome c_1 in the purified complex as well as that detected in membranes is in accordance with the expected calculated size. Also, the heterologously expressed cytochrome c_1 carrying a His_6-tag matches expectation. Most importantly, the N-terminus of cytochrome c_1 (see Table 1) has been identified in the isolated *Thermus* bc_1 complex by N-terminal sequencing (data not shown) and MALDI MS (a tryptic peptide starting at amino acid position 6, see Table 1).

Most of sequence of the cytochrome c_1 protein aligns, to any significant extent, only to the N-terminal half of the *c*-type component of the *D. radiodurans bc* complex. The C-terminal domain of the *T. thermophilus* cytochrome c_1, most likely protruding into the periplasm to interact with cytochrome c_{552} (see below), includes the highly conserved heme binding motif CxxCH.

An unusual spectral feature is the split absorption peak in the redox spectrum with a maximum at 555 nm and a shoulder at 548.5 nm (Fig. 4); its experimentally determined molar absorption coefficient of $\varepsilon_{555-620} = 15.3\ mM^{-1}\ cm^{-1}$, therefore, is lower than those published for other typical *c*-type cytochromes.

Based on analysis of the charge transfer band and further specified by sequence modeling to known *c*-type cytochrome domains, the sulfur of Met 212 is suggested as the sixth iron ligand in the *T. thermophilus* cytochrome c_1.

Considering all the differences of FbcC with respect to a classical cytochrome c_1, its precise evolutionary relationship is not completely clear at present. Nevertheless, from a functional point of view, we show here that the complex has an enzymatic activity of a quinol:cytochrome *c* oxidoreductase, and more specifically, its FbcC (heme-containing fragment) interacts in a kinetically competent manner with cytochrome c_{552} which in turn delivers electrons to the ba_3 oxidase. We therefore refer to this subunit of the complex as cytochrome c_1.

4.4. The additional fourth subunit FbcX

Next to the three known canonical subunits, the *T. thermophilus* bc_1 complex carries an additional small hydrophobic subunit, FbcX. The N-terminal region of the protein is detected by MALDI MS (see Table 1), providing clear evidence against any major posttranslational modification at the N-terminus. Therefore, four transmembrane helices (Fig. 2B) are suggested for this subunit.

We have no clues for a function of the FbcX subunit so far. A sequence alignment for this subunit only yields a hit for the C-terminus of the *D. radiodurans* cytochrome *c* subunit. We therefore speculate that this fourth subunit together with cytochrome c_1 (see above) of the *T. thermophilus* bc_1 complex is the product of a gene splitting/fusion event.

4.5. Reaction partners of the bc_1 complex

With the identification of the compounds of a typical cytochrome bc_1 complex, we can now envisage a minimal version of a functional ET chain in *T. thermophilus* membranes, reaching from NADH to oxygen. Our kinetic data obtained for the redox interactions of soluble fragments derived from complexes III and IV also provide clear evidence for specificity and efficiency in this part of the ET chain ([20] and this paper).

The kinetic measurements give an indication of the electron transfer routes in the respiratory chain of *T. thermophilus*. Starting from menaquinone-8 reduced by complex I, electrons are subsequently funneled into the bc_1 complex identified in this work. From its reduced cytochrome c_1, electrons are transferred to the soluble c_{552}, as evidenced by our stopped-flow experiments. The very fast reaction between these two cytochromes, yielding electron transfer rates of $10^7\ M^{-1}\ s^{-1}$, indicates a specific interaction. Any encounter between physiological electron transfer partners has to be highly specific to avoid side reactions, while maintaining high reaction rates which can only be reached if direct interactions between the molecules (e.g., salt bridges, van-der-Waals interactions, hydrophobic effects) are weak, avoiding extensive complex formation. The paucity of charges in the interaction between cytochrome c_1 and c_{552} shows the importance of non-electrostatic forces during the reaction, recalling the fact that electrostatic forces are weakened at elevated temperatures. A similar situation has been encountered for the reaction between *Thermus* cytochrome c_{552} and the soluble Cu_A domain of the ba_3 oxidase, where only a minor involvement of charges was observed [20].

From the reduced cytochrome c_{552}, the pathway of electrons branches at the level of terminal oxidases, as indicated by turnover experiments and stopped-flow kinetics using the soluble Cu_A fragment [20]: Cytochrome c_{552} is not an efficient substrate for the caa_3 oxidase as shown in kinetic experiments using ascorbate and TMPD [31], where addition of cytochrome c_{552} does not increase turnover. Whether electrons entering the caa_3 oxidase are transferred from cytochrome c_1 directly to the cytochrome c domain fused to subunit II of the caa_3 oxidase remains an interesting option (Fig. 7).

In recent years, *T. thermophilus* has evolved as a model organism for studying thermophilic proteins, with phylograms classifying this organism close to a common ancestral cell. Evolutionary studies focussing on Rieske proteins, as well as on the cytochromes *b*, place the members of the *Deinococcus*/*Thermus* group at a very early branching point [2]. Therefore the *T. thermophilus* bc_1 complex can be regarded as the progenitor, and should yield more insight into the general character and early composition of this enzyme. Using menaquinol-8 with a much lower redox potential than the ubiquinol pool used by many other bacteria and by mitochondria might reflect adaptations to an ur-atmosphere characterized by a lower oxygen tension.

With the identification of a *T. thermophilus* bc_1 complex, both on a genetic and a protein level, we are now in a position to specifically approach structure–function relations by site-directed mutagenesis; tagged complexes carrying mutations can be easily purified and separated from their wildtype counterparts. Moreover the *Thermus* expression system developed here provides the basis for detailed studies of thermostability of this unusual cytochrome bc_1 complex, and may be further exploited for the production of thermostable proteins for homologous and heterologous approaches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2005.03.008.

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