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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