Functional Dissection of the Multi-Domain Di-Heme Cytochrome c550 from Thermus thermophilus

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Abstract

In bacteria, oxidation of sulfite to sulfate, the most common strategy for sulfite detoxification, is mainly accomplished by the molybdenum-containing sulfite:acceptor oxidoreductases (SORs). Bacterial SORs are very diverse proteins; they can exist as monomers or homodimers of their core subunit, as well as heterodimers with an additional cytochrome c subunit. We have previously described the homodimeric SOR from Thermus thermophilus H88 (SORTTHB8); it identified its physiological electron acceptor, cytochrome c550 and demonstrated the key role of the latter in coupling sulfite oxidation to aerobic respiration. Herein, the role of this di-heme cytochrome c was further investigated. The cytochrome was shown to be composed of two conformationally independent domains, each containing one heme moiety. Each domain was separately cloned, expressed in E. coli and purified to homogeneity. Stopped-flow experiments showed that: i) the N-terminal domain is the only one accepting electrons from SORTTHB8; ii) the N- and C-terminal domains are in rapid redox equilibrium and iii) both domains are able to transfer electrons further to cytochrome c552, the physiological substrate of the ba3 and caa3 terminal oxidases. These findings show that cytochrome c550 functions as a shuttle electron, without working as an electron wire with one heme acting as the electron entry and the other as the electron exit site. Although contribution of the cytochrome c550 C-terminal domain to T. thermophilus sulfur respiration seems to be dispensable, we suggest that di-heme composition of the cytochrome physiologically enables storage of the two electrons generated from sulfite oxidation, thereby ensuring efficient contribution of sulfite detoxification to the respiratory chain-mediated energy generation.

Introduction

In addition to its natural occurrence in the environment, sulfite is an extremely important intermediary in sulfur metabolism, arising from a variety of reactions both in prokaryotes and eukaryotes [1–3]. The nucleophilicity and strong reducing capacity of sulfite account for its high toxicity. In the cell it can react with disulfide bonds causing protein inactivation and DNA damage. Although some microorganisms use sulfite as the sole electron/energy source [4,5], accumulation of sulfite in the cell generally leads to massive damage, so that both prokaryotic and eukaryotic cells require efficient sulfite detoxification systems.

The most common strategy for sulfite detoxification in bacteria and Archaea involves oxidation to sulfate, accomplished either directly or indirectly via the adenosine 5’-phosphosulfate reductase pathway [2,6]. Molybdenum-containing sulfite:acceptor oxidoreductases (SORs) catalyze the direct oxidation of sulfite to sulfate. They have been identified in mammals [7], birds [8], plants [9] and prokaryotes [10]. Two types of SORs have been identified to date: i) the sulfite oxidases (EC 1.8.3.1) that are able to utilize O2 as a direct electron acceptor, but also ferricyanide and sometimes cytochrome c3, and ii) the sulfite dehydrogenases (EC 1.8.2.1) unable to transfer electrons to O2. The first bacterial SOR was discovered almost half a century ago [11]. Although, since then, SORs have been shown to be widely distributed among bacteria, their exact physiological role is still elusive.

Compared to the vertebrate and plant enzymes, bacterial SORs are structurally much more diverse. The protein core consists of a molybdenum binding site and a dimerization domain. The enzymes can exist as monomers or homodimers of the core structure, as well as heterodimers with an additional cytochrome c subunit [10,12–16]. The SORs containing both the molybdenum cofactor and the additional cytochrome c subunit have been classified as Group 1 SORs, while members of the Group 2 contain only the molybdenum cofactor and are called ‘atypical’ SORs [17]. The name ‘atypical’ arises from the fact that most of these enzymes, if not all, cannot efficiently use horse heart cytochrome c as substrate and display higher activities when assayed with the artificial electron acceptor ferricyanide. To date, Group 1 includes only the cytochrome c-containing SOR isolated from Starkeya novella [10]. The SOR from Campylobacter jejuni, though originally defined as a two-subunit protein acting similarly to the S. novella enzyme, likely belongs to Group 2 SORs, as the
molybdenum- and the heme-containing subunits do not co-purify [10]. Similarly, all the other bacterial SORs so far described fall into Group 2 [12–16], thus calling for a revision of the term ‘atypical’.

With the exception of the enzyme from Deinococcus radiodurans, all characterized SORs were interestingly found to be encoded upstream their putative physiological electron acceptors (c-type cytochromes or other redox proteins [19]). Consistently, the genes coding for the c-type cytochromes were identified as electron acceptors for SORs from S. novella [10], C. jejuni [18], Sinorhizobium meliloti [20] and Thermus thermophilus [16] were all found downstream the relative SOR-encoding gene. These c-type cytochromes are also very diverse. They differ in size and heme content and this is an additional feature contributing to the complexity of SORs.

Several attempts have been made to elucidate how SOR-mediated sulfite oxidation is integrated in cell metabolism. It is postulated that Group 1 SORs are directly linked to the respiratory chain via their natural electron acceptor cytochromes [20]. In S. novella, cytochrome c550 [10] was tentatively suggested to enable the association between sulfite oxidation and aerobic respiration, based on the notion that cytochromes ε are natural substrates for cytochrome c oxidases [20]; the hypothesis however, remains to be tested as yet. Similarly, in C. jejuni electrons from sulfite oxidation were proposed to enter the respiratory chain downstream the bε complex via the natural substrate of cb oxidase [18]. Although such a scenario seems plausible, also in this case the exact electron transfer pathway and the redox proteins involved have not been identified. Only cell extracts, and not purified proteins, were used in these experiments and, indeed, one cannot exclude the involvement of additional, unidentified electron shuttles.

Recently, the connection to the respiratory chain was demonstrated for Group 2 SORs from S. meliloti [20] and T. thermophilus [16], whose natural electron acceptors (cytochrome c Smc04048 and cytochrome c550h, respectively) have been identified. In the former study [20], however, experiments were performed using isolated cell membranes and the association of the electron acceptor of SorT, cytochrome c Smc04048, with cytochrome oxidases remains to be proven. On the contrary, in the latter study on T. thermophilus the complete electron transfer pathway linking sulfite oxidation to oxygen reduction was unveiled [16]. Accordingly, the electrons generated upon sulfite oxidation by SOR_TTHB are transferred to the natural electron acceptor of the enzyme, cytochrome c550h and from here to cytochrome c552h, the physiological electron donor of the two terminal cytochrome c oxidases, ba3 and caa3.

Here, the role of T. thermophilus cytochrome c550 in coupling sulfite oxidation to cell respiration has been further investigated.

**Materials and Methods**

**Purification of Cytochrome c550 and the Cytochrome c Oxidases ba3 and caa3**

Native ba3- and caa3-type cytochrome c oxidases were isolated from T. thermophilus HB8 cells according to previously published procedures [21,22]. Native cytochrome c552 was purified according to Soulîmane and co-authors [23]. Purified proteins were concentrated by ultrafiltration, fast frozen in liquid nitrogen and stored at −80°C.

**Expression and Purification of SOR_TTHB and Cytochrome c550**

Expression and purification of the proteins were conducted as described previously [16].

**Determination of Cytochromes and Cytochrome Oxidases Concentration**

UV/vis absorption spectra were recorded with a Perkin Elmer Lambda 5 spectrophotometer. Concentration of the proteins was obtained from the dithionite reduced-minus oxidized spectra using the following extinction coefficients: e = 18000 M⁻¹ cm⁻¹ (λ = 550 nm) for cytochrome c550; e = 21000 M⁻¹ cm⁻¹ (λ = 552 nm) for cytochrome c552; e = 6300 M⁻¹ cm⁻¹ (λ = 613 nm) for ba3 oxidase and e = 24000 M⁻¹ cm⁻¹ (λ = 604 nm) for caa3 oxidase. The concentration of SOR_TTHB was determined using e = 67350 M⁻¹ cm⁻¹ (λ = 280 nm).

**Limited Proteolysis**

Cytochrome c550 was subjected to limited proteolysis by trypsin (Sigma-Aldrich) for 90 minutes at 37°C in 25 mM Tris-HCl pH 8.2, at a cytochrome:trypsin mass ratio of 100:1. To separate the proteolytic products, the reaction mixture was diluted with water and loaded on a Fractogel® TMAE 650(S) column (Merck, Germany) equilibrated with 5 mM Tris-HCl pH 8.2. The flowthrough was collected, the column washed with the equilibration buffer and finally bound proteins were eluted with the same buffer containing 150 mM NaCl.

**Construction of the Cytochrome c550 Domains Expression Plasmids**

The sequence encoding the mature N-terminal domain of cytochrome c550 (c550[N]) was amplified from T. thermophilus HB8 genomic DNA by PCR using the primers 5’-ATCTGAGCATGAGTCAGCCACCTCCTCCGAG-3’, containing NdeI restriction site (underlined), and 5’-CATGATCTCAAGT-CAAGCCAGGGTCTCCTGAGCTG-3’, containing XhoI restriction site (underlined). Similarly, the sequence encoding the mature C-terminal domain of cytochrome c550 (c550[C]) was amplified using the primers 5’-ATCTGAGCATGAGTCCGCAAACGCGAGCCGGTTGCTC-3’, containing NdeI restriction site (underlined), and 5’-CATGATCTCAAGT-TCATGAGCTTTGAGCCGCGGAG-3’, containing XhoI restriction site (underlined). The products were NdeI and XhoI cloned into the expression vector pET22b+ (Invitrogen) to yield the pET22bC550N and pET22bC550C vectors. These constructs permit the expression of the recombinant domains in E. coli, fused to the petB leader sequence for an optimal translocation to the periplasmic space.

**Expression and Purification of the Recombinant Cytochrome c550 N-terminal Domain (c550[N])**

The BL21 (DE3) E. coli strain was co-transformed with the pET22bC550N and pEC86 vectors [24], the latter containing the cytochrome maturation gene cluster necessary for the production of cytochrome c in E. coli under aerobic conditions [25]. The recombinant c550[N] was produced by growing the cells in LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 37°C for 24 h under shaking, and without protein expression inducers. Periplasmic proteins were prepared from fresh biomass. Cells were washed in PBS buffer (20 mM phosphate, 135 mM NaCl, 1 mM KCl, pH 7.4) and spun down at 8000×g for 20 min at 4°C. The pellet was resuspended in 100 mM Tris-HCl pH 8 buffer containing 0.75 M sucrose. Osmotic shock was induced by slowly adding 2 volumes of ice chilled 1 mM EDTA. Following 10 min incubation at room temperature, spheroplasts were prepared by incubation with 1 mg/ml lysozyme for 45 min at room temperature under gentle shaking. Following the addition of 25 mM MgCl2, and 50 μg/ml...
DNaSe1 to reduce the viscosity of the extract, intact spheroplasts were removed by centrifugation at 8000 x g for 10 min at 4°C. The supernatant containing the recombinant domain was extensively dialyzed against 5 mM Tris-HCl pH 8.0 and then loaded on a Fractogel® TMAE 630(S) (Merck, Germany) column equilibrated at 4°C with the same buffer. The protein was eluted with a gradient of NaCl (0–250 mM) and the fractions containing the protein were pooled, concentrated and desalted using a PD10 column (GE Healthcare, Germany) equilibrated with 5 mM Tris-HCl pH 8.0. The eluate was then loaded on a CaptoQ XL anion exchange column (GE Healthcare, Germany) equilibrated with the same buffer and eluted with a gradient of NaCl (0–150 mM). Fractions containing the domain were pooled, concentrated and finally purified by gel filtration on a Superdex 75 column (GE Healthcare, Germany) at 4°C with 5 mM Tris-HCl pH 8.0 buffer containing 150 mM NaCl. The isolated protein was concentrated by ultrafiltration, fast frozen in liquid nitrogen and stored at −80°C.

Expression and Purification of the Recombinant Cytochrome c550 C-terminal Domain (c550[C])

The C-terminal domain was expressed and initially extracted as described above for the N-terminal domain. The supernatant containing the c550[C] domain was extensively dialyzed against 5 mM Tris-acetate buffer pH 6.0 and then loaded on a CM Sepharose® (Merck, Germany) column equilibrated at 4°C with the same buffer. The protein was eluted with a gradient of NaCl (0–250 mM) and the fractions containing the protein were pooled, concentrated and finally purified by gel filtration on a Superdex 75 column (GE Healthcare, Germany) at 4°C with 5 mM Tris-HCl pH 8.0 buffer containing 150 mM NaCl. The last step was repeated twice. The isolated protein was concentrated by ultrafiltration, fast frozen in liquid nitrogen and stored at −80°C.

Analytical Size Exclusion Chromatography

Analysis of the association of cytochrome c550 domains was carried out by analytical size exclusion chromatography (SEC). 1.3 x 10⁻³ moles of each domain were incubated for one hour at room temperature in 10 mM Tris-HCl, 50 mM NaCl, pH 8.0 before injection on a Superdex 75 10/30 column at a flow-rate of 0.5 ml/min. Elution profiles were recorded at 280 nm. The oxidation of cytochrome full-length was analysed in a similar way.

Electron Transfer activity

Stopped-flow experiments were carried out with a thermostated instrument (DX.17 MV, Applied Photophysics, Leatherhead, UK), equipped with a 1-cm pathlength observation chamber. Reactions were investigated by monitoring the absorption changes at selected wavelengths. When necessary, ionic strength was adjusted by addition of KCl and the buffer was degassed with vacuum/N2 cycles. Data were analyzed using the software MATLAB (The Mathworks, South Natick, MA).

Reduction by SOR₁THB₁ of the cytochrome c550 and its domains was investigated anaerobically at 45°C in 100 mM Tris-HCl pH 8.0 buffer containing 0.1 mM EDTA. To prevent inhibition of SOR₁THB₁ resulting from prolonged incubation of the enzyme with a large excess of sulfite, in these experiments the stopped-flow instrument was used in the sequential mixing mode. Typically, 2 μM SOR₁THB₁ was pre-mixed with 4 mM sulfite and after 500 ms further mixed with increasing amounts of oxidized cytochrome c550, c550[N], c550[C] or a 1:1 mixture of the two domains. The reduction of the cytochrome or its domains was monitored at 418 nm or, in the case of a too high signal in the Soret region, at 555 nm. The turnover rates (TN) of reduction of cytochrome c550 and its domains were calculated by dividing the concentration (expressed in μM) of the c550 sample reduced at t = t₁/₂ by the half time of the reaction and the concentration of SOR₁THB₁ in the experiment (typically 0.5 μM after mixing).

The kinetics of electron transfer between ascorbate-reduced cyt c550 (or its domains) and cyt c552 was assayed anaerobically at 4°C. This low temperature was chosen to slow-down and thus better resolve in time the reactions. Experiments were carried out in 5 mM Bis-Tris pH 7.0 buffer under non-pseudo-first order conditions, i.e., at comparable concentrations of the two proteins. Observed rate constants (kobs) were therefore obtained by fitting the experimental time courses to the equations described in [26] for the analysis of bimolecular reactions assayed under second-order conditions.

The oxidation of cytochrome c552 and its domains by baa₁ or cata-oxidase was assayed at 25°C in 5 mM Bis-Tris pH 7.0 buffer complemented with 0.1% n-Dodecylβ-D-maltoside. Approximately 1.5 μM c550, c550[C], c550[N] or a 1:1 mixture of the two domains was pre-reduced with 300 μM ascorbate and stopped-flow mixed with air-equilibrated buffer containing either baa₁ (200 mM) or cata-oxidase (200 nM). The reaction was followed at 418 nm.

Results

The Cytochrome c550 is Composed of Two Conformationally Independent Domains

The recently identified periplasmic di-heme cytochrome c550 [16] as a whole shows poor similarity to known proteins. However, when analyzed separately, its N-terminal domain sequence containing one heme binding site presents a high homology to the subunit B of SOR from C. jejuni [18], whereas the C-terminal domain, containing the other heme binding site, exhibits a high sequence identity with c552 from T. thermophilus HB8. This led to the hypothesis that cytochrome c550 is likely organized in two distinct domains, each one possibly presenting an independent fold and a heme cofactor, with distinct roles in mediating electron transfer between SOR₁THB₁ and the respiratory chain, through cytochrome c552 [16].

Consistently, according to the DomPred server [27] cytochrome c550 consists of two domains, with a predicted boundary at residue 107 and a proline rich region (91–106 aa) likely representing a flexible inter-domain linker [28] (Figure 1A). While globular proteins, due to their native rigid structure, are typically resistant to proteolysis under physiological conditions, flexible inter-domain linkers can be substrates for proteases. This makes the limited proteolysis approach suitable to confirm the multi-domain organization of a protein [29]. Based on this notion, cytochrome c550 was subjected to limited proteolysis using trypsin. Overall, the cytochrome presents 19 putative cleavage sites for this protease (Figure 1A). While globular proteins, due to their native rigid structure, are typically resistant to proteolysis under physiological conditions, flexible inter-domain linkers can be substrates for proteases. This makes the limited proteolysis approach suitable to confirm the multi-domain organization of a protein [29]. Based on this notion, cytochrome c550 was subjected to limited proteolysis using trypsin. Overall, the cytochrome presents 19 putative cleavage sites for this protease (Figure 1A). Among these sites, the one in position K105 is of particular interest, as it is located within the predicted linker, being therefore potentially more accessible to the protease. On this basis, limited proteolysis of the recombinant cytochrome c550 is expected to yield the N- and C-terminal domains as the major cleavage products.
analyzed by SDS-PAGE. As expected, two major products of limited proteolysis were obtained in addition to the band corresponding to non-digested cytochrome (Figure 1B). The bigger fragment in the flowthrough can be attributed to \( c_{550}[C] \), while the shorter fragment eluting at 150 mM NaCl likely corresponds to \( c_{550}[N] \). This result confirms that the protein as a whole is strongly dipolar with a negatively charged \( c_{550}[N] \) and a positively charged \( c_{550}[C] \). The apparent molecular weights of the proteolytic products (7 kDa and 7 kDa) are smaller than those predicted for \( c_{550}[C] \) and \( c_{550}[N] \) (14 kDa and 9 kDa, respectively). This is most likely due to the presence of multiple trypsin cleavage sites within the protein, especially those located at the termini of the domains, more easily accessed by the protease (Figure 1A). The limited proteolysis did not disturb the core of the domains or the heme binding, as the two generated fragments exhibited UV-Vis spectroscopic properties identical to those of the individually expressed domains (see below). This indicates that the two proteolytic fragments correspond to the two domains, each of them being independently folded and associated with one heme cofactor.

The use of recombinantly produced, isolated polypeptidic domains has proven to be a valuable approach to investigate intra- and intermolecular electron transfer between redox centers in multi-domain proteins with largely overlapping spectral properties [30,31]. Therefore, to further confirm the existence of two independent domains in cytochrome \( c_{550} \) and to investigate their function, the N- and C-terminal parts of the protein were individually produced in \( E. coli \). The recombinant domains contained the PelB signal sequence to promote their translocation to the periplasmic space and fragments of the flexible linker in order to enhance the stability of both domains (Figure 1A). After protein expression and isolation of the periplasmic fractions, the two domains were purified by IEX and size exclusion chromatography (SEC), as described in the Materials and Methods section. The procedure yielded \( 95\% \) homogeneous fragments of approximately 9 kDa and 14 kDa corresponding to \( c_{550}[C] \) and \( c_{550}[N] \), respectively (Figure 2A). The UV-Vis spectra of the reduced \( c_{550}[N] \) and \( c_{550}[C] \) exhibited a Soret band centered at 415 nm and 417.5 nm, respectively, while \( c_{550}[C] \) showed also a composite \( a \) band (Figure 2B). This shows that the splitting of the signal does not arise from the presence of two hemes. Instead, it is the result of the transition between the ground state and two or more excited states close in energy. Overall, the spectral properties confirmed that one heme cofactor was successfully incorporated in each domain during recombinant expression in \( E. coli \).

**Figure 1. Domains of \( T. thermophilus \) cytochrome \( c_{550} \).** The schematic representation of the full-length cytochrome \( c_{550} \) (FL) with predicted trypsin cleavage sites (vertical lines) and the cloned \( c_{550}[N] \) and \( c_{550}[C] \) domains is shown in panel A. The predicted boundary of the two domains is indicated by an arrow. Panel B shows the SDS-PAGE analysis of cytochrome \( c_{550} \) after limited proteolysis with trypsin and IEX chromatography (Fractogel\textsuperscript{®} EMD TMAE(S)). Lanes: 1– molecular marker; 2– full-length cytochrome \( c_{550} \); 3– protein sample in the column flowthrough assigned to \( c_{550}[C] \); 4– protein sample eluting from the column at 150 mM NaCl assigned to \( c_{550}[N] \).

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Separation by IEX chromatography of the domains obtained by limited proteolysis of cytochrome c550 (Fig. 1B) argues against the formation of a stable complex between c550[N] and c550[C]. Consistently, when a 1:1 mixture of the two domains was incubated at room temperature for one hour and assayed by analytical SEC, c550[N] and c550[C] eluted separately as individual proteins (Figure 2C). All together the results presented above indicate that cytochrome c550 folds into two independent domains with distinct properties, each carrying a single heme group. These features may have implications with regard to the electron transfer activity of the cytochrome.

The N-terminal Domain of Cytochrome c550 Accepts the Electrons from SOR TTHB8

We have previously shown that cytochrome c550 is the physiological electron acceptor of the sulfite:cytochrome c oxidoreductase encoded by the ttha1326 gene in T. thermophilus HB8 [16]. Based on the high similarity between c550[N] and the SorB subunit of the sulfite:cytochrome c oxidoreductase from C. jejuni, it is very likely that this domain acts as an electron acceptor for SOR TTHB8. The 3D model of SOR TTHB8 was automatically built by means of the SWISS-MODEL server [32–34], using as a template the only available structure for a microbial SOR, i.e., the one of the S. novella enzyme (PDB ID: 2c9x, segment A; 33% identity to SOR TTHB8) (Figure 3A). In S. novella SOR, the formation of complementary electrostatic surfaces at the interface of the SorA and SorB subunit of sulfite:cytochrome c oxidoreductase has been observed [35]. Similarly, a positively charged surface area surrounding the pocket cradling the molybdopterin cofactor is present also in the SOR from T. thermophilus HB8 (Figure 3A), while the c550[N] presents an overall negative surface charge as shown by IEX chromatography. It is, therefore, reasonable to assume that c550[N] and SOR TTHB8 present analogies to typical SORs both in terms of function and type of interactions.

In order to test which domain of cytochrome c550 preferably interacts with SOR TTHB8, the electron transfer between the latter enzyme and the recombinant c550[N] or c550[C] was kinetically investigated by stopped-flow spectroscopy. In these experiments, a solution of SOR in the presence of an excess of sulfite was anaerobically mixed at 45°C with increasing amounts of oxidized full-length cytochrome c550, c550[N], c550[C] or a 1:1 mixture of the two domains. As shown in Figure 4A, compared to the full-length protein, c550[C] acts as a very poor electron acceptor for SOR TTHB8 even at a final concentration as high as 20 μM (not shown). In contrast, c550[N] is promptly reduced in this
experimental set up (Figure 4B), which confirms that the N-terminal domain of cytochrome c550 is the electron acceptor for SORTTHB8. Interestingly, a 1:1 mixture of the two cytochrome domains is also completely reduced, although at a slightly lower apparent rate (Figure 4B). This implies that c550[N] and c550[C] are in rapid redox equilibrium and can exchange electrons once reduction of the c550[N] has occurred. Figure 4C reports the average turnover rate of the reaction calculated as detailed in the Materials and Methods and plotted as a function of the concentration of c550[N], either isolated or as part of the full-length protein or mixed in a 1:1 ratio with c550[C]. Under all conditions a linear concentration dependence was observed. Interestingly, c550[N], either isolated or integrated in the full-length protein, is reduced at comparable rates, which further points to the N-terminal domain of cytochrome c550 as the electron acceptor for SORTTHB8.

Electron Transfer from Cytochrome c550 to Cytochrome c552

We have previously shown that cytochrome c550 passes the electrons generated during sulfite oxidation to the terminal oxidases of the respiratory chain via cytochrome c552. To assess which domain of cytochrome c550 takes part in this electron transfer, the oxidation of c550[N] or c550[C] by cytochrome c552 was tested. The reduced c550[N] or c550[C] were mixed anaerobically with oxidized c552 and the reaction was followed at 4°C at ionic strengths ranging from 2 to 152 mM. The reaction was studied under non pseudo-first order conditions and, accordingly, the experimental traces were fitted following the analysis described elsewhere [26]. From the results presented in Figure 5AB it can be concluded that, despite the low temperature, both c550[N] and c550[C] are able to rapidly exchange electrons with cytochrome c552. Table 1 shows the estimated forward (kF) and reverse (kR) rate constants of the reaction measured at the same ionic strength (12 mM). The observed differences in kF and kR are not significant due to the rather high experimental error in those measurements, partly arising from the large optical overlap among the investigated proteins. Based on the results, we conclude that both c550[N] and c550[C] exchange electrons with cytochrome c552 at rates similar to those previously measured with the full-length cytochrome c550 [16]. Interestingly, the reaction between c550[N] and c552 displays a similar ionic strength dependence to the full-length protein, whereas the dependence is less pronounced in the case of c550[C] (Figure 5C). This suggests that electrostatic forces play an important role in the interaction between c550[N] and c552, while apolar interactions may be involved in molecular recognition between c550[C] and c552.

In order to test this possibility, the 3D model of c550[C] was automatically built by means of the SWISS-MODEL server [32–34], using the X-ray structure of cytochrome c552 from T. thermophilus HB8 (PDB ID: 1c52; ~50% identity) as template (Figure 3BC). The heme cleft in the very well described cytochrome c552 is surrounded by a hydrophobic belt (Figure 3C) consisting of residues G13, C14, F26, V68, M69 and F72; this patch of residues likely participates in the interaction between the cytochrome and the terminal oxidase ba3 [36]. Interestingly, the model structure of c550[C] clearly shows that such hydrophobic residues (G11, C12, F24, V66, M67 and F70) are structurally conserved around the cleft (Figure 3B). It is therefore likely that molecular recognition between c550[C] and c552 is also mediated by this hydrophobic patch, in line with the modest ionic strength dependence reported in Figure 5C.
Electron Transfer to the Terminal Oxidases cca₃ and ba₃

When assayed separately, c₅₅₀[N], similarly to full-length c₅₅₂, is very slowly oxidized by either ba₃ or cca₃ oxidase, whereas c₅₅₀[C] is quickly oxidized by either of the two oxidases [Figure 6A,B]. Considering the high sequence similarity between c₅₅₀[C] and c₅₅₂ and the structurally conserved hydrophobic belt [Figure 3BC] likely participating in molecular recognition between c₅₅₂ and ba₃ oxidase, this finding is perhaps not unexpected. On the other hand, we have previously shown (and confirmed here) that the electron transfer between full-length c₅₅₀ and ba₃ or cca₃ is not efficient, unless mediated by c₅₅₂ acting as an electron shuttle [16]. Therefore, we suggest that the relatively fast direct electron transfer between c₅₅₀[C] and terminal oxidases here documented is of no physiological value. If the conserved hydrophobic patch mentioned above is involved in mediating electron transfer from c₅₅₀[C], in the full-length protein such a patch should not be accessible by large molecules, like ba₃ or cca₃ oxidase, though possibly still allowing interaction with much smaller molecules such as cytochrome c₅₅₂.

Interestingly, a 1:1 mixture of ascorbate-reduced c₅₅₀[N] and c₅₅₀[C] is also quickly and fully oxidized by ba₃ or cca₃ (Figure 6A,B), which further confirms that the two domains of c₅₅₀ are in relatively fast redox equilibrium. Expectedly, in the presence of 10 nM oxidized c₅₅₂, both c₅₅₀[N] and c₅₅₀[C] are promptly oxidized by ba₃ (Figure 6C,D) or cca₃ (data not shown), as previously observed with the full-length protein [16]. This further supports a fast electron transfer between either of the two domains of cytochrome c₅₅₀ and cytochrome c₅₅₂.

**Discussion**

While sulfite oxidizing enzymes (SOEs) in vertebrates and humans have been studied for over 40 years, significant progress in studying bacterial SOEs has only been made in the last decade. Bacterial SOEs are extremely diverse in terms of structure and oxidizing proteins. Discovery of novel SOEs is being constantly reported, but in most cases the relative electron acceptor was not identified. Hence, usually the link to the respiratory chain is not shown [12–14,18,20]. The complexity of those pathways is a real challenge for understanding how SOEs participate in cell metabolism and energy production. Recently, the complete sulfite oxidation pathway from *T. thermophilus* has been described [16]; it was shown that the electrons generated during sulfite oxidation are injected into the respiratory chain at the level of cytochromes c₅₅₂ via the di-heme cytochrome c₅₅₀. Here we have undertaken a detailed investigation of the role played by the novel cytochrome c₅₅₀ in linking sulfite oxidation to cell respiration.

Sequence analysis, limited proteolysis and individual expression of the N- and C-terminal regions of the cytochrome confirmed that overall the protein consists of two independent domains, each associated with one c-type heme. At physiological pH, the protein is most likely characterized by an asymmetric charge distribution: it has a negatively charged N-terminal domain and a positively charged C-terminal one. The detailed analysis of other di-heme c-type cytochromes revealed several features shared by c₄-type cytochromes. Cytochromes c₄ are periplasmic or membrane-bound members of class I cytochromes c with a molecular mass of ~20 kDa found in a variety of bacteria [37,38]. Analysis of the characterized cytochromes c₄ from *Vibrio cholerae* [39], *Pseudalteromonas haloplanktis* [40], *Pseudomonas aeruginosa*, *Azotobacter vinelandii* [41], *Acidithiobacillus ferrooxidans* [42] and *Pseudomonas stutzeri* [43,44], and the high resolution X-ray structures of the last two [45,46] show that, similarly to *T. thermophilus* c₅₅₀, these di-heme proteins are formed by two domains connected with a flexible, ~10 aa long linker.

While *T. thermophilus* c₅₅₀ exhibits only ~15% identity to cytochromes c₄, the latter cytochromes show relatively high sequence similarities not only one each other, but also between the two domains of the same cytochrome (though to a minor extent). This led to the hypothesis that cytochromes c₄ result from duplication of a common ancestral gene [45,46]. Given the low sequence conservation typically observed among multi-heme
Figure 5. Electron transfer analysis between c₅₅₀[N], c₅₅₀[C] and c₅₅₂. To investigate the electron transfer between c₅₅₀[N], c₅₅₀[C] and c₅₅₂, kinetic traces were collected after anaerobically mixing 3.2 μM ascorbate-reduced c₅₅₀[N] with 3.2 μM oxidized c₅₅₂ at λ = 418 nm and T = 4°C (A), or mixing 4.1 μM ascorbate-reduced c₅₅₀[C] with 5.2 μM oxidized c₅₅₂ at λ = 414 nm and T = 4°C (B). Panel C shows the ionic strength dependence of the reaction of c₅₅₀ (red), c₅₅₀[N] (blue) or c₅₅₀[C] (black) with c₅₅₂. Data were normalized for the rate constants measured at ionic strength = 2 mM.
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cytochromes $c$ [47], it would be tempting to postulate close relationships between $T.\ thermophilus\ c_{550}$ and cytochromes $c_4$. In the case of cytochrome $c_{550}$, however, the fusion of two distantly related cytochromes rather than gene duplication appears to be more likely; the domains indeed exhibit both remarkably different sequences and sizes (with $c_{550}[N]$ and $c_{550}[C]$ representing 1/3 and 2/3 of the full-length protein, respectively). It seems very likely that $c_{550}[C]$ originates from duplication and divergence of cytochrome $c_{552}$ [48]. Similarly, fusion of two distantly related cytochromes has been proposed also for the di-heme cytochrome $c$ subunit of the flavocytochrome $c$ sulfide dehydrogenase from $Chromatium\ vinosum$. This subunit exhibits $\sim$15\% identity to both cytochromes $c_4$ [49] and $T.\ thermophilus\ cytochrome\ c_{550}$, while showing a fold very similar to that of cytochrome $c_4$ [50].

Despite the low sequence similarity, the dipolar nature of $T.\ thermophilus$ cytochrome $c_{550}$ (resulting from an asymmetric charge distribution between the two domains) has been also reported for the cytochromes $c_4$ isolated from $P.\ stutzeri$ and $A.\ ferrooxidans$ [43,45,46]. It has been proposed that the dipolar nature of the cytochrome $c_4$ from $P.\ stutzeri$ is important for the interaction of the cytochrome with its redox partners. The positively charged C-terminal domain is proposed to interact with the negatively charged pocket of cytochrome $c$ oxidase, while negatively charged N-terminal domain with a reductase [45]. However, despite the extensive characterization of $P.\ stutzeri$ cytochrome $c_4$ (see references in [44]), its specific physiological function remains to be established as yet. Conversely, analysis of the cytochrome $c_4$ from $A.\ ferrooxidans$ showed the crucial role of a negatively charged residue E121 localized on the overall positively charged C-terminal domain in recognition of its electron donor, rusticyanin, while the Y63 localized in the negatively charged N-terminal domain seems to be responsible for the electron transfer from $c_4$ to cytochrome $c$ oxidase [51].

| Table 1. Forward ($k_F$) and reverse ($k_R$) rate constants estimated at 4°C and at ionic strength = 12 mM for the reaction of cytochrome $c_{550}$ and its domains with cytochrome $c_{552}$. |
|-----------------------------------------------|
| $k_F$ (M$^{-1}$ s$^{-1}$) | $k_R$ (M$^{-1}$ s$^{-1}$) |
| $c_{550}[N]$ | $\sim 10 \times 10^7$ | $8 \sim 30 \times 10^6$ |
| $c_{550}[C]$ | $\sim 7 \times 10^7$ | $3 \sim 7 \times 10^6$ |
| $c_{550}$ | $\sim 5.5 \times 10^7$ | $5 \sim 9 \times 10^6$ |

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Figure 6. Electron transfer between the domains of $c_{550}$ and terminal oxidases $b_3a_3$ and $ca_3$ in the absence (A,B) or in the presence (C,D) of cytochrome $c_{552}$. Oxidation by $b_3a_3$ (A) or $ca_3$ (B) oxidase of ascorbate-reduced $c_{550}$ (red), $c_{550}[C]$ (black), $c_{550}[N]$ (blue) or a 1:1 mixture of the two domain (green). In C and D, the oxidation of $c_{550}[N]$ or $c_{550}[C]$ by $b_3$ oxidase was investigated in the presence of 10 nM $c_{552}$ (magenta). T=25°C.

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The presented results here clearly show that the N- (but not the C-) terminal domain of *T. thermophilus* cytochrome c$_{550}$ binds to and accepts electrons fromSOR$_{THB8}$. Based on the 3D model generated for SOR$_{THB8}$ and IEX chromatography experiments on c$_{550}[N]$, recognition between these two proteins most likely involves electrostatic interactions between the positively charged Arg50 and the overall negative charge of c$_{550}[N]$. Electrostatic interactions have been also proposed to drive the reaction of SorT from *S. meliloti* with its negatively charged natural electron acceptor, cytochrome Sme0404 [20]. The importance of electrostatic interactions between SOR$_{THB8}$ and c$_{550}[N]$ is further supported by the presence of a conserved arginine residue (R50) in the C-terminal part of the di-heme cytochrome c$_{550}$ resembles the cytochrome c subunit of the flavocytochrome c sulfide dehydrogenase from *C. vinsonum* [50], where the N-terminal part of the di-heme cytochrome c tightly interacts with the flavin-containing enzymatically active subunit of the protein. Our results show that the two domains of cytochrome c$_{550}$ can rapidly exchange electrons once the c$_{550}[N]$ has been reduced by SOR$_{THB8}$. Furthermore, both domains are also able to reduce cytochrome c$_{552}$, the molecule connecting sulfite oxidation directly to the respiratory chain. The c$_{550}[C]$ most likely binds c$_{552}$ via hydrophobic interactions and electron transfer is therefore only modestly affected by the ionic strength. Hydrophobic interactions have also been proposed to be involved in the reaction of cytochrome c$_4$ from *A. ferrooxidans* [46,51] with its electron acceptor. Moreover they have also been shown to be particularly important in *T. thermophilus* [30] and in thermophilic organisms in general, as electrostatic interactions are weakened at high temperatures [53].

The finding that both domains of *T. thermophilus* c$_{550}$ can pass electrons to c$_{552}$ rules out the possibility that the protein acts as a wire, where one heme serves as the entrance and the other as the exit site for electrons. Our results show that in principle the pathway could be functional with only c$_{550}[N]$, as this domain is able by itself to shuttle electrons from SOR$_{THB8}$ to c$_{552}$. The c$_{550}[C]$ then, is necessary, but dispensable for c$_{552}$ to fulfill its function. Moreover, the presence of a second heme center in rapid redox equilibrium with both c$_{550}[N]$ and c$_{552}$ enables cytochrome c$_{550}$ to assist a two-electron transfer process, similarly to the di-heme subunit of flavocytochrome c sulfide dehydrogenase from *C. vinsonum* [50]. This confers to cytochrome c$_{550}$ the ability to store the two electrons generated during oxidation of a single sulfite molecule and inject them into the terminus oxidases of the respiratory chain via cytochrome c$_{552}$, thereby ensuring an efficient coupling between sulfite oxidation and the respiration-mediated energy production.

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**Author Contributions**

Conceived and designed the experiments: SR AG TS MA EF PS. Performed the experiments: SR MA EF PS. Analyzed the data: SR OKR AG TS MA EF PS. Contributed reagents/materials/analysis tools: SR OKR AG TS MA EF PS. Wrote the paper: SR OKR.

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