Different Interaction Modes of Two Cytochrome-c Oxidase Soluble CuA Fragments with Their Substrates*

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Cytochrome-c oxidase is the terminal enzyme in the respiratory chains of mitochondria and many bacteria and catalyzes the formation of water by reduction of dioxygen. The first step in the cytochrome oxidase reaction is the bimolecular electron transfer from cytochrome c to the homobinuclear mixed-valence CuA center of subunit II. In Thermus thermophilus a soluble cytochrome c552 acts as the electron donor to ba3 cytochrome-c oxidase, an interaction believed to be mainly hydrophobic. In Paracoccus denitrificans, electrostatic interactions appear to play a major role in the electron transfer process from the membrane-spanning cytochrome c552. In the present study, soluble fragments of the CuA domain and their respective cytochrome-c electron donors were analyzed by stopped-flow spectroscopy to further characterize the interaction modes. The forward and the reverse electron transfer reactions were studied as a function of ionic strength and temperature, in all cases yielding monoexponential time-dependent reaction profiles in either direction. From the apparent second-order rate constants, equilibrium constants were calculated, with values of 4.8 and of 0.19, for the T. thermophilus and P. denitrificans c552 and CuA couples, respectively. Ionic strength strongly affects the electron transfer reaction in P. denitrificans indicating that about five charges on the protein interfaces control the interaction, when analyzed according to the Brønsted equation, whereas in the T. thermophilus only 0.5 charges are involved. Overall the results indicate that the soluble CuA domains are excellent models for the initial electron transfer processes in cytochrome-c oxidases.

The aerobic electron transport chain of Paracoccus denitrificans represents a model system for the mitochondrial respiratory chain, where the terminal reaction, the reduction of dioxygen to water, is mediated by cytochrome-c oxidase (EC 1.9.3.1). The electrons for this reaction are donated by a c-type cytochrome (7) and enter the oxidase via the CuA center (for a recent review see Ref. 2), a highly conserved motif in subunit II of cytochrome-c oxidases of eukaryotes, aerobic bacteria, and in the nitrous oxide reductase of denitrifying bacteria. The CuA center resides in a periplasmic, solvent-exposed domain of subunit II and contains two copper ions in a mixed valence state, which give rise to the typical purple color of the isolated domain (3). The two copper atoms are bound by two cysteine residues forming thiolate bridges, two histidine residues, and as further ligands a methionine sulfur and a glutamate peptide carbonyl. Soluble domains of several bacterial cytochrome-c oxidases have been prepared including P. denitrificans (3), T. thermophilus (4), Paracoccus versutus (5), and Bacillus subtilis (6). Following electron transfer to the CuA center from cytochrome c, electrons are further transferred to the low-spin heme a (or b) in subunit I, in a very fast μs time-scale process, and finally to the binuclear heme a3-CuB site (on a ms time scale), where dioxygen is reduced to water. In P. denitrificans two c-type cytochromes have been suggested to mediate the ET2 processes between the bc1 complex and the terminal electron accepting enzymes, the aa3 and the cb567-type cytochrome-c oxidases, and the nitrite and the nitrous oxide reductases of the nitrate respiratory pathway. A soluble cytochrome c550 is believed to function as an electron donor in different respiratory pathways such as in methanol and methylamine oxidation or in denitrification (7). The kinetics of the electron transfer reaction between this cytochrome and a soluble CuA domain from P. denitrificans aa3 cytochrome-c oxidase have been studied (8). However, there is ample evidence that a different cytochrome, cytochrome c552, is the genuine mediator between the bc1 complex and aa3. This 18-kDa cytochrome (9) is composed of three functional domains: an N-terminal helical membrane anchor, a negatively charged spacer region, and a typical class I c-type heme domain. This cytochrome is believed to be the bona fide electron transfer shuttle protein between the bc1 complex and aa3, because (i) a ternary supercomplex consisting of these three components is isolated under certain detergent solubilization conditions from P. denitrificans membranes (10); (ii) electron transport from NADH to dioxygen in isolated membranes is blocked in deletion mutants lacking the c552-coding gene, but this inhibition may be overcome by mitochondrial cytochrome c (11), and (iii) specific antibodies directed against purified cytochrome c552 can block electron transport from NADH to oxygen in membrane activity assays (9).

At low ionic strength (i.e. below 10 mM), the reaction between mitochondrial cytochrome c and cytochrome-c oxidase involves the formation of a 1:1 stoichiometric complex, which may be isolated in vitro (12). Under these conditions, complex formation is believed to rate-limit the subsequent rapid intracomplex...

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‡ The abbreviations used are: ET, electron transfer; TEV, tobacco etch virus.

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ET process (13). From the strong ionic strength dependence of the reaction and from mutagenesis studies (14, 15) a two-step model has been proposed for the interaction of the proteins. Initially the orientation is mediated by long range electrostatic forces, followed by the fine-tuning of the interaction by hydrophobic patches within the docking site. In contrast to this, nearly no charged residues are found on the probable interaction interfaces of the corresponding proteins from T. thermophilus, as demonstrated by the recently solved crystal structures of the ba3 cytochrome-c oxidase (16) and its substrate (17), a soluble cytochrome spectroscopically identified as c552 (18). T. thermophilus is a Gram-negative, extremely thermophilic eubacterium found in hot springs with optimum growth temperature around 75 °C. Taking into consideration that the stability of electrostatic interactions is lower at higher temperatures, hydrophobic interactions become more favorable. Kinetic studies have shown that the turnover activity of cytochrome c552 with ba3 oxidase becomes faster as ionic strength is decreased (19). On the contrary, P. denitrificans aa3 cytochrome-c oxidase shows very low turnover rates under low ionic strength conditions, most likely because of the formation of a high affinity electrostatic complex (15). Elucidation of the cytochrome-cCuA electron transfer mechanism is complicated by the subsequent monomolecular ET events taking place along the reaction coordinate that complete the reduction of dioxygen to water. It was, therefore, of interest to isolate and compare both periplasmically oriented subunit II CuA domains from P. denitrificans and T. thermophilus and to study the electron transfer reactions with their cytochrome c552 electron donor counterparts. Differences between the two systems with respect to the relevant electron transfer reactions can be studied directly by stopped-flow spectroscopy without interference from heme α absorbance and by the subsequent electron transfer and energy transduction events. The results indicate that the expressed soluble CuA domains are excellent models for the initial electron transfer events in cytochrome-c oxidases.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification Procedures**—Expression of the CuA fragment of P. denitrificans subunit II, encoded by the ctaC gene (20), was carried out in Escherichia coli strain JM 109. The region representing amino acid residues 130–280 of the CuA domain was amplified by PCR. The subunit II-BamHIII-TEV primer with sequence agtagcggccgccactactacttctaacgagcagaggg (BamHI site underlined, TEV site in italics) was used as forward primer, coding for the TEV-protease recognition site (ENLYFQSL with cleavage between Q and S). As a reverse primer an oligonucleotide was used according to Lappalainen et al. (3), introducing a HindIII site, to allow for cloning into the expression vector pQE-30 (Qiagen). This construct provides considerably higher expression rates (up to 8 mg/liter) than the previous method (3). Because the His tag (coded on the parent vector) is not used for purification, nor is it present in the protein desirable, it was cleaved off in vivo by co-transforming pRK603 into this E. coli strain (21), thus providing constitutive expression of the TEV-protease. Cells were grown on minimal medium containing 40 g/liter glycerol, 7.5 g/liter KH2PO4, 5.3 g/liter NaH2PO4, 5.3 g/liter NH4Cl, 0.4 g/liter glucose, 1 mM MgSO4, 0.1 mM CaCl2, 10 mM/1 liter trace element solution 1 (22), 0.2 mM thiamin, 100 μg/ml ampicillin, 25 μg/ml kanamycin, in a 1-liter New Brunswick Microferm fermentor. Cells were induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at an optical density of 4.0. After 4 h cells were harvested, and preparation of inclusion bodies, protein refolding, copper insertion, and purification were carried out essentially as described previously (4). The cytochrome c552 soluble domain of P. denitrificans was expressed in E. coli according to the published protocol (23). Following a similar approach, the T. thermophilus cytochrome c552 gene was cloned into pET22b, a vector providing the pelB leader, which directs the protein to the periplasm.

Correct assembly and insertion of the catalyst into the apoprotein was achieved by outtransforming E. coli cells with the incomplete plasmid pEC86 (24). Purification was carried out according to Fee et al. (25). Protein concentration was determined by using the following extinction coefficients: Pd-CuA, ε450 nm, 552 nm = 19.4 mm−1 cm−1, Pd-CuA, ε305 nm, 552 nm = 3.0 mm−1 cm−1, Tt-CuA, ε450 nm, 552 nm = 21.0 mm−1 cm−1, Tt-CuA, ε552 nm, 552 nm = 3.1 mm−1 cm−1.

**Stopped-flow Spectroscopy and Experimental Protocol**—Kinetic experiments were carried out by using a thermostatted Applied Photonics stopped-flow apparatus (Leatherhead, United Kingdom) with a 1-cm observation chamber. The ET kinetics between the soluble CuA domains and cytochromes c were studied in both the forward (physiological) and reverse directions according to the following experimental protocol (Scheme 1), which was devised to (i) minimize the auto-oxidizability of the reduced proteins, and to (ii) better control the initial absolute concentration of the reduced proteins. According to Scheme 1, one of the partner proteins is initially anaerobically in the stopped-flow syringe by ascorbate (see vertical arrows), which at the chosen solution pH is a slow reactant (see below). Following complete reduction, the solution is mixed in the stopped-flow apparatus with an anaerobic solution containing the oxidized ET acceptor protein, and the time-dependent extinction changes followed at 552 nm (P. denitrificans) or 551 nm (T. thermophilus). Thus, in the forward direction cytochrome c is reduced by ascorbate and mixed with oxidized CuA, whereas in the reverse direction prereduced CuA is mixed with oxidized cytochrome c. In all experiments the buffer (20 mM Bis-Tris, pH 7.0, with ionic strength varied by appropriate amounts of KCl) was flushed with N2 in a glass gas-tight syringe (fitting the stopped-flow valve) for at least 15 min, and following addition of the protein of interest, the solution was flushed for an additional 15 min. Finally 0.5 mM sodium ascorbate was added from a 1.0 M stock solution. All experiments were performed at 8 °C.

Complete reduction was achieved after several minutes, as determined separately in stopped-flow experiments in which sodium ascorbate, at varying pseudo-first order concentrations, was mixed with either oxidized protein (see Fig. 1 and "Results and Discussion"). The apparent binuclear rate constants for reduction of the CuA domains both from P. denitrificans and T. thermophilus and the corresponding soluble cytochromes c552 were in the range of 60 to 330 M−1 s−1 (results not shown), 3–5 orders of magnitude smaller than the ET process of interest.

Under all the experimental conditions tested (cytochrome c concentration, ionic strength, and temperature), the forward and reverse ET apparent binuclear rate constants were determined by fitting the observed kinetic traces to a simple exponential relaxation process at different ferro- or ferricytochrome c concentrations (forward and reverse directions, respectively) followed by linear regression of the observed rate constants to the varied cytochrome c concentrations. Three or more kinetic traces were acquired for each specific experimental condition and averaged. True pseudo-first order conditions could not be completely achieved throughout the kinetic titration experiments because of the high cytochrome c (19.4–21.0 mm−1 cm−1) and low CuA (3–3.1 mm−1 cm−1) extinction coefficients on one hand, and to the relatively high reaction rates observed especially at low ionic strength for the P. denitrificans couple, which approached the time resolution of the stopped-flow apparatus (dead time of 1.3 ms, determined by using the myoglobin-carbon monoxide combination reaction) on the other. Apparent second-order rate constants were determined from the slopes of the linear portions of the pseudo-first order plots (see "Results and Discussion"). Data fitting was performed by using either the Matlab (The MathWorks Inc.) or Scientist (Micromath Scientific Software Inc.) softwares. The standard deviation of the fitted parameters never exceeded 10%.
Electron Transfer in Isolated Cu₅ Domains

RESULTS AND DISCUSSION

In the present investigation the kinetics of electron transfer between two genetically engineered ET couples from a mesophilic and a thermophilic species have been studied by stopped-flow spectroscopy. The experimental rationale has been to preclude one protein of each ET couple with the kinetically sluggish reductant ascorbate and to subsequently mix the proteins and follow the interprotein ET events at a suitable wavelength (see “Experimental Procedures”). At pH 7 the concentration of the true reductant, i.e. the ascorbate dianion, is very low (26, 27) and therefore not expected to interfere with the ET events taking place between the partners of interest. Fig. 1 depicts a typical example of the Paracoccus couple, studied in the forward, physiological direction (bottom panel), mixing of fully reduced Pd-c₅₅₂ with oxidized Pd-Cu₅, and in the reverse direction (top panel), in which oxidized Pd-c₅₅₂ is mixed with reduced Pd-Cu₅, followed at 551 nm, where the extinction of Pd-c₅₅₂ is dominant. In either direction, absorbance changes take place on a short time scale (0.2 and 0.1 s) indicating partial oxidation (bottom panel) or reduction (top panel) of Pd-c₅₅₂. These experiments were carried out by varying the ascorbate concentration from 0.25 to 1 mM to exclude any competition of the reductant with the interprotein ET reaction of interest. Indeed as ascorbate concentration is increased, no significant change in rate or amplitude is observed on the short time scale, and all time courses display a simple exponential behavior. On longer time scales (50 s), however, the time-dependent absorbance changes appear to be linearly correlated to ascorbate concentration (not shown). These observations suggest that in the fast phase (Fig. 1, left part of top and bottom panels) interprotein ET occurs, taking the proteins to a transient equilibrium state controlled by the protein-chemical structural details and driven by the redox potential of the two ET proteins. On longer time scales (Fig. 1, right part of top and bottom panels) the ascorbate reaction takes the partially oxidized equilibrium ET couple mixture to the fully reduced state as expected from the larger driving force of ascorbate. Similar results were obtained with the Thermus thermophilus ET couple (not shown).

We have systematically studied the cytochrome c₅₅₂ concentration dependence of the interprotein ET reaction with both couples as a function of ionic strength, using the ascorbate (0.25 mM after mixing) protocol described above. All observed fast phases could be fitted to a single exponential time course. The results of these experiments are shown in Figs. 2 and 3 (P. denitrificans couple) and Fig. 4 (T. thermophilus couple) in which the observed fitted rate constants are plotted as a function of cytochrome c₅₅₂ concentration. The pseudo-first order plots were linear with respect to the varied cytochrome c₅₅₂ concentration, although, especially at low cytochrome concentrations, there was some deviation from linear behavior. This is expected, because at low cytochrome c₅₅₂ concentrations the reaction mixture is not under true pseudo-first order conditions. Nevertheless from the slope of the linear portion of the plots the apparent second-order rates for the interprotein ET reaction could be estimated and are given in Tables I and II for the P. denitrificans and T. thermophilus couple, respectively, at different ionic strength values. As can be seen from the reported data, the ET reaction of the P. denitrificans couple is strongly dependent on ionic strength with second-order rate constants ranging from $-4 \times 10^6$ to $9 \times 10^4$ M$^{-1}$ s$^{-1}$ and from $0.04$, 0.07, and 0.11 s$^{-1}$; reverse reaction, $k_F$ = 39.7 s$^{-1}$; reverse reaction, $k_R$ = 83.3 s$^{-1}$). The fitted rate constants of the slow phase linearly depended on ascorbate concentration (forward reaction, $k_F$ = 0.04, 0.07, and 0.11 s$^{-1}$; reverse reaction, $k_R$ = 0.06, 0.1, and 0.16 s$^{-1}$, with ascorbate concentration varied as indicated above. In all cases the standard deviations of the fitted parameters were less than 2%. T = 8 °C.

![Fig. 1. Ascorbate dependence of the forward and reverse ET reaction in the Paracoccus couple.](image)
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2 *10⁷ to 6*10⁵ M⁻¹ s⁻¹, for the forward and reverse ET reactions, respectively, as ionic strength is increased from 10 to 200 mM (see Table I). The apparent equilibrium constant for the physiological direction is thus about 0.2 for the P. denitrificans couple and does not appear to vary significantly with ionic strength. Although this result indicates a higher stability of reduced Pd-c₅₅₂ over oxidized Pd-Cu₄, (i) this has also been observed previously (28) in the reaction between bovine heart cytochrome-c oxidase and yeast iso-1-cytochrome c, supporting our result; (ii) the reported redox potentials of the P. denitrificans couple are 270 and 240 mV for Pd-c₅₅₂ and Pd-Cu₄, respectively (3, 29), which yields an equilibrium constant of about 0.3, in close agreement with our findings; and (iii) the ensuing exergonic ET reactions to the low spin heme a and the a₃-CuB center that account for O₂ reduction drive the thermodynamically unfavorable reaction in the physiological direction.

In contrast to these findings the ionic strength dependence of the T. thermophilus ET couple is very modest with rates in excess of 10⁶ M⁻¹ s⁻¹ at the lowest ionic strength (see Fig. 4 and Table II). From these data an equilibrium constant of 4.8 was calculated for the Thermus protein pair, indicating that in this case the physiological direction is thermodynamically favored for the isolated domains. Several values of the redox potentials, depending strongly on the experimental conditions (temperature, ionic strength, pH), of the Thermus proteins have been reported (Th-Cu₄ 240 mV up to 266 mV; see Refs. 4 and 30; TT-c₅₅₂ 200 mV (25) and 230 mV (31)), complicating the analysis and the comparison with the reported equilibrium constant gained from the kinetic data. However, calculation of the equilibrium constant with any of these values always confirms the physiological direction to be favored.

From the data shown in Figs. 2–4, as well as Tables I and II, Fig. 5 was constructed in which the logarithm of the apparent bimolecular rate constant is plotted as a function of the square root of ionic strength of the solution. As predicted by the Brønsted law (32), shown below in Equation 1, where k is the observed bimolecular rate constant at ionic strength I, kₒ the bimolecular rate constant at I = 0, B a term whose value = −0.5 at 8 °C, is derived from Debye-Hückel equations, and zₐ and zₐ represent the ET-sensitive resident charges on the protein surfaces), the bimolecular rates in both directions decrease as the ionic strength is increased with slopes (the zₐzₐ product) of about −4.6 for the P. denitrificans ET couple.

\[
\log k = \log k_0 + 2Bz_\text{aa} \sqrt{I} 
\]  (Eq. 1)

This indicates that two to three effective charges of opposite sign on each protein interface interact in the hemeoxygen electron transfer reaction, which is totally consistent with recent protein-protein docking calculations (33). The extrapolated rates at I = 0 (kₒ = 1.2 *10⁷ and 5.6*10⁷ M⁻¹ s⁻¹ for the forward and reverse reactions, respectively) indicate that the ET reactions approach the limit imposed by diffusion, as observed with wild-type aa₃ (13). Overall the results strongly suggest that the fragments approach each other and orient according to long-range electrostatic forces, showing the same principle of interaction as the native wild-type partners.

So far direct contacts between the two Pd-c₅₅₂ and Pd-Cu₄ are unclear. In a computational approach by Flick and Helms (33), the complementarity of both protein surfaces were considered, followed by an energy minimization of the resulting complexes.

Using the minimal heme to Trp-121 (the electron entry site of Cu₄) distance as a further input parameter, two suggestions for an electron transfer complex resulted, clearly differing in the heme orientation. However, in both predicted complex structures, three ion pairs (involving three of the nine lysines on the cytochrome) could be identified. Consistently lysine 70 on the surface of the cytochrome was involved, and this residue also showed up in a recently performed chemical shift perturbation mapping experiment by NMR (34). Taken together, these data support the following scenario. In a very fast, diffusion-controlled process, electrostatic forces predominantly influence the formation of a loose encounter complex, allowing for some degree of freedom. Between two and three effective charges on each interaction protein surface provide the electrostatic setting for the initial attraction and steering of the two reaction partners. This is followed by a second step where the final (electron transfer) complex is generated mostly by specific, non-ionic contacts. A similar situation could be found for a cytochrome f-plastocyanin electron transfer couple from the cyanobacterium Phormidium laminosum, which has also been characterized by NMR (35) and double mutant cycle analysis by stopped-flow spectroscopy (36).

Fig. 5 also shows the ionic strength dependence of the T. thermophilus couple in the forward direction. The \( z_\text{aa}z_\text{aa} \) product (see Equation 1) is about −0.57 indicating that in the thermophilic couple the ET reaction is only slightly affected by electrostatic interactions. These results are in agreement with the surface properties of the high resolution x-ray structures of P. denitrificans aa₃ (37), T. thermophilus ba₃ (16), and of the corresponding structures of the substrate cytochrome partners (17, 38). Comparison of the possible interaction surfaces of both oxidases shows that of the conserved acidic residues of the aa₃ cytochrome-c oxidase proposed to be involved in cytochrome c binding (14, 15) only Asp-111 in the ba₃ oxidase from Thermus is present (16).

So far there is no direct experimental proof for the formation of a hydrophobically stabilized complex, but several lines of evidence support the notion that indeed mainly hydrophobic interactions govern the Thermus ET protein interaction. As ba₃ oxidase activity under turnover conditions is maximal at low ionic strength, Giuffrè et al. (19) exclude the formation of a high affinity electrostatically stabilized complex. In this system the cytochrome c off-rate could play a significant role and would be expected to limit the turnover rate as ionic strength is increased and as a hydrophobic complex is concomitantly stabilized. Our data presented here do not show any major depend-
ence on ionic strength, but it should be kept in mind that our experimental approach, in contrast to a turnover assay (19), only follows the initial steps of encounter and ET between the two proteins. Thus the fast kinetics observed here do not depend on a sluggish off-rate and therefore are only moderately affected by ionic strength (Fig. 5). We also note that the extrapolated second-order rate constant at $I = 0$ is already $-4 \times 10^6$ M$^{-1}$ s$^{-1}$ (see Fig. 5) at the temperature of the current experiment (8 °C) and is expected to further increase with temperature.

These considerations suggest that in contrast to the Paracoccus electron transfer couple, no electrostatically governed pre-orientation occurs within the Thermus protein couple. This is also supported by the electrostatic surface potentials, given in Fig. 6, indicating that opposite charges on the presumed interaction surfaces of the Paracoccus protein couple affect the ET process, whereas in the Thermus pair clearly hydrophobic amino acids dominate the interaction. Another confirmation for the different interaction modes of the two systems is the observation that the Thermus $c_{552}$ is not a efficient substrate for the $P$. denitrificans cytochrome $c_{552}$ oxidase (39).

Fig. 7 depicts the temperature dependence of the ET reaction for the Paracoccus protein couple in the 8 to 26 °C range. In this temperature range the forward reaction displays a simple Arrhenius-like behavior with an activation energy of 44.4 kJ mol$^{-1}$. Analysis according to the Eyring equation (41) yields an activation enthalpy of 42.0 kJ mol$^{-1}$ and an activation entropy of $-59.5$ J mol$^{-1}$ K$^{-1}$. On the other hand, the reverse reaction is distinctly non-linear over this temperature range with a breakpoint at $-14$ °C. Usually non-linear Arrhenius or Eyring plots arise from secondary reactions, which take place because of the deviation of activation parameters with rising temperature. Presently the most likely explanation is an intrinsic temperature-dependent conformational heterogeneity of the $C_{552}$ domain in the reduced state, which differs from an unfolding process, because the rate constants in the 14 to 26 °C range are still quite high. Separate analysis of the two temperature ranges yield the following activation enthalpy and entropy parameters, respectively: 8 to 14 °C range, 39.6 kJ/mol and $-63.4$ J mol$^{-1}$ K$^{-1}$; 14 to 26 °C range (dashed line in Fig. 7), 13.7 kJ/mol and $-153.5$ J mol$^{-1}$ K$^{-1}$. Thus, in the low temperature range both the forward and reverse reactions display the same activation enthalpy (i.e. the same slope) and differ in the entropy of activation, as analysis according to the Eyring equation suggests. Therefore, and at least in the low temperature range, the rate is determined by translational, rotational, and vibrational or solvent entropic contributions rather than changes in the ground state reagent and product energy levels relative to the transition state.

All together the data indicate, again, that for this reaction the reverse direction is favored, although in the complete cytochrome-c oxidase heme $a$ will serve as electron acceptor in a fast process, influencing the equilibrium between cytochrome $c$ and $C_{552}$. By extrapolating the absolute bimolecular rate constant for the Pd-c$552$-to-Cu$C_{552}$ electron transfer couple to 20 °C, one obtains a rate of $4.5 \times 10^6$ M$^{-1}$ s$^{-1}$, and it is possible to compare this to previously reported rate constants for the reaction of the Cu$C_{552}$ domain with either cytochrome $c_{550}$ from

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**Table I**

| Ionic strength (mM) | $k_{\text{forward}}$ ($s^{-1}$) | $k_{\text{reverse}}$ ($s^{-1}$) | $k_{\text{forward}}/k_{\text{reverse}}$ |
|---------------------|-------------------------------|-------------------------------|-----------------------------------|
| 10                  | 4.13 $\times 10^9$            | N.D.                          | N.D.                              |
| 15                  | 3.42 $\times 10^9$            | 17.7 $\times 10^9$            | 0.19                              |
| 25                  | 2.65 $\times 10^9$            | 10.6 $\times 10^9$            | 0.19                              |
| 35                  | 1.46 $\times 10^9$            | 8.1 $\times 10^9$             | 0.18                              |
| 50                  | 0.83 $\times 10^9$            | 5.0 $\times 10^9$             | 0.17                              |
| 100                 | 0.36 $\times 10^9$            | 1.9 $\times 10^9$             | 0.19                              |
| 200                 | 0.09 $\times 10^9$            | 0.6 $\times 10^9$             | 0.15                              |

**Table II**

| Ionic strength (mM) | $k_{\text{forward}}$ ($s^{-1}$) | $k_{\text{reverse}}$ ($s^{-1}$) | $k_{\text{forward}}/k_{\text{reverse}}$ |
|---------------------|-------------------------------|-------------------------------|-----------------------------------|
| 15                  | 3.50 $\times 10^9$            | N.D.                          | N.D.                              |
| 25                  | 3.35 $\times 10^9$            | 0.7 $\times 10^9$             | 4.8                               |
| 50                  | 3.27 $\times 10^9$            | N.D.                          | N.D.                              |
| 100                 | 2.62 $\times 10^9$            | N.D.                          | N.D.                              |
Electrostatic surface potentials for the Paracoccus and Thermus CuA domains and cytochromes c₅₅₂

Electrostatic potentials of the proposed interaction surfaces for P. denitrificans cytochrome c₅₅₂ (Protein Data Bank entry 1QL3) (A), P. denitrificans Cu₅₅₂ fragment (Protein Data Bank entry 1AR1) (B), T. thermophilus c₅₅₂ (Protein Data Bank entry 1C52) (C), and T. thermophilus Cu₅₅₂ (Protein Data Bank entry 1EHK) (D) are shown. Coloring is according to the calculated electrostatic potential with GRASP (40) with boundary values from −15 kT (intense red) to +15 kT (intense blue) for the Cu₅₅₂ proteins and from −5 to +5 kT for the cytochromes. The approximate location of the partially solvent exposed heme cofactors is shown in black text. The Cu₅₅₂ site is positioned under the protein surface within 5 Å from Trp-121, the electron entry site in P. denitrificans cytochrome-c oxidase (14, 15, 41); in the T₄-Cu₅₅₂ protein Phe-88 has been suggested to be the corresponding residue (16–18). Both residues are highlighted in black text.

**FIG. 6.** Electrostatic surface potentials for the Paracoccus and Thermus Cu₅₅₂ domains and cytochromes c₅₅₂.

**FIG. 7.** The temperature dependence of the ET reaction between Paracoccus fragments. The natural logarithm of the observed rate constants is plotted versus the reciprocal absolute temperature (varied from 8 to 26 ºC). – forward reaction (Pd-c₅₅₂ = 6 µM, Pd-Cu₅₅₂ = 10.8 µM after mixing); — reverse reaction (Pd-c₅₅₂ = 5 µM, Pd-Cu₅₅₂ = 10.7 µM after mixing). Experiments were performed in 20 mM Bis-Tris, pH 7.0, containing 10 mM KCl. Solid or dashed lines are the best fits obtained by analysis according to the Eyring equation (42). See text for details on the activation parameters.

P. denitrificans or horse heart cytochrome c (8). This analysis shows that the reaction with Pd-c₅₅₂ is about 3-fold faster than the reaction with Pd-c₅₅₆ (1.46×10⁶ M⁻¹s⁻¹) and 15-fold faster than the reaction with horse heart cytochrome c (3×10⁵ M⁻¹s⁻¹). This observation provides an independent kinetic argument that the c₅₅₂ protein is the favored electron donor for the aa₃ cytochrome-c oxidase from P. denitrificans. This is also supported by the fact that a supercomplex of bc₁ complex, cytochrome c₅₅₂, and aa₃ cytochrome oxidase can be found in Paracoccus membranes (10). Considering that the native cyto-

chrome c₅₅₂ has a membrane anchoring domain and an additional charged domain, it should be pointed out that these structural elements could further stabilize a supercomplex and may even allow higher electron transfer rates, without the constraints imposed by three-dimensional diffusion of the electron mediator.

Finally, the results presented in this investigation show that the kinetic processes taking place between the engineered proteins are truly bimolecular, i.e. with every collisional encounter resulting in a very efficient ET process. One may also assume that additional structural determinants present in the full-size proteins (complexes) may further modulate the specificity and efficiency of the interaction of oxidases with their substrates. All together the present study shows that the protein fragments studied are very good models of the initial ET events in oxidases without the complication of the ensuing ET processes taking place within the oxidases.

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**REFERENCES**

1. Droux, V., Reincke, B., Schneider, M., and Ludwig, B. (2002) Biochemistry 41, 10629–10634.
2. Schultz, B. E., and Chan, S. I. (2001) Annu. Rev. Biophys. Biomol. Struct. 30, 25–59.
3. Lappalainen, P., Aasa, R., Malmström, B. G., and Saraste, M. (1993) J. Biol. Chem. 268, 26416–26421.
4. Slutter, C. E., Sanders, D., Wittung, P., Malmström, B. G., Aasa, R., Richards, J. H., Gray, H. B., and Fee, J. A. (1996) Biochemistry 35, 3387–3395.
5. Salgado, J., Warmerdam, G., Ruhacsek, L., and Caners, G. W. (1998) Biochemistry 37, 7378–7389.
6. von Wachenfeldt, C., de Vries, S., and van der Oost, J. (1994) FEBS Lett. 340, 109–113.
7. Baker, S. C., Ferguson, S. J., Ludwig, B., Page, M. D., Richter, O. M. H., and van Spanning, R. J. (1998) Microbiol. Mol. Biol. Rev. 62, 1046–1078.
8. Lappalainen, P., Watmough, N. J., Greenwood, C., and Saraste, M. (1995) Biochemistry 34, 5824–5830.
9. Turba, A., Jetzek, M., and Ludwig, B. (1995) Eur. J. Biochem. 231, 259–265.
10. Berry, E. A., and Trumpower, B. L. (1985) J. Biol. Chem. 260, 2458–2467.
11. Turba, A. (1995) Molecularbiologische und biochemische Charakterisierung des Membran-burenenden Cytochrome C552 aus Paracoccus denitrificans, Ph.D. thesis, University of Frankfurt, Frankfurt, Germany.
12. Dehmers, J. K., Ferguson-Miller, S., and Margoliash, E. (1979) J. Biol. Chem. 254, 1173–1181.
13. Antalis, T. M., and Palmer, G. (1982) J. Biol. Chem. 257, 6194–6206.
14. Witt, H., Malatesta, F., Nicoletti, F., Brunori, M., and Ludwig, B. (1998) Eur. J. Biochem. 251, 629–644.
15. Drosou, V., Malatesta, F., and Ludwig, B. (2002) Eur. J. Biochem. 269, 2960–2968.
16. Soulimane, T., Buse, G., Beurenkov, G. P., Bartunik, H. D., Huber, R., and Than, M. E. (2000) EMBO J. 19, 1766–1776.
17. Than, M. E., Hof, P., Huber, R., Beurenkov, G. P., Bartunik, H. D., Buse, G., and Soulimate, T. (1997) J. Mol. Biol. 271, 629–644.
18. Soulimate, T., von Walter, M., Hof, P., Than, M. E., Huber, R., and Buse, G. (1997) Biochem. Biophys. Res. Commun. 237, 572–576.
19. Giuffre, A., Forte, E., Antonini, G., D'Itri, E., Brunori, M., Soulimate, T., and Buse, G. (1999) Biochemistry 38, 1057–1065.
20. Steiner, D., Steffen, C. G., Panauskus, G., Buse, G., and Ludwig, B. (1987) Eur. J. Biochem. 167, 431–439.
21. Kapust, R. B., and Waugh, D. S. (2000) Protein Expr. Purif. 19, 312–318.
22. Wingfield, P. T. (1998) in Current Protocols in Protein Science (Coligan, J. E., Dunn, B. M., Flegh, H. L., Speicher, D. W., and Wingfield, P. T., eds) John Wiley & Sons, Inc., New York.
23. Reinecke, B., Thony-Meyer, L., Dannehl, C., Odenwald, A., Aidim, M., Witt, H., Ruterjans, H., and Ludwig, B. (1999) Biochim. Biophys. Acta 1411, 114–120.
24. Arslan, E., Schulz, H., Zufferey, R., Kunzler, P., and Thony-Meyer, L. (1998) Biochem. Biophys. Res. Commun. 251, 744–747.
25. Fee, J. A., Chen, Y., Todaro, T. R., Bren, K. L., Patel, K. M., Hill, M. G., Gomez-Moran, E., Loeher, T. M., Ai, J., Thony-Meyer, L., Williams, P. A., Stura, E., Sridhar, V., and McRee, D. E. (2000) Protein Sci. 9, 2074–2084.
26. Al Ayash, A. I., and Wilson, M. T. (1979) Biochem. J. 177, 641–648.
27. Myer, Y. P., Thallam, K. K., and Pande, A. (1980) J. Biol. Chem. 255, 9666–9673.
28. Szundy, I., Cappuccio, J. A., Borov, N., Koviara, A., and Einarsdottir, O. (2001) Biochemistry 40, 2186–2193.
29. Schneider, M. (2000) Zielgerichtete Mutagenese am Cytochrom C552 aus Paracoccus denitrificans, Diploma thesis, University of Frankfurt, Frankfurt, Germany.
30. Inmoos, C., Hill, M. G., Sanders, D., Fee, J. A., Slutter, C. E., Richards, J. H., and Gray, H. B. (1996) J. Biol. Inorg. Chem. 1, 529–531.
31. Hon-Nami, K., and Oshima, T. (1977) J. Biochem. (Tokyo) 82, 769–776.
32. Brown, J. N., and La Mer, V. K. (1924), 1411, 114–120.
33. Hon-Nami, K., and Oshima, T. (1977) J. Biochem. (Tokyo) 82, 769–776.
34. Wienk, H., Maneg, O., Lücke, C., Pristov, F., Lihr, F., Ludwig, B., and Ruterjans, H. (2003) Biochemistry 42, 6000–6012.
35. Crowley, P. B., Otting, G., Schlaf-Bley, B. G., Canters, G. W., and Ubbink, M. (2001) J. Am. Chem. Soc. 123, 10444–10453.
36. Hart, S. E., Schlaf-Bley, B. G., Delon, C., Bendall, D. S., and Howe, C. J. (2003) Biochemistry 42, 4529–4536.
37. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature 376, 660–669.
38. Harwenga, A., Reinecke, B., Ruterjans, H., Ludwig, B., and Michel, H. (2000) J. Mol. Biol. 295, 667–678.
39. Drosou, V. (2002) Cytochrome C als Elektronenacceptor für die aa3-Oxidase aus Paracoccus denitrificans, Ph.D. thesis, University of Frankfurt, Frankfurt, Germany.
40. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins C1, 281–296.
41. Witt, H., Malatesta, F., Nicoletti, F., Brunori, M., and Ludwig, B. (1999) J. Biol. Chem. 274, 5132–5136.
42. Atkins, P. W. (1990) Physical Chemistry, 4th Ed., p. 851, Oxford University Press, Oxford.
Different Interaction Modes of Two Cytochrome-c Oxidase Soluble Cu₆ Fragments with Their Substrates
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