Rapid Electron Transfer within the III-IV Supercomplex in Corynebacterium glutamicum

Simone Graf1,2, Olga Fedotovskaya1, Wei-Chun Kao3, Carola Hunte3, Pia Ädelroth1, Michael Bott4, Christoph von Ballmoos2 & Peter Brzezinski1

Complex III in C. glutamicum has an unusual di-heme cyt. c1 and it co-purifies with complex IV in a supercomplex. Here, we investigated the kinetics of electron transfer within this supercomplex and in the cyt. aa3 alone (cyt. bc1 was removed genetically). In the reaction of the reduced cyt. aa3 with O2, we identified the same sequence of events as with other A-type oxidases. However, even though this reaction is associated with proton uptake, no pH dependence was observed in the kinetics. For the cyt. bc1-cyt. aa3 supercomplex, we observed that electrons from the c-hemes were transferred to CuA with time constants 0.1–1 ms. The b-hemes were oxidized with a time constant of 6.5 ms, indicating that this electron transfer is rate-limiting for the overall quinol oxidation/O2 reduction activity (~210 e−/s).

Furthermore, electron transfer from externally added cyt. c to cyt. aa3 was significantly faster upon removal of cyt. bc1 from the supercomplex, suggesting that one of the c-hemes occupies a position near CuA. In conclusion, isolation of the III-IV supercomplex allowed us to investigate the kinetics of electron transfer from the b-hemes, via the di-heme cyt. c1 and heme a to the heme a3-CuB catalytic site of cyt. aa3.

The respiratory chain in aerobic organisms is composed of a number of membrane-bound protein complexes through which electrons, originating from the oxidation of organic compounds, are transferred to finally reach O2. The free energy released in this process is employed to establish a proton electrochemical gradient across the membrane, which is used to synthesize ATP by the F1F0 ATP synthase or for secondary transmembrane transport. In mitochondria, Complex III (the cytochrome (cyt.) bc1-complex) of the respiratory chain links the two-electron oxidation of quinol (QH2) to the one-electron reduction of water-soluble cyt. c in the respiratory chain:

\[ \text{QH}_2 + 2\text{cyt. c}^{2+} + 2\text{H}_2\text{O} \rightarrow \text{Q} + 2\text{cyt. c}^{3+} + 4\text{H}_2\text{O} \]  
(1)

where the subscripts N and P refer to the more negative and positive sides of the membrane, respectively. Reduced cyt. c delivers electrons to Complex IV (cytochrome c oxidase, CytcO), which catalyzes the reduction of dioxygen to water:

\[ 4\text{cyt. c}^{3+} + 4\text{H}_2\text{O} + \text{O}_2 \rightarrow 4\text{cyt. c}^{2+} + 2\text{H}_2\text{O} \]  
(2)

Part of the respiratory-chain enzymes in mitochondria are organized in so-called supercomplexes1-11. There are also reports of supercomplexes in bacteria, for example in Paracoccus denitrificans. In this bacterium, depending on the detergent used, supercomplexes composed of respiratory-enzyme complexes III-IV or I-III-IV at variable stoichiometries were identified12-14. Furthermore, in several bacterial systems electrons could be transferred directly between complexes III and IV via a membrane-anchored cyt. c15-17.

Corynebacterium glutamicum is a rod-shaped, Gram positive soil bacterium, which harbors two different terminal oxidases; an aa3-type CytcO and a bd-type menaquinol oxidase18-20. The cyt. aa3 in C. glutamicum is a four-subunit protein complex, comprising subunits CtaD, C, E, and F. Mass spectrometric analyses of the purified

1Department of Biochemistry and Biophysics, The Arhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden. 2Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland. 3Institute of Biochemistry and Molecular Biology, ZBMZ, Faculty of Medicine, BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany. 4IBG-1: Biotechnology, Institute of Bio- and Geosciences, Forschungszentrum Jülich, Wilhelm-Johnen-Strasse, D-52425 Jülich, Germany. Correspondence and requests for materials should be addressed to P.B. (email: peterb@dbb.su.se)
Cytc revealed that instead of heme \( a \), the \( C. \) glutamicum contains heme \( a_s \) in the active site. Furthermore, the \( C. \) glutamicum Cytc harbors an extra charged amino-acid cluster near the cyt. \( c \)-binding domain of subunit II (CtaC), which was suggested to interact with the second cyt. \( c \) of the cyt. \( bc_1 \) complex. Complex III in \( C. \) glutamicum is a three-subunit protein, containing cyt. \( c_1 \) (QcrC), the Rieske iron-sulfur protein (QcrA), and cyt. \( b \) (QcrB) (Fig. 1A). The QcrC subunit contains two CXXCH heme-binding motifs, suggesting that this protein complex contains two \( c \)-type hemes, hence referred to as the di-heme \( c_1 \) cyt. \( bc_1 \) complex. Furthermore, \( C. \) glutamicum contains no other \( c \)-type hemes, which suggested that the second heme \( c \) in cyt. \( bc_1 \) shuttles electrons between complexes III and IV that form a tight supercomplex. Such a supercomplex was isolated, its shape was determined using electron microscopy and it was shown to exhibit quinol-oxidase activity:

\[
2QH_2 + O_2 + 8H^+ \rightarrow 2Q + 2H_2O + 8H^+ \quad (3)
\]

The involvement of the second cyt. \( c \) of cyt. \( bc_1 \) in electron transfer between cyt. \( c_1 \) and CuA, the electron acceptor of Cytc (see below), is also supported by mutagenesis data. Quinol-oxidase activity was also found for the cyt. \( bc_1 \)-aa3 supercomplex from Mycobacterium smegmatis, a bacterium also devoid of soluble cyt. \( c \).

The mechanism of the cyt. \( bc_1 \) complex involves a Q-cycle in which the net reaction results in oxidation of menaquinol and reduction of cyt. \( c \) (see Fig. 1A), linked to proton uptake from the \( N \) side and release to the \( P \) side of the membrane. The process is initiated by binding of a menaquinol at the quinone-binding site, \( Q_m \) located near the low-potential heme \( b_1 \) (see Fig. 1A). In the next step, one electron is transferred from the menaquinol, via the Rieske protein [2Fe–2S] cluster, to cyt. \( c_1 \) while one electron is transferred via heme \( b_1 \) and heme \( b_1' \) to a menaquinone bound at a second quinone-binding site, \( Q_N \). This bifurcated electron transfer yields reduced cyt. \( c_1 \), a semireduced menaquinone at the \( Q_m \)-site and release of two protons to the \( P \)-side of the membrane. After binding a second menaquinol at the \( Q_p \)-site the same process is repeated. The doubly reduced menaquinone at the \( Q_N \) site picks up two protons from the \( N \)-side of the membrane to form a menaquinol that is released into the membrane.
Cytochrome c oxidase, which belongs to a large family of enzymes called the heme-copper oxidases, catalyzes oxidation of cyt. c and reduction of O₂ to H₂O. Here, we refer to the C. glutamicum cytochrome aa₃ as a CytcO even though in the cyt. bc₁-cyt. aa₃ supercomplex it receives electrons from quinol, via the cyt. bc₁ complex and not from (a water-soluble) cyt. c. The heme-copper oxidases are classified according to sequence, phylogenetic, and structural analyses into three main classes, A, B and C²⁶,²⁷. Subunit I, the core subunit shared by all of the three oxidase types, contains a low-spin heme group and the catalytic site, which is composed of a copper ion, Cuₐ, and a high-spin heme (for review on structure and function of the CytcOₚ with O₂, see²⁸–³⁷). The fourth redox-active site, Cuₐ, is found in subunit II. The most studied CytcOs are those from bovine heart mitochondria and the bacterial aa₃, CytcOs from Paracoccus denitrificans and Rhodobacter sphaeroides, which all belong to the A-class. In these CytcOs, electrons delivered by cyt. c are transferred consecutively to the Cuₐ site, heme a and finally to the binuclear center composed of heme a₁ and Cuₐ. In these bacterial CytcOₚ protons are transferred to the catalytic site through two pathways denoted by the letters D and K after conserved residues Asp132 and Lys362, respectively (numbering refers to the R. sphaeroides aa₃-type CytcO).

Internal electron and proton-transfer reactions in CytcO from several organisms have been studied in the past (see e.g.²⁶,²⁷,²³,²⁴,²⁵). An experimental technique that yields particularly detailed information about the sequence and rates of these reactions is the so-called flow-flash technique, in which the oxidative part of a reaction cycle (single turnover) of the enzyme is monitored. In this approach, the oxidase is first fully reduced by four electrons and rates of these reactions is the so-called flow-flash technique, in which the oxidative part of a reaction cycle (single turnover) of the enzyme is monitored. In this approach, the oxidase is first fully reduced by four electrons and then oxidized (τ ≅ 30 μs), electrons are transferred from the cyt. bc₁ complex to the CytcO. Further electron transfer from the c and b hemes occurs over a slower time scale. For simplicity, all these electron transfers are indicated schematically in the lower part of this scheme (the time constants are given in Fig. 8). The pumped protons are not shown.

![Figure 2. Schematic illustration of the reactions in CytcO studied in this work.](image)

The four red circles represent the redox-active metal sites as indicated (filled - reduced). During preparation of the sample, the oxidized CytcO (O) is reduced by 4 electrons under an atmosphere of pure N₂ (anaerobic reduction) to yield the fully reduced CytcO (state R). The sample is then incubated under an atmosphere of CO, which binds to heme a₁ at the catalytic site forming R-CO (see dashed line). The reaction studied in this work (indicated by a solid line) is started by photo-dissociation of the CO ligand (laser flash), yielding the reduced CytcO (R), which allows O₂ to bind to heme a₁ forming state A with a time constant of ~10 μs at 1 mM O₂. An electron is then transferred from heme a₁ to the catalytic site forming the “peroxo” state called Pₐ with a time constant of ~30 μs. Next, a proton is taken up from solution forming the ferryl state F with a time constant of ~100 μs. In most oxidases studied to date, at the same time the electron at Cuₐ equilibrates with heme a. This electron transfer is not indicated in the figure because in the C. glutamicum CytcO the equilibrium is shifted towards reduced Cuₐ. In the final step of the reaction the electron from Cuₐ (or the Cuₐ-heme a equilibrium) is transferred to the catalytic site forming the oxidized CytcO (state O) in ~1 ms. In cyt. bc₁-CytcO supercomplex, as soon as Cuₐ is partly oxidized (τ ≅ 100 μs), electrons are transferred from the cyt. bc₁ complex to the CytcO. Further electron transfer from the c and b hemes occurs over a slower time scale. For simplicity, all these electron transfers are indicated schematically in the lower part of this scheme (the time constants are given in Fig. 8). The pumped protons are not shown.
Results

Sequence Alignment and Homology Modeling. To analyze the structural characteristics of CytO from C. glutamicum, we performed homology modeling of the highly conserved subunit I with the three-dimensional structure of that from the R. sphaeroides aa3-type CytO20 using the SwissModel program40-42 (Fig. 1B,C). The two protein sequences are ~40% identical and ~60% similar. On the basis of the sequence itself and this model, the C. glutamicum protein is identified as an A1-type CytO. It holds the active site tyrosine (Tyr269 in C. glutamicum)21 and Glu267 in the D pathway (Glu286 in R. sphaeroides). Furthermore, in the C. glutamicum CytO an Asp residue (Asp116) is found at the same location as Asp132, the entry point of the D pathway in the ΔC-DSt and the C-DSt. The quality of the preparations was assessed using SDS gel electrophoresis (Figure S1) and dithionite-reduced minus ferricyanide-oxidized difference spectra of the two samples (Figure S2). As expected, both spectra showed the heme a signature (605 nm), and the supercomplex preparation displayed additional peaks for b-heme (560 nm) and c-heme (550 nm) (Figure S2). The approximately similar height of the three alpha peaks indicates the presence of a 1:1 complex of cyt. bc1 (2 heme b and 2 heme c) and cytochrome aa3 (two heme a). The reduced CO-bound minus reduced difference spectrum (Figure S3) displayed the characteristic features of CO binding to heme a.

Next, the quinol-oxidase activity of the purified supercomplex was measured by following O2 consumption after addition of a pre-reduced menaquinone. This process involves quinol oxidation by the cyt. bc1 complex followed by electron transfer to CytO, where oxygen is reduced to water. The measured quinol-oxidase activity for the purified supercomplex was 210 ± 20 e- /s (SD, n = 4 measurements) (normalized to the total CytO), i.e. in the same range as the published value20. The activity dropped rapidly upon flash freezing and thawing the preparation. Therefore, the sample was kept at 4 °C, where no activity loss was observed in the time frame between purification and functional studies (typically ~1 day), but the preparation was stable up to 7 days, Figure S4).

Purification and multiple turnover activity. We have purified the cyt. bc1-aa3 supercomplex from strain ΔC-DSt and the aa3 oxidase from the cyt. bc1 deficient strain ΔQ-DSt. The quality of the preparations was assessed using SDS gel electrophoresis (Figure S1) and dithionite-reduced minus ferricyanide-oxidized difference spectra of the two samples (Figure S2). As expected, both spectra showed the heme a signature (605 nm), and the supercomplex preparation displayed additional peaks for b-heme (560 nm) and c-heme (550 nm) (Figure S2). The approximately similar height of the three alpha peaks indicates the presence of a 1:1 complex of cyt. bc1 (2 heme b and 2 heme c) and cytochrome aa3 (two heme a). The reduced CO-bound minus reduced difference spectrum (Figure S3) displayed the characteristic features of CO binding to heme a.

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CytO activity of the purified supercomplexes and pure CytO (without cyt. bc1) was also measured using ascorbate as electron source and either TMPD, or TMPD and water-soluble cyt. c as electron mediators (Fig. 3). For the cyt. bc1-CytO, the TMPD activity was 90 ± 10 e- /s and it increased to 130 ± 10 e- /s (SD, n = 3) upon addition of free cyt. c, i.e. both rates were lower than the coupled quinol activity. For pure CytO, the activity increased from 160 ± 10 e- /s to 440 ± 20 e- /s (SD, n = 3) upon addition of cyt. c, thus displaying a much greater stimulation by the soluble electron carrier.

Figure 3. O2-reduction activity upon addition of an electron donor to CytO. The measurements were done with either CytO or the cyt. bc1-CytO super complex using ascorbate as electron donor and, TMPD and cyt. c as electron mediators. The activity was determined by measuring the O2-reduction rate (e- /s/CytO). Conditions: 100 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM MgSO4, 0.015% (w/v) DDM, 2 mM sodium ascorbate, 0.2 mM TMPD, with or without 24 μM cyt.c.
The absorbance changes at 550 nm (Fig. 5E) are mainly attributed to oxidation of heme $c$ for which the standard deviation was 20% of the measured values (for both the pure Cyt$_{bc_1}$-CytcO complex and the intact membrane (Fig. 4A,B). This observation indicates the presence of two Cyt$_{bc_1}$-CytcO populations, also in the native membrane (see Discussion). The time constants for the fast and the slow phases were about the same for all samples (pure CytcO, the purified Cyt$_{bc_1}$-CytcO supercomplex and whole cells), i.e., 11 ± 3 ms and 130 ± 30 ms (SD, n = 15) (at 1 mM CO), respectively. The kinetic difference spectra of the fast and the slow components were similar (Fig. 4C,D).

The apparent binding affinity for CO to the binuclear center of the oxidase was determined by measuring the observed CO-recombination rates for the slower kinetic phase at different CO-concentrations, for both the oxidase alone and the purified supercomplex (Figure S5). The second-order rate constants, determined from a linear fit to the data in Figure S5, were $K_{obs} = 7.6 \pm 0.2 \times 10^3$ M$^{-1}$s$^{-1}$ and $9.4 \pm 0.2 \times 10^3$ M$^{-1}$s$^{-1}$ for the CytcO and cyt. $bc_1$-CytcO complex, respectively. The rate of the faster component was CO-concentration independent.

**Kinetics of CO rebinding after flash photolysis.** CO binds with high affinity to heme $a_1$ in the binuclear site in the reduced CytcO. Upon illumination with a short laser flash, CO dissociates instantaneously, but in the absence of oxygen rebinds to the binuclear site (CO recombination). The CO-recombination kinetics was biphasic where the relative contribution of the two components varied slightly between the purified Cyt$_{bc_1}$-CytcO complex and the intact membrane (Fig. 4A,B). This observation indicates the presence of two CytcO populations, also in the native membrane (see Discussion). The time constants for the fast and the slow phases were about the same for all samples (pure CytcO, the purified Cyt$_{bc_1}$-CytcO supercomplex and whole cells), i.e., 11 ± 3 ms and 130 ± 30 ms (SD, n = 15) (at 1 mM CO), respectively. The kinetic difference spectra of the fast and the slow components were similar (Fig. 4C,D).

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**Single-turnover measurements.** Figure 5 shows absorbance changes after flash-induced dissociation of the CO ligand from the reduced cyt. $bc_1$-CytcO supercomplex in the presence of O$_2$. At 605 nm (Fig. 5A), three kinetic phases were resolved. The initial decrease in absorbance, with a time constant of $\sim 25 \mu$s (The standard deviation of the time constants was typically 10% of the measured values (n = 7–17), except for the $P \rightarrow F$ reaction for which the standard deviation was 20% of the measured values (for both the pure CytcO and the supercomplex)), is attributed to oxidation of heme $a$, i.e. electron transfer from heme $a$ to heme $a$, which yields the $P$ state at the catalytic site (formation of state A was not resolved, see below). This component is also seen at 445 nm (initial decrease, Fig. 5B). At 605 nm, the decrease is followed by a small increase in absorbance in the time range 0.05–0.2 ms that is attributed to re-reduction of heme $a$ with a time constant of 120 $\mu$s concomitant with the $P \rightarrow F$ reaction at the catalytic site. The slowest absorbance decrease ($\tau \approx 1.7$ ms) at 605 nm is associated with oxidation of the CytcO $F \rightarrow O$, also seen at 445 nm (Fig. 5B). At 830 nm, oxidation of Cu$_A$ is observed as an increase in absorbance. The small initial increase in absorbance is associated with oxidation of Cu$_A$ during the $P \rightarrow F$ transition, i.e. $\tau \approx 120 \mu$s while the major oxidation component displayed a time constant of $\sim 1.7$ ms (Fig. 5D).

The absorbance changes at 550 nm (Fig. 5E) are mainly attributed to heme absorption, where a decrease in absorbance is associated with oxidation of the hemes. Two kinetic components with time constants of $\sim 120 \mu$s and $\sim 1.7$ ms, respectively, were observed, i.e. concomitant with electron transfer from Cu$_A$ to heme $a$ during the
PR → F reaction and during the F → O reaction. Finally, at 563 nm (Fig. 5F) after the unresolved initial drop in absorbance (presumably associated with CO dissociation), a further decrease in absorbance associated with oxidation of heme b (τ ≅ 6.5 ms) was observed, i.e. oxidation of heme b occurred after oxidation of the Cyt c and at 563 nm (F) oxidation of the b-hemes. The CO ligand was dissociated at t = 0. Experimental conditions: 100 mM Tris-HCl at pH 7.5, 4 mM ascorbate, 1 μM PMS, ~900 μM O2. In addition, dithionite was added to fully reduce the cyt. bc1 complex. The CytO concentration was ~1 μM. A laser artifact at t = 0 has been removed for clarity. The filled circle in (B) represents the extrapolated initial absorbance after the laser flash.  

Figure 5. Absorbance changes associated with the reaction of the reduced cyt. bc1-CytO complex and CytO with O2. Absorbance changes were recorded over time at 605 nm (A), 445 nm (B) both associated with oxidation of a-hemes, and at 445 nm with the pure CytO (C) (this trace was multiplied by a factor of two to yield approximately the same absorbance changes as those seen in (B). The absorbance changes at 830 nm (D) are associated with oxidation of CuA at 550 nm (E) oxidation of cyt. c, and at 563 nm (F) oxidation of the b-hemes. The CO ligand was dissociated at t = 0. Experimental conditions: 100 mM Tris-HCl at pH 7.5, 4 mM ascorbate, 1 μM PMS, ~900 μM O2. In addition, dithionite was added to fully reduce the cyt. bc1 complex. The CytO concentration was ~1 μM. A laser artifact at t = 0 has been removed for clarity. The filled circle in (B) represents the extrapolated initial absorbance after the laser flash.

The concentration of reacting CytO (i.e. CytO from which the CO ligand is dissociated) was estimated from the change in absorbance at t = 0 at 445 nm (Fig. 5B), which yields ~0.15 μM CytO (using an absorption coefficient (ε) of 82 mM⁻¹cm⁻¹). The absorbance at t = 0⁺, i.e. just after CO dissociation corresponds to that of reduced CytO. Consequently, the decrease in absorbance from this point until t ≅ 10 ms, when the reaction is essentially over, corresponds to the amount oxidized CytO (~0.10 μM, using ε = 164 mM⁻¹cm⁻¹). Consequently, ~0.05 μM (~35%) of the reacting CytO becomes re-reduced by the cyt. bc1 complex. From the absorbance changes at 550 nm and 563 nm we estimate that ~0.025 μM (ε = 19.1 mM⁻¹cm⁻¹) heme b and ~0.028 μM (ε = 22 mM⁻¹cm⁻¹) heme c, respectively, become oxidized, which together account for ~0.05 μM CytO that is re-reduced during the experiment. It should be noted that these estimations are only approximate because we were not able to accurately resolve O2 binding to the reduced heme a1 in all samples. In part, this problem is attributed to the requirement to add dithionite in order to fully reduce the cyt. bc1-CytO supercomplex. Because dithionite reduces O2 directly during mixing, the O2 concentration was lowered before initiation
of the reaction of CytO with O₂, thereby slowing the R → A reaction. Consequently, it was difficult to resolve the associated absorbance changes from those associated with the next, A → P₈ transition.

The single-turnover reaction was also studied with purified CytO (Fig. 5C). Here, heme a was oxidized with a time constant of ~2.1 μs (R → P₈), followed in time by re-reduction from Cu₄ with a time constant of ~90 μs (P₈ → F) and oxidation with a time constant of ~1.3 ms (F → O). These time constants were almost the same as those observed with the cyt. bc₁-CytO supercomplex. No changes in absorbance were observed at 550 nm nor 563 nm for the purified oxidase (data not shown). The end absorbance level at 445 nm was slightly lower with pure CytO than with CytO that is part of the supercomplex (c.f. Fig. 5B,C), which means that the former was more oxidized than the latter. This observation presumably reflects the re-reduction of CytO by the cyt. bc₁ complex in the latter. However, as mentioned above, it was difficult to quantitatively resolve the relative absorbance differences in the two samples because we were unable to confidently scale the two different traces to each other due to unresolved absorbance changes associated with O₂ binding.

Binding of water-soluble cyt. c to e.g. the bovine heart CytO₃ is highly dependent on the salt concentration, reflecting electrostatic interactions of the two proteins. In order to investigate whether or not the interactions of CytO with cyt. c in the supercomplex could be disrupted, we studied the reaction with O₂ at increasing ionic strengths (addition of KCl). As seen in Figure S6, the amplitude of the absorbance changes associated with the F → O reaction decreased slightly (indicating more re-reduction of CytO) rather than increasing with increased ionic strength (we expect more oxidation of CytO upon cyt. c dissociation), which indicates that the cyt. c-CytO interactions were not disrupted at high salt concentrations.

**Net Proton Uptake from Solution.** The protons required for the reduction of O₂ to water are taken up from the medium, a process that can be monitored during flow-flash experiments in the absence of buffer by use of a pH-sensitive dye. Using phenol red, we studied proton uptake during the reaction of the reduced cyt. bc₁-CytO supercomplex with O₂, following absorbance changes at 560 nm. As seen in Fig. 6, the absorbance increased over time, which indicates net proton uptake during the reaction. The process displayed two components with time constants of 130 μs (~25% of the total absorbance change) and 1.9 ms, i.e. they coincided with the F → P₈ → F transition.

**pH-dependence.** Reactions steps that are associated with proton uptake from solution (e.g. the F → O step of the reaction of reduced CytO with O₂) often display pH dependent rates. We therefore investigated the pH dependence of the reaction with O₂ of the reduced cyt. bc₁-CytO supercomplex and CytO alone (Fig. 7A-B). At 445 nm, for the supercomplex, only a slight pH dependence was observed for the F → O reaction rate. As the total amplitude of the oxidation also changed, the results presumably reflect a pH-dependence in the extent of re-reduction by the cyt. bc₁ complex, i.e. “down-stream” steps of the reaction. Also with the pure CytO, the F → O reaction rate was pH independent and all kinetic components displayed essentially the same amplitudes in the measured pH range (c.f. data at pH 7.5 and 8.5 in Fig. 7B).

**Na⁺-dependence.** Because we did not observe any significant pH-dependence in the reaction rates with O₂, we also investigated the Na⁺-concentration dependence to test the possibility that the CytO transports Na⁺ (see refs 48 and 49). As can be seen in Figure S7, the addition of Na⁺ had a slight effect on the time constant of the A → P₈ reaction, i.e. electron transfer from heme a to the catalytic site (inset Figure S7), but this reaction step is not linked to pumping in other A-type oxidases. No effect on the kinetics of the F → O reaction was observed.
Discussion

Purification and Activity. The purified cyt. bc₁-CytO complex displayed quinol-oxidase activity, i.e. electrons were transferred first from quinol to the cyt. bc₁ complex and then, via the two c-hemes²⁰, to the CytcO, which reduces oxygen to water. The activity was ~210 e⁻/s, which is in good agreement with previously published results²⁰. The data are also consistent with those obtained for the cyt. bc₁-CytcO supercomplex from Mycobacterium smegmatis²⁴, which exhibited quinol-oxidase activity even at high detergent concentrations, supporting the presence of the cyt. bc₁-CytcO supercomplex. Both the C. glutamicum cyt. bc₁-CytcO and CytcO preparations exhibited TMPD-driven O₂-reduction activities. In both cases the activity increased upon addition of soluble cyt. c. However, the increase was larger for the pure CytcO (a factor of ~2.8) than for the supercomplex (a factor of ~1.4) (but see data with the M. smegmatis²⁴). This observation suggests that in C. glutamicum cyt. c is more accessible for binding to the CytcO upon removal of the cyt. bc₁ complex, which indicates that in the supercomplex one of the c-hemes is located near the CytcO electron entry point (i.e. presumably near Cu₅). Thus, the results indicate that pure CytcO (i.e. with the cyt. bc₁ removed) is capable of binding soluble cyt. c in spite of the presence of an extra loop of charged amino acids located at the cyt. c binding site¹⁸ (this binding can only occur in the mutant where the cyt. bc₁ complex is removed, i.e. not in vivo). Formation of a stable cyt. bc₁-CytcO complex that is capable of transferring electrons directly from cyt. bc₁ to CytcO is also consistent with the rapid electron transfer from the b and c-hemes to CytcO (see below).

We note that the TMPD and cyt. c-oxidation activities measured here are higher than those presented previously¹⁸,²⁰. The discrepancy is presumably due to differences in experimental conditions. While in the earlier studies the rate was derived from changes in the concentration of reduced electron donor, here the data was obtained by measuring the O₂-reduction rate at a constant concentration of the reduced electron donor (with excess ascorbate).

Ligand Binding to CytcO. Results from earlier studies with e.g. the bovine heart oxidase indicate that upon pulsed illumination the CO ligand dissociates from heme a₁ and binds transiently to Cu₅ before it dissociates into solution²⁰:

\[
\begin{align*}
\text{Fe}_{a₁}{}^{(CO)} + \text{Cu}_{₅} & \overset{k_1}{\underset{k₂}{\rightleftharpoons}} \text{Fe}_{a₁}{}^{(CO)} \text{Cu}_{₅} \\
\text{Fe}_{a₁}{}^{(CO)} \text{Cu}_{₅} & \overset{k_₃}{\underset{k₄}{\rightleftharpoons}} \text{Fe}_{a₁}{}^{(CO)} + \text{CO}
\end{align*}
\]
Dissociation of CO from heme \(a_1\) in the dark is very slow (\(k_{-1} \approx 0.03 \text{s}^{-1}\)), but upon illumination the ligand moves to \(Cua\) in \(< 10 \text{ ns}\), if the light intensity of the pulse is strong enough (such as in this study). The dissociation rate constant from \(Cua\), \(k_{-2}\), is \(7 \times 10^{-5} \text{s}^{-1}\) with the bovine heart CytO\(2+\). Recombination of CO occurs via \(Cua\) with a second-order process (\(k_{2} \approx 1 \times 10^{4} \text{M}^{-1} \text{s}^{-1}\)). The rate for internal CO transfer from \(Cua\) to heme \(a_{2}\), \(k_{1}\), is \(\approx 1 \times 10^{3} \text{s}^{-1}\). The observed rate of CO recombination is approximately given by the fraction of \(Cub\) with bound CO (middle state in scheme 1) multiplied by the rate of CO transfer from \(Cub\) to heme \(a_{2}\).

\[
k_{\text{obs}} \approx \frac{k_{2}}{k_{2} + k_{1}}
\]

With the rate constants given above, we obtain \(k_{\text{obs}} \approx 0.12 \times 1000 \text{s}^{-1} = 120 \text{s}^{-1}\) (\(\tau \approx 8 \text{ ms}\)) at 1 mM CO.

The CO-recombination kinetics measured with the \(C.\ glutamicum\) CytO was biphasic (see Fig. 4A). The kinetic difference spectra of the two components with the purified supercomplex and the oxidase alone were similar (see Fig. 4C,D), indicating that both components are associated with CO binding to heme \(a_1\) after light-induced dissociation. Consequently, the data indicate the presence of two populations of CytO with different CO-recombination rates to heme \(a_1\). The smaller (\(\sim 25\%\)) CytO population, displayed a CO-concentration independent rate constant of \(\sim 90 \text{s}^{-1}\) (also the relative amplitude was independent on the CO concentration). Assuming the model in Eq. 4, this observation indicates that for this population the ratio \(k_{2}/(k_{2} + k_{-2})\) is equal to \(\sim 1\) at all CO concentrations used in this study and that \(k_{1} = 90 \text{s}^{-1}\). Alternatively, after photolysis from heme \(a_1\) and binding to \(Cua\), the CO ligand did not equilibrate with solution in this population.

The slower, major CO-recombination component displayed a CO-concentration dependent rate constant of \(6.7 \text{s}^{-1}\) at 1 mM CO. Assuming that \(k_{1}\) has the same value of \(90 \text{s}^{-1}\) for the two CytO populations, the ratio \(k_{2}/(k_{2} + k_{-2})\) \(\approx 0.07\). Thus, the difference between the two CytO populations reflecting the two time constants could be explained by different values of \(k_{2}/(k_{2} + k_{-2})\), i.e. by differences in CO binding to \(Cua\). Because CO-recombination was biphasic both in whole cells and in the detergent-purified samples, the presence of the two components is not an artifact caused by the purification of the supercomplex or the oxidase. Instead, we speculate that the two components reflect two CytO populations that are present in the native membrane and that could differ, for example, in the local structure of CuB resulting in different CO-binding affinities. Most likely these two CytO populations would display different reactivity towards the natural ligand and electron acceptor, \(O_2\). If the relative fraction of these two populations would be modulated by the cell, this mechanism could be used to regulate electron transfer through the respiratory chain.

**Reaction with \(O_2\).** The four-electron reduction of \(O_2\) to \(H_2O\) takes place in a number of distinct kinetic steps in which the CytO is gradually oxidized and \(O_2\) is reduced. We identified the kinetic components on the basis of a comparison to data obtained earlier with other well-studied oxidases\(^{36}\). With the pure CytO we observed electron transfer from heme \(a\) to the catalytic site and formation of the "peroxy" state, \(P_a\), with a time constant of \(\sim 21 \mu\text{s}\). Formation of the next, ferryl intermediate (F) displayed a time constant of \(90 \mu\text{s}\). Finally, the CytO was oxidized forming the oxidized state (O) with a time constant of \(1.3 \text{ ms}\). All these time constants are essentially the same as those observed previously with e.g. the well-studied CytO from bovine heart or \(R.\ sphaeroides\). Consequently, the differences in CO-binding kinetics between the earlier studied A-type oxidases and \(C.\ glutamicum\) CytO are apparently not reflected in the kinetics of \(O_2\) binding and reduction.

For the cyt. \(bc_1\)-CytO complex, all time constants for the different reaction steps were similar to those observed with the pure CytO, however, there were also notable differences reflecting intra-complex electron transfer. During the \(P_a\rightarrow F\) reaction (\(\tau \approx 120 \mu\text{s}\)) an electron is transferred from \(Cub\) to heme \(a\), which leaves \(Cub\) oxidized allowing electron transfer from cyt. \(c\) of the cyt. \(bc_1\) complex to \(Cub\). This electron transfer is rate-limited by proton uptake\(^{31}\) and does not occur at the same rate as when photochemically injected into \(Cub\)\(^{52}\).

As seen at 550 nm (Fig. 5E), cyt. \(c\) was partially oxidized over a time scale of \(\sim 100 \mu\text{s}\), which indicates that \(Cub\) was re-reduced concomitantly with the \(Cub\)-to-heme \(a\) electron transfer. This interpretation is also supported by the very small extent of net \(Cub\) oxidation (observed at 830 nm) over the 100-\(\mu\text{s}\) time scale (Fig. 5D, see the small increase in absorbance). In the next step of the reaction, \(F\rightarrow O\) (\(\tau \approx 1.7 \text{ ms}\)), the fourth electron is transferred to the catalytic site, which allows further electron transfer from heme \(c\) to the CytO, reflected in a further decrease in absorbance at 550 nm over the time scale of the \(F\rightarrow O\) reaction in CytO. The absorbance changes at 550 nm reflect oxidation of cyt. \(c\), but we could not determine the degree of oxidation of each of the two cyt. \(c\) separately. Most likely these two cyt. \(c\) are oxidized simultaneously, but not necessarily to the same degree. In the summarizing Fig. 8 we indicate that both cyt. \(c\) are oxidized over time scales of 100-\(\mu\text{s}\) and 1.7 ms (approximated by 2 ms in the figure).

We also observed further electron transfer from heme \(b\) (absorbance decrease at 563 nm, Fig. 5F), which reflects electron transfer from heme \(b\) to the CytO, but this electron transfer significantly lags behind (\(\tau \approx 6.5 \text{ ms}\)) that of the \(F\rightarrow O\) reaction (\(\tau \approx 1.7 \text{ ms}\)). As described above for cyt. \(c\), also for heme \(b\) oxidation we could not discriminate between hemes \(b_1\) or \(b_2\) and conclude only that heme \(b\) of the cyt. \(bc_1\) is oxidized over the 6.5-ms time scale. As outlined in the Results section, the amounts of oxidized heme \(c\) and heme \(b\) approximately equal the amount of CytO that becomes re-reduced during or after reaction with \(O_2\). Furthermore, the electron-transfer time constant from heme \(b\) to CytO (\(\tau \approx 6.5 \text{ ms}\)) is approximately compatible with the overall quinol-oxidation/ \(O_2\) reduction turnover rate of the cyt. \(bc_1\)-CytO supercomplex (approximately \(\sim 210 \text{ s}^{-1}\)). This rapid electron transfer with a time constant of \(\sim 6.5 \text{ ms}\) corresponds to a maximum electron-transfer rate over a distance of \(\sim 25 \AA\). Even though the distances connecting the heme \(c\) with their partners are not known for the \(C.\ glutamicum\) cyt. \(bc_1\)-CytO complex, this distance estimation is consistent with that between heme \(b\) and the iron-sulfur cluster in cyt. \(bc_1\) from e.g. \(S.\ cerevisiae\).
Asn207 by Asp resulted in an increase in the Glu286 pCyt structural variants show that very small changes in the D pathway structure, also at a distance from the Glu, may yield but not all residues “below” the Glu267 in the D pathway are conserved. The data with the strength above ~300 mM45.

To investigate the functional stability of the cyt. bc1-CytO supercomplex we investigated the reaction with O2 upon increasing the ionic strength (Figure S6). Neither the amplitudes nor rates of the observed absorbance changes at 445 nm were significantly altered even at the highest KCl concentration of 1.5 M. These results suggest that the purified supercomplex is stable and remains functionally intact. Furthermore, the interactions between one of the c hemes of the cyt. bc1 complex and CytO seem more stable than those observed for the water-soluble cyt. c from e.g. bovine heart as in the latter case the 1:1 cyt. c-CytO complex dissociates at ionic strength above ~300 mM45.

**pH (in)dependence of the Reaction with O2.** As seen in Fig. 7 we did not observe any significant pH-dependence in the kinetics of the F→O reaction, neither for the pure CytO nor for the cyt. bc1-CytO supercomplex. Slight differences were observed for the two samples (see inset to Fig. 7), which may be attributed to binding of cyt. bc1 to CytO in the supercomplex. The observation that the F→O rate is essentially pH-independent is surprising given that the reaction is associated with proton uptake (see Fig. 6) and therefore expected to display pH-dependent kinetics, as in other oxidases30,46,47. One possible explanation for pH-independent kinetics of a reaction step that is linked to proton uptake is that the pK in this pH dependence may be outside of the accessible pH range. Alternatively, the proton uptake may not be part of the rate-limiting step, however, this explanation is less likely based on results from earlier studies with other A- and B-type oxidases where proton uptake is rate limiting31.54–57.

In earlier studies with the *R. sphaeroides* CytO it has been observed that the pH dependence of the F→O rate is essentially pH-independent up to pH ~9 and then decreases with increasing pH with an apparent pK of 9.458. The F→O rate in *R. sphaeroides* CytO displayed a more complex pH dependence and was found to titrate with two pKs of ~9 and ~6, respectively30. The pK of ~6 was attributed to residue Glu286 within the D proton pathway, which is conserved in the *C. glutamicum* CytO (Glu267). In the *R. sphaeroides* CytO, replacement of e.g. Asn139 or Asn207 by Asp resulted in an increase in the Glu286 pK; for example in the Asn139Asp variant the pK increased to a value above the accessible pH range46,59,60. In other words, even though the reaction is associated with proton uptake in these structural variants, it did not display a pH-dependent kinetics. In the *C. glutamicum* CytO, many but not all residues “below” the Glu267 in the D pathway are conserved. The data with the *R. sphaeroides* structural variants show that very small changes in the D pathway structure, also at a distance from the Glu, may yield pH-independent kinetics. Thus, considering the differences in the environment of Glu267 in the *C. glutamicum* CytO it is possible that its pK is tuned to adopt a value that is higher than that of Glu286 in the *R. sphaeroides* CytO. However, more experiments with single point mutations in the D pathway are necessary to confirm this speculation.

**Concluding Remarks**

The reaction of the purified reduced CytO with O2 displayed the same sequence of electron transfers as that observed previously with other bacterial and mitochondrial A-type CytOs. However, in contrast to data obtained with the other oxidases, none of the reaction steps associated with proton uptake displayed any pH dependence rates, which is explained in terms of an elevated apparent pK of Glu267. The data indicate that one of the c hemes of cyt. bc1 is bound near CuA, at the same site where externally added water-soluble cyt. c would bind. Furthermore, the data indicate that the interaction of this heme c with CytO is strong in the cyt. bc1-CytO supercomplex and insensitive to changes in ionic strength. The second heme c of the cyt. bc1-CytO supercomplex provides a link for direct electron transfer between cyt. bc1 and CuA, the electron acceptor of CytO (see Fig. 8), which was also indicated from earlier mutagenesis data30. Electron transfer from heme c to the CytO occurred over time scales of approximately 100μs–2 ms, while re-reduction of heme c by heme b displayed a time constant.

**Figure 8.** A schematic picture showing the approximate time constants for the electron-transfer reactions. Note that the positions of the heme groups in this picture are not compatible with the X-ray structure (e.g.39); they have been adjusted for clarity. Because the data cannot discriminate between the two heme bs, we have indicated the oxidation time constants for each type of heme without specifying which heme that is oxidized. Redox reactions of the quinones are not observed in these experiments.
of ~6.5 ms, which suggest that this reaction may be rate limiting for the overall quinol-oxidation O₂-reduction turnover rate. In conclusion, the isolation of a stable supercomplex from C. glutamicum allowed us to investigate the kinetics of electron transfer all the way from heme b in the cyt. bc₁ complex to the catalytic site of CytCₐO, via the bridging c hemes.

Materials and Methods
If not stated otherwise, the chemicals were purchased from Sigma-Aldrich.

Corynebacterium glutamicum strains. The C. glutamicum strains used for purification of CytCₐO and the cyt. bc₁-CytC supercomplex were described before. The ΔC-Dₐ₈ strain refers to the 13032A ΔctaD strain transformed with the pJC1-ctaD₈ plasmid (Kan'), which serves as an expression plasmid for Strep-tagged CtaD; ctaD is expressed from its native promoter and contains 10 additional codons at the 3’-end (AAWSHPQFEK). The ΔQ-Dₐ₈ strain refers to the 13032ΔqcrCAB strain transformed with the pJC1-ctaD₈ plasmid.

Culture Conditions. The cells were cultivated at 30°C in all steps. Single C. glutamicum colonies were picked from BHI-Agar plates (33 g/l brain heart infusion broth, 15 g/l agar agar, 20 g/l D- (+)-glucose, 25 mg/l kanamycin) and inoculated into 10 ml BHI culture medium (33 g/l brain heart infusion broth, 20 g/l D- (+)-glucose, 25 mg/l kanamycin) at 220 rpm. After overnight growth the pre-culture was inoculated into 500 ml CGXII medium in a 2 l Erlenmeyer flask (at 160 rpm). After the optical density at 600 nm (OD₆₀₀) reached between 25 and 30, the cells were diluted 1:20 into 2 l of CGXII medium into a 5 l baffled Erlenmeyer flask (at 130 rpm) and cultivated to an OD₆₀₀ of 15–17 before harvest.

Membrane Preparation. The cells were harvested using a Beckman centrifuge equipped with the JLA 10.800 rotor at 7,500 rpm (~10,000 × g) for 30 min. The cells were homogenized in 4 ml cell lysis buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgSO₄, 50 mg/l avidin (iba lifescience), 1% (w/v) DDM (GLYCON Biochemicals)) at a protein concentration of 5 mg/ml, and incubated at 4°C for 45 min under slow stirring. Unsolubilized material was removed by ultracentrifugation at 150,000 × g for 20 min. The concentrated supernatant was diluted tenfold in solubilization buffer without DDM (yielding a final DDM concentration of 0.1% (w/v)) and concentrated again to reach a volume <15 ml. The concentrated supernatant was applied to a Gravity flow Strep-Tactin Superflow column (bed volume 5 ml, iba lifescience). Subsequently, the column was washed 3 times with 0.5 column volumes of washing buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgSO₄, 0.015% (w/v) DDM) and protein was eluted with up to 3 column volumes elution buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgSO₄, 0.015% (w/v) DDM, 2.5 mM D-desthiobiotin) and concentrated as described above. The protein samples (CytCₐO alone green colored, bc₁-CytC brown colored) were stored at 4°C.

The CytCₐO alone, as well as the cyt. bc₁-CytC supercomplex, could be purified from membranes originating from the ΔQ-Dₐ₈ and the ΔC-Dₐ₈ C. glutamicum strains, respectively, both having a Strep-tag on subunit I of the CytC. The SDS-PAGE for the two purifications (supplementary Figure S1) shows that the eluate of the supercomplex contains the three core subunits of CytC (CtaD, C, and E), along with the three subunits of the bc₁-complex (QcrC, A, and B), as well as some additional subunits, which were also co-purified with the supercomplex. Accordingly, the eluate for the oxidase purification from ΔQ-Dₐ₈ membranes contained only the three core subunits of the CytCₐO.

Dithionite-reduced minus ferricyanide-oxidized difference spectra were recorded at room temperature using a Cary100 UV-Vis Spectrophotometer. The CytCₐO concentration was determined from the reduced minus oxidized difference spectrum using the absorption coefficient Δε₆₉₀₋₆₃₀ = 3.2 mM⁻¹ cm⁻¹.

Quinone Reduction. Instead of ubiquinone, C. glutamicum, as a Gram-positive bacterium, employs menaquinones as an electron carrier in the membrane. To prepare reduced quinol, 3.7 mg 2,3-dimethyl-[1,4]naphthoquinone (Rare Chemicals GmbH) was dissolved in 1 ml N₂-saturated anhydrous cyclohexane to yield a 20 mM solution. The solution was mixed with 5 ml N₂-saturated 1 M sodium dithionite solution (in H₂O) and shaken vigorously. After phase separation, the organic phase containing the reduced 2,3-dimethyl-[1,4]naphthoquinol was removed and transferred to a 15 ml Falcon tube (all steps performed under a stream of N₂.). The cyclohexane was evaporated under an N₂ stream, while the sample was kept at ~40°C in a water bath. Subsequently, the reduced quinol was dissolved in N₂-saturated, acidified ethanol (ethanol with 10 mM HCl), aliquoted, and flash frozen in liquid nitrogen, and stored at −20°C.

Determination of enzyme activities. Oxygen consumption in multiple turnover experiments was measured using a Clark-type oxygen graph (Hansatech). The TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) -oxidase activity was measured in 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.015% (w/v) DDM, 0.2 mM TMPD, 2 mM sodium ascorbate. Both TMPD and ascorbate were added before the sample and background oxygen consumption was measured and the reaction was started by adding the protein sample. Cytochrome c driven oxidase activity was measured as above in the presence of ascorbate and TMPD, but bovine heart cyt. c (25 μM final concentration) was also supplied before addition of the sample. Quinol-driven oxidase activity was measured in...
the same buffer (100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.015% (w/v) DDM), but 20 μl of the reduced quinol solution (see above) was added before the protein sample. Background oxygen consumption was measured, followed by addition of the protein sample and monitoring the enzymatic oxygen consumption. When measuring the CytO activity with ascorbate/TMPD/cyt. c as a substrate, this activity was insignificant. However, in the presence of quinol the background O2-reduction rate increased significantly, due to quinol auto-oxidation, and at most it reached 70% of the rate measured after addition of the supercomplex.

Flash Photolysis and Flow-flash Experiments. The purified samples (typical protein concentrations were in the range of 2–3 μM and 1–1.5 μM for bc2c-CytO and CytO, respectively) were transferred into a Thunberg cuvette and the atmosphere was exchanged for N2 on the vacuum line. The sample was reduced by the addition of 1 μM phenazine methosulphate and 5 mM sodium ascorbate from the sidearm of the cuvette. In order to achieve complete reduction of all the redox-active centers in samples containing supercomplexes, 0.5–1 mM sodium dithionite (Merck Millipore) was added and the reduction state was confirmed spectrophotometrically. After complete reduction, the atmosphere was exchanged for CO on a vacuum line. To measure the pH dependence of the reaction of the reduced CytO or cyt. bc2c-CytO with O2, the sample was prepared in 10 mM Tris-HCl, the atmosphere was replaced consecutively by N2, and then CO, and the sample was reduced by 4 mM ascorbate, 1 μM PMS and 100 μM dithionite and pH 7.5. Samples at different, higher pH values were prepared by adding various volumes of 1 M Tris-HCl buffer at pH 10 followed by incubation for at least 1 hour. Alternatively, the sample in 10 mM Tris-HCl at pH 7.5, was reduced with 4 mM ascorbate, 1 μM PMS and 100 μM dithionite (under CO atmosphere) and then mixed with an O2-saturated solution at different pH values containing 100 mM buffer. The buffering capacity of the O2 solution was much higher (100 mM) than that of the enzyme solution (10 mM) so the final pH after mixing was determined by the former. The pH after mixing was measured using a pH meter.

The CO rebinding/recombination kinetics to the catalytic site was measured as a change in absorbance over time at several wavelengths after the photolysis by a ~10-ns laser flash (λ = 532 nm, Nd:YAG laser, Quantel; the flash-photolysis/flow-flash setup was purchased from Applied Photophysics, UK). The time resolution of the set-up was ~10^-7 s. The absorbance changes were then fitted to an exponential decay function using the ProK software from Applied Photophysics, UK.

In order to determine the CO concentration dependence of the CO-recombination, 1 ml of a protein sample was transferred into a Thunberg cuvette and treated as described above, except that after reduction it was left under a nitrogen atmosphere. The sample was covered with a layer of paraffin oil and CO was added in small aliquots of CO-saturated buffer (100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.015% (w/v) DDM) with a gas-tight Hamilton syringe through the paraffin layer. The CO-recombination kinetics was studied after each CO addition using a flash-photolysis set-up (Applied Photophysics, UK).

In flow-flash experiments, the reduced and CO-blocked protein sample was mixed 1:3 with oxygen-saturated buffer (~1.2 mM O2) in a flow-flash setup (Applied Photophysics, UK). About 200 ms after mixing (mixing time <10 ms) with the oxygenated buffer, CO was dissociated from the catalytic site by a short laser pulse (~10-ns laser flash (λ = 532 nm, Nd YAG-laser, Quantel). Changes in absorbance were recorded over time at different wavelengths. The data were fitted to a kinetic model using the ProK software from Applied Photophysics, UK.

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**Acknowledgements**

We would like to thank Dr. Emelie Svahn for technical assistance. This study was supported by grants from the Knut and Alice Wallenberg Foundation (KAW 2013.0006) and Swedish Research Council (to PB, CvB, PÅ). Support was also obtained from the Excellence Initiative of the German Federal and State Governments (EXC 294 BIOSS to CH) and the COST European Cooperation in Science and Technology, CM1306, “Understanding Movement and Mechanism in Molecular Machines.

**Author Contributions**

P.B., C.v.B. and P.Ä. planned the research; S.G. and O.F. performed the experiments; M.B. constructed the strains; P.B., S.G., C.v.B. and P.Ä. wrote the manuscript; W.-C.K. and C.H. assisted in development of methods for enzyme purification.

**Additional Information**

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

**How to cite this article:** Graf, S. et al. Rapid Electron Transfer within the III-IV Supercomplex in *Corynebacterium glutamicum*. *Sci. Rep.* 6, 34098; doi: 10.1038/srep34098 (2016).

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