Comparisons of subunit 5A and 5B isoenzymes of yeast cytochrome c oxidase

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Mitochondrial CcO (cytochrome c oxidase) is the terminal enzyme of the respiratory chain that catalyses electron transfer from cyt c (cytochrome c) to oxygen. The reaction is coupled to the translocation of protons across the mitochondrial inner membrane, forming a protonotive force used to drive ATP synthesis and giving an overall reaction of: 4 cyt c$^{2+}$ + 8 H$^+$ mitochondrial matrix + O$_2$→4 cyt c$^{3+}$ + 4 H$^+$ intermembrane space + 2 H$_2$O. Saccharomyces cerevisiae CcO is composed of at least 11 subunits. Subunits I, II and III are encoded by the mitochondrial genome and constitute the essential catalytic core where the metal is expressed at very low oxygen tensions. As a consequence, COX5A-deleted strains (Δcox5A) have no or only low levels of CcO under normoxic conditions rendering them respiratory deficient. Previous studies have reported that respiratory growth could be restored by combining Δcox5A with mutations of ROX1 that encodes a repressor of COX5B expression. In these mutants, 5B isoenzyme expression level was 30–50% of wild-type (5A isoenzyme) and exhibited a maximum catalytic activity up to 3-fold faster than that of 5A isoenzyme. To investigate the origin of this effect, we constructed a mutant strain in which COX5B replaced COX5A downstream of the COX5A promoter. This strain expressed wild-type levels of the 5B isoenzyme, without the complication of additional effects caused by mutation of ROX1. When produced this way, the isoenzymes displayed no significant differences in their maximum catalytic activities or in their affinities for oxygen or cytochrome c. Hence the elevated activity of the 5B isoenzyme in the rox1 mutant is not caused simply by exchange of isoforms and must arise from an additional effect that remains to be resolved.

Key words: complex IV, cytochrome c oxidase, Michaelis–Menten constant, oxygen affinity, subunit 5 isoform, turnover number.

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Subunit 5 of Saccharomyces cerevisiae cytochrome c oxidase (CcO) is essential for assembly and has two isoforms, 5A and 5B. 5A is expressed under normoxic conditions, whereas 5B is expressed at very low oxygen tensions. As a consequence, COX5A-deleted strains (Δcox5A) have no or only low levels of CcO under normoxic conditions rendering them respiratory deficient. Previous studies have reported that respiratory growth could be restored by combining Δcox5A with mutations of ROX1 that encodes a repressor of COX5B expression. In these mutants, 5B isoenzyme expression level was 30–50% of wild-type (5A isoenzyme) and exhibited a maximum catalytic activity up to 3-fold faster than that of 5A isoenzyme. To investigate the origin of this effect, we constructed a mutant strain in which COX5B replaced COX5A downstream of the COX5A promoter. This strain expressed wild-type levels of the 5B isoenzyme, without the complication of additional effects caused by mutation of ROX1. When produced this way, the isoenzymes displayed no significant differences in their maximum catalytic activities or in their affinities for oxygen or cytochrome c. Hence the elevated activity of the 5B isoenzyme in the rox1 mutant is not caused simply by exchange of isoforms and must arise from an additional effect that remains to be resolved.

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allows derepression of a set of hypoxic genes including this prevents levels fall and Hap1 and Hap2/3/4/5 are no longer activated.

Expression of COX5A under normal aerobic growth conditions. Respiratory growth competent
Deletion of COX5A. Respiratory growth deficient
COX5A cloned on a centromeric plasmid under the control of its own promoter.
Deletion of genomic COX5A. Respiratory growth competent
Replacement of COX5A by COX5B downstream of the COX5A promoter on the nuclear genome. Expression of COX5B under normal aerobic growth conditions. Respiratory growth competent
COX5B cloned downstream of COX5A promoter on a centromeric plasmid. Deletion of COX5A. Respiratory growth competent
Up-regulation of COX5B through deletion of its transcription repressor ROX1 in COX5A-deleted background. Weak respiratory growth
COX5A with its own promoter on a centromeric plasmid. Deletion of ROX1, the transcription repressor of COX5B. Respiratory growth competent
COX5B cloned downstream of the COX5A promoter on a centromeric plasmid. Deletion of ROX1, the transcription repressor of COX5B, in COX5A-deleted background. Respiratory growth competent

Materials and Methods

Chemicals were purchased from Sigma–Aldrich. Yeast iso-1 cyt c and iso-2 cyt c were a gift from B. Guiraud (CGM, CNRS, Gif-sur-Yvette, France).

Yeast strains

All strains (Table 1) were constructed from a modified S. cerevisiae strain W303-1B (ade2 HIS3 leu2 trp1 ura3) that expressed wild-type CcO with a His6 tag sequence attached to Cox13 [31]. Deletion and gene replacement were performed via homologous recombination of PCR products. The PCR products and gene replacements were confirmed by DNA sequencing. The plasmid pRS415-COX5A containing COX5A gene under the control of its promoter and terminator was a gift from D. Winge (University of Utah, Salt Lake City, UT, U.S.A.).

Growth conditions and mitochondrial membrane preparation

Respiratory growth competence was checked on YPG medium (1 % yeast extract, 2 % peptone, 2 % glycerol and 2 % agar). All strains were grown in YPGal (1 % yeast extract, 2 % peptone and 2 % galactose) and their mitos (mitochondrial membranes) were prepared by published protocols [31] and stored in 50 mM potassium phosphate and 2 mM EDTA, pH 7.4. Concentrations of CcO were measured from sodium dithionite reduced minus oxidized difference spectra at 605–621 nm with an absorption coefficient, Δε, of 26 mM⁻¹ cm⁻¹ or at 445–465 nm with a Δε of 204 mM⁻¹ cm⁻¹ (based on values for bovine CcO [32]). Protein contents were determined using the Bradford assay [33].

Preparation of cyt c

Horse heart, yeast iso-1 cyt c (isoform-1) and iso-2 cyt c (isoform-2) stock solutions were prepared by washing with 20× volume
of the reaction buffer (10 mM potassium phosphate, pH 6.6, and 50 mM KCl) using a 10 kDa Vivaspin centrifugal concentrator at 4°C and 10000 g. The cyt c concentrations were measured from visible absorption spectra of sodium dithionite reduced samples, using an ε of 27.7 mM⁻¹·cm⁻¹ at 550 nm [34].

**Determination of oxygen affinity**

The \( K_m \) of CcO for oxygen was measured using the myoglobin method [35]. Oxygen consumption rates at low oxygen concentrations were monitored by following the conversion of oxymyoglobin into myoglobin at 582–564 nm using a Shimadzu dual-wavelength spectrophotometer. Oxygenated myoglobin was prepared by reduction with sodium ascorbate, followed by separation into aerobic 10 mM potassium phosphate (pH 6.6) and 50 mM KCl with a Sephadex G-25 column (15 cm × 1 cm). The total myoglobin concentration was measured from a visible absorption spectrum of a dithionite reduced sample, using an ε of 12.92 mM⁻¹·cm⁻¹ at 555 nm [36] and samples were typically 85% in the oxyferrous form and 15% in the ferric form. The reaction was carried out in a stoppered cuvette with stirring at 25°C. The cuvette was filled with a total volume of 3.8 ml of 10 mM potassium phosphate (pH 6.6), 50 mM KCl, 0.05% DDM (n-dodecyl β-D-maltoside; Melford Laboratories), 2 mM sodium ascorbate, 40 \( \mu \)M TMPD (\( N,N,N',N' \)-tetramethyl-p-phenylenediamine, a redox mediator), 30 \( \mu \)M total myoglobin and mitos to give 1–3 nM CcO. After the baseline had stabilized, the reaction was initiated with 50 \( \mu \)M horse heart cyt c. The reaction was complete in 10–15 min. The ratio of oxyferrous/ferric myoglobin was used to determine both free oxygen concentrations and rates of oxygen consumption, using an oxygen dissociation constant of 1.34 \( \mu \)M [37]. These values were used to determine the \( K_m \) of CcO for oxygen by non-linear fitting of data to the Michaelis–Menten equation using the curve-fitting toolbox in Matlab™.

**Determination of turnover numbers and kinetic parameters for cyt c**

Steady-state oxygen consumption rates were measured in a stirred reaction vessel of a Clark-type O2 electrode at 25°C. Assays with whole cells were carried out using 12.5 mg/ml cells giving 10–25 nM CcO in 50 mM potassium phosphate (pH 7.2), 440 mM sucrose, 10 mM lactate, 1.8 mM CCCP (carbonyl cyanide m-chlorophenylhydrazone) and 1 \( \mu \)M valinomycin [38]. Assays with mitos were carried out using membranes containing 2–10 nM CcO in 10 mM potassium phosphate, pH 6.6, 50 mM KCl, 0.05% DDM, 2 mM sodium ascorbate and 40 \( \mu \)M TMPD. The assay conditions for wild-type yeast CcO oxidizing horse heart cyt c were optimized in WTCOX5A mitos for pH (range 5.8–7.4), ionic strength (using 0–130 mM KCl), DDM concentration (0–20%) and horse heart cyt c concentration. The optimum assay conditions were pH 6.6, 0.05% DDM and 50 mM KCl. Under these conditions 50 \( \mu \)M horse heart cyt c produced rates that were close to \( V_{\text{max}} \). These conditions were assumed to be also optimal for yeast iso-1 and iso-2 cyts c. Initially, a baseline was measured in the absence of cyt c and then the reaction was initiated by addition of 50 \( \mu \)M cyt c. Turnover numbers and rates are expressed in terms of the number of electrons transferred from cyt c per s per CcO (e⁻·s⁻¹). Rates at each cyt c concentration were averaged from two to four repeats. \( V_{\text{max}} \) and \( K_m \) values were determined using non-linear fitting of the Michaelis–Menten equation for single phase kinetics and with the sum of two Michaelis–Menten terms for plots that displayed biphasic kinetics using the curve-fitting toolbox in Matlab™.

**RESULTS**

**Cell growth and CcO expression levels**

All of the mutant strains constructed and analysed in the present study are listed in Table 1. The COX5B gene at its genomic locus was not deleted in these mutants since its expression relies on hypoxic conditions and hence is not expressed under the aerobic growth conditions used in the present study. Thus the COX5A-deleted strain could not grow on respiratory medium (YPG). In order to compare the catalytic activity of 5A and 5B isoenzymes in the COX5A-deleted strain, we replaced COX5A by COX5B both in the COX5A genomic locus and on a centromeric plasmid. In both cases, COX5B expression was under the control of the COX5A promoter, the 5B isoenzyme was expressed at normal levels under aerobic growth conditions, and the cells were respiratory growth competent. When the deletion of COX5A was combined with deletion of ROX1, encoding a transcription repressor of COX5B, the resulting mutant was also respiratory growth competent, as previously reported, although the growth in respiratory medium was slower than the wild-type control.

Strains were grown to late exponential phase (\( \log \)O = 0.9–1) in YPGal medium for 14–16 h at 28°C. All displayed a wild-type doubling time of 2.7–3.0 h. This yielded 11 g of wet mass of cells per litre of culture for all strains except \( \Delta \)ROX1\Delta COX5A, which yielded ~6 g. The difference in biomass was most probably due to the difference in respiration/fermentation ratio of \( \Delta \)ROX1\Delta COX5A when grown to late exponential phase in galactose medium. The doubling times were measured during early exponential phase where the strains might use more fermentation than respiration, thus no difference in doubling times could be observed between strains.

Reduced minus oxidized visible absorbance difference spectra of whole cells confirmed the presence of CcO at approximately 2 nmol of CcO per g of wet weight cells in COX5B, pCOX5B, \( \Delta \)ROX1\Delta COX5A and \( \Delta \)ROX1\Δ COX5A pCOX5B, consistent with the level in the WTCOX5A strain. The CcO level in \( \Delta \)ROX1\Δ COX5A cells was significantly less than 1 nmol g⁻¹, although difficult to quantify accurately in whole cell spectra.

Redox spectra of mitos allowed accurate quantification of CcO in all strains from either the visible band at 605–621 nm or the Soret band at 445–465 nm [32]. For comparison, Figure 1 includes a spectrum of \( \Delta \)COX5A mitos which have no assembled CcO. Mitos from WTCOX5A, COX5B, pCOX5B, \( \Delta \)ROX1\Δ COX5A pCOX5B and \( \Delta \)ROX1\Δ COX5A pCOX5B have approximately equivalent levels of CcO (0.23–0.31 nmol per mg of mitochondrial protein; nmol of CcO-mg⁻¹), whereas mitos from \( \Delta \)ROX1\Δ COX5A contained 5–6-fold less CcO (0.05 ± 0.01 nmol-mg⁻¹). However, the level of bc₁ complex remained fairly constant in mitos from all strains, including those derived from \( \Delta \)ROX1\Δ COX5A, at 0.76–1.2 nmol of bc₁ complex-mg⁻¹ (as measured from the visible band at 562–575 nm using an \( \Delta \varepsilon \) of 28 mM⁻¹·cm⁻¹) (Figure 1) [32]. Hence the ratio of CcO to bc₁ complex in \( \Delta \)ROX1\Δ COX5A mitos was also ~6-fold less (~0.04:1) than the ratio (0.25:1) in mitos derived from strains WTCOX5A, COX5B, pCOX5B, \( \Delta \)ROX1\Δ COX5A pCOX5A and \( \Delta \)ROX1\Δ COX5A pCOX5B.

**Turnover numbers in whole cells**

The steady-state turnover numbers of CcO in whole cells under uncoupled conditions are shown in Figure 2(A). Strains that expressed the 5B isoenzyme under the control of the COX5A promoter from either the nuclear genome (strain COX5B) or
Turnover numbers in mitos preparations

The uncoupled turnover numbers of CcO in whole cells will most probably be limited by additional factors, and so turnover numbers in isolated mitos were also compared using conditions that had been optimized for oxidation of horse heart cyt c (Figure 2B). In addition, turnover numbers were determined using yeast iso-1 cyt c and iso-2 cyt c under the same conditions (see the Discussion section). For all strains under these conditions, activities were fastest with horse heart cyt c, followed by yeast iso-2 cyt c. In agreement with whole cell turnover values, CcO in mitos isolated from the ΔROX1ΔCOX5A strain had an approximately 2-fold greater turnover number compared with that in all other strains, irrespective of the cyt c substrate used (Figure 2B). However, the CcO activity per mg of mitochondrial protein was ~3-fold less than that in the other strains (140 e·s⁻¹·mg⁻¹ compared with 380 e·s⁻¹·mg⁻¹) because of the lower level of CcO per mg of protein.

Determination of \( K_m \) for oxygen

Rates of oxygen consumption in the concentration range of \( K_m \) for oxygen of CcO were determined by the oxymyoglobin method [35]. \( K_m \) and \( V_{max} \) values for WTCOX5A, COXSB and ΔROX1ΔCOX5A mitos are summarized in Table 2 and Michaelis–Menten plots are shown in the Supplementary Figure S1. All strains exhibited approximately the same \( K_m \), although the \( V_{max} \) of ΔROX1ΔCOX5A was at least 1.5-fold greater compared with that of WTCOX5A and COXSB.

DISCUSSION

Incorporation of subunit 5 (A or B) is essential for the stable expression of optically detectable functional CcO [20,39] and
Comparisons of subunit 5A/5B isoenzymes

Figure 3 Michaelis–Menten plots of WTCOX5A (○), COX5B (■) and ΔROX1ΔCOX5A (×) mitos using horse heart cyt c (A), yeast iso-1 cyt c (B) and yeast iso-2 cyt c (C)

Reaction conditions were the same as in Figure 2(B), but the rate was measured at multiple cyt c concentrations (1.25–350 μM). \( V_{\text{max}} \) and \( K_m \) values (see Table 3) were determined using non-linear fitting of the Michaelis–Menten equation for data displaying single phase kinetics (A) and two Michaelis–Menten terms for those with biphasic kinetics (B and C).

Table 2 Comparison of oxygen affinities and \( V_{\text{max}} \) of WTCOX5A, COX5B and ΔROX1ΔCOX5A mitos

The \( K_m \) and \( V_{\text{max}} \) values were determined using non-linear fitting of the Michaelis–Menten equation to plots shown in Supplementary Figure S1. Error values are 95% confidence intervals given by the \( t \)-distribution.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Strain (mitos)</th>
<th>( V_{\text{max}} ) (μM O₂/s per μM Cyt c)</th>
<th>( K_m ) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>WTCOX5A</td>
<td>184 ± 12</td>
<td>0.85 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>COX5B</td>
<td>154 ± 9</td>
<td>0.74 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ΔROX1ΔCOX5A</td>
<td>302 ± 21</td>
<td>0.86 ± 0.2</td>
</tr>
</tbody>
</table>

hence the level of detectable CrO is a direct measure of the level of functionally incorporated subunit 5. It has also been shown that the COX5B isoform is not expressed under aerobic growth conditions [24]. Hence, for those strains used to generate the 5A isoenzyme of CrO, the optically detected CrO will have only subunit 5A since they were grown aerobically. For those strains used to generate the 5B isoenzyme, 5A isoenzyme was absent since the COX5A gene was replaced entirely by COX5B.

COX5A deletion (Δcox5A) results in a lack of respiratory competence [24] and absence of CrO (Figure 1). Respiration-competent mutants of such a Δcox5A strain had mutations in ROX1, a repressor of COX5B, that resulted in the enhanced expression of COX5B [24]. The 5B isoenzyme was up to 3-fold more catalytically active than the 5A isoenzyme. The results of the present study with the ΔROX1ΔCOX5A mutant confirm this observation. A 2-fold faster 5B isoenzyme activity was also observed in a COX5A-deleted strain with wild-type ROX1 and with COX5B cloned on a high copy plasmid [30]. However, in all of these strains, the level of CrO was significantly diminished in comparison with wild-type levels: 34% of the control in the strain with both Δcox5A and rox1 mutations [29]; and less than 50% in the Δcox5A strain with higher copy number of COX5B [30]. It was also substantially decreased in the ΔROX1ΔCOX5A strain described in the present study.

In order to circumvent such complications, we generated COX5A-deleted mutants in which the COX5B was placed downstream of the COX5A promoter on the genomic locus, similarly to the chimaeric constructs described in [39]. In addition, COX5A-deleted strains with or without ROX1 deletion were transformed with a centromeric plasmid to express 5A or 5B isoforms under the control of the COX5A promoter. This enabled expression of 5A or 5B isoforms at wild-type levels and at normal oxygen concentrations and with the same status (present...
or deleted) of ROX1. In these constructs, 5A and 5B isoenzymes failed to show any difference in their $K_m$ values for oxygen (∼0.8 ± 0.1 μM), that are in a similar range to those (1 μM [40] and 0.95 μM [41]) reported for bovine Cyt C. This latter comparison was carried out since their expression is differentially regulated by oxygen concentration. Similarly, the $V_{max}$ and $K_m$ values for cyt c from horse heart showed no differences between WT COX5A and COX5B but were ∼1.5-2-fold greater in $V_{max}$ in the 5B isoenzymes of Cyt C expressed in the ΔROX1ΔCOX5A strain. The $V_{max}$ and $K_m$ values of WT COX5A with horse heart cyt c reported in the present study are in a range consistent with Geier et al. [42] ($V_{max}$ 1773 s$^{-1}$, $K_m$ 15.3 μM).

Equivalent $V_{max}$ and $K_m$ measurements were made using $S. cerevisiae$ cyt c (Figure 2B and Table 3) since two isoforms are differentially expressed depending on the oxygen levels. Iso-1 cyt c is predominantly expressed under normal oxygen levels and iso-2 cyt c under hypoxic conditions [25,26]. Once again no significant difference in $V_{max}$ was observed between WT COX5A and COX5B (Figure 2B and Table 3). The $V_{max}$ and $K_m$ values with yeast iso-1 and iso-2 cyt c are much greater than those reported ($V_{max}$ < 40 s$^{-1}$; $K_m$ < 10 μM; [28]). Apart from the overall assay conditions and possibly the method used to prepare mitos, these differences in $V_{max}$ and $K_m$ could be due to the higher and more physiologically relevant (0.5 mM cyt c [43]) cyt c concentration range used in the present study (∼300–1.25 μM) compared with the range (20–0.05 μM) used by Allen et al. [28]. WT COX5A, COX5B and ΔROX1ΔCOX5A strains shared the same $K_m$ values for cyt c (Table 3), as was also observed by Allen et al. [28].

Noticeably, for all strains, the $V_{max}$ values were in the order: horse heart > iso-2 > iso-1 cyt c (Figure 2B and Table 3). This finding conflicts with the previous report of faster activity with iso-1 cyt c compared with iso-2 cyt c [28]. However, this is probably accounted for by the different conditions of measurement, that in the present study were optimized in pH and ionic strength for horse heart cyt c. More significantly, it was reported that the isoenzymes showed different relative rates with the cyt c isoforms, with selectivity ‘dampened’ from 4-fold to 1.6-fold when the physiologically relevant aerobic (Cyt C isoenzyme 5A/iso-1 cyt c) and hypoxic (Cyt C isoenzyme 5B/iso-2 cyt c) isoforms were paired together [28]. However, no preferences for specific cyt c isoforms were evident in the 5A and 5B isoenzymes in the present study and, if anything, the opposite effect was observed when comparing WT COX5A/iso-1 cyt c and ΔROX1ΔCOX5A/iso-2 cyt c pairs (Figure 2B and Table 3).

Biphasic kinetics (Figure 3 and Supplementary Figure S2) were observed for yeast cyt c, consistent with previous data [28], whereas horse heart cyt c exhibits single phase kinetics consistent with the findings in [42]. The phenomenon has been well-documented for many types of Cyt C and with different types of cyt c (including yeast CrC) [44,45]. Several models have been proposed to explain the biphasic behaviour. One model is that there are two catalytic sites on Cyt C that can bind cyt c with different affinities [44]. A second proposal is that there is a non-catalytic binding site for cyt c, either very close to the catalytic site and so allowing direct interaction with substrate [46], or at a distance and acting allosterically [47]. The transition of high-affinity to low-affinity phase, was suggested to arise from an increase in the dissociation rate constant of ferricyt c (the rate-limiting step) when the regulatory site was bound by a second cyt c. Most recently, it was proposed that oxidized cyt c also acts as a competitive inhibitor of Cyt C, which is in addition to the effects of binding of cyt c to a second regulatory site [48]. For the purpose of the present study, a comparison of WT COX5A, COX5B and ΔROX1ΔCOX5A isoenzymes has been made with the high-velocity/low-affinity phase data since it is the phase that may predominate under physiological conditions since cyt c concentration in the intermembrane space is at 0.5 mM [43] and it is observed for horse heart, yeast iso-1 and iso-2 cyt c (Table 3).

To summarize, yeast strains that express the 5A or 5B isoenzymes to the same levels under aerobic growth conditions, with or without the deletion of the ROX1 gene, show definitively that these isoenzymes display similar turnover numbers and affinities for oxygen and cyt c. Hence the origin of the elevated activity of the 5B isoenzyme reported previously [28,29] and confirmed in the present study with the equivalent ΔROX1ΔCOX5A strain must be caused by a secondary effect. Two possible explanations are discussed in the present paper.

Rox1 is a transcription repressor of many hypoxic genes [49,50]. It is possible that its loss could cause increased 5B isoenzyme activity through separate effects that might lead to post-translational modification of subunit 5B, for example phosphorylation or binding/unbinding of ATP/ADP [4,51]. However, elevated 5B isoenzyme activity has been previously observed when expressed from a high copy number plasmid both in the presence of ROX1 [30] and in a roxl1-mutated strain [28]. Furthermore, in the present study the 5B isoenzyme had the same activity (and expression level) as the 5A isoenzyme when expressed both with (in the COX5B strain) and without (in the ΔROX1ΔCOX5A/COX5B strain) a functioning Rox1 (Figure 1). All of the above argues against a secondary effect of ROX1 deletion as the prime cause of the increased activity of the 5B isoenzyme when produced at low oxygen tensions or in strains such as ΔROX1ΔCOX5A.

Elevated activity of the 5B isoenzyme has been observed only when its expression level was significantly lower than in wild-type strains, as is also the case for the ΔROX1ΔCOX5A strain reported. Since the level of bc1 complex remains relatively constant, this results in a proportionately lower Cyt C/bc1 ratio. It has been shown that a proportion of Cyt C in yeast mitochondria can be isolated as Cyt C–bc1 supercomplexes [52–54]; the proportion of Cyt C in such supercomplexes will presumably be governed by the Cyt C/bc1 ratio. It seems feasible that the interaction between complexes may induce allosteric effects that elevate the catalytic activity of Cyt C, an effect that becomes more evident when the Cyt C/bc1 ratio is low since this will favour a greater proportion of the Cyt C forming Cyt C–bc1 supercomplexes (simply from an equilibrium constant point of view). Interestingly, the III1IV2 supercomplex structure of yeast Cyt C shows that subunit 5 is close to the interface with the bc1 complex [54]. The extent of
supercomplex formation, and the allosteric effect that it induces, could be different with the 5A and 5B isoforms, providing a novel possible functional basis for the existence of the isoforms.

**AUTHOR CONTRIBUTION**

Peter Rich, Brigitte Meunier and Raksha Dodia were involved in the concept, design and interpretation of data. Brigitte Meunier constructed and characterized all of the mutant strains and Raksha Dodia carried out all biochemical and kinetic experiments. Christopher Kay carried out the fitting of the kinetic data. The paper was written by Raksha Dodia with contributions from all authors.

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