Loss of a Conserved Tyrosine Residue of Cytochrome b1 Induces Reactive Oxygen Species Production by Cytochrome bc1

Dong-Woo Lee†1, Nur Selamoglu‡1, Pascal Lanciano§2, Jason W. Cooley‡12, Isaac Forquer†3, David M. Kramer‡3, and Fevzi Daldal‡3

From the †Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the §Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164

Production of reactive oxygen species (ROS) induces oxidative damages, decreases cellular energy conversion efficiencies, and induces metabolic diseases in humans. During respiration, cytochrome bc1 efficiently oxidizes hydroquinone to quinone, but how it performs this reaction without any leak of electrons to ATP production through oxidative phosphorylation that relies on the respiratory NADH:dehydrogenase chain (1). Production of reactive oxygen species (ROS) leads oxidative disruption of its catalytic [2Fe-2S] cluster. Hence, this Tyr residue is essential in controlling unproductive encounters between O2 and catalytic intermediates at the quinol oxidation site of cytochrome bc1 to prevent ROS generation. Remarkably, the same Tyr to Cys mutation is encountered in human mitochondrial disorders and in Plasmodium species that are resistant to the anti-malarial drug atovaquone. These findings illustrate the harmful consequences of this mutation in human diseases.

In most organisms, the ubiquinolcytochrome c oxidoreductase (cytochrome bc1 or complex III) is a central enzyme for ATP production through oxidative phosphorylation that relies on the proton motive force (ΔμH+) generated by the respiratory chain (1). Production of reactive oxygen species (ROS) leads oxidative damages of cellular components and in eukaryotes induces apoptosis (2, 3). Most cellular ROS are thought to emanate from the respiratory NADH:dehydrogenase (i.e. complex I) and cytochrome bc1 (see Fig. 1a for Rhodobacter capsulatus structure) under compromising physiological conditions (4, 5). Cells use antioxidant enzymes (e.g. superoxide dismutase or glutathione peroxidase) to prevent oxidative damages, but upon extensive ROS generation, harmful damage occurs. Indeed, cellular redox homeostasis, regulated by the rate of electron flow through the respiratory chain and O2 availability, is tightly coupled with the global metabolism (4, 5). For example, recent studies show that cytochrome bc1 is involved in stabilization and activation of hypoxia-induced factors like HIF-1α by mitochondria-generated ROS under hypoxic conditions (6, 7).

Mitochondrial DNA mutations are known causes of clinical syndromes (e.g. LHON, Pearson syndrome, and exercise intolerance) or provide predisposition for inherited and common diseases (e.g. aging, cardiomyopathy, cancer, diabetes, and neurodegenerative diseases) (8, 9). Mutations in mitochondrial or nuclear DNAs associated with mitochondrial fission and fusion (10), ascribed to ROS generation, lead to progressive dysfunction of mitochondria and loss of energy efficiency (11). For example, the Tyr to Cys mutation at position 278 (position 302 in R. capsulatus; see Fig. 1b) of cytochrome bc1 of human mitochondrial cytochrome bc1 (complex III) causes severe exercise intolerance and “multi-system disorder” (12). Remarkably, the same mutation in this conserved residue (Fig. 1c) is also encountered in cytochrome bc1 of malarial parasites (e.g. Plasmodium falciparum or Plasmodium yoelii) that are resistant to the antimalarial drug atovaquone (13, 14). These clinical findings highlight that specific amino acids of cytochrome bc1 are critical for human mitochondrial diseases (12, 15), for resistance of parasites to therapeutic agents (16), and for catalytic activity of the enzyme in bacteria (17) and yeast (18, 19), yet our understanding of the mechanisms underlying these disparate defects remains elusive.

Cytochrome bc1 oxidizes reduced quinone (QH2)4 via an unstable semiquinone intermediate at a catalytically active (i.e. Qo) site. Its function depends on an elaborate mechanism with two “one-electron” transfer steps, one to a high potential chain (comprising the iron-sulfur protein and cytochrome c1) and another to a low potential chain (formed of the hemes b1 and b14 of cytochrome b) to generate ΔμH+ across the membrane with minimal energy expenditure (20–22). Several models (23–25) have been proposed to rationalize the efficient and safe occurrence of these electron transfer steps in the presence of O2.

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‡1 Present address: Industrial Biotechnology and Bioenergy Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea.
§2 Present address: Dept. of Chemistry, University of Missouri, Columbia, MO 65211.
†3 To whom correspondence should be addressed: Dept. of Biology, University of Pennsylvania, Philadelphia, PA 19104. Tel.: 215-898-4394; Fax: 215-898-8780; E-mail: fdaldal@sas.upenn.edu.

4The abbreviations used are: QH2, reduced quinone (hydroquinone); ROS, reactive oxygen species; MOPS, 4-morpholinepropanesulfonic acid; IAM, idoacetamide; DBH2, decylbenzo-hydroquinone; Stg, stigmatellin; SOD, superoxide dismutase.
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However, how the reactive intermediates of the Qₐ site are shielded from O₂ to avoid oxidative damages remains unknown (26). We hypothesized that specific amino acids at the Qₐ site might provide protective mechanism(s) against oxidative damage while ensuring catalytic efficiency. Using the bacterial enzyme, here we show that mutating a specific Tyr residue of cytochrome b subunit of cytochrome bc₁ greatly enhances ROS production. Remarkably, the Tyr to Cys substitution cross-links together the cytochrome b and the iron-sulfur subunits of cytochrome bc₁ and renders the bacterial enzyme sensitive to O₂ by oxidative disintegration of its catalytic [2Fe-2S] cluster. These findings demonstrate the occurrence of “protective residues” in cytochrome bc₁, like this Tyr of cytochrome b that controls ROS generation. They also illustrate how specific mutations on such critical residues cause broad cellular defects extending from human mitochondrial diseases (12) to parasite resistance to therapeutics (16).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Genetic Techniques—**Escherichia coli and *R. capsulatus* strains were grown as described earlier (27). Mutations at Tyr302 of *R. capsulatus* cytochrome b were introduced by using the QuikChange site-directed mutagenesis kit (Stratagene) with the plasmid pPET1 as a template and appropriate mutagenic primers. The L.66-kb Xmal-StuI DNA fragment of pPET1 bearing the mutation was exchanged with its counterpart in pMTS1, yielding the plasmids pMTS1:Y302X (where X indicates Ala, Cys, Phe, Ser, or Val), which were crossed into the strain MT-RBC1 lacking cytochrome bc₁, as described earlier (27–31). The presence of only the desired mutation was confirmed by DNA sequence analysis of plasmids.

**Preparation of Aerobic and Anaerobic Membrane Samples—**Aerobic chromatophore membranes were prepared in 50 mM MOPS (pH 7.5), 100 mM KCl, 100 mM EDTA, 1 mM caproic acid, and protein concentrations determined as described earlier (28, 29). For anaerobic membrane samples, an anaerobic chamber (MBraun-USA Inc., Stratham, NH) at 20 °C was used with degassed, argon-flushed buffers containing 5 mM sodium dithionite. The membranes were prepared under argon flushing and stored in gas-tight serum vials with septa (National Scientific Co.). For sulphydryl alkylation experiments, 100 mM idoacetamide (IAM) or N-ethylmaleimide was added prior to cell disruption.

**Biochemical and Biophysical Techniques—**Protein samples were solubilized in 62.5 mM Tris-HCl (pH 6.8), 4% SDS, 100 mM dithiothreitol, 25% glycerol, and 0.01% bromophenol blue incubated at 60 °C for 10 min, separated by SDS-polyacrylamide gel electrophoresis, and stained with Coomassie Brilliant R-250, and immunoblots were performed using monoclonal and polyclonal antibodies specific for *R. capsulatus* cytochrome bc₁ subunits as described in Ref. 29. Cytochrome bc₁ activity was measured using decylenbenzo-hydroquinone (DBH₂) cytochrome c reductase assay as described in Refs. 27 and 29. For all of the strains, at least three independent cultures were used, three independent measurements per culture were performed, and the mean values ± standard deviations are indicated. Optical difference spectra for b- and c-type cytochromes were recorded, and *R. capsulatus* cytochrome bc₁ was purified and stored at −80 °C as described (28, 29). Light-activated kinetic spectroscopy was performed as described in Ref. 30 using a dual wavelength spectrophotometer. In all cases, the membranes contained 0.3 μM of reaction center as determined by a train of 10 light flashes separated by 50 ms at an *Eₐ* of 380 mV, and an extinction coefficient εₐ₉₅₄₅₄ of 29 mm⁻¹ cm⁻¹. Transient cytochromes c (at 550–540 nm) and b (at 560–570 nm) reduction kinetics exhibited by membranes resuspended in 50 mM MOPS buffer (pH 7.0), 100 mM KCl, and 100 mM EDTA at an ambient redox potential of 100 mV were monitored. Antimycin A, myxothiazol, and stigmatellin (Stg), inhibitors of cytochrome bc₁, were used at 5, 5, and 1 μM, respectively. EPR spectroscopy was performed using a Bruker ESP-300E (Bruker Biosciences), equipped with an Oxford Instruments ESR-9 helium cryostat (Oxford Instrumentation Inc.) as described in Ref. 31. As needed, the membranes (25–30 mg of protein/ml) were reduced by incubation on ice for 10 min with 20 mM ascorbate in the presence or absence of 100 μM Stg or 100 μM famoxadone. Spectra recording conditions were for the [2Fe-2S] cluster: modulation frequency, 100 kHz; microwave power, 2 milliwatts; sample temperature, 20 K; microwave frequency, 9.443 GHz; modulation amplitude, 12 G, and for the b hemes: modulation frequency, 100 kHz; sample temperature, 10 K; microwave frequency, 9.59 GHz; modulation amplitude, 10 G.

**Superoxide Measurements—**The rate of superoxide production was determined using manganese-SOD-sensitive and Stg-sensitive cytochrome c reduction assays (32). First, in the presence and absence of manganese-SOD (150 units/ml SOD from Sigma), (−SOD/−Stg) and (+SOD/−Stg) cytochrome c reduction rates were determined by using DBH₂:cytochrome c reductase assays (27, 29). Then (+SOD/+Stg) and (−SOD/+Stg) rates of cytochrome c reduction were determined using membranes inhibited by 10 μM Stg to obtain cytochrome bc₁-specific rates. The amounts of superoxide produced independently of cytochrome bc₁ were excluded by subtracting from (−SOD/+Stg) the (+SOD/+Stg) values. The absence of enhancement of ROS production by the addition of antimycin A in the presence of Stg provided an extra control for cytochrome bc₁-independent ROS generation. The ROS that is specifically produced by the cytochrome bc₁ was then determined by subtracting from [(−SOD-Stg) − (+SOD-Stg)] the [(−SOD+Stg) − (+SOD+Stg)] values. In this way, the total cytochrome c reduction rates observed, only the portion that is sensitive to Stg and to manganese-SOD was attributed to superoxide originating specifically from cytochrome bc₁ Qₐ site. Antimycin A-induced ROS productions were measured in similar ways except that 20 μM antimycin A was added.

The cumulative amounts of superoxide production were measured via H₂O₂ formation using the Amplex® Red-horse-radish peroxidase method (Molecular Probes, Inc.). As above, DBH₂:cytochrome c reduction assay mixtures with and without Stg were supplemented with 50 μM Amplex Red, 150 units/ml manganese-SOD and 0.1 units/ml of horseradish peroxidase and incubated at room temperature for 1 min. The amount of the fluorescent resorufin formed from Amplex Red at the end of the incubation period was measured using a PerkinElmer Life Sciences LS-5B luminescence spectrophotometer and 530- and
590-nm excitation and emission wavelengths, respectively. Fluorescence production was linear during the incubation period, and the amount of \( \text{H}_2\text{O}_2 \) production was calibrated using the standards provided in the Amplex Red kit. Of the total amounts of \( \text{H}_2\text{O}_2 \) produced, only the portions that were sensitive to Stg were considered as superoxide generated by cytochrome \( bc_1 \). Antimycin A-induced superoxide productions were measured in the same way except that 20 \( \mu \text{M} \) antimycin A was added to the assay mixture (29).

**Mass Spectrometry Analyses—**SDS-PAGE samples were excised and subjected to in-gel digestions, whereas purified cytochrome \( bc_1 \) samples were digested in-solution using trypsin and, as needed, GluC, as described (33–35). Peptides were analyzed with a Thermo LCQ Deca XP+ MS/MS spectrometer coupled to a LC Packings Ultimate Nano HPLC system controlled by Thermo Xcalibur 2.0 software. Thermo Bioworks 3.3 software was used to perform SEQUEST searches against the \( R. \text{capsulatus} \) protein database as described earlier (33–35). The results were filtered using standard values for \( X_{corr} \) (1.5, 2.0, and 2.5 for \( m/z \) of +1, +2, and +3, respectively) and \( \Delta C_N \geq 0.1 \), and relevant spectra were inspected manually for validity of the assignments.

**Chemicals—**All of the chemicals were as described earlier (29).

**RESULTS**

**Mutation of Cytochrome b Tyr302 to Cys Renders Cytochrome \( bc_1 \) Activity Sensitive to \( O_2 \)—**The structures of cytochrome \( bc_1 \) (36–38) reveal that the conserved Tyr302 residue of \( R. \text{capsulatus} \) cytochrome \( b \) (Tyr278 and Tyr279 in human or bovine and yeast numbers, respectively) is near the \( Q_\text{o} \) site, where \( \text{QH}_2 \) oxidation occurs (Fig. 1, \( b \) and \( c \)). This residue is part of the docking niche of the iron-sulfur protein subunit of the enzyme and is H-bonded to the backbone of a Cys residue of this subunit to position correctly its \( [2\text{Fe-2S}] \) cofactor at the \( Q_\text{o} \) site. The role of this Tyr residue on \( \text{QH}_2 \) oxidation has been analyzed with \( R. \text{capsulatus} \) and \( R. \text{eroides} \) and famoxadone (Table 1). Thus, like the homologous \( R. \text{sphaeroides} \) (17) or yeast (18, 19) mutants, all \( R. \text{capsulatus} \) \( Y302X \) mutants also assembled cytochrome \( bc_1 \), but the \( Y302A, Y302S, \) and \( Y302C \) substitutions had comparatively lower activities than the \( Y302V \) and \( Y302F \) substitutions using membranes prepared in air from aerobically grown cells (Fig. 2a). Remarkably, however, these activities changed when cells were grown by anoxicogenic photosynthesis, and the membranes were prepared in the absence of \( O_2 \). Wild type cells showed little decrease under \( -O_2 \) conditions with respect to cytochrome \( bc_1 \) activity (~70% of +\( O_2 \) activity, possibly because of total membrane protein content changes and because of anaerobic preparations). A similar pattern was also observed with \( Y302F \) and \( Y302V \) substitutions. In contrast, the \( Y302A, Y302S, \) and \( Y302C \) mutants displayed an opposite pattern with increased cytochrome \( bc_1 \) activity under \(-O_2 \) as compared with +\( O_2 \) conditions (Fig. 2a). In particular, the \( Y302C \) substitution showed a marked enhancement (~3–4-fold increase from +\( O_2 \) to ~\(-O_2 \) conditions), but as expected, it did not reach wild type activity levels because of its partially defective \( Q_\text{o} \) site (like all \( Y302X \) mutants). When corrected for the corresponding wild type activities observed under +\( O_2 \) and \(-O_2 \) conditions (15 and 56% of wild type, respectively), the \( Y302C \) enhancement appears even more (~3–4-fold) pronounced.

**Performance of light-activated, single-turnover cytochrome \( c \) and \( b \) reduction kinetics (30) established that the \( O_2 \)-sensitive catalytic step in the \( Y302C \) mutant was the bifurcated electron transfer event during \( \text{QH}_2 \) oxidation. Although rapid reduction kinetics of cytochromes \( c \) and \( b \) were seen with +\( O_2 \) membranes of aerobically grown wild type and \( Y302V \) mutant, they were undetectable with similar +\( O_2 \) \( Y302C \) samples (Fig. 3a, upper and lower traces, respec-
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tively; see also the schematic drawings underneath of them). However, rapid kinetics were readily seen with Y302C membranes prepared in the absence of O₂ using anaerobically grown cells, demonstrating that Qₒ site catalysis became O₂-sensitive in Y302C mutant.

Y302C Substitution Induces Oxidative Disruption of the [2Fe-2S] Cluster of Cytochrome bc₁—Because the cytochromes b and c contents of all Y302X mutants were similar (Table 1), EPR spectroscopy was used to examine their iron-sulfur protein [2Fe-2S] cluster (31). The mutants showed altered EPR spectra and gₓ and gᵧ values different from those (1.80 and 1.89, respectively) of the wild type, revealing that substitution of Tyr₃₀² perturbed the Qₒ site and the environment of the [2Fe-2S] cluster (Fig. 3b, left panel). When samples prepared under +O₂ and −O₂ conditions using appropriately grown cells were compared, drastic changes were seen with the Y302C mutant in respect to the iron-sulfur protein [2Fe-2S] cluster amounts, as reflected by the EPR gₓ signals (Fig. 3b middle and right panels). For the wild type, EPR spectra of the [2Fe-2S] cluster of −O₂ or +O₂ grown cells were similar to one another (and the observed amplitudes of the gₓ and gᵧ signals were in agreement with the respective cytochrome bc₁ activities shown in Fig. 2a). On the other hand, the amplitudes of the iron-sulfur protein [2Fe-2S] cluster gₓ and gᵧ signals of Y302C samples were very low under +O₂, but significantly higher under −O₂ conditions (Fig. 3b, middle and right bottom panels). Based on the gᵧ signal amplitudes, +O₂ and −O₂ grown Y302C cells contained ~14 and 53%, respectively, of the iron-sulfur protein [2Fe-2S] cluster as compared with the wild type enzyme (31). These percentage values roughly match the corresponding relative enzyme activities for Y302C (Fig. 2a, at 15 and 56% of wild type activities) obtained for +O₂ and −O₂ conditions, suggesting that a loss of cytochrome bc₁ activities correlates with a loss of the [2Fe-2S] cluster in Y302C mutant. This finding was unexpected, because the [2Fe-2S] cluster of wild type cytochrome bc₁ has a high redox midpoint potential, and its oxidative disruption does not occur readily upon exposure to air (31, 39).

Loss of Tyr₃₀² Enhances ROS Generation During Qₒ Site Catalysis—Native cytochrome bc₁ produces insignificant amounts of ROS except under conditions that interfere with bifurcated electron transfer at the Qₒ site (e.g. in the presence of inhibitors like antimycin A or high membrane potential, ΔΨₒ) (22, 26, 32, 40). The unusual oxygen sensitivity of the [2Fe-2S] cluster of the iron-sulfur protein in Y302C seen above (i.e. the decreased gₓ signal in Fig. 3b) led us to examine ROS production by the Y302X mutants. Manganese-SOD-sensitive cytochrome c reduction assays were used to monitor Stg-sensitive

![FIGURE 2. Cytochrome bc₁ activities of WT and Y302X mutants in the presence and absence of O₂ and biochemical properties of WT and Y302X mutant variants. a, cells were grown, and membranes were prepared, either in the presence (+O₂, filled bars) or absence (−O₂, open bars) of air. In each case, cytochrome bc₁ (Cyt bc₁) activities are expressed as percentages of the wild type activity determined under +O₂ conditions. Wild type activities were 3.5 and 2.7 μmol of cytochrome c reduced min⁻¹ mg⁻¹ of membrane protein under +O₂ and −O₂ conditions, respectively. Mean values ± standard deviations are shown. b, variations in the wild type cytochrome bc₁ activities under +O₂ and −O₂ conditions derive from the changing total membrane protein contents and different sample preparation methods and are not to be compared directly. b, dithionite-reduced (black) or ascorbate-reduced (gray) minus ferricyanide-oxidized optical difference spectra of aerobically prepared membrane fractions (0.4 mg of total proteins) from +O₂ grown WT and Y302X mutant strains. The 550- and 560-nm peaks correspond to the Xₒ and X_y signals of Y302C samples were very low under +O₂, but significantly higher under −O₂ conditions (Fig. 3b, middle and right bottom panels). Based on the gᵧ signal amplitudes, +O₂ and −O₂ grown Y302C cells contained ~14 and 53%, respectively, of the iron-sulfur protein [2Fe-2S] cluster as compared with the wild type enzyme (31). These percentage values roughly match the corresponding relative enzyme activities for Y302C (Fig. 2a, at 15 and 56% of wild type activities) obtained for +O₂ and −O₂ conditions, suggesting that a loss of cytochrome bc₁ activities correlates with a loss of the [2Fe-2S] cluster in Y302C mutant. This finding was unexpected, because the [2Fe-2S] cluster of wild type cytochrome bc₁ has a high redox midpoint potential, and its oxidative disruption does not occur readily upon exposure to air (31, 39).

| TABLE 1 | Properties of Y302X mutants under aerobic conditions |
|----------|-----------------------------------------------------|
| Strain   | Wild type | Y302A | Y302C | Y302F | Y302S | Y302V |
| Relevant phenotype | Ps⁺ | 12.3 ± 0.6 | 13.4 ± 0.9 | 14.3 ± 0.6 | 15.5 ± 1.8 | 12.7 ± 0.5 |
| Membrane protein content (mg/ml) | 13.4 ± 0.1 | 2.7 ± 0.2 | 2.6 ± 0.2 | 2.5 ± 0.2 | 2.4 ± 0.2 | 2.7 ± 0.2 |
| Membrane cytochrome c content (nmol/mg) | 2.4 ± 0.2 | 1.7 ± 0.1 | 1.7 ± 0.2 | 1.7 ± 0.2 | 1.6 ± 0.2 | 1.6 ± 0.2 |
| Membrane cytochrome content (nmol/mg) | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.7 ± 0.2 | 1.7 ± 0.2 | 1.6 ± 0.2 | 1.6 ± 0.2 |
| Relative cytochrome c reductase activity (%) | 100 | 15.4 ± 1.4 | 14.8 ± 2.9 | 77.4 ± 8.4 | 4.0 ± 1.3 | 41.6 ± 4.3 |
| No inhibitor | 0.4 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| Stigmatellin | 0.8 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 |
| antimycin A | 0.5 ± 0.1 | 0.8 ± 0.1 | 1.4 ± 1.0 | 3.7 ± 1.2 | 9.7 ± 1.9 | 7.7 ± 3.0 |

Ps⁺ refers to anaerobic photosynthetic (−O₂) growth ability.

Cytochromes b and c contents of membranes were determined using optical redox difference spectra.

Cytochrome bc₁ activity refers to the 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone (DBH₂):cytochrome c reductase activity expressed as a percentage of the wild-type activity, which was approximately 3.5 μmol of cytochrome c reduced min⁻¹ mg⁻¹ of membranes prepared in the presence of air using aerobically grown cells. Each assay was conducted at least in triplicate.

The data are the means ± standard deviations.
(i.e. cytochrome $bc_1$-specific) ROS generation. Under these conditions, native cytochrome $bc_1$ produced detectable amounts of ROS exclusively upon exposure to antimycin A (Fig. 4a, observed as a decrease of cytochrome $bc_1$ activity by $\sim$12% upon manganese-SOD addition). In contrast, all of the Y302X mutants produced large amounts of ROS (6−13% decrease of cytochrome $bc_1$ activity upon manganese-SOD addition) even in the absence of antimycin A (Fig. 4a). In all cases, this production was further enhanced (to $\sim$10−23%) by the addition of antimycin A.

In addition, cumulative ROS production by Y302X mutants was also tested using Amplex Red-based assays. The data demonstrated that Y302X mutants produced ROS in the absence of antimycin A at amounts similar to those seen with a native enzyme inhibited with antimycin A (Fig. 4b). Thus, loss of Tyr$^{302}$ led to electron leakage from the Qo site to O$_2$ in a way...
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**FIGURE 4.** Rate of superoxide production, and cumulative superoxide production, by native (WT) and Y302X variants of cytochrome bc₁, in the absence and presence of antimycin A. a, rate of superoxide production by native (WT) and Y302X = Ala, Cys, Phe, Ser, or Val variants of cytochrome bc₁, in the absence (filled bars) and presence (open bars) of antimycin A was monitored via their EPR signals, as described in “Experimental Procedures” and Fig. 1 legend. b, cumulative superoxide production was determined using membranes prepared in the presence of air from aerobically grown cells, incubated for 1 min under the DBH2:cytochrome bc₁ oxidase conditions, and accumulated amounts of ROS produced were measured by the Amplex red assay. In this assay, H₂O₂ formation in the presence of excess manganese-SOD was coupled to horseradish peroxidase-mediated Amplex red conversion to resorufin. Of the total amounts of H₂O₂ produced, only the portions that were sensitive to Stg are considered as superoxide generated by cytochrome bc₁.

independent of antimycin A effect. This finding indicated that Tyr³⁰² of cytochrome bc₁ has a protective role in suppressing ROS generation mediated by cytochrome bc₁ Q₈ site.

**Arresting Q₈ site Catalysis, or Alkylating Cys-302, Protects the [2Fe-2S] Cluster against Oxidative Damage—**Although all Y302X mutants produced ROS, oxidative disintegration of the [2Fe-2S] cluster was observed only with Y302C mutation (Fig. 3b). We examined −O₂-grown Y302C cells after treatment with Stg to inactivate cytochrome bc₁ Q₈ site, prior to O₂ exposure to elucidate how the [2Fe-2S] cluster was inactivated (Fig. 5a). Monitoring the Y302C [2Fe-2S] cluster as a function of exposure time to O₂, using the EPR g₁ signal, revealed that the [2Fe-2S] cluster of uninhibited Y302C decreased by 50% of its initial value in 50 h of exposure to O₂, whereas Stg-treated samples showed no effect. Thus, inhibiting Q₈ site catalysis and burying the iron-sulfur protein head domain into cytochrome b₁ surface prevented oxidative disruption of cytochrome bc₁ [2Fe-2S] cluster in Y302C mutant.

Because no cysteine residue is present in R. capsulatus cytochrome b (41), we suspected that the newly gained thiol group in Y302C mutant might be responsible for the oxidative disruption of the [2Fe-2S] cluster. We treated −O₂-grown Y302C cells with IAM to alkylate the thiolate anion at position 302 and monitored the [2Fe-2S] cluster disintegration by EPR g₁ signal as a function of exposure time to O₂. Upon exposure to air, untreated Y302C lost the [2Fe-2S] cluster g₁ signal and the cytochrome bc₁ activity during the initial 50 h, whereas IAM-treated membranes retained ~80% of both of these features (Fig. 5a, and b). EPR spectra of the same samples exhibited no significant variations with respect to hemes b₁ and b₄₁ amounts, which were used as internal controls via their EPR g₄ transitions (Fig. 5c). Upon extension of the exposure time to O₂ up to ~250 h, the g₁ signal amplitude of untreated Y302C decreased to 30%, but IAM-treated membranes retained ~70% of their initial levels (data not shown). We noted that the rate of cytochrome bc₁ activity loss induced by IAM was more rapid than that of the EPR g₁ transition, possibly because of inhibitory hindrances inflicted by alklylation of the Y302C residue at the Q₈ site. Similar to IAM, treatment with the bulkier alkylating reagent N-ethylmaleimide also protected the [2Fe-2S] cluster from oxidative damage, but it induced a more rapid loss of cytochrome bc₁ activity (data not shown). We concluded that introduction of a thiol group at position 302 of cytochrome b rendered bacterial cytochrome bc₁ O₂-sensitive via oxidative disruption of its iron-sulfur protein [2Fe-2S] cluster upon exposure to air.

**An Intersubunit Disulfide Bond Inactivates Cytochrome bc₁ and Destabilizes Its [2Fe-2S] Cluster**—To further investigate the basis of O₂ sensitivity described above, native, O₂-sensitive Y302C, and O₂-tolerant Y302V cytochrome bc₁ enzymes were purified and subjected to comparative tandem mass spectrometry analyses. Appropriate protease digestions allowed detection of the peptide Tyr₃⁰⁴/Val₃⁰⁶ that encompasses position 302 of cytochrome b (Fig. 6) as expected, this position corresponded to Tyr and Val in the native and Y302V enzymes, respectively, but no such peptide could be detected in Y302C samples, despite searches for ROS-induced cysteine modifications (e.g. sulfenic, sulfinic, or sulfonic acids) (42). However, a shorter peptide Tyr/Val₃⁰⁴ corresponding to a cleavage product immediately before position 302 was observed in all cases (Fig. 6). This suggested that this position might be modified in Y302C mutant enzyme.
Next, the possibility of an intersubunit disulfide bond formation between the iron-sulfur protein, which is located structurally close to position 302 of cytochrome bc1 (37, 38), was considered. Such an intersubunit cross-link was suspected to be incomplete based on the EPR data related to the destruction of the [2Fe-2S] cluster (Fig. 5a). Purified native and Y302C cytochrome bc1 samples were extracted with Triton X-114 for cytochrome bc1 enrichment and subjected to SDS-PAGE, mass spectrometry, and Western blot analyses. Unlike the Y302C samples under nonreducing conditions, a 70-kDa protein band that disappeared upon treatment with DTT was found (Fig. 7, left panel). Using both mass spectrometry (Fig. 7, right panel) and specific antibodies (not shown), the DTT-sensitive 70-kDa band was shown to contain both the 22-kDa iron-sulfur protein and 48-kDa cytochrome bc1 subunits.

Furthermore, extensive MS/MS analyses found additional modified cysteine containing peptide fragments of the iron-sulfur protein (supplemental Fig. S1). The Cys138 and Cys155 residues form an intramolecular disulfide bond that is near the position 302 of cytochrome bc1. In the light of these data, we tentatively propose that in the Y302C enzyme, upon exposure to O2, position 302 of cytochrome bc1 self-inactivates cytochrome bc1, i.e., Cys138 and Cys155, Cys155 is closer to O-Tyr302 is 5 or 11 Å (supplemental Fig. S2), Cys138 and Cys155 of the iron-sulfur protein are the ligands of the [2Fe-2S] cluster and of the intramolecular disulfide bond, respectively, in the case of yeast structure (Protein Data Bank code 3CX5), the corresponding distances are ~4 and 10 Å (supplemental Fig. S2). Cys138 and Cys155 of the iron-sulfur protein are the ligands of the [2Fe-2S] cluster [FeS] core and of the intramolecular disulfide bond forming cysteines (i.e., Cys138 and Cys155), Cys138 is closer to Tyr302 of cytochrome bc1. In the light of these data, we tentatively propose that in the Y302C enzyme, upon exposure to O2, possibly activated by ROS production via the Qo site, cytochrome bc1 Y302C attacks the Cys138–Cys155 disulfide bond of the iron-sulfur protein to yield an intersubunit disulfide bond between these subunits. The resulting intersubunit disulfide bond then self-inactivates cytochrome bc1, and loss of the intramolecular disulfide bond of the iron-sulfur protein compromises its [2Fe-2S] cluster stability to induce its oxidative disruption (Fig. 8).
We emphasize that this attractive proposal should remain hypothetical until the resolution of the structure of Y302C enzyme.

DISCUSSION

Respiratory energy conservation by cytochrome bc₁ depends on an elaborate bifurcated electron transfer reaction that occurs in the presence of O₂ (22, 43). Unless the catalytic steps associated with oxidation of reduced quinone are compromised, the native enzyme safely carries out this reaction without any ROS production. Various factors, like inhibitors that disable reduction of b hemes (32) or increased ΔW₁ (44) and changes in pO₂ (45), trigger ROS production by cytochrome bc₁. Serendipitously, studying cytochrome bc₁ of a facultative anoxygenic photosynthetic bacterium, R. capsulatus, we found that the Y302C mutant preserved its activity in the absence of O₂ but lost it progressively upon exposure to O₂ because of oxidative disintegration of its [2Fe-2S] cluster cofactor. This finding illustrates that specific amino acid(s) of cytochrome bc₁, like Tyr³⁰² of cytochrome b, might play key roles in preventing undesirable electron leakage to O₂ during Qₒ site catalysis. In the case of R. capsulatus, substituting Tyr³⁰² by other amino acids decreased cytochrome bc₁ activity and enhanced ROS production, but only the Tyr to Cys mutation self-inactivated cytochrome bc₁. In the latter case, we tentatively propose that formation of an intersubunit disulfide cross-link, probably via ROS-induced cysteine redox chemistry (42), destabilizes the iron-sulfur protein [2Fe-2S] cluster by eliminating its own stabilizing intramolecular disulfide bridge (Fig. 8, top panel). The role of Tyr³⁰² of cytochrome b in correctly positioning the iron-sulfur protein [2Fe-2S] cluster at the Qₒ site to confer optimal catalytic activity has been discussed in earlier studies for bacterial (16, 17) and yeast (18, 19) cytochrome bc₁. However, in these studies, oxygen sensitivity of the enzyme or oxidative disintegration of its iron-sulfur protein [2Fe-2S] cluster has not been described. Although no Y302C mutant was examined in R. sphaeroides (17), it was reported that the homologous yeast Y279C mutant produced superoxide (18), but oxidative disruption of its iron-sulfur protein [2Fe-2S] cluster was not described (18, 19). It is noteworthy that R. capsulatus native cytochrome b has no Cys residue, whereas the yeast homologue has several, and one of them (Cys³⁴²) is located near Tyr²⁷⁹ of cytochrome b (supplemental Fig. S2, bottom panel). The distance that separates Tyr²⁷⁹ and Cys³⁴² in R. sphaeroides (17) is large (~10 Å) in the yeast native cytochrome bc₁ structure. However, neither the corresponding distance in the Y279C mutant nor the occurrence of an intracytochrome b disulfide bond not affecting the [2Fe-2S] cluster stability is currently known.

Interestingly, the bovine and chicken native cytochrome b subunits also contain several Cys residues, but they lack a homologue of Cys³⁴² or any other Cys residue near its Tyr²⁷⁸ (Fig. 8, bottom panel). Thus, as in R. capsulatus, in bovine, chicken, and possibly human cytochrome bc₁, the iron-sulfur protein Cys residues remain as the likely candidates to yield an
intersubunit disulfide bond upon Cys substitution of Tyr278 in cytochrome bc1.

How a Tyr at a specific position of cytochrome bc1 interferes with ROS production is not obvious. We note that replacing Tyr with other amino acids (including Phe) eliminates a key hydroxyl group at position 302 of cytochrome b and enhances antimycin A-independent ROS production by the Qo site and also produce more ROS to endanger host cell viability. Indeed, permanent ROS production induces lethal intracellular disulfide bond formation upon Cys substitution of Tyr278 in cytochrome b.

ROS Production and Signaling by Cytochrome bc1

Besides the bacterial Y302C, homologous human Y278C and malarial Y268C cytochrome bc1 mutants also exhibit decreased Qo site activities (12, 13). However, whether they produce ROS remains unknown. Extrapolation of the information gained using the bacterial cytochrome bc1 to the evolutionarily conserved organelle homologues suggests that human mitochondrial cytochrome bc1 mutants might produce ROS, and cytochrome bc1 variants with Tyr to Cys mutations might self-inactivate via oxidative disruption of their [2Fe-2S] clusters. Although this inference awaits experimental validation, it also suggests that resistance to the antimalarial drug atovaquone by the parasite might be acquired at the expense of increased O2 sensitivity and concomitant decrease of energetic efficiency of the parasite. Recent findings indicate that malarial cytochrome bc1 might have different selective pressure than most other systems (46) to allow the survival of O2-compromised malarial atovaquone resistant parasites in microaerobic human host environment. Regarding the human cytochrome bc1, all Y278X but C substitutions would have decreased activity and kill host cells by continuous ROS production. Only the self-inactivating Y278C allele of cytochrome bc1 would cease ROS production via oxidative disruption of the [2Fe-2S] cluster and allow somewhat better host cell viability. However, even in this case, the hampered catalytic efficiency of the Y278C variant, combined with progressive loss of heteroplasmic state and differing energy threshold requirements of various tissues, would cause progressively increasing mitochondrial dysfunctions. These include exercise intolerance (15), ischemic cardiomyopathy (47), reperfusion injury (48), and multi-system disorder (12).
ROS Production and Signaling by Cytochrome bc₁

Clearly, reversible modification of a critical hydroxyl group of cytochrome bc₁ might be beneficial as a ROS signal initiator, but its permanent loss by mutation would steer mitochondria to a disastrous destiny via continuous oxidative damage and cell death.

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