

Time-resolved generation of a membrane potential by *ba*₃ cytochrome *c* oxidase from *Thermus thermophilus*

Evidence for reduction-induced opening of the binuclear center

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Abstract *ba*₃-type cytochrome *c* oxidase purified from the thermophilic bacterium *Thermus thermophilus* has been reconstituted in phospholipid vesicles and laser flash-induced generation of a membrane potential by the enzyme has been studied in a μ s/ms time scale with Ru(II)-tris-bipyridyl complex (RuBpy) as a photoreductant. Flash-induced single electron reduction of the aerobically oxidized *ba*₃ by RuBpy results in two phases of membrane potential generation by the enzyme with τ values of about 20 and 300 μ s at pH 8 and 23°C. Spectrophotometric experiments show that oxidized *ba*₃ reacts very poorly with hydrogen peroxide or any of the other exogenous heme iron ligands studied like cyanide, sulfide and azide. At the same time, photoreduction of the enzyme by RuBpy triggers the electrogenic reaction with H₂O₂ with a second order rate constant of $\sim 2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The data indicate that single electron reduction of *ba*₃ oxidase opens the binuclear center of the enzyme for exogenous ligands. The fractional contribution of the protonic electrogenic phases induced by peroxide in cytochrome *ba*₃ is much less than in bovine oxidase, pointing to a possibility of a different electrogenic mechanism of the *ba*₃ oxidase as compared to the oxidases of the *aa*₃-type.

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1. Introduction

The respiratory chain of a Gram-negative thermophilic bacterium, *Thermus thermophilus*, contains two terminal oxidases *caa*₃ and *ba*₃, both of which belong to the so-called heme/copper superfamily. The *ba*₃ oxidase is expressed during growth under low oxygen tension and catalyses oxidation of cytochrome *c*₅₅₂, a highly specific electron donor [1,2], by molecular oxygen and formation of water. The enzyme contains two hemes (low-spin heme B and high-spin heme A) and two copper centers (binuclear Cu_A and mononuclear Cu_B). Cytochrome *ba*₃ was first isolated by Zimmerman et al. [3]

as a single-polypeptide enzyme with all the four metal redox sites located in subunit I. However, later on, it was shown to contain a weakly staining 18 kDa subunit II carrying the Cu_A redox center [4].

Although the *T. thermophilus* *ba*₃-type oxidase belongs to the heme/copper superfamily, the gene sequence [4] indicates it to be one of the most distant members of the class, grouping with other exotic oxidases like cytochrome *ba*₃ from the halo-alkaliphilic archeon *Natronobacter pharaonis* [5] and quinol oxidase Sox-ABCD from *Sulpholobus acidocaldarius* [6]. Interestingly, the enzyme lacks most of the highly conserved amino acid residues that form the so-called input proton channels in other oxidases but nevertheless pumps protons, although with a relatively low efficiency of 0.4–0.5 H⁺/e[−] [7].

The binuclear center of *ba*₃ reveals a number of odd features [3,8], including an unusual pattern of reactivity towards exogenous heme ligands. For instance, in cytochrome *ba*₃, cyanide binds with the ferrous but not with the ferric heme *a*₃ [8], while ligation to the ferric state is much stronger in other oxidases. Also CO affinity for Cu_B in the reduced *ba*₃ may be 50–100 times higher than in the bovine oxidase [2,8].

Crystal structures of the *aa*₃-type cytochrome *c* oxidase (COX) from *Paracoccus denitrificans* [9,10] and bovine heart [11,12] are known and a three dimensional model of *T. thermophilus* *ba*₃ oxidase is about to be published. Analysis of functional deviations of *ba*₃ oxidases from ‘canonical’ behavior and relating it to the structural differences between the *aa*₃ and *ba*₃ oxidases may allow us to understand which elements of the enzyme molecule are strictly indispensable for energy-coupled dioxygen activation in terminal oxidases.

Time-resolved measurements of intraprotein charge translocation provide an insight into the core of the oxidase electrogenic mechanism. Rapid kinetics studies of $\Delta\psi$ generation by *aa*₃-type COX from mitochondria [13–17] and bacteria [18] have resolved a number of individual electrogenic steps within the catalytic cycle of the enzyme. Here, we report initial results of our investigation into the electrogenic mechanism of *ba*₃ oxidase. The enzyme differs very significantly from the *aa*₃ COXs.

2. Materials and methods

2.1. Chemicals

Trisbipyridyl Ru(II) chloride hexahydrate (RuBpy) was from Aldrich. 30% Hydrogen peroxide (‘Suprapur’ grade) was from Merck. Other chemicals used for preparation of COX and experiments were as described earlier [2,15].

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Abbreviations: COX, cytochrome *c* oxidase; P, F and O, peroxy, ferryl-oxo and oxidized states of cytochrome *c* oxidase; RuBpy, Ru(II)(2,2′-bipyridyl)₃Cl₂

2.2. Preparations

ba_3 -type COX was isolated in Aachen from *T. thermophilus* HB8 cells as described in [2] and reconstituted into phospholipid vesicles made from asolectin (phosphatidyl choline type IIS from Sigma) by a standard cholate dialysis method used earlier with bovine COX [19]. caa_3 COX from *T. thermophilus* was purified as described [20].

2.3. Measurements

Absorption spectra have been recorded in an Aminco-SLM 2000 dual wavelength/split beam spectrophotometer in semi-micro cells with a 1 cm optical pathway. Time-resolved photo-induced generation of the membrane potential by liposome-reconstituted ba_3 COX was measured electrometrically as earlier with bovine COX [13] using the RuBpy/aniline photoreduction system described by Nilsson [21]. RuBpy was photo-excited by short pulses of light from a Nd-YAG laser (Quantel 471) operating in a double frequency mode ($\lambda = 532$ nm, pulse half-width 15 ns, flash energy ~ 50 mJ/flash). Photoelectric traces were resolved into individual exponents using a software package GIM developed by Alexander L. Drachev (subroutine 'Discrete' based on a Fourier-transform method of analyzing multi-exponential decays by Provencher [22]). All experiments were carried out at room temperature.

3. Results

Hydrogen peroxide reacts rapidly with aa_3 -type COXs, bringing about formation of the peroxy and/or ferryl-oxo states of COX (compounds P and/or F), depending on the exact conditions [23–25]. At H_2O_2 concentrations of 1 mM or higher, a steady-state is achieved in less than 1 min after mixing.

Quite a different reaction pattern is observed with the ba_3 -type oxidase. The spectral changes in the first 5–10 min after hydrogen peroxide addition are very small and the response grows slowly on a time scale of hours. The H_2O_2 -induced difference spectrum reveals a maximum at 437 nm and a minimum at 413 nm in the Soret band and a broad maximum around 580 nm with a trough at 660 nm in the visible band. Such a line-shape resembles peroxide reaction with heme a_3 in other oxidases and may indicate formation of the ferryl-oxo state (compound F). However, the size of the difference spectrum attained in the Soret band even after 3 h is still only about $15 \text{ mM}^{-1} \text{ cm}^{-1}$ (peak to trough), i.e. less than 1/3 of the responses typical of aa_3 oxidases from bovine heart or *Rhodobacter sphaeroides* (e.g. [26]).

It is to be noted that the H_2O_2 -induced spectral changes of ba_3 oxidase are promoted considerably by weak reductants (Azarkina, unpublished) and in particular by aniline included

in the reaction buffer in Fig. 1A to match conditions of the photoelectric experiments described below. In the absence of aniline, the hydrogen peroxide-induced absorption changes of ba_3 -COX were even slower than in Fig. 1.

In accordance with the sluggish reaction of ba_3 -COX with hydrogen peroxide, the aerobically oxidized enzyme reacts poorly with other binuclear center ligands tested (Fig. 1B, spectra a–c) as compared to an aa_3 -type oxidase from the same bacterium (spectra d and e) or bovine COX (not shown).

Fig. 2 shows generation of $\Delta\psi$ by liposome-reconstituted cytochrome ba_3 . In the absence of additions, flash-induced photoreduction of the cytochrome by RuBpy is followed by rapid build up of a membrane potential (negative inside the

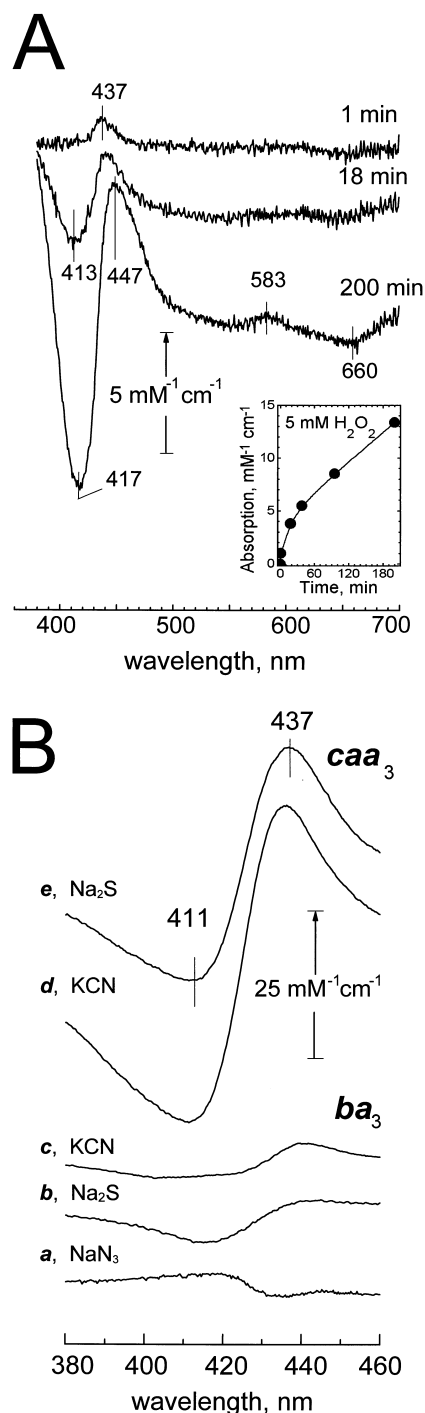


Fig. 1. Interaction of the oxidized cytochrome ba_3 with hydrogen peroxide and other ligands. (A) Reaction with H_2O_2 . Spectrophotometric cells contained $2 \mu\text{M}$ cytochrome ba_3 in 5 mM Tris-acetate buffer pH 8 with 10 mM aniline (the medium matches the conditions of the photoelectric measurements). The absolute spectrum of the aerobically oxidized sample was recorded and used as a baseline subtracted from subsequent spectra. 5 mM H_2O_2 has been added and the spectra recorded at the indicated time intervals (only three spectra of the series are shown). The inset gives the kinetics of the absorption changes development (the peak to trough amplitude of the difference spectra in the Soret band is plotted versus time). (B) Reaction with azide, cyanide and sulfide. $1.2 \mu\text{M}$ COX in 50 mM Tris-HCl, pH 7.8, with 0.2 mM EDTA and 0.0045% dodecyl maltoside. (a–c) ba_3 -type oxidase from *T. thermophilus*, (e and d) absorption changes induced by cyanide and sulfide in case of an aa_3 -type oxidase (caa_3 -COX from *T. thermophilus*) are shown for comparison. The difference spectra have been recorded at room temperature in 5 min after addition of the ligands at final concentrations of: Na_2S , 2 mM; KCN, 5 mM; NaN_3 , 5 mM.

vesicles) which consists mainly (85%) of two exponential phases with τ values of about 20 and 300 μ s and an amplitude ratio of about 3:2, respectively. The exact τ values of the 300 μ s phase varied slightly in different experiments and, for a particular trace, depended somewhat on the details of a fitting procedure with the maximal scatter in the range 250–420 μ s. There is also a minor slow phase of $\Delta\psi$ generation that is difficult to interpret unambiguously at the moment. H_2O_2 brings about an additional electrogenic phase that roughly equals the peroxide-independent part of the response (traces b and c). This phase is fully reversed by catalase (data not shown). Note that the traces have been recorded in 2–3 min after peroxide addition, i.e. at the time at which no reaction of peroxide with the enzyme is revealed spectrophotometrically (Fig. 1A). The inset in Fig. 2 compares an electrogenic response of ba_3 -COX (at 40 mM H_2O_2) with a typical response of bovine COX coupled to a $\text{F} \rightarrow \text{O}$ transition.

As shown in Fig. 3, the rate of the H_2O_2 -induced phase is proportional to the concentration of peroxide in the range studied (0.5–80 mM) with a second order rate constant of $\sim 2000 \text{ M}^{-1} \text{ s}^{-1}$. In contrast, the amplitude of the peroxide-dependent increment of $\Delta\psi$ as resolved by curve fitting depends only slightly on $[\text{H}_2\text{O}_2]$ (e.g. 32% at 4 mM peroxide, 46% at 40 mM peroxide, see legend to Fig. 2). Even this modest growth can be apparent since at concentrations of hydrogen peroxide below ca. 5 mM, the peroxide-induced

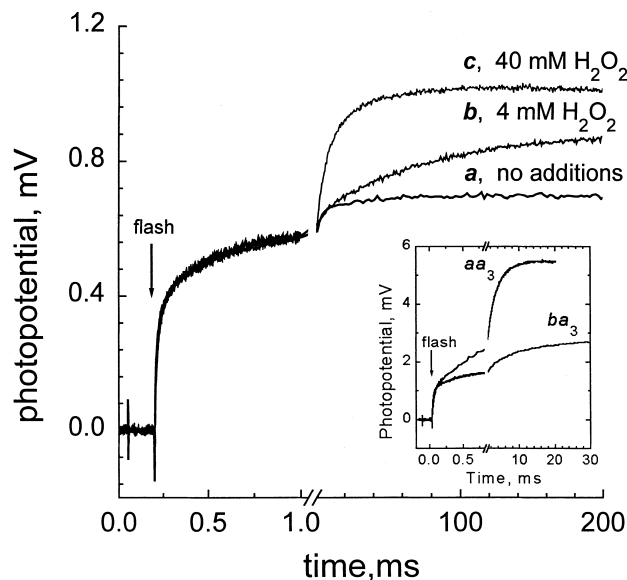


Fig. 2. Flash-induced membrane potential generation by cytochrome ba_3 . Reaction buffer contains 5 mM Tris-acetate buffer pH 8, 40 μ M RuBpy as photo-activated reductant and 10 mM aniline to reduce photo-oxidized RuBpy. The photoelectric traces have been recorded in the same sample in the absence of other additions (a) and 1–2 min after addition of H_2O_2 at the indicated final concentrations (b and c). The photoelectric curves have been resolved into exponential phases with the following parameters. Trace a (no H_2O_2): $\tau_1 = 21 \mu\text{s}$, $\alpha_1 = 50\%$, $\tau_2 = 0.25 \text{ ms}$, $\alpha_2 = 34\%$, $\tau_3 = 10 \text{ ms}$, $\alpha_3 = 16\%$. Trace b (4 mM H_2O_2): $\tau_1 = 25 \mu\text{s}$, $\alpha_1 = 39\%$, $\tau_2 = 0.42 \text{ ms}$, $\alpha_2 = 28\%$, $\tau_3 = 57 \text{ ms}$, $\alpha_3 = 32\%$. Trace c (40 mM H_2O_2): $\tau_1 = 20 \mu\text{s}$, $\alpha_1 = 31\%$, $\tau_2 = 0.42 \text{ ms}$, $\alpha_2 = 23\%$, $\tau_3 = 11.6 \text{ ms}$, $\alpha_3 = 46\%$. The inset compares the photoelectric responses of *T. thermophilus* ba_3 (curve c from the main panel at 40 mM H_2O_2) and bovine aa_3 oxidase (typical $\text{F} \rightarrow \text{O}$ transition at 4 mM H_2O_2 and pH 8, e.g. [13]). The traces have been normalized arbitrarily by the magnitude of the response at 100 μs .

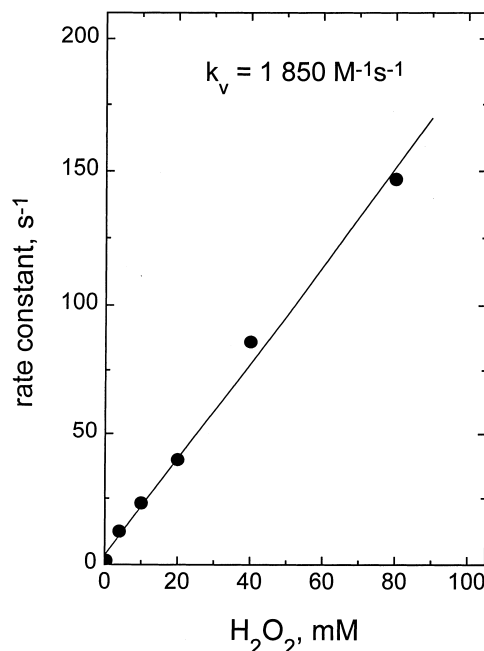


Fig. 3. Second order character of the slow electrogenic phase induced by H_2O_2 . Conditions as in Fig. 2. The photoelectric response was recorded at increasing concentrations of H_2O_2 . The traces were deconvoluted into individual exponents and the rate constant of the peroxide-induced slow phase is plotted versus the peroxide concentration. The points are fitted by a straight line with a slope of $1.85 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$.

phase is so slow that it does not reach its true maximum because of the passive discharge of the membrane potential.

4. Discussion

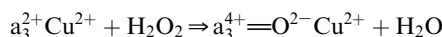
4.1. Reduction-triggered bimolecular reaction with hydrogen peroxide

Electrogenic responses of ba_3 -COX induced by single electron photoreduction by RuBpy differ significantly from those observed earlier for the aa_3 oxidases. The differences are most obvious when the peroxide-dependent parts of the photoelectric responses are concerned. In COX from bovine heart or *R. sphaeroides*, addition of peroxide converts the enzyme to the F state and brings about a large additional electrogenic phase associated with proton translocation coupled to the $\text{F} \rightarrow \text{O}$ transition of heme a_3 (Fig. 2, inset; [13,15,18]). At a H_2O_2 concentration high enough for full conversion of the enzyme to the F state ($> 1 \text{ mM}$ at pH 8), neither the rate nor the amplitude of the peroxide-induced electrogenic part of the response depend on the peroxide concentration.

The effect of hydrogen peroxide on the photoelectric response of ba_3 -COX is strikingly different. First, H_2O_2 induces the additional electrogenic phase under conditions at which it shows virtually no reaction with ferric ba_3 in the spectrophotometric experiments. Second, the rate of the peroxide-induced electrogenic phase is proportional to the H_2O_2 concentration indicating a bimolecular reaction. We propose that in the photoelectric experiments, the added hydrogen peroxide does not react with the oxidized enzyme prior to the flash, but its interaction with the binuclear center is triggered by flash-induced single electron reduction of COX.

The H_2O_2 -induced oxidation of the singly reduced ba_3 is

likely to involve two electron reduction of peroxide to water, concomitant with generation of the ferryl-oxo state of heme a_3 :



Such a reaction will be analogous to H_2O_2 reaction with ferrous mono-heme peroxidases resulting in the generation of compound II.

H_2O_2 reaction with photo-reduced ba_3 as measured here ($k_v \sim 2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) is 2–4-fold faster than binding of peroxide with the ferric high-spin heme in other oxidases such as bovine COX [24] or *Escherichia coli* quinol oxidases of the *bo*-[27,28] and *bd*-type [29] ($k_v \sim 500\text{--}1000 \text{ M}^{-1} \text{ s}^{-1}$). On the other hand, it is about 10-fold slower than peroxide interaction with the ferrous heme a_3 in the mixed-valence of ferrous bovine COX ($3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) [30]. In principle, the apparent second order rate constant observed in our experiments could be consistent with the value of Gorren et al. [30] if we assume that (i) H_2O_2 reacts rapidly ($k_v = 3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) with the fraction of singly photo-reduced ba_3 oxidase in which the electron photo-injected by RuBpy locates at heme a_3 iron, (ii) electron equilibration among the four redox centers is much faster than hydrogen peroxide binding, (iii) the equilibrium fraction of the enzyme with heme a_3 reduced is about 10%.

In conclusion, the data obtained allow us to suggest that the binuclear center in the oxidized ba_3 -COX does not react with H_2O_2 but opens for interaction upon single electron reduction of the enzyme. In this connection, ba_3 oxidase may resemble di-heme cytochrome *c* peroxidase from *P. denitrificans* (see [31] and references therein) and *cd*₁ nitrate reductase [32].

4.2. Attribution of the electrogenic phases

The photoelectric measurements resolve three electrogenic phases induced by photoreduction of ba_3 oxidase in the presence of H_2O_2 . The rapid (20 μs) component in cytochrome ba_3 photoelectric traces is faster than the 40–50 μs electrogenic phase observed with the bovine COX [13,14], where it can be assigned unambiguously to reduction of low-spin heme *a* by Cu_A , but it fits nicely to the 15–20 μs electric phase associated with the $\text{Cu}_A \rightarrow$ heme *a* step in bacterial aa_3 -type COX [18]. Therefore, the 20 μs electrogenic phase in ba_3 -COX can be assigned provisionally to reduction of the low-spin heme *b* by Cu_A .

The 300 μs intermediate phase is likely to be associated with re-oxidation of heme *b* by the binuclear center. According to our preliminary data, this phase fully disappears upon prolonged pre-incubation of the enzyme with cyanide, binding of which with heme a_3 (ferrous) prevents electron transfer to the binuclear center. Also, time-resolved spectrophotometric measurements of heme *b* oxido-reduction in ba_3 -COX are consistent with this interpretation (results to be reported elsewhere). Electrogenicity of this phase could report vectorial intraprotein movement of protons coupled to reduction of the binuclear center.

As to the peroxide-induced slow electrogenic phase, two simple interpretations may be considered. First, oxidation of heme a_3^{2+} by H_2O_2 can by itself be linked to vectorial translocation of protons. As suggested in [18,33], heterolytic cleavage of hydrogen peroxide following its binding (or formation)

in the binuclear center of COX can be coupled to electrogenic uptake of two protons required for water formation and preceding generation of the compound I-type ‘peroxy’ intermediate P_{607} . Second, it is possible that oxidation of a_3 by peroxide is not linked per se to vectorial proton movement, but this irreversible reaction drives complete intraprotein electron transfer from the photo-reduced heme *b* to heme a_3 , whereas this transfer is only partial in the absence of peroxide (cf. discussion of the second order rate constant value for ba_3 interaction with H_2O_2 in Section 4.1). Since the $b \rightarrow a_3$ redox reaction is likely to be electrogenic (the 300 μs electrogenic phase, see above), reaction with peroxide may reveal the full amplitude of this phase (with the apparent rate constant limited by peroxide interaction with the enzyme).

4.3. Magnitude of the peroxide-dependent electrogenic phase: relevance to proton pumping

The ba_3 -COX from *T. thermophilus* lacks most of the residues believed to play the key role in the input ‘proton channels’ in other heme/copper oxidases. In particular, the D-channel associated with the proton pumping peroxidase phase of the COX catalytic cycle [18,33] appears to be fully missing. Although the ba_3 -COX generates $\Delta\psi$ under steady-state conditions and even pumps protons, the reported H^+/e^- stoichiometry is low (ca. 0.5) [7].

In the aa_3 -COX, the net contribution of the several electrogenic phases induced by addition of H_2O_2 and associated with proton translocation is about 4-fold that of the cyanide-insensitive ms phase [13,15], whereas in the ba_3 oxidase, the peroxide-induced electrogenic phase is only 1.5–2-fold larger than the 20 μs phase (cf. inset in Fig. 2). On a phenomenological level, this finding complies with the low pumping efficiency of ba_3 oxidase [7] and may indicate a different mechanism of proton translocation in cytochrome ba_3 with a lower H^+/e^- stoichiometry as compared to other COXs. However, the ba_3 oxidase from *T. thermophilus* has been studied to a rather limited extent so far and we must be cautious with the conclusions. In particular, the electrogenic function of the enzyme has been investigated here and in [34] at conventional room temperature, while the activity optimum of ba_3 -COX is above 60°C [2]. A possibility of functional inhomogeneity of cytochrome ba_3 at room temperature has to be kept in mind. For instance, it is not possible to exclude that only part of the enzyme photo-reduced by RuBpy reacts with exogenous peroxide in the photoelectric experiments. Conceivably, this could explain the low contribution of the peroxide-induced electrogenic phase. Experiments are in progress to check this possibility.

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