Elementary steps of proton translocation in the catalytic cycle of cytochrome oxidase

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Abstract

Proton translocation in the catalytic cycle of cytochrome c oxidase (CcO) proceeds sequentially in a four-stroke manner. Every electron donated by cytochrome c drives the enzyme from one of four relatively stable intermediates to another, and each of these transitions is coupled to proton translocation across the membrane, and to uptake of another proton for production of water in the catalytic site. Using cytochrome c oxidase from Paracoccus denitrificans we have studied the kinetics of electron transfer and electric potential generation during several such transitions, two of which are reported here. The extent of electric potential generation during initial electron equilibration between CuA and heme α confirms that this reaction is not kinetically linked to vectorial proton transfer, whereas oxidation of heme α is kinetically coupled to the main proton translocation events during functioning of the proton pump. We find that the rates and amplitudes in multiphase heme a oxidation are different in the O→E and P→F steps of the catalytic cycle, and that this is reflected in the kinetics of electric potential generation. We discuss this difference in terms of different driving forces and relate our results, and data from the literature, to proposed mechanisms of proton pumping in cytochrome c oxidase.

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1. Introduction

Cytochrome c oxidase is a biological energy transducer, which catalyzes the reduction of molecular oxygen to water, consuming four electrons and four protons in the process. The free energy released in this chemical reaction is used to create and maintain an electrochemical proton gradient across the inner mitochondrial or bacterial cell membrane, which may subsequently be used to power the synthesis of ATP—the main energy source for cell functions.

The one-electron carrier cytochrome c serves as a donor for the enzyme and delivers electrons, via the metal centres CuA, and heme α to the binuclear heme a3/CuB catalytic site, which cycles through intermediate states (Fig. 1) [1,2]. The “relaxed” oxidized catalytic site (state Ω) accepts one electron and turns into the E state. This transformation is coupled to proton uptake into the binuclear site from the negative (N) side of the membrane through the K-pathway, but this is not accompanied by proton pumping [3,4]. The entrance of the next electron into the binuclear site is favoured by binding of an oxygen molecule, which transforms the site into the Pɪ intermediate as a result of four-electron reduction of bound O2. In addition to two electrons delivered to the binuclear site from outside, two more electrons are borrowed from the binuclear center itself —
Fig. 1. Catalytic cycle of cytochrome c oxidase. The active states are shown in orange. The transitions between each of them are coupled to proton pumping. The relaxed states are shown in blue. Their reduction is not coupled to proton pumping.

one from heme a2-iron, and another from Tyr280. The transition of the relaxed E state to P_M is also coupled to proton uptake from the N-side of the membrane, but like the previous step, it is not linked to proton pumping [4,5]. This step of the catalytic cycle transfers the enzyme into the active state, which means that each subsequent reaction step of the cycle (P_M→F, F→O_H, O_H→E_H and E_H→P_M; Fig. 1) consists of transfer of one electron to the binuclear site, accompanied by net uptake of a “substrate proton” and coupled to pumping of another proton across the membrane [5]. The cycle described above is drawn as a sequence of orange states in Fig. 1 and applies for continuous turnover.

The two sequential pumping steps of the reaction cycle after binding of O_2 have been extensively investigated using a convenient experimental model (the flow-flash method), where the reaction with O_2 is initiated by photolysis of carbon monoxide bound to the fully prereduced enzyme [6,7]. With the bovine enzyme, such experiments [8] showed that during the P_R to F transition there is proton translocation across the membrane with a time constant of ca. 100 μs. The same is true for the next step (the F to O_H transition), but now the proton translocation processes are more than 10 times slower. The rates of proton translocation in these two transitions differ also by their sensitivity to pH [9–11] and solvent isotope replacement [12,13]. While the P_R to F transition does not depend on pH (up to pH 9), and has a very weak kinetic H/D isotope effect, the F→O_H transition exhibits significant pH-dependence and a very strong kinetic isotope effect. Such a large difference raises a very important question: is the mechanism of proton translocation different, or is the difference somehow connected to the different chemistry at the binuclear site? On the other hand, results from single-electron photoinjection experiments [14] argued against such a large difference between the rates of these two transitions. By contrast to the flow-flash results, these workers found that the rates of membrane potential generation during the P_M→F transition were only slightly faster than during F→O_H, and that the former rates were considerably slower than the rates during P_R→F in flow-flash experiments. Perhaps the most obvious explanation is that in the flow-flash experiments the reaction is started from an unusual (fully reduced) state of the enzyme [15,16]. In order to test whether these kinetic differences are a result of the experimental model used, or due to the inherent chemistry in the different parts of the catalytic cycle, we have used the electron injection technique successfully employed in the past [14,17–19]. The main emphasis in our experiments was on spectroscopic assignment of electron transfer steps, and comparison of these steps to the electrogenic events measured electrophoretically.

2. Experimental approach

To deliver one electron to the enzyme we employ the photochemical electron injection technique pioneered for CcO studies by Nilsson in 1992 [17], and first applied in conjunction with electrometry by the Konstantinov group [18]. This method is based on photoexcitation of tri[2,2′-bipyridyl]ruthenium [II] (RubiPy), which binds to CcO at low ionic strength. As a result of photoexcitation, each laser flash delivers an electron from RubiPy to Cu_A in a time faster than 0.5 μs (the life-time of the RubiPy excited state). In our experiments the quantum yield of such electron injection into P. denitrificans aa3 oxidase varies between 10 and 20%. To reach our aim, we prepared enzyme in two different states (see below) and followed the electron transfer and electric potential generation kinetics by time-resolved techniques described earlier [20–23].

2.1. Preparation of intermediate states

2.1.1. O_H state

For optical measurements anaerobic fully-reduced CcO was mixed in a stopped-flow module (SFM-300) with oxygen-saturated buffer (2 mM Tris (pH 8), 0.05% DM, 20 mM aniline and 400 μM RubiPy) resulting in enzyme oxidation and formation of O_H. Immediately after the mixing (Δt=5 ms) a laser flash induced electron injection from RubiPy to CcO. For electrometry, the O_H state was obtained by oxidation of the fully reduced CO-bound CcO. The electron injection was in this case separated by 100 ms intervals (see [5]).

2.1.2. P_M state

A solution of activated 40 μM CcO (3 mM Bis–Tris propane (pH 9), 0.05% DM, 20 mM aniline) was incubated in anaerobic 100% CO atmosphere resulting in formation of mixed-valence (two-electron reduced) enzyme (COMV). COMV enzyme was equilibrated with oxygen producing the P_M state as was verified by its optical absorption spectrum. At this step 200 μM RubiPy was introduced into the sample to make it ready for electron injection. The P_M state for electrometric examination was made differently to maximize the yield. Attached to the measuring membrane CcO-containing vesicles [8] were incubated in 3 mM Bis–Tris propane (pH 9), 50 μg/ml catalase, 0.3 μM hexamaminoruthenium, 50 mM glucose, 20 mM aniline, and 100% argon atmosphere. Then, to ensure complete anaerobiosis and to create reductive conditions for the enzyme, 3.5 mg/ml glucose oxidase was added to the sample and atmosphere replaced for 100% CO. The CcO was fully reduced, and then re-oxidized to the COMV state by addition of 7 mM ferricyanide, the redox potential was measured by a platinum redox-electrode. At a redox potential +400 mV the P_M state was formed by injection of 100 μl of oxygen-saturated water with 20 mM aniline and 1.7 mM RubiPy, directly towards the measuring membrane, and the first flash of light induced CO dissociation from the enzyme with formation of P_M. Subsequently a series of laser flashes with 100 ms intervals was given for electron injection. It should be noted that in contrast to the corresponding procedure applied for fully reduced enzyme [5], the first laser flash to the COMV enzyme will not only fully dissociate CO to form the P_M state in the reaction with O_H, but will also cause ca. 15–20% electron injection via photoexcitation of RubiPy that will generate the corresponding proportion of a “contaminating” F state. Therefore, a small proportion of the F→O_H transition will occur together with the main P_M→F step.
2.2. The $\text{O}_H \rightarrow \text{E}_H$ reaction

Photochemical electron injection into the enzyme at any state results, first, in very fast reduction of CuA, followed by concomitant reduction of heme $a$ and oxidation of CuA. We observe a time constant ($\tau$) of $\sim 10$ $\mu$s for the latter reaction in CcO from $P. denitrificans$ (Fig. 2A). The extent of this electron transfer in the $\text{O}_H \rightarrow \text{E}_H$ transition is about $60$–$70\%$, which yields an equilibrium constant in this step of $ca. 2$ between heme $a$ and CuA. A corresponding small $10$ $\mu$s phase (only about $10\%$ of the full amplitude; contrast below) is also seen as the first event of membrane potential generation. This first phase has been ascribed to electrogenic electron transfer from CuA to heme $a$ ($5,18,24,31$). However, in the context of the present paper it is important to note that the CuA to heme $a$ electron transfer is not complete in this reaction, as was often assumed in the past, because this critically affects calibration of the amplitude of the corresponding phase of membrane potential formation (see below).

Reduction of heme $a$ is followed by two phases of oxidation ($\tau \sim 125$ and $\sim 900$ $\mu$s), which is complete at the end of the reaction. These rates of electron transfer fit well with the kinetics of membrane potential formation ($\tau \sim 10, 110$, and $770$ $\mu$s; Fig. 2B; contrast [24]), with amplitudes of $10, 28$ and $46\%$ of total, but with a remaining phase of $16\%$ amplitude with $\tau \sim 5.3$ ms that does not have a counterpart in the electron transfer kinetics.

From these results it is clear that two main steps of vectorial proton transfer are coupled to two phases of heme $a$ oxidation by the binuclear site. Below, we will discuss the implications of these data on the mechanism of proton translocation by CcO.

2.3. The $\text{P}_M \rightarrow \text{F}$ reaction

The P intermediate may occur in two different forms, $\text{P}_M$ or $\text{P}_R$ [25]. $\text{P}_M$ is a transient state, observed during the reaction of fully reduced enzyme with oxygen [26]. The lifetime of this state is $\sim 50$ $\mu$s for the $P. denitrificans$ enzyme, during this time it relaxes to the F state [27]. In comparison to the stable $\text{P}_R$ state, $\text{P}_M$ is characterised by the presence of an extra electron in the binuclear site. The $\text{P}_M$ state can be created by several ways: in a reaction of the two-electron reduced enzyme with oxygen, from the oxidized enzyme by a low concentration of hydrogen peroxide [28], or by steady-state formation of $\text{P}_M$ by incubation of oxidized enzyme with a mixture of oxygen and carbon monoxide.

Photochemical electron injection into $\text{P}_M$ also results in very fast ($\tau \sim 10$ $\mu$s) reduction of heme $a$ by CuA as for electron injection into $\text{O}_H$ (Fig. 2A). The extent of this electron transfer is $60$–$70\%$, which shows that the functional redox potential of heme $a$ in this state is again close to the $E_m$ of CuA. However, as seen from Fig. 2A, and differently from the $\text{O}_H \rightarrow \text{E}_H$ transition, most of heme $a$ oxidation now occurs almost monophase with $\tau \sim 90$ $\mu$s. In fact, the low-amplitude $900$ $\mu$s phase can be ascribed to “contamination” from a small fraction of the $\text{F} \rightarrow \text{O}_H$ transition (see Experimental approach). The main proton translocation events, as judged by the kinetics of electric potential generation, follow the heme $a$ oxidation kinetics, also showing a faster rate than in the $\text{O}_H \rightarrow \text{E}_H$ case. Thus, the increased rate of heme $a$ oxidation during $\text{P}_M \rightarrow \text{F}$ is accompanied by an increased rate of proton transfer.

Electrogenic proton transfer associated with photoreduction of $\text{P}_M$ to $\text{F}$ as measured in this work is only about twice slower than the charge translocation phase of $\text{F}$ formation during oxidation of the fully reduced $P. denitrificans$ CcO by oxygen [27]. However, formation of the $\text{F}$ state from the fully reduced enzyme is governed by electron transfer from $100\%$ reduced heme $a$ to the binuclear site, whilst in the photoreduction experiment the electron for $\text{F}$ formation is initially distributed between CuA and heme $a$. Such a smaller occupancy of reduced heme $a$ in the electron injection experiment can explain the slower rate. Therefore, the rate constants of $\text{F}$ formation are essentially the same in the two experimental approaches.

Whilst our finding for electron injection into the $\text{P}_M$ state is thus consistent with the flow-flash data, it differs considerably from that of the Moscow group [14], who reported time constants of $0.3, 1$–$1.5$ and $6$–$7.5$ ms for the protonic phases of membrane potential formation in the bovine enzyme. This difference might be due to the source of enzyme, but that does not explain why the flow-flash [8] and electron injection data [14] yield so different results for the bovine enzyme.

2.4. Amplitudes of membrane potential formation

The electron injection technique, combined with time-resolved electrometric monitoring of charge translocation across the membrane with incorporated CcO, was introduced by the Moscow group in 1993 [18], and has turned out to be a powerful means of tracking translocation of electrical charge equivalents in real time during functioning of the proton-pumping CcO (cf. [4,5,19,23,24,27]). One of the major potential assets of this technique is the kinetically distinct early electron transfer from CuA to heme $a$, which has been used to calibrate the electrometric response of the subsequent protonic reaction phases. According to the crystal structure [29,30] this electron transfer takes place across $1/3$ of the membrane dielectric. It does not seem to be kinetically associated with any vectorial proton transfer, because its rate is unaffected by deuteration of water [24,31], or by pH [32]. As summarised by Siletzky et al. [14,24], the amplitude of this phase has been reported to be $20\%$ (bovine enzyme) or even $30\%$ ($R. sphaeroides$ and $P. denitrificans$ enzymes [31]) of the total electrometric amplitude during the $\text{F} \rightarrow \text{O}_H$ and $\text{P}_R \rightarrow \text{F}$ transitions. Both these reactions are believed to involve translocation of two electrical charge equivalents in total.
Fig. 3. Scheme of the proton translocation cycle. (A) Fast (τ = 10 μs) electron re-equilibration between Cu₄ and heme α. For all investigated cases the equilibrium constant of this reaction is not far from unity. (B) Second electron re-equilibration between Cu₄, heme α and binuclear site coupled to proton transfer from the N-side of the membrane. (C) Final electron trapping at Cu₅ by a second proton transfer from the N-side of the membrane. (D) Protein relaxation and release of the “prepumped” proton towards the P-side.
such a case, electron injection will largely reflect electron transfer to the barrier. Indeed, Ruitenberg et al. \[31\] concluded somewhat surprisingly that the would constitute translocation of a single charge across 40 – 60% of the dielectric distance, which is not plausible. Hence, the high relative electrometric amplitude of the Cu₅ → heme a electron transfer is clearly inconsistent with the relative position of Cu₅ and heme a in the structure, unless the medium between these centres is especially nonpolar, which does not seem to be the case. A second possibility is that the Cu₅ → heme a electron transfer is kinetically coupled to a vectorial proton transfer reaction that would increase the amplitude, but this is contradicted by the insensitivity of the reaction to pH and deuterium exchange \[24,31\], and the reaction has indeed been ascribed to pure electron transfer all along \[18\]. The third possibility is that the CeO would pump protons only with very low efficiency in these experiments, but that is also unlikely considering that high proton-pumping efficiency has been described for these preparations. For these reasons, the extensive research summarised by Siletsky et al. \[24\] (and see \[19\]) is associated with a real dilemma related to the very high relative amplitude of membrane potential formation during electron transfer from Cu₅ to heme a. In contrast to this, we find that the relative electrometric amplitude attributable to Cu₅ → heme a electron transfer in CeO from \textit{P. denitrificans} is much less, of the order of 10% of total \[5\] and above), at least for the OH → E₄₅ transition, with a time constant of about 900 \(\mu s\). According to results with \textit{Paracoccus} enzyme in vesicles \[19\], treatment with H₃O₂ yielded only ca. 25% of the F state, and this is also our experience with vesicular oxidase that has not been “pulsed” from the reduced state with O₂. In such a case, electron injection will largely reflect electron transfer to the “resting” O state (Fig. 1), rather than into F, which would easily account for the high relative amplitude of the fast electrometric reaction phase, because reduction of the O state is not associated with proton translocation \[4,5,31\].

2.5. The proton translocation cycle

Many models in the past \[33–36\] have proposed that reduction of heme a initiates the proton pumping sequence. It is less clear from these models, however, whether heme a reduction is thought to be kinetically linked to uptake of the pumped proton. Several facts contradict such kinetic linkage, as summarised above. Moreover, the theoretically calculated electron transfer rate constant, with the assumption of a standard reorganization energy for proteins \[37\], precisely matches the experimentally observed rate without proton coupling. In other words, electron transfer across such a distance with coupled proton transfer should be much slower \[38\]. All of this indicates that reduction of heme a is not kinetically linked to proton uptake. Here, the electron transfer from Cu₅ to heme a is seen in the electrometric response as a distinct phase with the same rate constant, and an amplitude of \(\sim 10\%\) of the complete charge translocation event (2 charges across the membrane). Taking into account that only 60% of the injected electron is transferred in this phase, we can define the distance perpendicular to the membrane plane that the electric charge travels during this phase. Measured as a fraction of the membrane dielectric, this distance is 0.1 + 0.2/0.6 (= 0.33), which is the distance between Cu₅ and heme a determined from the X-ray structure \[29,30\]. We may therefore conclude that there are indeed no other charge movements across the dielectric during this phase (Fig. 3A). However, our data are still consistent with thermodynamic coupling, where reduction of heme a increases the pK₅ of a pump site (see below).

Our description of the proton translocation events differs from that proposed by Siletsky et al. \[24\] in some of the mechanically important details, even though we agree that the pumped proton is transferred before transfer of the substrate proton to the binuclear site \[39; contrast \[40\]. In our model, proton transfer to the pump site starts prior to electron transfer from heme a to the binuclear site, but in kinetic conjunction with that event. The electron tunneling time constant of electron transfer between heme a and the binuclear site is in the nanosecond to picosecond range \[41,42\], but the observed first phase of heme a oxidation is much slower: for example, in the OH → E₄₅ transition \(\tau \) is about 125 \(\mu s\). Electron transfer to the binuclear site is slowed down because without a proton the redox potential of the binuclear site is so low that the electron (being in quasi-equilibrium with the binuclear site) stays at heme a \[43\]. This equilibrium is shifted towards the binuclear site when the proton arrives close to the binuclear site to partially compensate for the charge of the electron (Fig. 3B).

This means that proton transfer precedes electron transfer to the binuclear site, as we have proposed previously \[39\], rather than \textit{vice versa} \[16,24\]. The amplitude of the electrometric phase with a time constant of \(\sim 110 \mu s\) is \(\sim 30\%\) of the full response in the OH → E₄₅ case. Hence, taking into account the fractions of electron redistribution in quasi equilibrium, we can calculate that in this phase the proton moves across \(\sim 2/3\) of the membrane dielectric (red arrows in Fig. 3B). Therefore, we conclude that the first phase of heme a oxidation by the binuclear site is coupled to proton translocation through the major part of the membrane dielectric, all the way from the aqueous N-side to the pump site above the hemes. In this phase the destination of the proton cannot be an oxygenous ligand of the binuclear site (“substrate proton”), because then all the energy would be released, and the binuclear site would be converted into the final product. The latter is observed only in the next reaction step.

In the next optical phase (Fig. 3C) the oxidation of heme a is completed in our example of the OH → E₄₅ transition, with a time constant of about 900 \(\mu s\), and ends up with formation of the E₄ state where the electron has moved completely to Cu₅ (unpublished data). This electron transfer is possible only because during this phase the redox potential of Cu₅ becomes higher than the redox potential of all other centers due to uptake of the substrate proton. Such a change is very likely due to protonation of an OH⁻ ligand of Cu₅ \[44\]. As we can see from the electrometric response, the corresponding electrogeneric phase is also large (\(\sim 40\%\) of the complete response). The amplitude of this phase consists of two parts: (i) the remaining fraction of the electron in heme a moves to the binuclear site; and the pumped proton travels through the dielectric from the N-side, as just described, (ii) the “chemical” proton is taken up from the N-side, and the electron moves to its final destination. Because we know the quantity of electrons taking part in this latter phase from the optical data, we can calculate that the proton, which arrives to the binuclear site, is again transferred all the way from the aqueous N-side (red arrows Fig. 3C).
The final phase of the electrometric response (τ \sim 5.3 ms, 16% amplitude) does not have a corresponding optical phase. This means that the nature of the chemical product in the binuclear site does not change during that phase. It is possible that protein relaxation, such as proposed for bacteriorhodopsin [45], forms a proton-conducting output channel for proton exit towards the P-side, and this relaxation occurs with the time constant of the last electrogenic phase.

The heme α oxidation and membrane potential kinetics appear quite different in the \( P_M \rightarrow F \) case, where a fast phase (τ \sim 100 µs) dominates the reaction (Fig. 2), which seems to contradict the notion arrived from the \( O_2 \rightarrow E_{hH} \) case that uptake of the pumped and consumed protons occurs as kinetically separable events (cf. [24]). However, we have recently shown [23,27] that the corresponding \( P_a \rightarrow F \) reaction is preceded by internal proton transfer into the pump site (τ \sim 30 µs), which accompanies electron transfer from heme α to the binuclear site, and that the subsequent \( P_a \rightarrow F \) step merges the major electrogenic events of both proton-pumping and uptake of the substrate proton essentially into a single phase. A possible reason for this is discussed below.

3. Why are the rates of proton translocation cycles different?

In the experiments presented above, the rate of reduction of the binuclear center, or oxidation of heme α, differ significantly. To explain such a difference, and also the different sensitivity of these rates to pH and hydrogen isotope substitution, we should consider the coupling between proton and electron transfer.

A scheme of such coupling is depicted in Fig. 4 (a similar scheme for a simpler system was analyzed earlier [46]). The electron tunneling rate constant \( k_{e-e} \) between hemes α and αβ is in the time range of nanoseconds [41,42]. The proton tunneling rate constant \( k_{pH} \) for proton transfer through the proton conducting pathways is not known, but could be in the same range. In spite of the potential for fast electron and proton transfer, the real process is much slower due to low occupancy of the intermediate states. The oxidized unprotonated binuclear center (Fig. 4, state I), and the binuclear center with the electron (state II), would be in quasi-equilibrium with an equilibrium constant \( (K_{eq})^{e-} \), determined by the midpoint redox potential of the unprotonated acceptor (\( E_{eq}^{pH} \)):

\[
K_{eq}^{e-} = 10^{\frac{E_{eq}^{pH}}{2.303K_b}}
\]

On the other hand, the oxidized unprotonated binuclear center (Fig. 4, state I) and the binuclear center with the proton (state III) would be in quasi-equilibrium with an equilibrium constant \( (E_{eq}^{pH}) \), which is determined by the pH of the oxidized acceptor (\( pK_a \)):

\[
K_{eq}^{H^+} = 10^{\frac{pK_a}{2.303pH}}
\]

The rate of the appearance of the reduced protonated binuclear site (state IV) is the sum of the reaction rates through the two reaction branches:

\[
k_{app} = K_{eq}^{e-} \times k_{H^+} + K_{eq}^{H^+} \times k_e.
\]

From this simple model it is clear that the rate of oxidation of heme α by the binuclear site would in both kinetic phases be determined by the midpoint redox potential of the electron acceptors and the \( pK_a \) of the groups accepting protons during the two sequential processes. For example, we may compare the rates of the \( P_M \) to \( F \) and \( F \) to \( O \) transitions [21] considering experimentally determined midpoint potentials. The \( E_m \) values for the \( P_M/F \) and \( F/O \) redox couples were obtained from redox titrations in mitochondria at high protonmotive force [47]. The effect of protonmotive force may be corrected for using the current knowledge that each of these two transitions is coupled to translocation of two electrical charge equivalents across the membrane [5], and this yields \( E_m \) values of 817 mV and 762 mV for the \( P/F \) and \( F/O \) redox couples, respectively [48]. Hence, there is a ca. 60 mV difference in the midpoint potentials of the final acceptor which can easily explain why the \( P_M \rightarrow F \) step is faster than \( F \rightarrow O \). The higher midpoint redox potential of the acceptor not only speeds up the reaction, but also channels it through state II in our scheme. This branch of the reaction would not depend on pH, or the nature of the hydrogen isotope. At the same time, when the acceptor \( E_m \) is lower, this not only slows down the reaction, but can also channels it through another pathway (the state III branch), the rate of which would be sensitive to pH and the hydrogen isotope.

We conclude that the difference in the rates of heme α oxidation and their sensitivity to pH and hydrogen isotope in the different parts of the catalytic cycle (Fig. 2) need not reflect a difference in proton translocation mechanism, but may be explained by the different driving forces for the electron transfer reactions.

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