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Review

Electrochemistry of heme–thiolate proteins ☆

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Abstract

Heme–thiolate proteins (HTPs) play critical biological roles by catalyzing challenging chemical reactions. The ability of HTPs to selectively oxidize inert substrates under mild conditions has led to much research aimed at the development of useful in vitro oxidation technology. Very complex electron transfer machinery is required to support HTP chemistry, and electrochemical methods provide many of the needed components. The challenge is to find a system that has good electrode-enzyme electronic coupling that, in turn, would drive catalytic turnover at relatively high rates. Several systems reviewed herein have shown promise in experimental work on components that could be part of a molecular machine for the selective oxidation of organic substrates.

Keywords: Electrochemistry; Heme–thiolate proteins; Electron transfer

Heme–thiolate proteins (HTPs) (cytochromes P450, nitric oxide synthase, and chloroperoxidase) are oxidoreductases that perform essential functions in living systems. The hallmark reaction catalyzed by these enzymes is substrate oxidation, converting R–H to R–OH. In vivo, oxidation reactions are critical for a broad range of biological processes, from hormone synthesis [1][1] to pro-drug activation [2].[2] In vitro, exploiting HTP activity would greatly contribute to stereo- and regiospecific syntheses of high-value molecules [3,4].[3] HTP activity also has applications in bioremediation [5].

Thiol ligation to the heme iron is postulated to be the critical factor that modulates the heme redox properties to activate dioxygen for substrate oxidation in HTPs [6,7]: two electrons are delivered by native reductase proteins to reduce dioxygen, forming water and a high-valent iron-oxo species capable of performing oxygen atom transfer. The nature of this iron–oxo (ferryl) complex, referred to as compound I, is not definitively known. Whatever the true nature of compound I, it is clear that electron transfer (ET) is key to generating the catalytically active species [8–15].

Electrochemical methods have proven particularly useful for characterizing HTP redox chemistry. Generally, direct protein electrochemistry is difficult. The intervening peptide medium results in relatively weak electronic coupling between buried cofactors and external reductants

☆ *Abbreviations:* HTP, heme–thiolate protein; ET, electron transfer; Py, *N*-(1-pyrene)iodoacetamide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; DDAB, didodecyldimethylammonium bromide; Co(sep), cobalt(III) sepulchrate; M_{ox}, 1,1′-dicarboxycobaltocenium hexafluorophosphate; Rh, Cp*Rh(bpy)(H_2O)Cl_2; BPG, basal plane graphite; H_4B, tetrahydrobiopterin; P450 CAM, cytochrome P450 from *Pseudomonas putida*; P450 BM3, flavocytochrome P450 from *Bacillus megaterium*; Pdx, putidaredoxin; hBM3, cytochrome P450 BM3 heme domain.

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[1] Hydroxylation of androgens to estrogens by oxidoreductases is essential for development of female sexual characteristics.

[2] Site-specific hydroxylation of the anticancer pro-drug cyclophosphoamide is required to generate the bio-active molecule.

[3] As an example, hydroxylated alkanes can be used to synthesize surfactants. The physical and chemical properties of the surfactant will be entirely dependent on the site of oxidation and the nature of subsequent chemical modifications.

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[16–18]. For HTPs, the heme is usually buried deep within the polypeptide, which means that the success of an electrochemical method depends on finding a way to increase electronic coupling between the heme and the electrode. Notably, direct electrochemistry at an unmodified edge-plane graphite electrode has been achieved with P450 from *Pseudomonas putida* (P450 CAM); however, the enzyme-electrode system was not well coupled, as evidenced by slow and quasi-reversible ET [19].

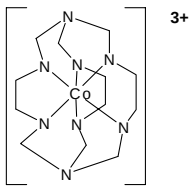
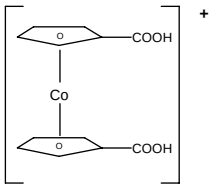
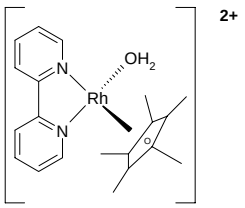
Electrochemical mediators

In vivo, the ultimate source of electrons for HTP catalysis is NAD(P)H. This requirement for NAD(P)H is perhaps the greatest obstacle preventing in vitro applications of HTPs for selective oxidations. NAD(P)H is expensive, decomposes over time, and is difficult to recover once oxidized. While electrochemical [20], chemical [21], enzymatic [22], and whole-cell systems [23] for regenerating NAD(P)H are available, these approaches are often inefficient or difficult to implement on a practical scale.

Harnessing HTP activity for in vitro applications is best approached with electrochemical systems utilizing soluble mediators. Of the HTPs, extensive work has been conducted with the P450s. Estabrook et al. [24] discovered a promising system, which utilized a platinum electrode and the water-soluble cobalt(III) sepulchrate (Co(sep)) cage complex ($E^\circ = -550$ mV vs. Ag/AgCl) as the electron shuttle (Table 1). Co(sep)-mediated catalysis was demonstrated with a variety of P450s (mammalian and bacterial), with rates approaching that of NAD(P)H-driven systems: as an example, reactions with P450 from *Bacillus megaterium* (P450 BM3) and lauric acid yielded turnover rates of 110 min^{-1} (as compared with 900 min^{-1} for reactions with NADPH) [24]. Although a good start, practical limitations of the Co(sep) system include production of reactive oxygen species [25], difficulty in synthetically manipulating Co(sep) to tune the mediator to different reaction conditions (e.g., solvent, pH), and aggregation and precipitation at functional Co(sep) concentrations (typically, 1–10 mM) [26].

Inspired by Estabrook's work, we searched for an alternative mediator for P450. Previous work with glucose oxidase utilized a ferrocene derivative to mediate enzyme oxidation [27], demonstrating that a mediator could be designed for a given application. For P450 reduction, we decided to try the analogous reductant cobaltocene as a scaffold to construct a suitable mediator. Anticipating that the dicarboxy derivative could improve water solubility and disfavor aggregation, we synthesized 1,1′-dicarboxycobaltocenium hexafluorophosphate (M_{ox}) (Table 1) and evaluated its ability to mediate bioelectrochemical catalysis with P450 BM3 [28]. The surrogate mediator was observed to reduce both the FAD and FMN in the P450 BM3 reductase domain, as well as the iron in the heme

Table 1
Electrochemical mediators for HTP catalysis

Mediator	Structure	Best rate
Cobalt(III) sepulchrate		110 min^{-1} with P450 BM3 and lauric acid
1,1′-Dicarboxycobaltocenium		16.5 min^{-1} with P450 BM3 and lauric acid
Putidaredoxin	2Fe–2S protein, 11.6 kDa	36 min^{-1} for P450 CAM with camphor
$Cp^*Rh(bpy)(H_2O)Cl_2$		6.4 mM h^{-1} for StyA with styrene; HTP activity not determined

domain. Electrolysis reactions with the holo protein resulted in lauric acid hydroxylation (16.5 nmol product nmol^{-1} enzyme min^{-1}). Similar ET and catalysis were observed in reactions with just the BM3 heme domain (hBM3) and the metallocene (1.8 nmol product nmol^{-1} enzyme min^{-1}). However, as with the Co(sep) system, our M_{ox} system could not overcome the problem of direct dioxygen reduction by the reduced mediator. This problem was most evident from the observed coupling efficiency, which revealed that only 2% of the total current passed resulted in product formation.

Vilker [29] described an electrochemical system with P450 CAM using putidaredoxin (Pdx), the native ET partner, as the electrochemical mediator. Direct reduction of Pdx with an antimony-doped tin oxide electrode initiated the ET cascade that ultimately resulted in dioxygen activation and camphor hydroxylation. An average turnover rate of 36 min^{-1} and 2600 total turnovers were reported. Notably, reactor performance was optimal when the concentration of Pdx was approximately two orders of magnitude larger than P450 CAM. Once again, the co-substrate dioxygen proved problematic: the working electrode had to be screened with a platinum mesh to disproportionate peroxide formed from direct dioxygen reduction, while the necessary dioxygen for catalysis was generated in situ through water oxidation at another electrode.

Clearly, the key obstacle in the mediated systems is dioxygen reduction, with futile redox cycling of the system and production of reactive oxygen species. Recent studies by Hollman et al. [30–32] have demonstrated the efficacy of Cp*Rh(bpy)(H_2O)Cl_2 (Rh) (Table 1) as a recyclable hydride transfer mediator, capable of regenerating nicotinamide- (NADH, NADPH) and isoalloxazine-based (FAD, FMN) cofactors in solution. The key advantage was the relatively low rate of uncoupling from direct reduction of dioxygen; this was demonstrated in reactions using Rh to reduce the flavins in the catalytic cycle of styrene monooxygenase, which had a coupling efficiency of 60% while displaying substrate turnover rates comparable to those of the native system [32]. Regarding Rh activity with HTPs, preliminary studies with wild type P450 BM3 revealed no catalytic activity [33], possibly owing to poor Rh-FAD interaction. In this instance, mutagenesis [34,35][4] may be able to improve the protein-mediator interaction, resulting in ET and catalytic turnover.

Electrochemical wiring of HTPs

Direct electrochemical wiring of HTPs can be accomplished through covalent modifications of the protein. Attaching a molecular electronic relay to the protein surface can result in a system well coupled for ET from the electrode to the heme. The difficulty here is in choosing the precise position of protein modification: the wire needs to be attached at a site that facilitates ET.

Shumyantseva et al. [36] reported a technique that covalently attached a synthetic flavin cofactor to the surface of two mammalian P450 heme domains, P450 2B4 and P450 1A2. The flavin functioned as an electronic relay, mimicking the contact that the native reductase would make with the heme domains. Reduction of the flavin at a rhodium–graphite electrode resulted in ET through the protein sheath and into the heme. Although ET rates were not reported, they did observe electrode-driven catalysis at rates comparable to NADPH-driven systems (e.g., approximately 0.5 turnovers min^{-1} for aminopyrene N-demethylation by P450 2B4).

Gilardi et al. [37] utilized flavodoxin as the electrochemical wire for reduction of hBM3. A chimeric gene consisting of hBM3 and flavodoxin was created by fusing the 3′ end of the hBM3 gene to the 5′ end of the flavodoxin gene through a short nucleotide that encoded the native linker between hBM3 and its reductase. The linker allowed the two proteins some separation so they could fold independently and retain native solution dynamics. Modeling studies revealed that the closest approach between the two cofactors is 12 Å, placing the molecules in good proximity for ET. Photochemical experiments on the resulting fusion protein using deazariboflavin to reduce flavodoxin revealed flavin to heme ET at 370 s^{-1}, verifying that the two proteins were electronically coupled. Electrochemical experiments at a glassy carbon electrode resulted in enhanced ET to hBM3.

Our own work on electrochemically wired HTPs led to a system well coupled for ET with hBM3 [38]. We followed Sevrioukova et al. [39] who observed rapid photoreduction of hBM3 (2.5×10^6 s^{-1}) by covalently tethering a ruthenium diimine to an engineered cysteine (N387C) on hBM3. The position of the Ru complex was selected to mimic the interaction between hBM3 and its reductase; indeed, the rapid ET rate suggests that the complex was attached at a position well coupled to the heme. It occurred to us that wiring the N387C hBM3 mutant to an electrode through the engineered cysteine could also yield high electron tunneling rates. Previously, Katz [40] had utilized *N*-(1-pyrene)iodoacetamide (Py) (thiol specific) to anchor and electronically connect a photosynthetic reaction center to a basal plane graphite (BPG) electrode. Thus, we made the hBM3 N387C mutant, attached Py to the cysteine, and successfully achieved ET employing a BPG electrode. The voltammogram in Fig. 1 displays the $Fe^{III/II}$ couple at −340 mV (vs. Ag/AgCl). AFM images reveal that only pyrene-wired enzyme molecules are adsorbed to the electrode, resulting in protein clusters on the surface 2–5 nm high and 30–40 nm wide. The enzyme-electrode system undergoes rapid and reversible electron transfer: k^0 was estimated to be 650 s^{-1}, underscoring the relatively strong coupling we achieved through the Cys^{387} wired enzyme. Voltammetry in the presence of dioxygen resulted in large catalytic reduction currents. Independent analytical assays

[4] As examples, separate studies with different NADPH-specific P450 reductases demonstrated that selected mutations were able to increase the specificity for NADH, causing a switch in cofactor preference.

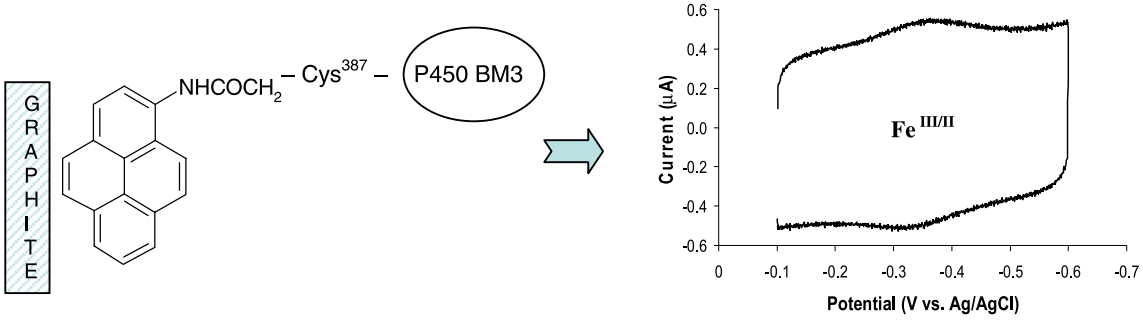


Fig. 1. The P450 BM3 heme domain was anchored to a graphite electrode using a pyrene tether, resulting in reversible access to the heme $Fe^{III/II}$ redox couple.

using fluorescence and rotated-disk electrode techniques demonstrated that the wired enzyme was catalyzing four-electron reduction of dioxygen to water. However, catalytic substrate oxidation was not observed. Instead of dioxygen activation, we believe our system rapidly reduces dioxygen to water without forming catalytically active ferryl species. Our findings are analogous to those reported by Anson et al. [41] in which dioxygen reduction to water by porphyrin systems was observed.

Electrode films for HTP electrochemistry

Some of the most promising bioelectrochemical systems employ the use of protein films on conductive supports to achieve relatively strong electronic coupling between redox enzymes and electrodes. Organic films on electrode surfaces, either covalently attached or chemisorbed, are systems that can be used for general electrochemical investigations of HTPs.

Polyelectrolyte films have been used with various HTPs for electrochemical studies. The polyions are co-adsorbed with the protein in distinct layers: alternate layer-by-layer adsorption of the molecules is achieved by multiple rounds of soaking the electrode in a polyion solution, washing, and then soaking in an enzyme solution. Polystyrenesulfonate layers have been used to study electrochemistry with mammalian P450 1A2 in experiments that yielded catalytic turnover of styrene [42]. Styrene oxidation was also studied in films made of P450 CAM and DNA [43]; in this case, styrene oxide-mediated DNA damage was found. Generally, styrene oxidation rates in these systems are on the order of 10 turnovers h^{-1}.

Lei et al. [44] studied electrochemistry with P450 CAM using sodium montmorillite (carbon-based polyanionic clay) to coat the surface of an electrode. The anionic clay was proposed to interact electrostatically with a region on the surface of P450 CAM that was positively charged; this region is also presumed to be the binding site of the native reductase for P450 CAM. Rapid, reversible ET (k^0 approximately 150 s^{-1}), as well as catalytic reduction of dioxygen was observed. In more recent work, Shumyantseva et al. [45] co-filmed P450 2B4 with montmorillite and the non-ionic detergent Tween 80. Tween 80 keeps P450 2B4 monomeric: the slow ET in the absence of Tween 80 was attributed to protein aggregation, blocking charge transfer. Differential catalytic currents for P450 2B4 substrates aminopyrine and benzphetamine paralleled enzymatic specificity for these substrates, demonstrating biosensor potential for the system.

Fantuzzi et al. [46] utilized self-assembled mercaptan films on gold to achieve ET with mammalian P450 2E1. Indeed, alkanethiol monolayers on gold electrodes are commonly used to provide surfaces that interact favorably with various proteins to achieve ET [47]. For P450 2E1, the gold-mercaptan surface was further derivatized using either maleimide or poly(diallyldimethylammonium chloride). While electronic coupling was not as strong as in the aforementioned systems (k^0 between 2 and 10 s^{-1}), catalytic substrate oxidation was observed. This type of system is not practical for large-scale substrate turnover [48],[5] although it could potentially be used for biosensing.

Surfactant film voltammetry

Rusling has pioneered the use of surfactant films on carbon surfaces for direct electrochemistry of heme proteins [49–51]. The surfactant is deposited onto the electrode, resulting in the formation of bilayers and micelles into which the protein is incorporated. The end result is a system that supports rapid and reversible ET between the electrode and the enzyme; typically, standard rate constants between 50 and 300 s^{-1} are observed. Comprehensive reviews on thin film electrochemistry are available [52,53].

Protein-surfactant film voltammetry is now a routine method for studying the redox chemistry of HTPs: flavocytochromes [54], engineered mutants [55], as well as bacterial [56] and mammalian [57] variants, have been investigated. Notably, Farmer's group has conducted extensive investigations of the thermophillic P450 from *Sulfolobus solfataricus* [5,58]. In addition to the $Fe^{III/II}$ redox couple, they have

[5] There are several difficulties with scaling up this system, most of which are related to properly preparing the gold surface. See Tanimura et al. for a discussion on this problem.

also observed $Fe^{II/I}$ (−1040 mV vs. Ag/AgCl) and have demonstrated that the Fe^{I} species is capable of catalytic dehalogenation of certain substrates. Indeed, at elevated temperatures (>50 °C) they were able to achieve efficient dechlorination of carbon tetrachloride, producing methane.

Unraveling the mechanism of nitric oxide synthase (NOS) currently represents a major challenge. NOS converts L-arginine to citrulline and NO, with NADPH and O_2 as co-substrates [59]. It is well established that the catalytic cycle involves two turnovers of the enzyme. The first turnover converts L-arginine to *N*-hydroxyarginine: although envisaged as a P450-like hydroxylation, the reaction is dependent on one-electron oxidation of the cofactor tetrahydrobiopterin (H_4B) [60]. The second turnover is thought to involve a ferric peroxide nucleophile, but H_4B is also required for this turnover in an unknown way. Although many details of the NOS mechanism remain to be elucidated, it is certain that ET reactions are key steps in the catalytic cycle.

Two recent studies have exploited surfactant film voltammetry to shed light on the redox properties of NOS. Working with neuronal NOS (nNOS), Bayachou and Boutros confined the enzyme within didodecyldimethylammonium bromide (DDAB) films on the surface of graphite electrodes and interrogated the system using square wave voltammetry [61]. Two redox couples were observed: the $Fe^{III/II}$ redox couple at −197 mV (vs. Ag/AgCl) and an unidentified redox couple at −1092 mV. Adding H_4B caused the $Fe^{III/II}$ redox potential to shift ca. 100 mV to a higher potential, analogous to the shift when this cofactor is added to nNOS in solution. They also demonstrated that ET is coupled to proton transfer, as the pH dependence was found to be −54 mV/pH unit.

We have conducted cyclic voltammetry studies of inducible NOS (iNOS) in DDAB films on graphite electrodes (Fig. 2) [62]. Redox couples E_1 (−191 mV) and E_2 (−1049 mV) correspond to heme $Fe^{III/II}$ and $Fe^{II/I}$ (notably, E_2 is similar to the unidentified low-potential couple observed for nNOS). A closer inspection of Fig. 2 reveals that the E_1 cathodic wave is slightly broader than the other waves. Voltammetry at variable scan rates

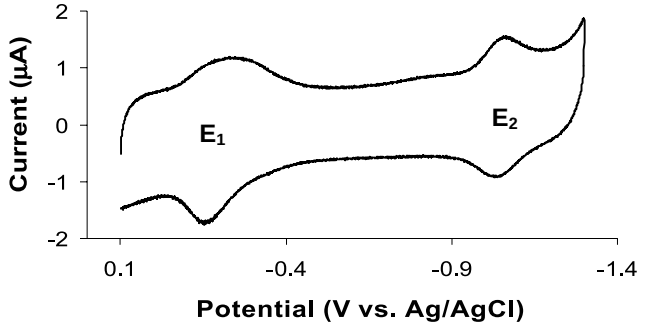


Fig. 2. Cyclic voltammogram of NOS in a DDAB film on a graphite electrode (pH 7, 200 mV s^{-1}). Note the unusual width of the E_1 cathodic peak.

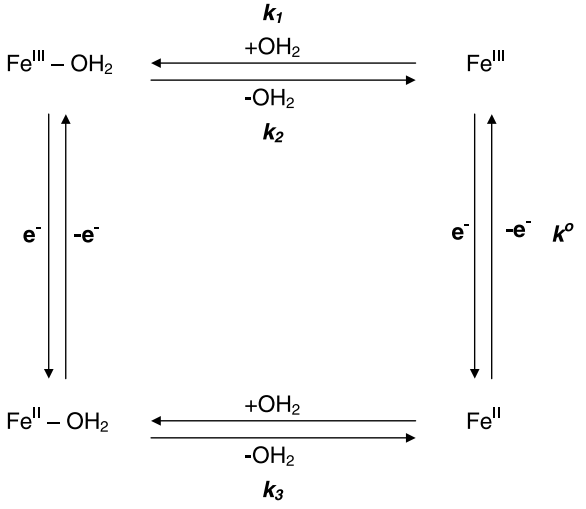


Fig. 3. Square scheme for interpretation of iNOS electrochemistry in DDAB.

and different pH's have allowed us to resolve this peak into two distinct cathodic processes separated by ca. 133 mV. Our analysis led us to believe that the two cathodic peaks represent reduction of water-free (five-coordinate) and water-bound (six-coordinate) iNOS hemes. We were able to explain our results with a classic square scheme, reproduced in Fig. 3. Using digital simulation to model the data, we estimated values for k_1, k_2, and k_3 in Fig. 3 to be 1, 0.5, and >100 s^{-1}. In the catalytic cycle of iNOS, the first ET event is proposed to be the rate-limiting step for substrate turnover, occurring at 1 s^{-1} [63]. k^0 for the 5-coordinate heme, estimated experimentally to be 370 s^{-1}, indicates that well-coupled pathways exist between the protein surface and the heme; thus, electron tunneling is unlikely to be rate-limiting in catalysis. Notably, the kinetics of the Fe^{III} equilibrium with water (k_1, k_2) are on the order of the catalytic rate. It would appear that water dissociation from Fe^{III} and the accompanying potential shift may function as a potential gating mechanism for iNOS catalysis [64].

The future of HTP electrochemistry

Enormous benefits could come from exploiting HTP activity in vitro, and work along these lines remains a central theme in the field. Several reviews have outlined the various uses of HTPs (mostly the P450s) in biotechnology, as well as the challenges and potential solutions of capturing this oxygenase activity in vitro [65–70]. Perhaps, the greatest obstacle in the way of practical application is the intricate ET machinery: this includes lengthy ET chains that utilize several intermediate proteins and cofactors, each of which makes a specific interaction in order to achieve the required ET for subsequent catalysis. This problem has motivated studies aimed at replacing the ET machinery required for HTP catalysis, some of which we have described.

Electrochemical methods represent the simplest way of providing the necessary reducing equivalents for HTP catalysis. The earliest reports of HTP electrochemistry were approximately 30 years ago; determination of the redox potential of rabbit liver P450 with electrochemical mediators [71] was followed shortly by direct electrochemistry with a mercury electrode [72]. Since then great advances have been made in HTP electrochemistry. However, despite these innovations a practical system has yet to be found. We can step back at this point and ask: why not? First, direct electrochemical wiring is not practical, owing to the difficult step of protein modification, which is both inefficient and harsh for a (generally) marginally stable HTP. Regarding thin-film systems, these are best suited for biosensors, perhaps something that can be used on a chip for screening purposes. However, confining HTPs to surfaces often calls into question the integrity of the protein: is the protein properly folded, does it retain its native catalytic and substrate discriminatory properties, and what is the long-term stability of the system? Finally, mediated electrochemical HTP systems are the simplest and easiest to apply for large-scale biocatalysis. Unfortunately, the problem of uncoupling through dioxygen reduction by the mediators has not been satisfactorily addressed. Thus, although we have come a long way over the last 30 years, there is still much hard work to do before an electrochemically based HTP system makes its mark in the real world.

Acknowledgments

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