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Revealing the Membrane-Bound Catalytic Oxidation of NADH by the Drug Target Type-II NADH Dehydrogenase

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Supporting Information

ABSTRACT: Type-II NADH:quinone oxidoreductases (NDH-2s) are an important element of microbial pathogen electron transport chains and an attractive drug target. Despite being widely studied, its mechanism and catalysis are still poorly understood in a hydrophobic membrane environment. A recent report for the *Escherichia coli* NDH-2 showed NADH oxidation in a solution-based assay but apparently showed the reverse reaction in electrochemical studies, calling into question the validity of the electrochemical approach. Here we report electrochemical catalysis in the well-studied NDH-2 from *Caldalkalibacillus thermarum* (*Cth*NDH-2). In agreement with previous reports, we demonstrated *Cth*NDH-2 NADH oxidation in a solution assay and electrochemical assays revealed a system artifact in the absence of quinone that was absent in a membrane system. However, in the presence of either immobilized quinone or mobile quinone in a membrane, NADH oxidation was observed as in solution-phase assays. This conclusively establishes surface-based electrochemistry as a viable approach for interrogating electron transfer chain drug targets.

The regeneration of NADH is an essential process for all known life. At the cellular level, this is carried out by respiratory enzymes such as quinone oxidoreductases, found in the electron transport chain or other dehydrogenases.[1] NADH dehydrogenases such as respiratory complex I (type I NADH dehydrogenases, NDH-1) and type-II NADH dehydrogenases (NDH-2) have a significant contribution to keeping the $NADH/NAD^+$ balance in the living cell.[2] Unlike NDH-1s, NDH-2s are nonproton pumping enzymes, substantially contributing to a membrane electrical potential ($\Delta\psi$), but not a proton gradient (ΔpH).[3] Moreover, NDH-2 is proposed to be an enzyme with critical function upon infection in several pathogenic organisms (e.g., *Mycobacterium tuberculosis*,[3] *Staphylococcus aureus*,[4] and *Trypanosoma brucei*[5]), with some organisms having multiple, seemingly identical copies. Importantly, NADH-2 is not found in higher animal life, and for that reason, it has been proposed as a possible new drug target for the rational design of antibiotics.[6]

Despite many attempts to understand the diverse functional roles of quinone oxidoreductases, mechanistic details remain difficult to resolve. This is due to the need for detergents in solution phase assays,[7] the complex nature of the dielectric membrane environment,[8] and the lack of available tools to accurately study them. Unfortunately, there are several pitfalls when studying membrane proteins and hydrophobic substrates, such as quinones. For example, there is a requirement for solubility agents such as dimethyl sulfoxide and detergents when working in the solution phase.[7] Physiological context is essential to understand membrane protein processes and crucial for targeted drug development. One of the most powerful methods to study quinone oxidoreductases in membranes is electrochemistry, allowing direct access to a membrane-bound quinone pool.[9] However, a recent article about the *Escherichia coli* NDH-2 reported NADH oxidation in a solution-based assay, but apparently showed the reverse reaction in electrochemical studies.[10] This calls into question the validity of the electrochemical approach.

Here, we report electrochemical catalysis in the well-studied NDH-2 from *Caldalkalibacillus thermarum* (*Cth*NDH-2). In agreement with previous reports, we demonstrated *Cth*NDH-2 NADH oxidation in a solution assay.[11] Protein film voltammetry assays revealed a systematic artifact reaction in the absence of quinone, an issue that was solved using the membrane platform in this communication. In the presence of quinones, NADH oxidation was observed as in solution-phase assays. This establishes a viable approach for interrogating electron transfer chain drug targets. Furthermore, our studies hint toward a co-operative mechanism involving two quinone-binding sites, supporting previously reported models.[6,12]

RESULTS AND DISCUSSION

Catalytic Oxidation of NADH Requires Oxidized Quinones. Initially, *Caldalkalibacillus thermarum* NDH-2 (*Cth*NDH-2) catalysis was investigated in the solution phase using a detergent-solubilized system, as it is extensively reported in literature[4−6,11,13] (Figure 1A). Predictably, kinetics followed a Michaelis−Menten model (Figure 1B, also see Figure S1A−C), with a K_M for MD of 48.6 mM and a k_{CAT} of 572 U/mg.

Emulating the study by Salewski et al. on the *Escherichia coli* NDH-2[10] (*Ec*NDH-2), we performed a study with immobilized NDH-2 on an electrode. First, *Cth*NDH-2 was adsorbed onto a

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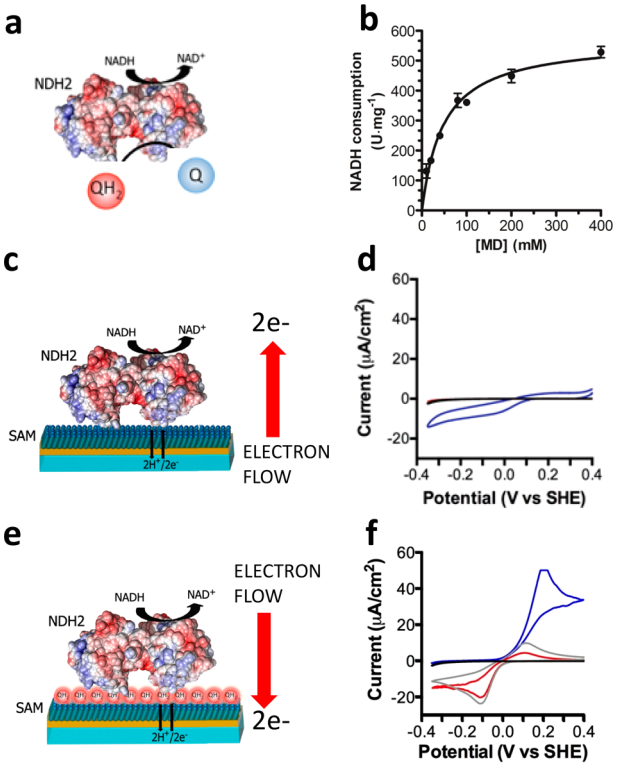

Figure 1. Oxidation of NADH by *Caldalkalibacillus thermarum* NDH-2 (*Cth*NDH-2) using different approaches. (A, C, and E) Schematics of the biochemical/bioelectrochemical experimental systems used (a surface cartoon of the *Cth*NDH-2, PDB ID: 4NWZ, is shown in every case). (A) Soluble phase system; (C) immobilization on a template-stripped gold electrode (TSG) modified with a 6-mercaptohexanol self-assembled monolayer (6MH SAM); (E) immobilization on a menadione-modified 6MH-modified TSG. Panel B shows Michaelis–Menten kinetics in soluble phase, at 25°C, of the systems in panel A. Measurements were conducted in a 20 mM MOPS and 30 mM Na_2SO_4 buffer (pH 7.4), measuring absorbance decay of NADH at 340 nm. Panels D and F show cyclic voltammograms of experiments conducted using the systems shown in panels C and E, respectively. (D) 6-MH SAM only (black); SAM with immobilized *Cth*NDH-2 and 600 μM NADH (blue). (F) 6MH SAM only (black); SAM with immobilized menadione (gray); 6MH SAM with immobilized menadione and *Cth*NDH-2 (red); 6MH SAM with immobilized menadione and *Cth*NDH-2 in the presence of 600 μM NADH (blue). *Cth*NDH-2 was rendered using PyMol (Delano Scientific). All cyclic voltammetry measurements (CVs) were conducted in a 20 mM MOPS and 30 mM Na_2SO_4 buffer (pH 7.4) using a 10 mV/s scan rate at 25 °C. Experiments were performed in duplicate, and representative plots are shown and plotted following IUPAC convention.

gold electrode modified with a self-assembled monolayer (SAMs) of 6-mercaptohexanol (6-MH) or 6-MH modified with menadione (MD Figure S2A; also see Figure 1C,E). As previously observed by Salewski et al. with the *Ec*NDH,[10] no FAD cofactor redox catalysis was observed; yet a reductive wave was observed upon NADH addition (Figure 1D). In contrast, when *Cth*NDH-2 was immobilized on the MD-modified SAM, a catalytic oxidative wave was observed (Figure 1F). The onset of catalysis was in unity with the onset of the oxidative MD peak in the absence of NADH (Figure 1F) and resulted in a disappearance of the MD reductive peak. This result suggests that NADH transfers electrons through *Cth*NDH-2 to FAD, then on to the MD immobilized on the electrode. Hence, we consider this oxidative wave to be the electrocatalytic activity of the unidirectional *Cth*NDH-2, a catalysis that undoubtedly occurs via MD.

This conclusively demonstrates that the reported electrochemical activity of EcNDH-2[10] is not NADH oxidation and is an artifact of the system employed. We propose the reason for the observations in Salewski et al.[10] was that the EcNDH-2 was immobilized on the surface, blocking access to the active site to soluble quinones. Conversely, we immobilize the MD on the electrode; then, we immobilize the CthNDH-2 on top of this layer, thus circumventing this artifact. However, it is noteworthy that the catalytic wave exhibited in Figure 1F is diffusion-limited; therefore, an imperfect method to measure such an enzymatic activity. This flawed result is likely because of the necessity for an electron acceptor to be in closer proximity to the FAD cofactor (i.e., the Q_I site), as shown in several NDH-2 crystal structures.[11,12,14] We then anticipated that diffusion limitation could be solved by using a mobile quinone in a lipid membrane. This approach is much like the solution-phase system but uses a physiological quinone with an isoprenoid tail.

Lipid-Bilayer Electrochemistry Reveals Non-Diffusion-Limited NADH Oxidation. To address this issue, we tested two lipid membrane systems where *Cth*NDH-2 is embedded in a native-like lipid environment containing menaquinone-7 (MQ_7) at 25 °C. For this, we used a state-of-the-art planar tethered lipid bilayer system (tBLM; Figure 2A). tBLM formation was confirmed using electrochemical impedance spectroscopy, which showed a drop of capacitance to below 1.0 $\mu F/cm^2$ after the addition of *Cth*NDH-2 (proteo)liposomes, in which MQ_7 was embedded in the lipid phase (Figure 2B). Initial electrochemical measurements lacking any one component (i.e., either *Cth*NDH-2, MQ_7, or addition of NADH) revealed oxidative and reductive peaks at potentials of 0.055 V and −0.25 V, respectively, in the presence of MQ_7 but not in its absence (Figure 2C). Importantly, no artifact currents that were present in the absence of quinone were observed (see Figure 1D), confirming our proposition this was indeed an artifact current.

This analysis confirmed that our system was functional and that any catalytic signal measured would be valid. A 10 mV/s scan rate was chosen because cytochromes bo_3[15] and cymA[16] were both functional at this rate of electron removal/addition from a membrane-bound quinone pool. NADH addition to a bilayer containing *Cth*NDH-2 and MQ_7 subjected to cyclic voltammetry resulted in an oxidative catalytic wave originated at 0.055 V, producing a substantial current of 48 $\mu A/cm^2$ which could be inhibited by the addition of the known quinone oxidoreductase inhibitor HQNO (Figure 2D), supporting the systems utility as a drug-screening platform. The catalytic wave was not diffusion-limited, indicating that any limitations found in the system used in Figure 1E,F had been resolved.

A Two Quinone-Binding Site Model? Our results showed significant differences between the systems we used to study the *Cth*NDH-2 and offers insight into the catalytic mechanism. The NDH-2 from *S. cerevisiae* has been solved with two different quinones: UQ_2[14] and UQ_4[12]. These crystal structures revealed critical information about the quinone binding sites. When UQ_2 was used, a single quinone was bound in a deep binding pocket with the quinone headgroup within 3.4 Å of the bound FAD. Conversely, when UQ_4 was used, two UQ_4 molecules were bound (Figure 3A). The Q_I site is closest to the FAD and proposed to be critical for correct

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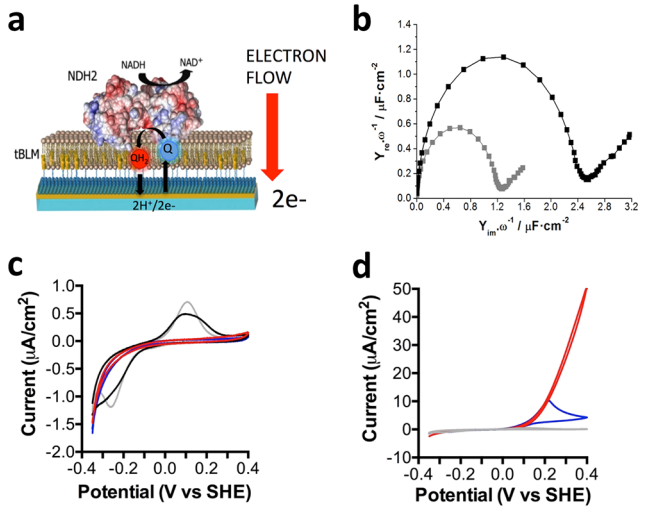

Figure 2. Direct oxidation of NADH by *Caldalkalibacillus thermarum* NDH-2 (*Cth*NDH-2) via menaquinone-7 (MQ_7) using an electrochemical approach. (A) Schematics of the biolectrochemical experimental system used. A surface cartoon of the *Cth*NDH-2 (PDB ID: 4NWZ) is shown reconstituted in a tethered supported *Escherichia coli* polar lipid bilayer (ECPL tBLM) with membrane-incorporated MQ_7 (red and blue circles); lipids are shown in brown. (B) Electrochemical impedance spectroscopy measurements (EIS) demonstrating the process of membrane formation in panel A. 6MH/eo3-cholesteryl SAM before membrane formation (black) and after membrane formation (gray). (C, D) A complete system is required to observe NDH-2 catalytic oxidation of NADH in a membrane. (C) ECPL membrane without MQ_7 in the presence of 600 μM NADH (red); ECPL membrane with CthNDH-2 without MQ_7 in the presence of 600 μM NADH (blue); ECPL membrane with MQ_7 in the presence of 600 μM NADH (gray); ECPL membrane with *Cth*NDH-2 and MQ_7 in the absence of NADH (black). (D) ECPL membrane with *Cth*NDH-2 and MQ_7 in the absence of NADH (gray); in the presence of 600 μM NADH (red); and in the presence of 600 μM NADH and 100 μM HQNO (inhibitor introduced using DMSO). *Cth*NDH-2 was rendered using PyMol (Delano Scientific). All cyclic voltammetry measurements (CVs) were conducted in a 20 mM MOPS and 30 mM Na_2SO_4 buffer (pH 7.4) using a 10 mV/s scan rate at 25 °C. Experiments were performed in duplicate and representative plots are shown and plotted following IUPAC convention.

function, but as shown in our immobilized MD approach (see Figure 1F), electrons can clearly "hop" to the MD immobilized on the electrode, seeming in support of a distal Q_{II} site as suggested by Feng et al. (2012).[12] While the physical distance of the "electron hop" of approximately 7.1 Å is within an electronic coupling distance (H_{DA}) and also acceptable within the physical bounds dictated by Marcus theory[17] (where β in the Franck–Condon term is less than 15 Å), it is clearly suboptimal as demonstrated by the diffusion limitation observed (see Figure 1F).

This evidence suggests that, in a native lipid environment, two quinone-binding sites may be formed by quinones, possibly in interaction with lipids. Our results confirm the accessibility of menadione in solution, which we propose to access the Q_I site easily, not visibly affected by diffusion limitation (Figure 3B). On the other hand, we observe diffusion limitation when the substrates are tethered to the electrodes, preventing MD molecules from accessing the deeper Q_I site (Figure 3C, also see Figure 1F). In such a case, the electron transfer happens, but it is slowed down due

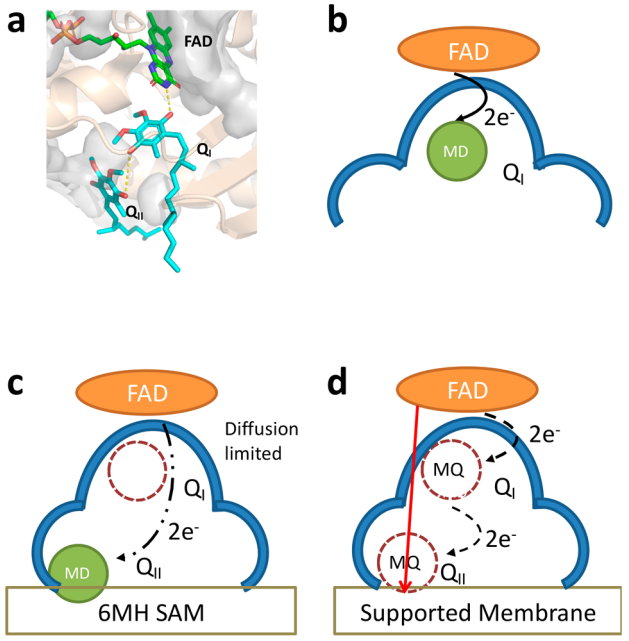

Figure 3. Proposed mechanism for quinone NADH oxidation by *Cth*NDH-2, based on crystallographic evidence. (A) Structure of the *Saccharomyces cerevisiae* type-II NADH dehydrogenase two quinones bound (PDB ID: 4G74). Cartoon depiction of the structure was rendered using PyMol (Delano Scientific). FAD and UQ are labeled and represented as stick models in green and blue, respectively, the protein polypeptide in brown. (B–D) Schematic interpretation of the reaction mechanisms: (B) mobile quinone mechanism in solution phase; (C) immobilized quinone mechanism in a membrane, with two quinone-binding sites; (D) proposed mobile quinone mechanism in a membrane, with two occupied quinone-binding sites.

to the distance between atoms. In this conjecture, we propose a hypothetical reaction mechanism involving an electron hop between the two described quinone-binding sites (e.g., Q_I and Q_{II}, see Figure 3D).

CONCLUSION

Our study here conclusively shows *Cth*NDH-2 to consume NADH in both solution-phase and electrochemical assays. We reveal that, in the presence of either immobilized quinone or mobile quinone in a membrane, NADH oxidation was observed as in solution-phase assays. We conclude that reductive current by NDH-2 family proteins is an artifact, only occurring in the absence of quinone, hence not the true electrochemical catalytic profile. This highlights the need to study membrane protein drug targets in membrane environments and defines the best current electrochemical platform for this task.

Unexpectedly, these results may also offer a tantalizing new insight into the catalytic mechanism of NDH-2. This study indicates that the mechanism of *Cth*NDH-2 differs from the ones previously reported.[13] Instead, a co-operative mechanism involving two quinone-binding sites may occur, but this may indeed rely on the presence of lipids and the use of long-isoprenoid chain quinones to allow the aforementioned co-operativity.

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■ ASSOCIATED CONTENT

Supporting Information

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Methodology, SDS-PAGE, and structures of menaquinone species and inhibitors used in this study (PDF)

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Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript. D.G.G.M. conceptualized the study, gained funding, and performed electrochemical experiments. A.G.-H. performed solution-phase experiments. Both D.G.G.M. and A.G.-H. purified and reconstituted protein. D.J.T. synthesized the eo3-cholesteryl. A.G.-H. and D.G.G.M. contributed equally.

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Notes

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■ ABBREVIATIONS

NDH-2, type-II; NADH, quinone oxidoreductase; NADH, nicotinamide adenine dinucleotide; UQ, ubiquinone; MQ, menaquinone; ECPL, *Escherichia coli* polar lipids; Q_I site, FAD-proximal quinone binding site; Q_{II} site, FAD-distal quinone binding site; SAM, self-assembled monolayer.

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