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Structural Basis for Inhibition of ROS-Producing Respiratory Complex I by NADH-OH

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Abstract: *NADH:ubiquinone oxidoreductase, respiratory complex I, plays a central role in cellular energy metabolism. As a major source of reactive oxygen species (ROS) it affects ageing and mitochondrial dysfunction. The novel inhibitor NADH-OH specifically blocks NADH oxidation and ROS production by complex I in nanomolar concentrations. Attempts to elucidate its structure by NMR spectroscopy have failed. Here, by using X-ray crystallographic analysis, we report the structure of NADH-OH bound in the active site of a soluble fragment of complex I at 2.0 Å resolution. We have identified key amino acid residues that are specific and essential for binding NADH-OH. Furthermore, the structure sheds light on the specificity of NADH-OH towards the unique Rossmann-fold of complex I and indicates a regulatory role in mitochondrial ROS generation. In addition, NADH-OH acts as a lead-structure for the synthesis of a novel class of ROS suppressors.*

Respiratory complex I (NADH:ubiquinone oxidoreductase) is the major entry point for electrons into the electron-transport chains that power ATP synthesis.[1–4] Its dysfunction is associated with several human neurodegenerative disorders and it is a major source of reactive oxygen species (ROS), implying a role in ageing.[5–8] Complex I couples electron transfer from NADH to ubiquinone to the translocation of protons across the membrane, contributing to the proton motive force. The FMN cofactor, the primary electron acceptor and site of NADH oxidation, was shown to catalyze a side reaction leading to ROS production.[9,10] The vast majority of competent complex I inhibitors such as rotenone, rollastatin, and piericidin are hydrophobic and act at the (ubi)quinone-binding site.[11] NADH-OH is the only known potent inhibitor that acts at the NADH-binding site of the complex and suppresses NADH oxidase activity in submicromolar concentrations.[12] Importantly, the binding of NADH-OH also fully suppresses ROS production by the complex.[13] The inhibitor is highly specific with respect to complex I: it displays an approximately six orders of magnitude lower affinity to other enzymes containing a Rossmann-fold for dinucleotide binding, such as malate and lactate dehydrogenases. NADH-OH is formed in alkaline, oxygenized NADH solutions and was suggested to differ from the original dinucleotide by a modification of the nicotinamide moiety that amounts to an additional hydroxylation.[12] However, the presence of tautomeric and rotameric forms in aqueous solution[12] and the rapid decay of the free inhibitor have impeded the elucidation of the atomic structure of the inhibitor by NMR spectroscopy.

Here, we report the structure of NADH-OH determined by X-ray crystallography of a complex between NuoEF, a fragment of the *Aquifex aeolicus* complex I that contains the NADH oxidation site,[14] and the inhibitor. The fragment comprises subunits NuoE and NuoF as well as the FMN cofactor and two Fe/S clusters[14] and has a nonphysiological NADH:ferricyanide oxidoreductase activity that is used in functional assays.[15] The structure was obtained at 2.0 Å resolution, thereby enabling the identification of key amino acid residues specific and essential for its binding. The data point towards a regulatory role of NADH-OH for mitochondrial ROS generation.

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NADH-OH was produced as previously described and enriched from the reaction mixture by ion-exchange chromatography.[13] The procedure yields a number of compounds with similar physicochemical properties in addition to NADH-OH. Attempts to separate the inhibitor from these compounds either by reverse-phase or ion-exchange HPLC failed. To overcome this challenge, we inverted the process commonly used for affinity purification of enzymes. The crude mixture of the dinucleotide derivatives was incubated with NuoEF that was found to exclusively bind NADH-OH from the mixture (Figure S1). The non-bound compounds were separated from the enzyme–inhibitor complex by ultrafiltration. The inhibitor was then dissociated from the complex at pH 11 and separated from the denatured enzyme by ultrafiltration. Note that NADH-OH is very stable under alkaline conditions.

The inhibitory action of NADH-OH prepared by the novel procedure was compared to that described in the literature[12] by measuring the inhibition of the NADH oxidase activity of bovine heart mitochondria.[12] A K_i value of 10 nM was observed (Figure S2), similar to the value of 12 nM reported.[12] Thus, the original preparation of NADH-OH contained virtually the same amount of active substance as the preparation using NuoEF to purify the inhibitor. NADH-OH was also capable of inhibiting bacterial complex I from *Escherichia coli* in a competitive manner to NADH (Figure S2). The K_i value was determined to be 46 nM.

Attempts to crystallize NuoEF directly after incubation with the reaction mixture failed repeatedly, most likely because of the presence of multiple NuoEF conformations caused by the binding of other compounds at various positions. Attempts to soak crystals of NuoEF with purified NADH-OH were also not successful because of an immediate, macroscopic destruction of the crystals. However, co-crystallization of the purified inhibitor with the protein in the presence of PEG 8000, ethylene glycol, and 0.1M MES/imidazole buffer at pH 6.5 led to crystals that diffracted to 2.0 Å resolution. Data sets were collected at beam line X06SA at the Swiss Light Source (Villingen, Switzerland) at 100 K. The NuoEF:NADH-OH complex crystallized in space group $P2_1$ with unit cell dimensions of $a = 96.0$ Å, $b = 63.8$ Å, $c = 121.4$ Å, and $\beta = 105.7°$. The asymmetric unit contained two heterodimers of NuoEF (Table S1). Although the space group is different from the one reported earlier,[14] the composition of the asymmetric unit is maintained. The atomic model has been deposited in the Protein Data Bank (PDB) under accession number 6SAQ.

The initial model derived from data processing showed additional electron density close to the FMN, which was attributed to bound NADH-OH (Figure 1). To clarify its structure, a model was fitted to the difference density. The electron density could be consistently attributed only to an adenosine, pyrophosphate, and the nicotinamide ribose of NADH, but no electron density from the nicotinamide ring itself was detectable. Instead, the moiety had undergone an oxidative ring opening to form an aliphatic chain containing a carboxamide, a hydroxy, and an additional formyl group. The planar geometry of the aliphatic chain and the C40/C41 and C42/C43 distances are consistent with double bonds at

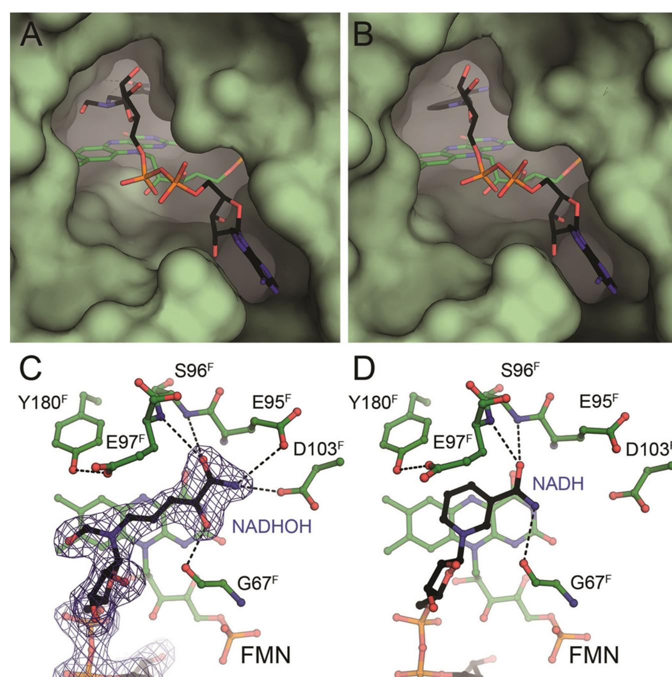

Figure 1. Structure of NADH-OH (A, C) and NADH (B, D) bound to NuoEF. A) shows a surface representation of the active site of NuoF (green) with the bound inhibitor and B) the one with bound NADH. FMN is shown in light green and with color-coded atoms. C) and D) show ball-and-stick models of NADH-OH (C) and NADH (D), as derived from the experimentally determined electron difference density (black mesh). Strong hydrogen bonds to the protein backbone and individual residues are indicated. Note the two additional hydrogen bonds of the amide nitrogen atom of NADH-OH to E95 and D103.

these positions (Figure 2). The derived molecular mass of the NADH-OH molecule is 696 Da, in accordance with the original publication.[12] Unexpectedly, the structure of the inhibitor is entirely different from earlier proposals, from which NADH-OH was expected to contain two additional oxygen atoms at the nicotinamide ring. As a consequence of its instability when not bound to the enzyme, it was not possible to obtain NMR data from NADH-OH, as it decayed during measurements. However, we employed a new method, namely CE-ESI-MS, to demonstrate the chemical identity of NADH-OH, both purified by chromatographic means and by binding to NuoEF (Figure S3). Both samples and a mixture of both eluted with the same retention volume by CE and resulted in identical mass spectra (Figure S3).

The structure of NuoEF with bound NADH has been reported.[14,16] A tight and a loose mode for dinucleotide binding to the active site of complex I was described,[14] and among these, NADH-OH binds to the tight position. Binding of NADH-OH did not lead to major conformational changes in NuoEF, although the hydrogen-bonding network and some hydrophobic interactions around the nucleotide binding site were slightly altered (Figure 1). The adenosine diphosphate moieties of NADH-OH and NADH undergo similar interactions with the protein (Figure 3). The adenine ring undergoes hydrophobic interactions with Phe71^F, Phe79^F, Tyr205^F, Pro206^F, and Val207^F, and additionally forms hydrogen bonds with three water molecules. The pyrophosphate group forms

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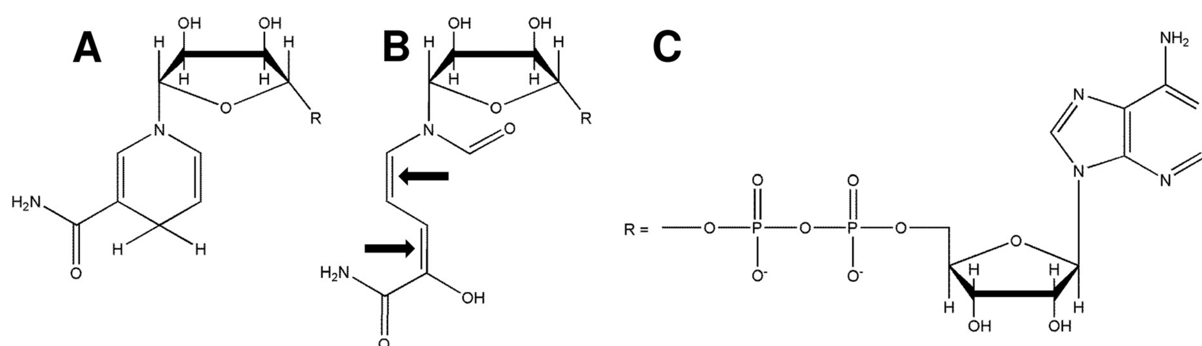

Figure 2. Structures of NADH and NADH-OH. NADH (A) and NADH-OH (B) both consist of the adenosine moiety and the pyrophosphate bridge (C). The nicotinamide moiety of NADH has been opened in NADH-OH under oxidative conditions, giving rise to a linear chain that retains the distal amide group and the conjugated system, but is now substituted with a hydroxy and formyl group (B). The double bonds at C40/41 and C42/C43 are marked by black arrows.

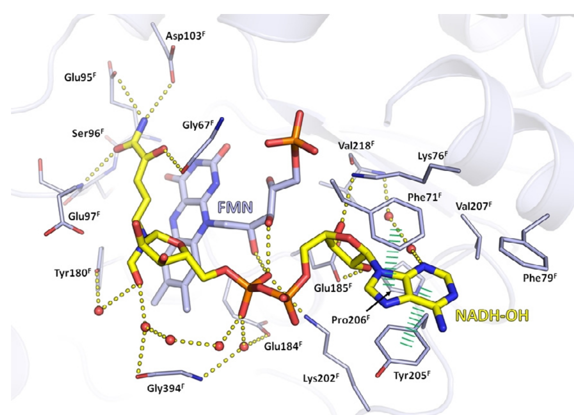

Figure 3. Interactions between NADH-OH and complex I. NADH-OH (yellow sticks) establishes numerous interactions with both NuoF and FMN (light blue sticks). Hydrogen bonds are indicated with yellow dashes, π stacking interactions with green stapled dashes. The protein backbone is shown schematically in gray.

hydrogen bonds to Glu185^F and Gly394^F bridged by a water molecule and two hydrogen bonds to the FMN. The pyrophosphate moiety of NADH-OH shows a slightly bent conformation, thereby enabling an additional hydrogen bond to Lys202^F. The adenine ribose of NADH-OH builds an additional hydrogen bond to Lys76^F. The nicotinamide ring of NADH displays several hydrophobic interactions with the protein and the FMN.[14] In NADH-OH, the new aliphatic chain is stabilized mainly by hydrophobic interactions and hydrogen bonds with Asp103^F and Glu95^F. These two acidic amino acid residues change their positions within the active site upon NADH-OH binding. The hydrogen bonds to the backbone carbonyl group of Gly67^F and to the backbone amino group of Glu97^F are significantly shorter and, therefore, stronger compared to those involved in the binding of NADH. In addition, O49 of NADH-OH interacts with two structural water molecules held in place by the carbonyl backbone of Gly394^F and the oxygen atom of the Tyr180^F residue (Figure 3). Thus, the higher affinity of complex I to NADH-OH than to NADH can be explained by a better occupancy of the binding pocket and novel hydrogen-bonding interactions between the modified nicotinamide moiety and the protein. The interactions of NADH-OH with the protein are summarized in the Figure S4. All positions refer to the *A. aeolicus* enzyme.

Other NAD-dependent enzymes such as malate dehydrogenase, lactate dehydrogenase, and alcohol dehydrogenase were not inhibited by NADH-OH at nanomolar concentrations,[12] although these enzymes similarly bind nucleotides at a Rossmann-fold domain. Complex I, however, is characterized by a slight variation of the "classical" XYZ arrangement of the Rossmann-fold.[14,16–18] As a consequence of this, FMN and the pyridine nucleotide are bound by the same motif. Modeling the structure of NADH-OH in the nucleotide binding pockets of lactate and malate dehydrogenase showed that the modified and open form of the former nicotinamide moiety cannot be accommodated without causing steric clashes (Figure 4). The double bonds in the aliphatic chain of the modified nicotinamide of the inhibitor restrict the flexibility of this part of the chain, thereby hampering high-affinity binding. The unusual Rossmann-fold domain of NuoF is ideally suited for the specific binding of NADH-OH. We suggest that the tightly binding inhibitor leaves no space for oxygen to diffuse to the flavin, leading to the suppression of ROS production.[13]

The two novel and strong hydrogen bonds to Asp103^F and Glu95^F contribute mainly to the tight binding of NADH-OH to complex I (Figure 1 and Figure 3). Remarkably, these residues are fully conserved in complex I (Figure S5), but they are not present in other human proteins with a Rossmann-fold (Figure S6). Only two other proteins contain Glu95^F in the Rossmann-fold, which implies that this residue does not have a structural function. Thus, the conservation of Asp103^F and Glu95^F in complex I may serve an additional purpose, namely the specific binding of NADH-OH as a means to regulate ROS production. NADH-OH is very stable when bound to complex I, but degrades within hours at room temperature in solution at neutral pH, thus losing its capability to inhibit NADH oxidation and ROS production by complex I.[12] The high affinity to complex I and its short lifetime suggest NADH-OH to be a regulator of ROS production in mitochondria in vivo through a feedback-type regulation. Mitochondrial dioxygenases that catalyze oxidative ring opening reactions are, in principle, capable of

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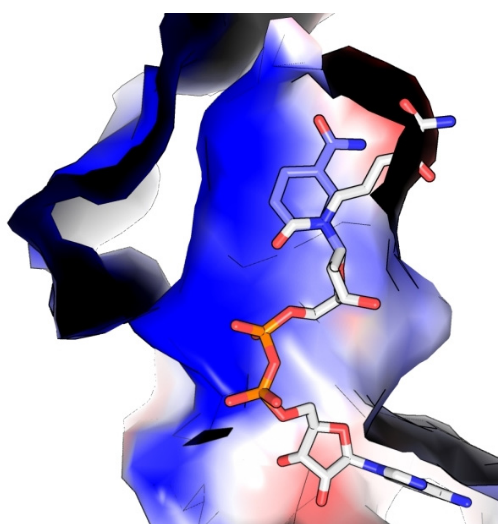

Figure 4. NADH-OH modeled in the NADH binding site of lactate dehydrogenase (open conformation, PDB: 4l4s). The cocrystallized bound conformation of NADH (blue) fits well into the active site of lactate dehydrogenase. Modeling of NADH-OH (white) onto the position of bound NADH in the active site of lactate dehydrogenase shows that the distal amide group of the modified nicotinamide group of NADH-OH would clash with the protein. The presence of the double bonds results in the flexibility of NADH-OH being limited so that it will likely not fit into a classical Rossmann-fold domain.

producing NADH-OH. These enzymes might be activated under oxidative stress conditions. A first screen revealed the presence of several putative mitochondrial dioxygenases that could potentially catalyze the oxidative opening of the nicotinamide ring (Table S2). None of the putative dioxygenases are known to specifically interact with complex I, which might not be necessary because of its high affinity towards NADH-OH. The enzymatic formation of NADH-OH from the reduced nicotinamide dinucleotide in mitochondria would lead to suppression of ROS formation by complex I. After dissociation from the active site, NADH-OH will degrade and irreversibly lose its ability to inhibit ROS production. When the cell overcomes oxidative stress, no NADH-OH will be produced and complex I will remain catalytically active. This mechanism should prevent the overproduction of ROS in the mitochondria of healthy cells. In bacteria, a conformational change in the NADH binding site prevents further reduction of the complex when the quinone pool is mostly reduced.[14] The conformational switch is induced by the reduction of the Fe/S cluster N1a in proximity to the flavin. However, in mitochondria, cluster N1a of complex I is not reduced by NADH,[19] which forces the cells to find another solution to the problem, probably the production of NADH-OH under oxygen stress conditions.

Taking the two specific and strong hydrogen bonds to Asp103^F and Glu95^F into account, simple derivatives of the oxidatively opened form of the nicotinamide moiety could be synthesized that specifically bind to mitochondrial complex I, while neglecting other NAD-dependent oxidoreductases that also contain a Rossmann-fold. A strategy for the synthesis of such compounds will focus on the identification of an optimal moiety that addresses the interactions of the opened nicotinamide with complex I. At the same time, an extension of the molecule to include interactions established by the pyrophosphate group appears feasible.

NADH-OH specifically blocks NADH oxidation and ROS production in nanomolar concentrations by interactions with key amino acid residues specific for respiratory complex I. Besides a possible role in regulating ROS production, the NADH-OH structure might serve as a lead structure for the design of a new class of ROS suppressors.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: electron transport · inhibitors · NADH:ubiquinone oxidoreductase · reactive oxygen species · structural biology

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