A novel strong competitive inhibitor of complex I

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Abstract Alkaline incubation of NADH results in the formation of a very potent inhibitor of complex I (NADH:ubiquinone oxidoreductase). Mass spectroscopy (molecular mass equal to 696) and absorption spectroscopy suggest that the inhibitor is derived from attachment of two oxygen atoms to the nicotinamide moiety of NADH. The inhibitor is competitive with respect to NADH with a $K_i$ of about $10^{-8}$ M. The inhibitor efficiently suppresses NADH-oxidase, NADH-artificial acceptor reductase, and NADH-ubiquinone reduction reactions catalyzed by submitochondrial particles, as well as the reactions catalyzed by either isolated complex I or the three subunit flavoprotein fragment of complex I.

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1. Introduction

Complex I (NADH:ubiquinone oxidoreductase) provides the entry point for electrons from NADH to the respiratory chain of mitochondria and bacteria. The enzyme transfers electrons from NADH to a non-covalently bound FMN cofactor and then via a series of at least eight iron-sulfur (FeS) clusters to ubiquinone which is the terminal electron acceptor. This 2e$^-$ transfer reaction is coupled to the translocation of 4H$^+$ across the mitochondrial inner membrane which contributes to the proton motive force used by the mitochondrial ATP synthase to generate ATP. The best characterized complex I is that from beef heart mitochondria and its subunit composition and properties have been elegantly reviewed by John Walker [1]. The mammalian enzyme is now known to contain 46 different subunits with a total molecular mass of about 980 kDa [2] making it one of the largest of the cellular machines. Treatment of bovine heart mitochondrial complex I with chaotropic agents such as sodium perchlorate results in solubilization of a “minimal” catalytically active fragment, the so-called FP (flavoprotein) composed of only three subunits (51, 24, and 10 kDa) [3]. FP contains flavin and two iron-sulfur clusters and catalyzes rotenone-insensitive NADH oxidation by artificial electron acceptors. Despite the fact that FP can not catalyze ubiquinone reduction it can serve as a useful model for studying the molecular mechanism of NADH dehydrogenation at the active site.

There are two main types of activities catalyzed by the enzyme: NADH:ubiquinone and NADH:artificial acceptor reductase reactions. The former includes the complete sequence of electron transfer reactions from the NADH dehydrogenating site via the prosthetic groups of complex I to the ubiquinone reduction site. The reaction is sensitive to specific inhibitors of Q reduction (rotenone and piericidin) and associated with vectorial proton translocation through the mitochondrial membrane. NADH:acceptor reductase reactions are evidently abbreviated compared to the natural electron pathway. Two main acceptors, ferricyanide and hexaamineruthenium (III) are usually used for measuring activity of complex I [4]. These activities in contrast to NADH:ubiquinone reduction are insensitive to rotenone and piericidin and are not linked to proton translocation.

Despite the considerable progress recently achieved in understanding the structure, molecular composition, and evolution of complex I, knowledge of the molecular mechanism of substrate dehydrogenation, intramolecular electron transfer, and the coupling of electron transfer with vectorial proton transfer still remains a matter of speculation. Little is known about regulation of complex I in the cell. More than a decade ago it was proposed that the mechanism of complex I regulation is based on its slow and reversible transition from an active form to a deactive form [5–7]. An alternative mechanism of complex I regulation in cells has been suggested by Papa and co-workers. These workers suggested that phosphorylation of the 18 kDa subunit of complex I by a mitochondrial cAMP-dependent protein kinase [8] modulates the enzyme activity in cells [9].

Interaction with naturally occurring analogues is one of the most common mechanisms of enzyme regulation in vivo. Complex I is, however, almost inert to NADH analogues. Rotenone and piericidin – classical inhibitors of complex I, act at or close to the quinone-binding site(s) of the enzyme [10–12] and suppress quinone reduction at a 1:1 stoichiometry [10,12]. ATP, ADP, AMP and even NAD$^+$, which appear to be the closest natural analogues of the substrate, inhibit

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complex I at very high (millimolar) concentrations [13,14]. The only substrate analogue which has relatively high affinity for the NADH-binding site of complex I is ADP-ribose. It was shown by Vinogradov’s group that ADP-ribose inhibits NADH oxidation with a $K_{i}$ value of approximately 30–40 $\mu$M [15]. The affinity of ADP-ribose, however, is not sufficient to suppress complex I activity in the presence of a high concentration of NADH moreover, to the best of our knowledge there are no data showing the presence of ADP-ribose in mitochondria.

In this work, we describe a novel and very potent specific competitive inhibitor of complex I. The inhibitor which we have termed NADH-OH may be structurally similar to NADH. The high affinity of the inhibitor for the NADH binding site of complex I also should make it useful for studies of the mechanism of NADH oxidation by complex I.

2. Materials and methods

2.1. Preparations of complex I and other assays

Submitochondrial membranes (SMP) were isolated from rat hearts as described in [16]. Complex I and FP were the kind gift of Dr. Y. Hatefi (The Scripps Research Institute, La Jolla, USA). Protein concentration was determined with the Biuret reagent. All chemicals were obtained from Sigma Chemical Company.

2.2. Preparation of the inhibitor

NADH (20 mM) was incubated aerobically for 40 days at 4°C in 50 mM KOH and 50% methanol. Incubation of NADH under a nitrogen atmosphere suppressed formation of the inhibitor. 40 ml of the solution was applied to a 5 ml Q-Sepharose column (Amersham Biosciences) equilibrated with 5 mM potassium phosphate (K-Pi) (pH 12). Elution of NADH-OH at pH 12 was with a linear K-Pi gradient from 5 to 500 mM. The fractions containing NADH-OH were eluted from the column at approximately 350–400 mM K-Pi and after authentic NADH. The fractions containing NADH-OH were collected and rechromatographed under the same conditions as above. All experiments were conducted at 4°C using a Thermo Finnigan SpectraSystem 2000 HPLC system with a photodiode array detector. Data were collected from a Thermo Finnigan SpectraSystem UV 6000LP PDA detector and analyzed with Microsoft Excel.

2.3. Enzyme essays

Enzyme activities were assayed at 25°C in 100 mM K-Pi (pH 7.4), 0.1 mM EDTA, 120 $\mu$M NADH (NADH-oxidase) and 2 mM hexaamineruthenium (III) chloride (HAR) and 5 $\mu$M rotenone (NADH:HAR reductase). NADH-oxidase reactions were initiated by addition of the enzyme preparations and were followed by the decrease of NADH absorbance at 340 nm ($\varepsilon_{340}=6.22$ mM$^{-1}$ cm$^{-1}$). Other details of the assays are indicated in the figure legends.

2.4. Mass spectrometer conditions

Mass spectrometric measurements were carried out on a Finnigan LCQ Classic ion trap instrument (ThermoFinnigan, San Jose, CA) equipped with its standard heated capillary electrospray source. The source was operated in the negative ion mode. Mass spectra were recorded in a row scan mode in the mass range from m/z of 400–800. All mass spectra were obtained by signal averaging for 1 min at a scan rate of 3 microscans/scan. Solutions of NADH-OH were admitted by direct infusion with a 100 $\mu$l Hamilton gas-tight syringe (Holliston, MA) at a flow rate of 3 $\mu$l/min.

3. Results

Incubation of NADH under alkaline conditions as described in the Materials and Methods results in accumulation of an inhibitor of complex I over time. Incubation of SMP with NADH exposed to alkaline incubation results in a strong suppression of the NADH-oxidase reaction of complex I. In order to determine the cause of inhibition the inhibitor was purified from non-modified NADH and other products of NADH degradation using anion exchange HPLC at high pH as described in Section 2. The fractions containing NADH-OH were rechromatographed and analyzed by HPLC (Fig. 1A) and by electrospray ionization (ESI)-mass spectrometry (see Fig. 1B). As evident from the data in Fig. 1 the compound elutes as a single peak (Fig. 1A) and had a molecular mass of 696 Da (Fig. 1B). Authentic NADH has a molecular mass of 663.8 Da, so NADH-OH has an additional mass of 32 Da as compared to authentic NADH.

Using SMP we investigated the kinetics of complex I inhibition by the inhibitor which we have termed NADH-OH. As shown in Fig. 2A addition of NADH-OH to the assay results in rapid (less than a minute) suppression of NADH-oxidase activity. Preincubation of SMP with the inhibitor, prior to initiation of the reaction, results in complete inhibition of the initial rate of NADH oxidation followed by slow activation of the enzyme (Fig. 2B). Analysis of the first order kinetics of the enzyme activation in a semi-logarithmic plot (the insert) yielded an activation constant ($k_{act}$) of 0.1 min$^{-1}$.

To measure the $K_{i}$ for the competitive inhibition observed we used analysis of the dependence of the apparent $K_{m}$ for NADH on the concentration of NADH-OH. $K_{m}$ values were estimated from analysis of the dependence of the reaction rate on NADH concentration in double reciprocal plots. Establishing the equilibrium between the enzyme, NADH, and the inhibitor is not instantaneous, therefore the rate of the enzymatic reaction was measured 5 min after addition of the enzyme to the assay containing NADH-OH and NADH, when steady state conditions have been reached. As seen in Fig. 3 the dependence of the apparent $K_{m}$ on NADH-OH concentration is linear, indicating a purely competitive character of the inhibition with respect to NADH. The linear dependence in the figure is described by the following equation: $K_{m(app)} = K_{m}(1 + [\text{NADH-OH}]/K_{i})$, where [NADH-OH]/$K_{i}$ corresponds to a slope of the line on the graph. A $K_{i}$ value approximately equal to 10$^{-8}$ M for competitive inhibition of the enzyme with NADH-OH was estimated from this graph. The low $K_{i}$ value is consistent with the slow release of the inhibitor as shown in Fig. 2B. The kinetic data presented strongly suggest that NADH-OH is a tightly bound competitive inhibitor of complex I.

Being competitive with respect to NADH the inhibitor also suppresses both NADH-ubiquinone reductase activity and NADH-artificial electron acceptor reductase reactions of complex I. The same kinetic patterns of NADH-OH release from the enzyme as shown in Fig. 2B for NADH-oxidase were obtained for inhibition of NADH-ferricyanide and NADH-hexaamineruthenium (III) reductase reactions (not presented).

Identical results were also obtained by measuring the effect of the inhibitor on NADH-oxidase activity using an oxygraph (data not shown). We have also demonstrated that the NADH-OH is characterized by the same affinity for different preparations of NADH-dehydrogenase, i.e., membrane bound complex I, purified bovine complex I, or the 3 subunit flavoprotein fragment (FP). The rates of inhibitor binding and dissociation from all the above enzyme preparations were identical.
We have shown that interaction with NADH-OH results in bleaching of the FP enzyme. The absorption spectrum of FP is largely due to the FMN moiety of the enzyme. In addition to FMN the FP enzyme contains two iron-sulfur centers (centers N3 and N1a) [17], however, their contribution to the absorption of the enzyme in the visible spectral region is minor. As seen in Fig. 4A, addition of NADH-OH to the enzyme results in about a 10% decrease of the initial absorption of the FP at 445 nm. The effect of NADH-OH on the absorption spectra is weaker than that induced by NADH (Fig. 4B). The effect of NADH on the spectrum is mainly due to reduction of the flavin and iron-sulfur clusters of FP. Analysis by EPR shows that NADH-OH does not cause reduction of the flavin to its radical form nor any of the iron-sulfur clusters and its presence also prevents reduction of the iron-sulfur redox centers by NADH (data not shown). The effect of NADH-OH on the absorption spectra is thus not associated with reduction of the enzyme and is one of strongest among those reported for ligands binding to various flavoproteins [18]. The latter is indicative of close molecular contact between the flavin and NADH-OH in the active site of the enzyme.

Formation of NADH-OH from NADH under alkaline conditions and the molecular mass of the compound equal to that of NADH plus 32 suggest that at high pH two oxygens are added to NADH by an unknown mechanism to the nicotinamide group of the dinucleotide. This suggestion was further...
supported by data from absorption spectroscopy. As seen in Fig. 5A at high pH the inhibitor is characterized by an absorption spectrum with maxima at 260 and 335 nm. The spectrum changes with pH between 4.5 and 12. Stepwise pH reduction from 10.5 to 6.5 results in decrease of absorption in the 330–350 nm region and a peak shift to 300 nm. Authentic NADH in contrast to NADH-OH shows no differences in this range. The pH-dependence of the inhibitor’s absorption at 340 nm (around the alkaline maximum) is shown in Fig. 5B. The pK value estimated for this transition is equal to 8.8.

An extensive search, (over 5 million chemical compounds) using the ChemNavigator and SciFinder Scholar databases, looking for NAD$^+$ and NADH derivatives with two additional oxygen atoms attached to the nicotinamide moiety was undertaken. No structures fitting this criterion were found in these databases. The only relevant compounds found were the following nicotinamide derivatives lacking adenine and ribose: 2,5-dihydroxynicotinamide (Registry Number, 37747-23-2) [19], 4,6-dihydroxynicotinamide (Registry Number, 5466-41-1) and 2,6-dihydroxynicotinamide (Registry Number, 71380-91-1) [20]. These compounds are characterized by a different pK of the hydroxyl group; the pK values calculated for the 2,4-, 4-, 6- and 2,6-derivatives using advanced chemistry development Software Solaris V4.76 are correspondingly equal to 4.50 ± 1.00, 7.82 ± 0.53, and 8.84 ± 0.40 (see SciFinder). As the pK of NADH-OH is equal to 8.8 (see Fig. 5B), which corresponds very well to the pK of the 2,6-dihydroxynicotinamide derivative, we speculate that the oxygen atoms attach to the nicotinamide moiety of the inhibitor at positions 2 and 6 as shown in Fig. 5C. However, without a definitive structure of the inhibitor this conclusion must be taken with caution.

The results presented in Fig. 6 show that NADH-OH is capable of rapid reduction of electron acceptors, such as ferricyanide and dichlorophenol-indophenol (DCIP), respectively, indicating the reductive nature of the inhibitor. The reaction between ferricyanide and the inhibitor (see Fig. 6A) results in a dramatic decrease of NADH-OH absorption in the 300–360 nm region. The rate of NADH-OH oxidation by ferricyanide is much faster (about two orders of magnitude) than the rate of NADH oxidation by the dye. This might indicate a lower midpoint redox potential of NADH-OH compared to that of NADH or that the product of the one electron oxidation of NADH-OH is more thermodynamically stable compared to that of the NAD+ radical. The redox reaction between NADH-OH and DCIP was used (see Fig. 6B) to estimate the extinction coefficient NADH-OH. As seen in the figure, reduction of DCIP by NADH-OH results in bleaching of the dye in the 550–560 nm region and a decrease of the inhibitor’s absorption in the 310–380 nm region. The spectra immediately after and 10 min after, mixing the dye and the inhibitor

![Fig. 3. The dependence of the apparent K_m for NADH on the concentration of NADH-OH. The activity was assayed in 100 mM KPi (pH 7.4), 100 µM EDTA at 340 nm. The dependence of the reaction rate on NADH concentration at a constant concentration of NADH-OH (indicated on the axes) was analyzed using double reciprocal coordinates (Lineweaver–Burk plots) to estimate the apparent K_m values. The value of K_m measured in the absence of the inhibitor was equal to 12 µM. The dependence of apparent K_m on NADH-OH concentration is shown on the figure. The continuous line is a best fit for K_m(app) = (K_m + [NADH-OH]/K_i) equation with the K_i equal to 0.012 µM (1.2 x 10^{-8} M).](image)

![Fig. 4. Absorption changes induced in FP by NADH-OH and by NADH. Trace 1 in both panels (A) and (B) shows the spectrum of FP (0.45 mg/ml) alone. The FP in trace 1 was then mixed with 4 µM NADH-OH (trace 2 on panel (A)) and 100 µM NADH (trace 2 on panel (B)). Spectra were recorded using an HP diode array spectrophotometer 2 min after the addition of NADH-OH or NADH. The insert in panel (B) is a difference spectrum obtained by subtraction of spectrum1 from 2.](image)
are shown in Fig. 6B (traces 1 and 2, respectively). The difference spectrum (10 min minus zero min spectrum) is depicted in the insert to Fig. 6B. There is almost no spectral overlap between the dye and the inhibitor; the inhibitor does not absorb in the 550–650 nm region and the differential absorption of oxidized and reduced DCIP forms at 335 nm (NADH-OH maximum) is minor. We estimated the extinction coefficient of NADH-OH by comparing absorption changes at 600 nm extinction coefficient of DCIP at alkaline pH at 600 nm is 22000 M$^{-1}$ cm$^{-1}$ [21] and at 335 nm (NADH-OH maximum); the coefficient at 335 nm is approximately equal to 25000 M$^{-1}$ cm$^{-1}$.

4. Discussion

We have discovered a very potent and specific inhibitor of complex I which appears to be a derivative of NADH. The inhibitor is spontaneously formed in alkaline solutions of the reduced dinucleotide. It is known that at high pH NAD$^+$ rapidly decomposes and a number of dinucleotide derivatives are formed [22–24]. Our data indicate that products obtained by alkaline treatment of NADH differ from the products of NAD$^+$ degradation and have much higher inhibition capacity compared to those obtained by alkaline treatment of NAD$^+$ (data not shown). In contrast to NAD$^+$, NADH is rather stable at high pH. Thus, prolonged (days) incubation is needed to produce detectable amounts of the inhibitor. NADH-OH seems to be the strongest competitive inhibitor of complex I NADH oxidation reported so far. Its affinity is more than 3 orders of magnitude higher than that of ADP-ribose [15], and 5 orders higher than that of NAD$^+$ [13]. In addition to its high affinity the inhibitor is characterized by high specificity with respect to complex I. We have examined other NADH utilizing enzymes such as malate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase, alternative
NADH-dehydrogenases of bacterial membranes and have seen no effect of NADH-OH on the activity of these enzymes (data not shown). Selective inhibition of complex I may indicate a different organization of the NADH-binding site of complex I and soluble NADH-dependent dehydrogenases. Based on data of mass and absorption spectroscopy at various pH a tentative structure of NADH-OH has been suggested. In order to confirm the proposed structure and determine the absolute atomic structure NMR spectroscopy of the compound will be required. The yield of NADH-OH is rather low and only sub-milligram quantities of the compound have been produced. Complete NMR analysis of the compound, however, will require tens of milligrams of the compound. Improvement in methodology for production and purification of the inhibitor in order to obtain more material for the structural analysis is underway. The structural similarity of NADH-OH and NADH and the high potency and specificity of the inhibitor with respect to complex I raise the possibility that it is a natural derivative of NADH. It may be possible that it could be produced in mitochondria by enzymatic hydroxylation of the nicotinamide ring of NADH under certain metabolic conditions. If indeed the inhibitor can be produced in cells this could provide important insights for understanding the mechanism of complex I regulation in vivo.

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