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On the mechanism of rotenone-insensitive reduction of quinones by mitochondrial NADH:ubiquinone reductase

The high affinity binding of NAD^+ and NADH to the reduced enzyme form

Narimantas K. Čėnas, Daiva A. Bironaitė and Juozas J. Kulys

Institute of Biochemistry, Lithuanian Academy of Sciences, 232021 Vilnius, Mokslininku 12, Lithuania, USSR

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NADH acts as an incomplete competitive inhibitor for 5,8-dioxy-1,4-naphtoquinone during its rotenone-insensitive reduction by mitochondrial NADH:ubiquinone reductase. NAD^+ and ADP-ribose act as incomplete mixed-type inhibitors. K_i of NAD^+ and NADH towards quinone are about one order less than towards ferricyanide. The bimolecular rate constant of the reduction of the enzyme by NADH in the quinone reductase reaction is about 2 times less than that of ferricyanide reductase reaction. These data indicate that the reduction site of 5,8-dioxy-1,4-naphtoquinone is close to NAD^+/NADH and ferricyanide binding site. It seems that during the steady-state reduction of ferricyanide and 5,8-dioxy-1,4-naphtoquinone these oxidizers react with NADH:ubiquinone reductase reduced to different extents.

NADH:ubiquinone reductase; Electron acceptor; Steady-state kinetics

1. INTRODUCTION

Mitochondrial NADH:ubiquinone reductase (complex I; EC 1.6.99.3) contains FMN, 8–9 iron-sulfur clusters and 3–4 molecules of protein-bound ubiquinone [1–5]. During the reaction, NADH reduces FMN, which further transfers the electrons to clusters N-1b, N-3, N-4 and N-2. The reduction of ubiquinone by cluster N-2 is prevented by rotenone and piericidin which bind near this cluster and the ubiquinone-binding protein [6–8]. However, the reduction of ferricyanide and soluble quinones involves other center(s) and is completely or partially insensitive to ubiquinone inhibitors [9–11]. The double substrate inhibition of ferricyanide reduction [10] and the competitive inhibition of arylazido-β-alanyl NAD^+ towards NADH and ferricyanide bind at identical or strongly overlapping sites close to FMN. The different effects of mercurials and crosslinking agents on reduction of ferricyanide and soluble quinones [9–13] indicate that these oxidizers are reduced at nonidentical sites.

The studies of the mechanism of the rotenone-insensitive reduction of quinones by mitochondrial complex I are of some interest, since this reaction seems to be responsible for the redox cycling of quinones, including antitumour quinone antibiotics, which leads to the 'oxidative stress' [11]. The data in this paper lead to the conclusions that the site of reduction of quinones is close to NAD^+/NADH and the ferricyanide binding site, and that the different redox states of complex I are responsible for the observed steady-state kinetics of reduction of quinones and ferricyanide.

Correspondence address: N.K. Čėnas, Institute of Biochemistry, Lithuanian Academy of Sciences, 232021 Vilnius, Mokslininku 12, Luthuania, USSR

Abbreviations: TN, turnover number; TN/K_m, biomolecular rate constant

2. MATERIALS AND METHODS

NADH:ubiquinone reductase from bovine heart mitochondria was prepared according to Hatefi and Rieske [1]. NADH, NAD^+, rotenone (Serva), ADP-ribose (Sigma), 5,8-dioxy-1,4-naphtoquinone (Fluka AG) were used as received. Potassium ferricyanide (Reakhim, USSR) was recrystallized from water. The reaction rates were monitored accordong to the decrease of NADH absorption ($\Delta\varepsilon_{340} = 6.2\ mM^{-1} \cdot cm^{-1}$) using a Hitachi-557 spectrophotometer at 25°C. 0.1 M and 0. 4 M K-phosphate buffer solutions (pH 7.0) containing 1 mM EDTA were used. The kinetic parameters of the reaction - turnover number (TN) and the bimolecular rate constant (TN/K_m) - correspond to the reciprocal intercepts and slopes of the Lineweaver-Burke plots. TN corresponds to the number of NADH oxidized by FMN/1 s, assuming that complex I contains 1.2 nmol of FMN/mg of protein [10].

3. RESULTS AND DISCUSSION

The oxidation of NADH by 5,8-dioxy-1,4-naphtoquinone, catalyzed by complex I is completely insensitive to 2 μM rotenone. At a fixed concentration of NADH the data on the initial rates linearize in the Lineweaver-Burke coordinates. At saturating concentrations of substrates TN_{max} is close to $100\ s^{-1}$. TN/K_m

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of quinone does not depend on the concentration of NADH (25–75 μM) and is close to 7×10^5 $M^{-1} \cdot s^{-1}$ (0.1 M phosphate) and to 9×10^5 $M^{-1} \cdot s^{-1}$ (0.4 M phosphate). The further decrease of NADH concentration increases TN/K_m of quinone. This is most evident when 0.4 M phosphate is used (Fig. 1). These data indicate that NADH acts as a competitive inhibitor for 5,8-dioxy-1,4-naphtoquinone. The nonlinear dependence of the slopes of the Lineweaver-Burke plots on the concentration of NADH (Fig. 2) indicates that the inhibition is incomplete. K_i of NADH is 12 μM in 0.4 M phosphate and is considerably lower when 0.1 M buffer is used.

The reduction of fericyanide by complex I follows a 'ping-pong' mechanism. TN_{max} of the reaction is 2.5×10^3 s^{-1} (5×10^3 s^{-1} on the one-electron basis) which is close to previously reported value [10]. TN/K_m for NADH is 1.4×10^7 $M^{-1} \cdot s^{-1}$ and that for ferricyanide $- 4 \times 10^6$ $M^{-1}s^{-1}$ (on the one-electron basis) in 0.1 M phosphate. NADH acts as a linear competitive inhibitor for ferricyanide (K_i = 50 μM) (Fig. 2), evidently competing for its binding site in the reduced complex I [10]. The use of 0.4 M phosphate decreased TN/K_m for NADH and ferricyanide by 3 and 2.1 times, respectively, increased K_i of NADH to ca. 200 μM (Fig. 2), and did not change TN_{max}.

NAD^+ and ADP-ribose act as mixed-type inhibitors for NADH, ferricyanide and 5,8-dioxy-1,4-naphtoquinone. At saturating concentrations of oxidizer, NAD^+ acts as a competitive inhibitor for NADH (Fig. 3). So the mixed inhibition of NAD^+ is caused by the competition for a NADH binding site in the oxidized form of reductase and the competition for an oxidizer binding site in the reduced form [14]. The data of Fig. 3 indicate that TN/K_m for NADH in quinone reductase reaction is about 2 times lower than that in ferricyanide reductase reaction. This is especially evident in the

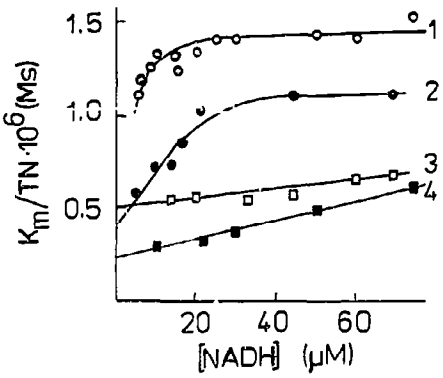

Fig. 2. The dependence of reciprocal TN/K_m of oxidizer on NADH concentration: 5,8-dioxy-1,4-naphtoquinone (1,2), ferricyanide (3,4; TN/K_m calculated on the one-electron basis). 0.1 M phosphate (1,4); 0.4 M phosphate (2,3).

presence of 6 mM NAD^+ or when 0.4 M phosphate is used. The data of Fig. 4 point out that NAD^+ linearly increases the reciprocal TN/K_m of ferricyanide (K_i = 1.5 mM), whereas the nonlinear Dixon plots are observed in the case of 5,8-dioxy-1,4-naphtoquinone. It is evident that TN/K_m of quinone reaches an almost constant value at high concentrations of NAD^+ irrespectitive of NADH concentration used, and that K_i of NAD^+ increases when the concentration of NADH is increased (Fig. 4). The reaction remains insensitive to rotenone in the presence of 6 mM NAD^+. It seems that at concentrations of NADH significantly exceeding K_i (Fig. 2), TN/K_m of 5,8-dioxy-1,4-naphtoquinone is determined by the NAD^+/NADH ratio (Fig. 5). It is not related to the redox potential imposed by the pair NAD^+/NADH, since the redox-inactive ADP-ribose also acts as an incomplete inhibitor for quinone, decreasing TN/K_m by a limiting factor of 1.6 (saturating concentration of ADP-ribose – 0.5 mM, concentration of NADH – 30 μM, 0.1 M phosphate). TN/K_m of ferricyanide is not determined by the NAD^+/NADH ratio,

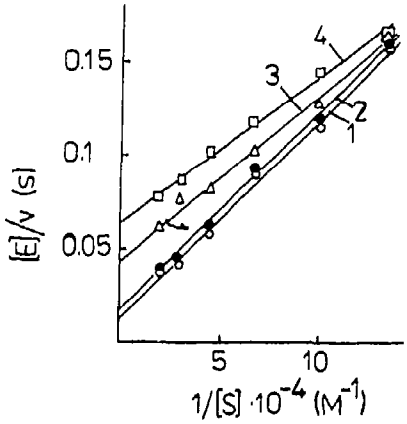

Fig. 1. The rates of NADH:ubiquinone reductase catalyzed oxidation of NADH by 5,8-dioxy-1,4-naphtoquinone. NADH concentrations, 70 μM (1), 45 μM (2), 18 μM (3) and 10 μM (4). 0.4 M phosphate, pH 7.0.

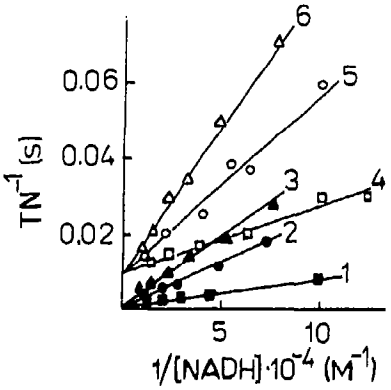

Fig. 3. The dependence of TN (at a saturating concentration of oxidizer) on NADH concentration of ferricyanide (1–3) and quinone reductase reactions (4–6). 0.1 M phosphate (1,4), 0.4 M phosphate (2,5), 0.1 M phosphate + 6 mM NAD^+ (3,6).

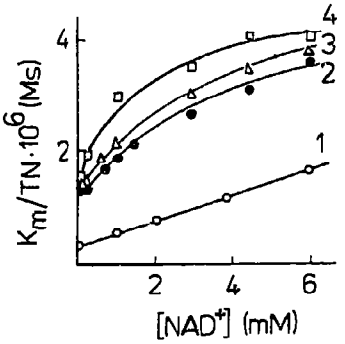

Fig. 4. The depencence of reciprocal TN/K_m of ferricyanide (1, calculated on the one-electron basis) and 5,8-dioxy-1,4-naphtoquinone (2-4) on NAD^+ concentration. Concentration of NADH, 100 μM (2), 30 μM (1,3) and 10 μM (4), 0.1 M phosphate.

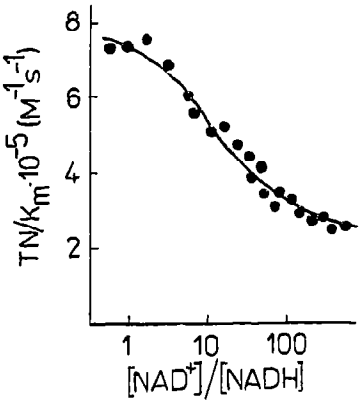

Fig. 5. The dependence of TN/K_m of 5,8-dioxy-1,4-naphtoquinone on the ratio NAD^+/NADH. Concentrations of NADH, 10-100 μM, 0.1 M phosphate.

since at a constant ratio of NAD^+/NADH = 10, TN/K_m of ferricyanide are $2.8 \times 10^6\ M^{-1} \cdot s^{-1}$ and $7.1 \times 10^5\ M^{-1} \cdot s^{-1}$, when 10 μM and 100 μM of NADH are used.

The data presented indicate that, contrary to ferricyanide, 5,8-dioxy-1,4-naphtoquinone can oxidize the complex of NADH with the reduced form of reductase. The oxidation rate is ca. 2.4 times slower than that of free reductase (Fig. 2). The incomplete inhibition of NADH towards quinone may go unnoticed at low or intermediate ionic strength due to the low K_i of NADH. The increase of K_i at high ionic strength (Figs. 1,2) is in accordance to our results [15] showing that the rise of ionic strength diminishes the affinity of NADH:ubiquinone reductase for negatively charged reagents (NADH, ferricyanide). Since K_i of NADH in ferricyanide reductase reaction is raised about 4 times on increasing the buffer concentration from 0.1 M to 0.4 M (Fig. 2), K_i of NADH in quinone reductase reaction may be close to 3 μM in 0.1 M phosphate. The data of Fig. 4 indicate that 5,8-dioxy-1,4-naphtoquinone may oxidize the complex of NAD^+ with the reduced reductase as well, and that NAD^+ competes for a NADH binding site in the reduced form. The complex with NAD^+ is oxidized about 3 times slower than the complex with NADH (Fig. 5). Since the half-maximal inhibition of reaction is observed at NAD^+/NADH = 10-20 (Fig. 5), K_d of the reductase-NAD^+ complex is in the range of 30-60 μM. These data indicate that the quinone binding site is distinct but is not distant from NAD^+/NADH binding site. The observed incomplete inhibition may be caused by partial overlapping of binding sites or the induced conformational changes. Another interesting point is that K_i of NADH and NAD^+ in the quinone reductase reaction are about one order lower than that of ferricyanide reductase (Figs. 2,4,5). It seems that this phenomenon is caused by the dissimilar affinity for NAD(H) of the various redox states of the enzyme, as was previously suggested for soluble NADH dehydrogenase [16]. Additionally this assumption may be supported by the different TN/K_m of NADH in quinone and ferricyanide reductase reactions (Fig. 3). So it is possible that the different redox states of NADH:ubiquinone reductase are responsible for the observed steady-state kinetics of quinone and ferricyanide reduction.

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