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The rotenone-insensitive reduction of quinones and nitrocompounds by mitochondrial NADH : ubiquinone reductase

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The rotenone-insensitive reduction of quinones and aromatic nitrocompounds by mitochondrial NADH : ubiquinone reductase (complex I, EC 1.6.99.3) has been studied. It was found that these reactions proceed via a mixed one- and two-electron transfer. The logarithms of the bimolecular rate constants of oxidation (TN/K_m) are proportional to the one-electron-reduction potentials of oxidizers. The reactivities of nitrocompounds are close to those of quinones. Unlike the reduction of ferricyanide, these reactions are not inhibited by NADH. However, they are inhibited by NAD^+ and ADP-ribose, which also act as the mixed-type inhibitors for ferricyanide. TN/K_m of quinones and nitrocompounds depend on the $NAD^+/NADH$ ratio, but not on NAD^+ concentration. They are diminished by the limiting factors of 2.5–3.5 at $NAD^+/NADH > 200$. It seems that rotenone-insensitive reduction of quinones and nitrocompounds takes place near the $NAD^+/NADH$ and ferricyanide binding site, and the inhibition is caused by induced conformational changes after the binding of NAD^+ or ADP-ribose.

Introduction

Quinones and aromatic nitrocompounds are widely used as antitumour, bactericidal and cytostatic agents [1–6]. These physiological activities are often determined by their ability to undergo the one-electron reduction to corresponding anion-radicals, which are readily reoxidizable by oxygen. This leads to the formation of superoxide and other activated forms of oxygen and results in the 'oxidative stress' – the peroxidation of lipids and modification of proteins and DNA. As a rule, NAD(P)H-oxidizing flavin dehydrogenases-electrontransferases and low-potential iron-sulfur redox proteins are responsible for the one-electron reduction of these compounds [1–6].

Mitochondrial NADH : ubiquinone reductase (complex I, EC 1.6.99.3) consists of approx. 26 polypeptides and contains FMN, 8–9 iron-sulfur clusters and 3–4 molecules of protein-bound ubiquinone [7–17]. The redox equivalents of NADH are transferred to FMN, which further reduces clusters N-1b, N-3, N-4 and N-2. The last cluster reduces ubiquinone. The inhibitors of the complex rotenone and piericidin bind near the cluster N-2 and the ubiquinone-binding protein, respectively, and prevent the reduction of ubiquinone [11–13,15]. Another inhibitor, rhein (4,5-dioxy-anthraquinone-2-carbonic acid), competes for a NADH-binding site [18].

Besides ubiquinone, NADH : ubiquinone reductase may reduce the artificial oxidizers, ferricyanide [19] and soluble quinones, including the antitumour anthracycline antibiotics [2,7,14,20]. The latter are reduced to their anion-radicals. The reduction of soluble electron acceptors is completely or partially insensitive towards piericidin and rotenone and takes part in the substrate side of cluster N-2. The double substrate inhibition of the reaction indicates that ferricyanide is reduced at the NADH binding center close to FMN [19]. However, little is known about the mechanism of the rotenone-insensitive reduction of soluble quinones, and, especially, nitrocompounds.

The aim of the present work was to evaluate the mechanism of rotenone-insensitive reduction of quinones and nitrocompounds by mitochondrial complex I. The relationship between the reaction rates and redox potentials of oxidizers, the ratio of the one- vs.

Abbreviations: TN, turnover number; TN/K_m, bimolecular rate constant; E_7^1, one-electron reduction potential at pH 7.0; $E_{m,7}$, midpoint potential at pH 7.0.

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two-electron reduction and the differences between kinetic and inhibition patterns of reduction of quinones and nitrocompounds and those of reduction of ferricyanide were studied.

Materials and Methods

NADH : ubiquinone reductase from bovine heart mitochondria was prepared according to Hatefi and Rieske [7]. Its activity, determined as a rate of rotenone-sensitive reduction of ubiquinone-1 [7] was 0.75 μmol of NADH oxidized/min per mg of protein (concentration of NADH, 100 μM; ubiquinone-1, 50 μM, 25 ° C). Cytochrome *c*, NADH, NAD^+, rotenone (Serva), 5-oxy-1,4-naphthoquinone, tetramethyl-1,4-benzoquinone, ADP-ribose, superoxide dismutase (Sigma), 2,5-dimethyl-1,4-benzoquinone, rhein (Aldrich), 5,8-dioxy-1,4-naphthoquinone (Fluka), ubiquinone-1 (Ferak), adriamycin (Carlo Erba), nifuroxim and nitrofurantoin (Minmedprom, U.S.S.R.) were used as received. 1,4-Benzoquinone, 2-methyl-1,4-benzoquinone, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone and 9,10-phenanthrenequinone (Reakhim, U.S.S.R.) were purified by sublimation in vacuum or were recrystallized from benzene or ethanol. Nitrobenzene was distilled in vacuum, 4-nitroacetophenone, 4-nitrobenzaldehyde and 4-nitrobenzoic acid (Reakhim) were recrystallized from benzene or ethanol. Potassium ferricyanide was recristallized from water.

All experiments, unless specified, were carried out in a 0.1 M potassium phosphate buffer solution (pH 7.0) containing 1 mM of EDTA without any preincubation of the enzyme with phospholipids and their absence in solution. The reaction rate was monitored according to the decrease of NADH absorption ($\Delta\epsilon_{340} = 6.2\ mM^{-1}\ cm^{-1}$) using a Hitachi-557 spectrophotometer. The concentration of NADH was 10–100 μM; quinones, 10–200 μM; ferricyanide, 0.15–1.0 mM; nitrocompounds, 30 μM–2.0 mM. The reaction rates were also measured according to the decrease of NADH fluorescence intensity at 440 nm (excitation wavelength, 340 nm; concentration of NADH, 10–30 μM) using a MPF-4 spectrofluorimeter (Hitachi) when the absorption of oxidizer in the 340 nm region was significant. The benzosemiquinone- or nitroradical-mediated reduction of cytochrome *c* by complex I was monitored using $\Delta\epsilon_{550} = 20\ mM^{-1}\ cm^{-1}$ [21]. The reaction mixture in this case contained 20–100 μM of benzoquinone or nitrocompound and 35 μM of cytochrome *c*. 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-Burk plots. TN, with an exception of cytochrome *c* reduction, corresponds to the number of NADH oxidized by FMN/1 s, assuming that complex I contains 1.2 nmol of FMN/mg of protein [19]. The NADH-oxidase and NADH-cytochrome *c* reductase activities of the complex (0.15 s^{-1} and 0.28 s^{-1}, respectively) were subtracted from the reaction rates.

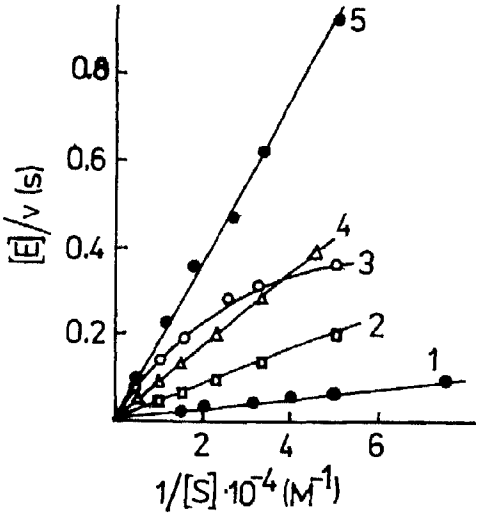

Fig. 1. The dependence of the enzymatic NADH oxidation rate on the concentration of electron acceptor: 5,8-dioxy-1,4-naphthoquinone (1), 1,4-benzoquinone (2), ubiquinone-1 (3), 2-methyl-1,4-benzoquinone-1 (4), ubiquinone-1 in the presence of 2 μM rotenone (5). Concentration of NADH, 20 μM.

Results

The NADH : ferricyanide reductase reaction of complex I follows a 'ping-pong' mechanism. TN of the reaction makes up $2.5 \cdot 10^3\ s^{-1}$ ($5.0 \cdot 10^3\ s^{-1}$ on the one-electron basis) that is close to the value given in Ref. 19. TN/K_m for NADH is $1.4 \cdot 10^7\ M^{-1}\ s^{-1}$, and that for ferricyanide $4.0 \cdot 10^6\ M^{-1}\ s^{-1}$ (on the one-electron basis). NADH acts as a competitive inhibitor for ferricyanide ($K_i = 50\ \mu M$). The reaction is not inhibited by ferricyanide above 1 mM, evidently, due to higher ionic strength of solution comparing to Ref. 19. According to our previous work [22] the increase of ionic strength decreases the affinity of complex I for NADH and ferricyanide.

Contrary to the reduction of ferricyanide, the reactions of NADH : ubiquinone reductase with quinones and nitrocompounds are not inhibited by NADH (10–100 μM). The data on the initial rates at varied concentrations of acceptors linearize in the Lineweaver-Burk coordinates (Fig. 1). However, a deviation from linearity with a gradual decrease in the slope of the Lineweaver-Burk plot is observed when ubiquinone-1 is used, which disappears in the presence of 2 μM rotenone (Fig. 1). TN for the most effective oxidizers are close to 100 s^{-1} (1,4-benzoquinone, 2,5-dimethyl-

1,4-benzoquinone, 2-methyl-1,4-benzoquinone) and to 60–70 s^{-1} (5-oxy-1,4-naphthoquinone, 5,8-dioxy-1,4-naphthoquinone, 9,10-phenanthrenequinone). They do not depend on the concentrations of NADH used. The other electron acceptors possess considerably higher K_m values and it is difficult to establish their TN precisely. TN/K_m for electron acceptors does not depend on the NADH concentration. They are presented in the Table I.

Rotenone (2 μM) exhibits an insubstantial influence on the reduction kinetics of acceptors, except for the reduction of ubiquinone-1 (Fig. 1). It diminishes the TN/K_m value for tetramethyl-1,4-benzoquinone and for 2,5-dimethyl-1,4-benzoquinone by 30 and 10%, respectively. The other oxidizers are almost completely insensitive to rotenone. The other inhibitor, rhein (30 μM), does not change TN/K_m of 1,4-benzoquinone and 5,8-dioxy-1,4-naphthoquinone.

On analogy to the data of our previous paper [22] NAD^+ acts as a mixed-type inhibitor for both substrates in the NADH:ferricyanide reductase reaction of complex I. As is seen from Fig. 2, at a fixed concentration of NADH, NAD^+ diminishes both TN of the reaction and TN/K_m of ferricyanide. The lines in the double-reciprocal coordinates intersect above the x-axis and left to the y-axis. When NADH is used as the variable substrate, the character of inhibition is similar, although the lines intersect below the x-axis. The inhibition is linear both with respect to slopes (Fig. 3A) and intercepts in the Lineweaver-Burk coordinates. ADP-ribose (0.2–1.0 mM) inhibits the reduction of ferricyanide in a similar way. In the presence of 1.0 mM ADP-ribose TN/K_m is diminished 4-times (Fig. 3B), and TN 8-times.

The reduction of quinones and nitrocompounds by NADH:ubiquinone reductase is also inhibited by NAD^+, but the inhibition character differs from that of ferricyanide reduction. NAD^+ diminishes the TN/K_m ratio of quinones, but the inhibition deviates from linearity in the Dixon plots (Fig. 3A). K_i of NAD^+ decreases at lower concentrations of NADH, but at high NAD^+ concentrations TN/K_m reaches almost a constant value which does not depend on the concentration of NADH. Thus, TN/K_m for quinone acquires a constant value both in the absence of NAD^+ and at a high NAD^+/NADH ratio. As follows from Fig. 4, TN/K_m values for 1,4-benzoquinone and 5,8-dioxy-1,4-naphtoquinone drop to almost constant at NAD^+/NADH > 200. NAD^+ diminishes TN of reduction of 5,8-dioxy-1,4-naphtoquinone to 20 s^{-1} at NAD^+/NADH = 200, however, it is difficult to determine its influence on that parameter for the other acceptors possessing lower TN/K_m values. ADP-ribose diminishes TN/K_m (Fig. 3B) and TN of 5,8-dioxy-1,4-naphthoquinone by a limiting factor of 1.6. An analoguous effect of NAD^+ is observed for the reduction of 4-nitrobenzaldehyde and rotenone-insensitive reduction of ubiquinone-1. At NAD^+/NADH = 200 TN/K_m values for these acceptors are diminished 3.5-fold. This is not characteristic of ferricyanide, since at constant

TABLE I

The bimolecular rate constants (TN/K_m) and K_m of rotenone-insensitive oxidation of NADH:ubiquinone reductase and the one-electron reduction potentials of oxidizers (E_7^1) (pH 7.0, 25 °C)

Oxidizer	TN/K_m ($M^{-1} s^{-1}$)	K_m (μM)	E_7^1 (V) (Refs. 3, 30–35)
Quinones			
5,8-dioxy-1,4-naphthoquinone	$8.3 \cdot 10^5$	80	−0.11
9,10-phenanthrenequinone	$8.0 \cdot 10^5$	90	−0.12
5-oxy-1,4-naphthoquinone	$5.7 \cdot 10^5$	120	−0.09
1,4-benzoquinone	$2.6 \cdot 10^5$	~400	0.09
2,5-dimethyl-1,4-benzoquinone	$2.3 \cdot 10^5$	~400	−0.08
2-methyl-1,4-benzoquinone	$1.2 \cdot 10^5$	–	0.01
1,4-naphthoquinone	$1.0 \cdot 10^5$	–	−0.15
ubiquinone-1 [a]	$5.6 \cdot 10^4$	–	−0.24
2-methyl-1,4-naphthoquinone	$3.7 \cdot 10^4$	–	−0.20
tetramethyl-1,4-benzoquinone [a]	$1.7 \cdot 10^4$	–	−0.26
adriamycin	$9.0 \cdot 10^3$	–	−0.33
Nitrocompounds			
nifuroxim	$3.3 \cdot 10^4$	–	−0.26
4-nitrobenzaldehyde	$1.2 \cdot 10^4$	–	−0.33
nitrofurantoin	$3.2 \cdot 10^3$	–	−0.26
4-nitroacetophenone	$7.5 \cdot 10^2$	–	−0.36
nitrobenzene	$1.1 \cdot 10^2$	–	−0.48
4-nitrobenzoic acid	$6.5 \cdot 10$	–	−0.43

[a] Determined in the presence of 2 μM of rotenone.

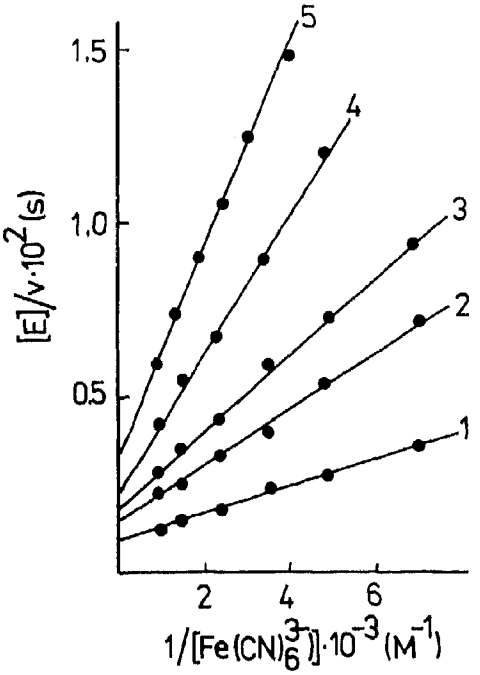

Fig. 2. The effect of NAD^+ on the enzymatic oxidation of NADH by ferricyanide. Concentration of NAD^+, 0 (1), 1.0 mM (2), 2.0 mM (3), 4.0 mM (4), 6.0 mM (5). Concentration of NADH, 45 μM. The reaction rates are expressed on the one-electron basis.

ratio of NAD^+/NADH = 10 TN/K_m values for ferricyanide are different, when 10 and 100 μM of NADH are used. They make up $2.8 \cdot 10^6$ M^{-1} s^{-1} and $7.1 \cdot 10^5$ M^{-1} s^{-1}, respectively.

Since the formation of free radicals of quinones under the action of NADH:ubiquinone reductase has been reported [2] it is of some interest to measure the efficiency of a one-electron transfer. According to Yamazaki et al. [21], at pH < 7.2 the rate of cytochrome *c* reduction by 1,4-benzohydroquinone is negligible and benzosemiquinone, formed in a one-electron process reduces cytochrome *c* at a high rate ($k = 1.5 \cdot 10^6$ M^{-1} s^{-1}). At high concentrations of cytochrome *c* and at low rates of quinone reduction almost 90% of benzosemiquinone dissociated from the enzyme are trapped by cytochrome *c*. The percentage of one-electron flux is expressed as the ratio between the rate of cytochrome reduction in the presence of benzoquinone and the doubled rate of NADH oxidation. We performed the experiments at pH 6.5 and found that the percentage of the one-electron flux in the reduction of benzoquinone makes up 25%. The rates of NADH oxidation under these conditions did not exceed 0.3 μM s^{-1}. The aerobic reduction of nitrocompounds is also accompanied by the reduction of cytochrome *c*. The percentage of one-electron flux, expressed analoguosly to that of mediated by 1,4-benzosemiquinone,

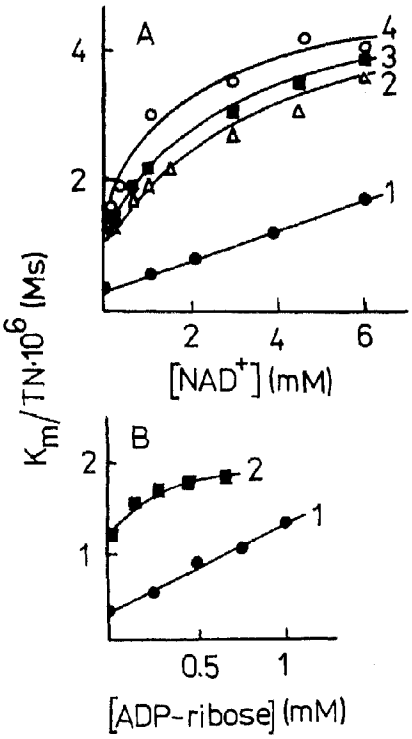

Fig. 3. (A) The dependence of TN/K_m of ferricyanide (1) and 5,8-dioxy-1,4-naphthoquinone (2–4) on the concentration of NAD^+. Concentration of NADH, 30 μM (1,3), 100 μM (2) and 10 μM (4). The reaction rate is expressed on the one-electron basis (1). (B) The dependence of TN/K_m of ferricyanide (1) and 5,8-naphthoquinone (2) on the concentration of ADP-ribose. Concentration of NADH, 30 μM.

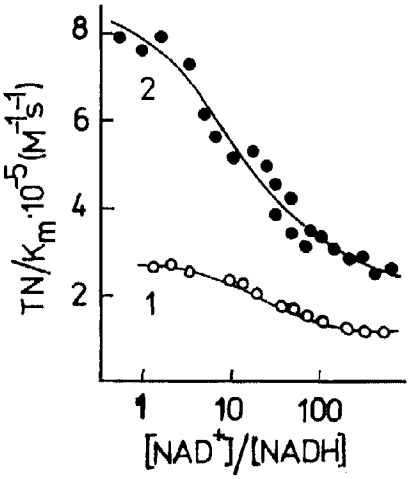

Fig. 4. The dependence of TN/K_m of 1,4-benzoquinone (1) and 5,8-dioxy-1,4-naphthoquinone on the ratio NAD^+/NADH. Concentrations of NADH, 10–100 μM.

makes up 45% (nifuroxim), 65% (4-nitrobenzaldehyde) and 70% (nitrofurantoin). The rate of the reduction of cytochrome *c* in the presence of nitrocompounds was inhibited by 30–50% by superoxide dismutase (60 μg/ml). That shows that cytochrome *c* is reduced by nitro anion-radicals, being in the equilibrium with redox pair $O_2/\dot{O}_2^-$ [5,6]. The efficiency of one-electron reduction of all compounds tested did not depend on the NAD^+/NADH ratio and was not influenced by rotenone.

Discussion

The reduction of soluble quinones, including ubiquinone-1 by isolated mitochondrial complex I (Fig. 1) is less sensitive to the action of inhibitors of ubiquinone-binding site, comaring to that of submitochondrial particles [20]. This may be determined by the diminished phospholipid/protein ratio which increases the affinity of quinones to the rotenone-sensitive site [10]. It is evident that ubiquinone-1 is reduced at two sites of the isolated complex I, one possessing higher TN and lower TN/K_m (rotenone-insensitive), and an other sensitive to rotenone and possessing lower TN, but higher TN/K_m (Fig. 1). Other quinones are reduced at the rotenone-insensitive center.

The double inhibition by NADH and ferricyanide [19], which is not characteristic of reduction of quinones, and different effects of mercurials and cross-linking agents towards these reactions [14,20] indicate that these classes of oxidizers are reduced at nonidentical sites. This is further supported by the inhibition analysis of these reactions (Figs. 2–4). The inhibition of NADH: ferricyanide reductase reaction by NAD^+ (Fig. 2) is consistent with the 'ping-pong' scheme with a single binding site for all reagents and products [23,24]. It indicates that NAD^+ competes for an NADH binding site in an oxidized form of a complex and binds to the reduced active center, acting as a competitive inhibitor for ferricyanide. The possibility of NAD^+ to reoxidize the reduced active center is not excluded. However, it must be treated cautiously since the redox inactive ADP-ribose inhibits in an analoguous way. Contrary to this, TN/K_m of quinones does not depend on the inhibitor concentration, but on the NAD^+/NADH ratio Figs. 3A, 4). One must note that the half-maximal inhibition of quinone reductase activity takes place at an NAD^+/NADH ratio, corresponding to an apparent $E_{m,7}$ of −280 to −290 mV (Fig. 4) that lies within the range of standard potentials of N-1b, N-3 and N-4 iron-sulfur cluster of NADH: ubiquinone reductase (Table II). One can suppose that the cluster possessing this $E_{m,7}$ plays the major role in the reduction of quinones, and the residual activity is provided by the center of $E_{m,7} > -240$ mV (Fig. 4), if the equilibration with the redox pair NAD^+/NADH is rapid enough. The estimation of the E_m values of these centers seems hardly possible due to discrepancy of potentiometric data for various preparations (Table II) and uncertainty whether linear or branched electron-transfer takes place between the clusters [17,27]. First, let us consider the linear electron-transfer between the clusters arranged according to their redox potential increase, e.g., N-1b, N-4, N-3, N-2. If clusters N-1b, N-3 and N-4 equilibrate with the NAD^+/NADH couple rapidly enough, then the quinone reductase activity should be inhibited by the imposed redox potential without any residual activity, as a single component (Fig. 4). $E_{m,7}$ imposed must correspond to the lowest potential of a cluster at the beginning of the electron-transfer chain. However, the presence of a residual activity (Fig. 4) may be better explained by the arrangement of the components not according to their potentials, or by the branching of electron flux. Recently, the evidence has been presented about the possible location of N-4 aside the main electron-transfer chain [27]. In this case, N-4 seems to be the possible candidate for the residual quinone reductase activity.

On the other hand, the inhibition of quinone reduction by the redox-inactive ADP-ribose (Fig. 3B) indicates the possibility of another mechanism. In this case, quinones are reduced close to the pyridine nucleotide and ferricyanide binding site. The data of Fig. 4 may indicate the competition of pyridine nucleotides for a binding to a reduced complex I affecting the quinone-reducing center by induced conformational changes. One must take into account that it requires the presence of the binding site of NADH possessing a high affinity ($K_d \ll 10$ μM) since at that concentration of NADH the dependence of TN/K_m on NAD^+/NADH is still observed (Figs. 3, 4). However, this discrepancy may be explained assuming that affinity for NADH may vary for complex I, reduced to the different extents.

TABLE II

The data on potentiometry of clusters N-1b, N-3 and N-4 of various preparations of NADH: ubiquinone reductase

Cluster	E_m (mV) [a]
N-1b	−260 [b]; −245 [c]; −287 [d]; −318 [e]; −335 [f];
N-3	−245 [b]; −245 [c]; −305 [d]; −265 [e];
N-4	−245 [b]; −245 [c]; −295 [d]; −285 [e];

[a] pH-independent [26].
[b] Purified complex I (pH 8.0), titrated by redox mediators [25].
[c] Pigeon heart mitochondria (pH 7.0), titrated by redox mediators [26].
[d] The same as b, titrated by NAD^+/NADH [26].
[e] Electron-transport particles (pH 7.0), titrated by NAD^+/NADH [27].
[f] Purified complex I (pH 8.0), titrated by redox mediators [28].

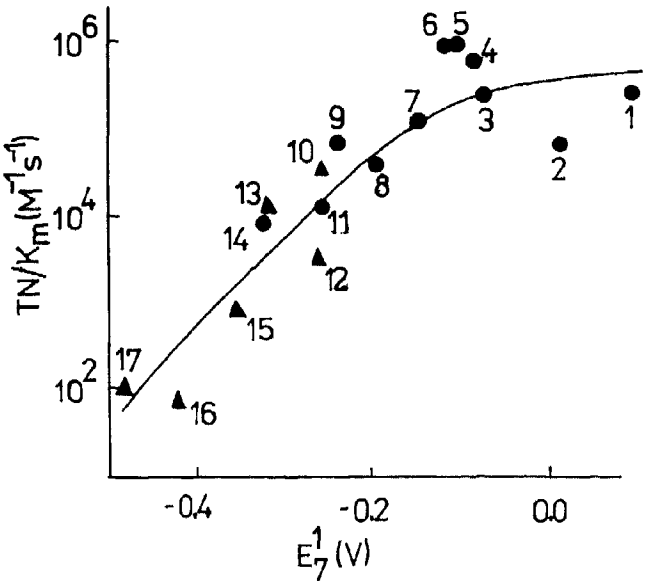

Fig. 5. The dependence of TN/K_m of oxidizers in the rotenone-insensitive oxidation of complex I on their one-electron reduction potential: 1,4-benzoquinone (1), 2-methyl-1,4-benzoquinone (2), 2,5-dimethyl-1,4-benzoquinone (3), 5-oxy-1,4-naphthoquinone (4), 5,8-dioxy-1,4-naphthoquinone (5), 9,10-phenanthrenequinone (6), 1,4-naphthoquinone (7), 2-methyl-1,4-naphthoquinone (8), ubiquinone-1 (9), nifuroxim (10), tetramethyl-1,4-benzoquinone (11), nitrofurantoin (12), 4-nitrobenzaldehyde (13), adriamycin (14), 4-nitroacetophenone (15), 4-nitrobenzoic acid (16), nitrobenzene (17).

The data presented here show that isolated complex I reduces quinones and nitrocompounds according to a mixed mechanism. The efficiency of one-electron reduction of benzoquinone is markedly lower than that for soluble low-molecular-weight NADH dehydrogenase, obtained by the method of Pharo et al. [21], where almost a 100% efficiency of one-electron reduction was observed. However, there exists a dependence of the $\log TN/K_m$ of oxidizers on their one-electron reduction potentials (E_7^1) (Fig. 5), similar to that predicted by the outer-spherical one-electron-transfer model [29]. One may suppose, that the mixed reduction of quinones proceeds via the initial one-electron step. Further, the semiquinone may dissociate from the active center or receive the second electron from the enzyme, being in the bound state. It seems that some structures of quinones may result in their increased reactivity (e.g., the proximity of oxy- and carbonyl groups of 5-oxy-1,4-naphthoquinone and 5,8-dioxy-1,4-naphthoquinone). Another interesting feature is that the reactivities of nitrocompounds correlate to the reactivities of quinones in the wide interval of E_7^1 (Fig. 5), and the efficiency of their one-electron reduction is even higher, than that of benzoquinone. It shows that quinoidal compounds are not specific oxidizers for the rotenone-insensitive reactions of complex I.

Acknowledgements

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