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NADH OXIDATION BY QUINONE ELECTRON ACCEPTORS

N.K. ČĖNAS, J.J. KANAPIENIENĖ and J.J. KULYS

Institute of Biochemistry, Lithuanian Academy of Sciences, Vilnius (U.S.S.R)

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The rate constants of NADH oxidation by quinones are increased with the oxidation potential increase: $\log k_{ox}$ ($M^{-1} \cdot s^{-1}$) = $-0.25 + 12.2\ E_7^0$ (V) for *o*-quinones and $\log k_{ox}$ ($M^{-1} \cdot s^{-1}$) = $-3.06 + 13.5\ E_7^0$ (V) for *p*-quinones (pH 7.0, 25°C). It is assumed that the oxidation proceeds via the hydride-ion transfer. The rate constants of NADH oxidation by single-electron quinone acceptors are also increased with the oxidizer potential increase; $\log k_{ox}$ ($M^{-1} \cdot s^{-1}$) = $-0.64 + 9.34\ E_7^0$ (V) and correlate with the constants of NADH oxidation by quinone radicals obtained earlier (Grodkowski, J., Neta, P., Carlson, B.W. and Miller, L. (1983) J. Phys. Chem. 87, 3135–3138). Single-electron transfer is the limiting stage of the process.

Introduction

The importance of biochemical reactions of NAD(P)H oxidation by flavins and carbonyl compounds caused a great interest in the study of the mechanism of these reactions [1–10]. It is assumed that 1,4-dihydropyridines are oxidized by flavins, and model quinone compounds by means of hydride transfer [1,5,9,10] with a possible formation of intermediate complexes [2,3,5]. Limiting stages of the processes are not uniquely enough determined, since there exist weighty arguments both for single-stage [1,9,10] and multistage hydride transfer [11,12]. Since flavins and phenylene-diimines show no relation between the acceptor two-electron oxidation-reduction potentials and its oxidative constant [2,4,7,8], it is assumed that the limiting stage of the process is not the electron transfer but the formation of preequilibrium complex [2,4] or the transfer of hydrogen atom [9,10]. Contrary to this, a linear dependence of the logarithm of NADH oxidation rate constant on the redox potential of *o*-quinones [6] exists which indicates the electron transfer as a possible limiting stage. For this reason, the reactions of NADH oxidation by quinones require a more thorough study, since such a dependence is also observed for the reactions of single-electron oxidation of NADH by phenothiazine and phenoxyl radicals [13].

The present paper deals with the reactions of NADH oxidation by *o*- and *p*-quinones and quinone radicals and studies the mechanism of the processes.

Experimental

Materials

NADH (Reanal, Hungary), tetracyano-*p*-quinodimethane (TCNQ), dichlorophenolindophenol and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) (Chemapol, Czechoslovakia) were used as received. Lithium salt of the anionic radical tetracyano-*p*-quinodimethane ($TCNQ^-$) was synthesized according to

Abbreviations: TCNQ, tetracyano-*p*-quinodimethane; TMPD, *N,N,N′,N′*-tetramethylphenylene-diamine; $TCNQ^-$, tetracyano-*p*-quinodimethane.

Ref. 14. 1,4-benzoquinone, 2-methyl-1,4-benzoquinone, 2-chloro-1,4-benzoquinone, 2,5-dioxybenzoic acid, 2-cyano-1,4-hydroquinone, 9,10-phenanthrenequinone, 1,2-naphthoquinone, sodium 1,2-naphthoquinone-4-sulphonate, 3-chloro-4,5-dioxyphenyl acetic acid (USSR) were recrystallized from benzol or ethanol. Reagents for buffer solutions and nitrogen were of highest purity.

Measurements

The rate of NADH oxidation by 1,4-benzoquinone, 2-methyl-1,4-benzoquinone and 2-chloro-1,4-benzoquinone was measured amperometrically with a polarograph LP-7e (Czechoslovakia) in a three-electrode circuit. 2–0.5 mM NADH was added to the acceptor solution (0.05 mM), and the current drop at the plato-potential of the acceptor reduction was recorded. The rate of NADH oxidation by 2-cyano-1,4-benzoquinone, 2-carboxyl-1,4-benzoquinone, $TMPD^+$, (3-chloro-1,2-benzoquinone-5-yl) acetic acid was measured by means of cyclic voltammetry using a polarograph LP-9 (Czechoslovakia) with a recorder XY-4103). Rate constants were calculated according to Ref. 15, recording the catalytic current value of NADH oxidation at the mediator oxidation potential. The concentration of the reduced form of the acceptor reached 0.1–0.05 mM, the concentration of NADH, 1–0.4 mM, the potential scan rate, 5–10 $mV \cdot s^{-1}$. The oxidation rate of NADH by the other acceptors was studied spectrophotometrically with a spectrophotometer Specord ultra violet VIS (GDR). The acceptor concentration was 0.1–0.03 mM. 2–0.5 mM NADH was introduced into the cell. Using TCNQ and 1,2-naphthoquinone-4-sulphonate equimolar (0.2–0.04 mM) concentrations of NADH and the acceptor were used. The solutions used were: 0.1 M potassium phosphate-citrate buffer (pH 7.8–5.0) and 0.1 M lithium phosphate-citrate buffer solution for TCNQ derivatives. The solution temperature was 25 ± 0.1 °C. Before measurements the solutions were thermostatted and bubbled with nitrogen for 15 min. The measurements were carried out in an anaerobic medium.

Complex formation of NADH with 1,4-benzoquinone and 2-methyl-1,4-benzoquinone was studied fluorimetrically with a spectrofluorimeter MPF-4 (Hitachi, Japan). The quenching of NADH fluorescence was studied at 470 nm in the presence of quinones (0.5–2.4 mM). A 0.03 mM solution of NADH in ethanol containing 2% water was used. The excitation wavelength was 370 nm, solution temperature 20 °C.

Results and Discussion

Amperometric and spectrophotometric measurements show that the concentration of quinones is decreased according to the first order on the introduction of large excess of NADH. The obtained constants of pseudofirst order are proportional to the NADH concentration. The second order constants based on the data of cyclic voltammetry do not vary with the NADH concentration. It indicates that the oxidation of NADH by quinone acceptors follows a bimolecular mechanism with the rate constants presented in Table I.

Reactivity increase with the increase in redox potential is characteristic of *o*- and *p*-quinones at pH 7.0 (Fig. 1). This dependence can be described by the following correlation equation:

o-quinones:

$$\log k_{ox}(M^{-1} \cdot s^{-1}) = -0.254 \pm 0.61 + (12.2 \pm 2.16)\, E_7^0\,(V)$$

$$(R = 0.9846) \quad (1)$$

p-quinones:

$$\log k_{ox}(M^{-1} \cdot s^{-1}) = -3.06 \pm 1.11 + (13.5 \pm 3.5)\, E_7^0\,(V)$$

$$(R = 0.9903) \quad (2)$$

Rate constants for *o*-quinones correlate well with the constants for other *o*-quinones obtained earlier (Fig. 1) [6], the reactivity of *p*-quinones is more than two orders smaller.

The reactivity of single-electron quinoidal acceptors (TCNQ, $TCNQ^-$, $TMPD^+$) occupies an intermediate position between *o*- and *p*-quinones (Fig. 1). Oxidation rate constants correlate with the earlier obtained constants of NADH oxidation by phenothiazine and phenoxyl radicals [13]:

$$\log k_{ox}(M^{-1} \cdot s^{-1}) = -0.6355 \pm 0.386 + (9.34 \pm 0.62)\, E_7^0\,(V)$$

$$(R = 0.9978) \quad (3)$$

TABLE I

RATE CONSTANTS OF NADH OXIDATION BY QUINOIDAL ELECTRON ACCEPTORS AT pH 7.0 AND AT 25 °C

Acceptor	k_{ox} ($M^{-1} \cdot s^{-1}$)	E_7^0 (V)	References for potential values
TCNQ	650	0.36	24
$TCNQ^-$	3.2	0.11	24
$TMPD^+$	50	0.27	13
(3-Chloro-1,2 benzoquinone-5-yl) acetic acid	2100	0.34 *	–
1,2-Naphthoquinone-4-sulphonate	330	0.215	6
1,2-Naphthoquinone	20	0.14	25
9,10-Phenanthrenequinone	0.9	0.02	25
2-Cyano-1,4-benzoquinone	200	0.39 *	–
2-Carboxy-1,4-benzoquinone	110	0.37 *	–
2-Chloro-1,4-benzoquinone	12	0.31	25
1,4-benzoquinone	2.5	0.27	25
2-Methyl-1,4-benzoquinone	0.5	0.21	25
Dichlorphenolindophenol	0.9	0.21	26

* Data on cyclic voltammetry in water medium.

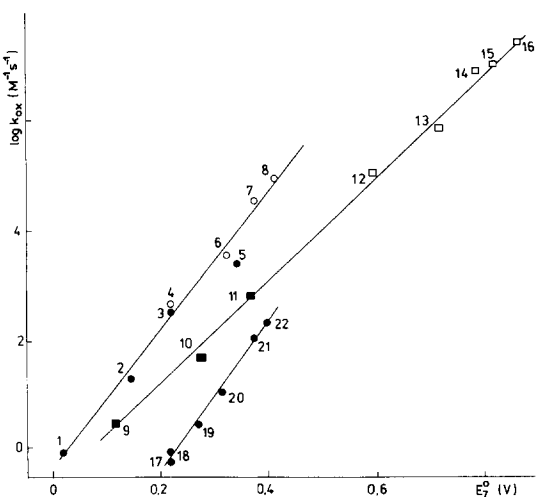

Fig. 1. Dependence of the rate constant of NADH oxidation on the potential of quinone electron acceptor. 9,10-Phenanthrenequinone (1), 1,2-naphthoquinone (2), 1,2-naphthoquinone-4-sulphonate (3, 4), (3-chloro-1,2-benzoquinone-4-yl)acetic acid (5), 4-methyl-1,2-benzoquinone (6), 4-aminoethyl-1,2-benzoquinone (7), 4-aminomethyl-1,2-benzoquinone (8), TCNQ (9), $TMPD^+$ (10), $TCNQ^-$ (11), *p*-methoxyphenoxyl (12), promazine$^+$ (13), chloropromazine$^+$ (14), *m*-benzosemiquinone (15), promethazine$^+$ (16), dichlorophenolindophenol (17), 2-methyl-1,4-benzoquinone (18), 1,4-benzoquinone (19), 2-chloro-1,4-benzoquinone (20), 2-carboxy-1,4-benzoquinone (21), 2-cyano-1,4-benzoquinone (22). pH 7.0, 25 °C. (4) and (6)–(8) are the data from Ref. 6, (12)–(16) are from Ref. 13.

The rate of NADH oxidation by *o*- and *p*-quinones does not change over the pH range 5–7.8 (Fig. 2). Over the pH range 5.5–7.8, TCNQ oxidizes NADH at the constant rate, but the oxidation rate of $TCNQ^-$ increases with the pH decrease, the proportionality coefficient $\Delta \log k_{ox}/\Delta$ pH equals 0.5 (Fig. 2).

In the presence of 25% ethanol in the solution, the oxidation rate of NADH by quinones decreases 2.5–3-times, though it remains the same with TCNQ. In 98% ethanol solution, the oxida-

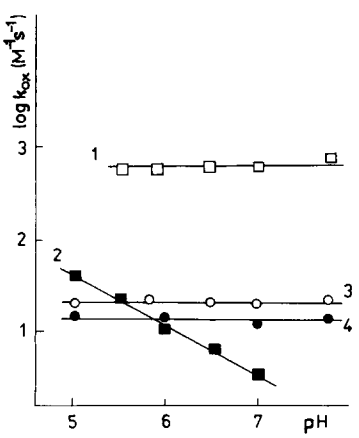

Fig. 2. The pH dependence of the NADH oxidation rate by quinoidal electron acceptors: TCNQ (1), $TCNQ^-$ (2), 1,2-naphthoquinone (3), 2-chloro-1,4-benzoquinone (4), 25 °C.

tion of NADH by 1,4-benzoquinone and 2-methyl-1,4-benzoquinone is approx. 100-times slower than in aqueous medium.

After the addition of quinones to NADH solution in 98% ethanol a weak (15–20%) quenching of NADH fluorescence occurs. It may be accounted for by the formation of a complex between NADH and acceptor, and the constants of the complex formation, calculated according to the Stern-Volmer equation [16], are 60 and 90 M^{-1} for 1,4-benzoquinone and 2-methyl-1,4-benzoquinone, respectively. However, in aqueous media one cannot observe any quenching, therefore a probable complex formation between NADH and quinones does not occur and determined second-order rate constants reflect the bimolecular process.

For single-electron acceptors the linear dependence of the oxidation constant logarithm on the acceptor potential indicates the electron transfer as a possible limiting stage during NADH oxidation. According to Marcus' theory used for outer-spherical electron transfer [17], the rate constant of electron transfer between the reagents (k_{12}) can be expressed by the equation:

$$k_{12} = (k_{11}k_{22}Kf)^{1/2} \quad (4)$$

where k_{11} and k_{22} are the internal exchange constants for every reagent, K is the equilibrium contant, $f \approx 1$. In our case, k_{12} and K are the rate and equilibrium constants, respectively, for electron transfer from NADH to the acceptor, k_{11} and k_{22} are the rates of electron exchange between reduced and oxidized forms in each redox couple that takes part in the reaction. If the reagent with k_{11} interacts with a series of reagents for which k_{22} is constant, then a linear dependence of $\log k_{12}$ on the potential difference of reagents (ΔE) is observed:

$$\log k_{12} = 8.4\, n\, \Delta E + \text{constant} \quad (5)$$

where n is the number of electrons transferred. (In biological redox systems such a dependence is observed for glucose oxidase oxidation by quinones [18] and cytochrome c reduction by hydroquinones [19]).

For single-electron quinoidal acceptors, TCNQ, $TCNQ^-$ and $TMPD^+$, the proportionality coefficient $\Delta \log k_{ox}/\Delta E_7^0$ equals 9.35 (Eqn. 3), which is close to the value predicted by Marcus for single-electron transfer (Eqn. 5). Oxidation constants correlate with those of other quinone radicals oxidizing NADH in a single-electron way [13] (Fig. 1). It is also known that in the analogous reaction, 1,4-dihydrpbenzylnicotinamide oxidation by tetracyanoethylene, a single-electron transfer takes place [20]. So it follows that during NADH oxidation by single-electron quinone acceptors the single-electron transfer acts as the limiting stage. The pH dependence on NADH oxidation rates by TCNQ derivatives supports the assumption made in Ref. 13 that the limiting stage of the single-electron oxidation of NADH proceeds according to the following scheme:

$$NADH - e^- \rightarrow NADH^+ \quad (6)$$

and the reaction standard potential (0.3–0.28 V) [21] does not depend on pH. Our data on cyclic voltammetry indicate that the TCNQ reduction over the pH range studied takes place according to:

$$TCNQ + e^- \rightarrow TCNQ^- \quad (7)$$

$$TCNQ^- + e^- + H^+ \rightarrow TCNQH^-$$

If the standard potential of $NADH^+/NADH$ does not depend on pH, then the pH changes must not affect the TCNQ oxidation rates, since the potential difference of redox pairs $TCNQ/TCNQ^-$ and $NADH^+/NADH$ does not change. Besides, on changing pH from 7.0 to 5.0, the potential of redox pair $TCNQ^-/TCNQ^-$ increases by 0.12 V, and at pH 5.0 k_{ox} for $TCNQ^-$ must increase 10-times in comparison with pH 7.0, as it follows from Eqn. 5. Such dependences are observed experimentally (Fig. 2).

The behaviour of NADH oxidation by quinones differs in some aspects from that of single-electron acceptors. So the proportionality coefficient $\Delta \log k_{ox}/\Delta E_7^0$ in Eqns. 1 and 2 considerably exceeds 8.4. Irrespectively of the increase in the potential difference of quinones and NADH at lower pH, the k_{ox} value of quinones does not change markedly (Fig. 2). It is similar to the pH dependence of flavin reactivity for which the single-stage hydride transfer is most probable in NADH oxidation [1,9,10]. The reactivity of quinones as well as of flavins is decreased on

decreasing the solution dielectric constant by means of ethanol introduction [1,4] in which respect they differ from TCNQ. So it follows that the linear dependence of $\log k_{ox}$ vs. E_7^0 is not related to electron transfer as a limiting step. Eqns. 1 and 2 do not reflect the reactivity increase due to a more effective complex formation too, since the formation of weak complexes between NADH and quinones occur only in ethanolic solutions where the dielectric constant is lower. On the other side, it is known that a linear dependence exists between the redox potentials of substituted quinones and the Taft constants of substituents [22]. It is also known that the hydride transfer reactions in oxidizing a number of 1-substituted 1,4-dihydropyridines by flavins can be described by the Taft equation [23]. So one can assume that Eqns. 1 and 2 are the modified Taft equations for hydride transfer during the oxidation of NADH by quinones. High reactivity of *o*-quinones may be accounted for by the structural analogy of reactive centres of *o*-quinones and flavins in which the site of hydride acceptation *N* [5] is adjacent to carbonyl group O (4) [14].

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