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# Electron transfer reactions of *Anabaena* PCC 7119 ferredoxin:NADP$^+$ reductase with nonphysiological oxidants

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**Abstract**

The mechanism of single-electron oxidation of ferredoxin-NADP$^+$ reductase (FNR) (EC 1.18.1.2) from cyanobacterium *Anabaena* PCC 7119 by quinones, aromatic nitrocompounds and inorganic complexes has been studied. In steady-state experiments, the logarithms of bimolecular rate constants of reduction of quinones and nitroaromatics increase with an increase in their single-electron reduction potential, the reactivities of nitroaromatics being markedly lower than of quinones. The absence of inhibition of reaction by ferredoxin and insignificant ionic strength effects suggest that positively charged ferredoxin binding site of FNR is not involved in reduction. In stopped-flow kinetics of oxidation of photoreduced enzyme by 5,8-dihydroxy-1,4-naphthoquinone, the oxidation of FADH$^\cdot$ to FAD proceeds much slower than oxidation of FADH$^-$ to semiquinone. The patterns of reaction inhibition by NADP$^+$ and 2′,5′-ADP also suggest that oxidation of FAD semiquinone is a rate-limiting step in oxidative half-reaction of steady-state experiments. The analysis of reaction kinetics within the framework of 'outer-sphere' electron transfer model gives the values of electron self-exchange constants of FAD/FADH$^\cdot$ couple and site-to-surface distances, 0.82–0.95 nm, that seem overestimated in view of available data on the accessibility of FAD to solvent. A possible explanation of poor reactivity of FAD/FADH$^\cdot$ redox couple of ferredoxin:NADP$^+$ reductase in comparison to FADH$^\cdot$/FADH$^-$ couple is that oxidation of FADH$^-$ to semiquinone re-presents a 'pure' electron transfer, whereas oxidation of FADH$^\cdot$ to FAD is electron transfer coupled to a slower proton transfer.

*Keywords:* Ferredoxin:NADP$^+$ reductase; *Anabaena*; Quinones; Aromatic nitrocompounds; Inorganic complexes; Electron transfer mechanism

## 1. Introduction

Ferredoxin:NADP$^+$ reductase (FNR, EC 1.18.1.2) is a FAD containing enzyme that participates in the reductive side of the photosynthetic chain of higher plants and cyanobacteria, transferring electrons from reduced ferredoxin to NADP$^+$ [1]. The primary structure of ferredoxin:NADP$^+$ reductase from cyano-

Abbreviations: FNR, ferredoxin:NADP$^+$ reductase; $k_{cat}$, catalytic constant; $k_{cat}/K_m$, bimolecular rate constant; $E_7^1$, single-electron reduction potential at pH 7.0; $\nu_{ET}$, limiting rate constant of electron transfer; $4\Delta G^{\neq}(0)$, reorganization energy; $k_{11}$, $k_{22'}$, reagent electron self-exchange constants; $R_{p'}$ distance of electron transfer

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bacterium *Anabaena* PCC 7119 is about 50% homologous to enzymes from higher plants [2]. A standard redox potential of FAD of *Anabaena* FNR ($E_7^0$), −0.344 V, spectral properties of neutral FAD semiquinone [3] and dissociation constants of enzyme complexes with NADP(H) [4] are similar to corresponding parameters of spinach FNR [5,6]. Recently, a three-dimensional structure of *Anabena* FNR has been obtained at 0.18 nm resolution [7], closely resembling the structure and FAD environment of spinach enzyme [8].

Although extensive literature exists on the mechanism of electron transfer between FNR and ferredoxin [5,9–15], relatively little is known about the mechanisms of single-electron reduction of low-molecular weight oxidants and redox active xenobiotics by FNR. The physiological activity of quinoidal and nitroaromatic xenobiotics is often determined by their ability to be reduced in a single-electron way by low-potential NAD(P)H-oxidizing flavin dehydrogenases and iron-sulfur proteins [16–18]. Reoxidation of the radicals formed by oxygen results in redox cycling of parent compound and formation of activated oxygen species. Ferredoxin:$NADP^+$ reductase reduces quinones [19], aromatic nitrocompounds [20], viologen derivatives [21] and heteropentalenes [22] in a single-electron way, and is supposed to be at least partly responsible for their herbicidal action [21,22]. It has been shown that the steady-state rates of reduction of nitroaromatics and viologen derivatives by FNR depended mainly on their single-electron reduction potential ($E^1$) values [20,21], indicating the absence of marked structure specificity and pointing out to a possibility of an 'outer-sphere' electron transfer mechanism. However, these studies were performed using limited number of compounds with a narrow range of redox potentials, ca. 0.2 V [20,21]. For this reason, a more detailed analysis of these reactions within the framework of an 'outer-sphere' electron transfer, linking observed reaction rates with structural and thermodynamic parameters of FNR, is required. Besides, the mechanism of single- vs. two-electron transfer during reduction of quinones by flavoenzymes remains an important and insufficiently studied problem of flavin catalysis [23].

The aim of this work was the analysis of electron transfer reactions of *Anabaena* PCC 7119 ferredoxin-$NADP^+$ reductase with various classes of non-physiological oxidants (quinones, aromatic nitrocompounds and inorganic complexes) within the framework of 'outer-sphere' electron transfer model [24,25].

## 2. Materials and methods

### 2.1. Materials

Ferredoxin-$NADP^+$ reductase and ferredoxin were purified from *Anabaena* PCC 7119, and their concentrations determined as described earlier [26]. $NADP^+$, NADPH, 2′,5′-ADP, cytochrome *c*, superoxide dismutase, methylviologen and commercially available quinones were obtained from Sigma or Serva, adriamycin was obtained from Carlo Erba. Aromatic nitrocompounds and inorganic complexes were obtained from Reakhim (Russia) and purified as described previously [27]. 3-Glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone and 2,3-diglutathionyl-1,4-naphthoquinone were generous gifts of Dr. K. Ollinger (University of Linköping, Sweden). The formulas of some uncommon redox agents used in this work, are presented in Fig. 1. Nifurtimox and megazol were generous gifts of Dr. M. Wittner (Albert Einstein College of Medicine, New York), chinifur was synthesized by Dr. N.M. Sukhova (Institute of Organic Synthesis, Riga, Latvia). *N*-[(4-Nitroimidazole-5-yl)-carbonyl]hydrazone of acetaldehyde was

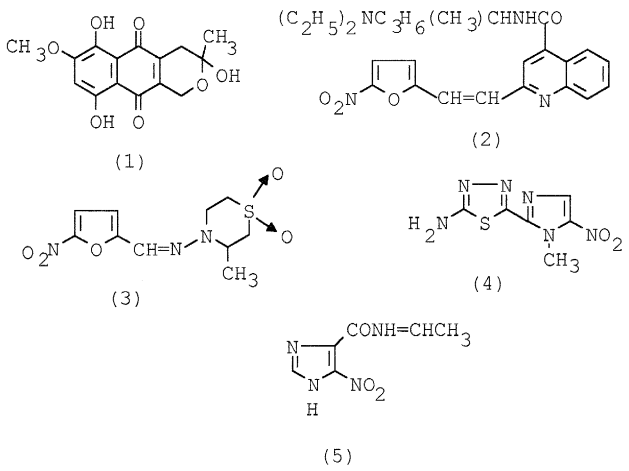


Fig. 1. Structural formulae of electron acceptors: fusarubin (1), chinifur (2), nifurtimox (3), megazole (4), *N*-[(4-nitroimidazol-5-yl)carbonyl]acetaldehyde (5).

synthesized by Drs. I.S. Selezneva and V.F. Gryazev (Ural Polytechnic Institute, Ekaterinburg, Russia). Fusarubin and 2,5-dihydroxy-1,4-naphthoquinone (Fig. 1) were generous gifts of Drs. A.G. Medentsev and V.K. Akimenko (Institute of Biochemistry and Physiology of Microorganisms, Pushchino, Russia). 5-Deazariboflavin was a kind gift of Dr. M. Medina (University of Zaragoza).

### 2.2. Methods

Kinetic experiments were carried out in 0.1 M phosphate buffer solution (pH 7.0) containing 1 mM EDTA at 25°C. For experiments with varied ionic strength, the 0.01 M–0.4 M phosphate buffer solutions (pH 7.0) were used. NADPH:acceptor reductase reaction rates of FNR were monitored according to the decrease of NADPH absorption ($\Delta_{340} = 6.2$ $mM^{-1} \cdot cm^{-1}$), using Kontron Unikon 860 or Hitachi 557 spectrophotometers. Kinetic parameters of the reaction, catalytic constant ($k_{cat}$), and bimolecular rate constant ($k_{cat}/K_m$) correspond to the reciprocal intercepts and slopes of the Lineweaver-Burk plots, $k_{cat}$ corresponds to the amount of NADPH oxidized by enzyme FAD per second. The rate of $O_2$ consumption was monitored using Clark electrode. The reduction of cytochrome *c* (50 μM) in the presence of 250 μM NADPH, quinones or ferredoxin (0.1 μM–1.0 μM) was monitored using $\Delta_{550} = 20$ $mM^{-1} \cdot cm^{-1}$.

The presteady-state anaerobic reoxidation of reduced FNR by 5,8-dihydroxy-1,4-naphthoquinone was monitored according to the decrease of absorption at 600 nm and increase of absorption at 460 nm, using stopped-flow system SX.17 MV from Applied Photophysics. The anaerobical conditions were established by repeated cycles of evacuation and flushing with argon, which have been purified by passage over a heated BASF catalyst. FNR was photoreduced in the presence of 5-diazariboflavin (1–2 μM) and 10 mM EDTA.

## 3. Results

The reduction of quinones by *Anabaena* ferredoxin:$NADP^+$ reductase follows a 'ping-pong' scheme, resulting in a series of parallel lines in Lineweaver–Burk plots at varied concentrations of quinones and fixed concentrations NADPH (data not shown). $k_{cat}$ of reduction of benzoquinone and naphthoquinone compounds extrapolated to infinite concentration of NADPH and oxidant, was equal to 100 $s^{-1}$ (on a two-electron base), and 25 $s^{-1}$ for fusarubin (Fig. 1) and adriamycin. $K_m$ of NADPH at the saturated concentrations of oxidant is close to 40

Table 1
The bimolecular rate constants ($k_{cat}/K_m$) of reduction of quinones by ferredoxin:$NADP^@$ reductase and the single-electron reduction potentials of oxidants ($E_7^1$) (0.1 M phosphate, pH 7.0)

| Oxidant | $k_{cat}/K_m$($M^{-1}$ $s^{-1}$) | $E_7^1$ [a](V) |
|---|---|---|
| 2,3-Dichloro-1,4-naphthoquinone | $2.0 \cdot 10^6$ ($6.7 \cdot 10^6$) [b] | −0.036 |
| 5-Hydroxy-1,4-naphthoquinone | $1.2 \cdot 10^6$ ($7.5 \cdot 10^6$) [b] | −0.09 |
| 5,8-Dihydroxy-1,4-naphthoquinone | $9.0 \cdot 10^5$ ($5.7 \cdot 10^6$) [b] | −0.11 |
| 9,10-Phenanthrenequinone | $2.2 \cdot 10^6$ ($7.1 \cdot 10^6$) [b] | −0.12 |
| 1,4-Naphthoquinone | $6.0 \cdot 10^5$ ($1.6 \cdot 10^6$) [b] | −0.15 |
| 2,3-Diglutathionyl-1,4-naphthoquinone | $2.7 \cdot 10^5$ | −0.15 |
| 5-Hydroxy-2-methyl-1,4-naphthoquinone | $6.7 \cdot 10^5$ | −0.16 |
| 2-Methyl-3-glutathionyl-1,4-naphthoquinone | $6.2 \cdot 10^5$ | −0.16 |
| 2-Methyl-1,4-naphthoquinone | $4.6 \cdot 10^5$ | −0.20 |
| Tetramethyl-1,4-benzoquinone | $2.0 \cdot 10^5$ | −0.26 |
| Fusarubin | $6.2 \cdot 10^5$ | −0.26 |
| Adriamycin | $6.3 \cdot 10^4$ | −0.33 |
| 2,5-Dihydroxy-1,4-naphthoquinone | $1.0 \cdot 10^4$ | −0.35 |
| 2-Hydroxy-1,4-naphthoquinone | $6.7 \cdot 10^3$ | −0.41 |

[a] The values of redox potential are from [18,27].
[b] Correspond to a 'slower' phase of quinone reduction in the presence of ferredoxin (Fig. 2).

Table 2
The bimolecular rate constants ($k_{cat}/K_m$) of reduction of aromatic nitrocompounds by ferredoxin:NADP@ reductase and the single-electron reduction potentials of oxidants ($E_7^1$) (0.1 M phosphate, pH 7.0)

| Oxidant | $k_{cat}/K_m(M^{-1} \cdot s^{-1})$ | $E_7^1$ [a](V) |
|---|---|---|
| Chinifur | $6.0 \cdot 10^4$ | −0.199 |
| Nifuroxim | $7.5 \cdot 10^3$ | −0.255 |
| Nitrofurantoin | $1.2 \cdot 10^4$ | −0.265 |
| Nifurtimox | $1.3 \cdot 10^4$ | −0.285 |
| 4-Nitrobenzaldehyde | $8.3 \cdot 10^3$ | −0.325 |
| 4-Nitroacetophenone | $1.0 \cdot 10^3$ | −0.355 |
| *N*-[(4-Nitroimidazol-5-yl)carbonyl]acetaldehyde | $5.0 \cdot 10^3$ | −0.367 |
| Megazole | $1.0 \cdot 10^3$ | −0.378 |
| 4-Nitrobenzoic acid | $2.5 \cdot 10^2$ | −0.425 |
| Nitrobenzene | $1.0 \cdot 10^1$ | −0.485 |
| Metronidazole | $1.1 \cdot 10^1$ | −0.485 |

[a] The values of redox potentials are from [17,18,27].

μM, which is similar to earlier reported values [28]. The bimolecular rate constants ($k_{cat}/K_m$) of quinones and nitroaromatics together with their single-electron reduction potentials ($E_7^1$) are presented in Tables 1 and 2.

In 0.1 M K-phosphate pH 7.0, $K_{m(app)}$ of ferredoxin in ferredoxin-dependent reduction of cytochrome *c* by FNR, was equal to 0.5 μM. Addition of this ferredoxin concentration stimulated quinone reductase activity of FNR at low benzo- or naphthoquinone concentrations ($< 10$ μM), causing a biphasicity of Lineweaver–Burk plots (Fig. 2). The kinetics of a 'faster' phase (high concentrations and lower $k_{cat}/K_m$ of oxidant) were almost indistinguishable from the data in the absence of ferredoxin. The $k_{cat}$ for a 'slower' phase (low concentrations and higher $k_{cat}/K_m$ of oxidant) was equal to 20–25 $s^{-1}$, and corresponded to about 50% of cytochrome *c* reduction rate at this ferredoxin concentration. The biphasicity of Lineweaver–Burk plots in the presence of ferredoxin is analogous to kinetics of quinone reductase reaction of NADPH:adrenodoxin reductase in the presence of adrenodoxin [29], and could be explained by existence of additional pathway of quinone reduction, via reduced ferredoxin. Thus, the formation of electrostatic complex of FNR and ferredoxin does not inhibit quinone reductase reaction, moreover, it provides an additional pathway of quinone reduction. The values of $k_{cat}/K_m$ of several quinones corresponding to a 'slower' phase of kinetics in the presence of ferredoxin, are given in Table 1.

At fixed concentration of quinone and varied concentration of NADPH, $NADP^+$ and redox inactive 2′,5′-ADP acted as competitive vs. NADPH inhibitors, increasing the slopes in Lineweaver–Burk plots and do not affecting intercepts. $K_i$ of $NADP^+$ and 2′,5′-ADP were equal to 27 μM and 30 μM, respectively. At fixed concentration of NADPH (250 μM), $NADP^+$ and 2′,5′-ADP (250–1000 μM) acted

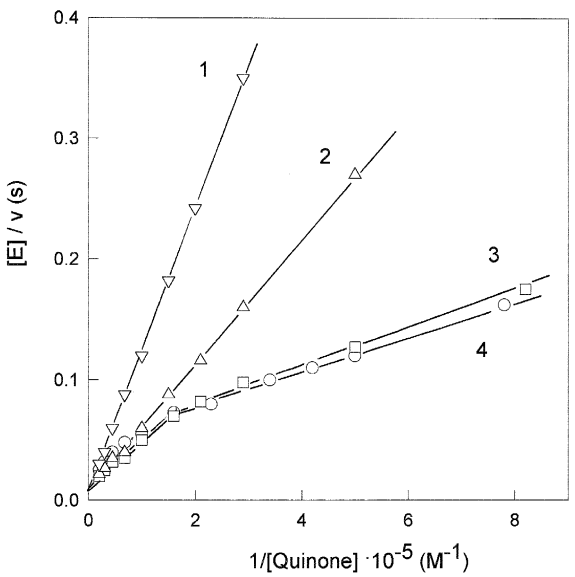


Fig. 2. Kinetics of reaction of 5-hydroxy-1,4-naphthoquinone (1,4) and 2,3-dichloro-1,4-naphthoquinone (2,3) with ferredoxin:$NADP^+$ reductase in the absence (1,2) and in the presence of 0.5 μM ferredoxin (3,4). 0.1 M phosphate, pH 7.0, 250 μM NADPH.

as uncompetitive inhibitors vs. electron acceptor (5-hydroxy-1,4-naphthoquinone or 5,8-dihydroxy-1,4-naphthoquinone) with $K_i = 250$ μM, yielding a series of parallel plots in Lineweaver–Burk coordinates (data not shown).

By analogy with previous observations [20], the rates of enzymatic oxidation of NADPH by quinones and nitroaromatics were equal to the rates of $O_2$ consumption. In parallel experiments, the reduction of added cytochrome *c* has been observed, approximately at doubled rates of NADPH oxidation. This process was almost completely inhibited by superoxide dismutase (30 μg/ml).

The single-electron oxidation of two-electron reduced FNR by quinones should proceed in two steps, i.e., oxidation of $FADH^-$ to $FADH^{\cdot}$, and oxidation of $FADH^{\cdot}$ to FAD. In order to evaluate the slower step in the overall process, we have performed the stopped-flow kinetic studies of reoxidation of photoreduced FNR by 5,8-dihydroxy-1,4-naphthoquinone. In the course of reaction, a partial increase in absorbance at 460 nm and 600 nm has been observed during instrument dead time, 3 ms. The further increase in absorbance at 460 nm and decrease in absorbance at 600 nm obeyed first-order kinetics (Fig. 3A). The amplitude of observed absorbance changes at 600 nm was consistent with formation and subsequent decay of 80–85% of stoichiometrical amount of FAD semiquinone, using an assumed value of $\Delta A_{600} = 5\ mM^{-1} \cdot cm^{-1}$ [3,5] (Fig. 3A). The absorbance change at 460 nm was by 30–40% less than expected using an assumed value $\Delta A_{460} = 7\ mM^{-1} \cdot cm^{-1}$ (the absorbance difference between oxidized and single-electron reduced spinach FNR [5]), evidently due to a parallel absorbance decrease during reduction of 5,8-dihydroxy-1,4-naphthoquinone. The observed first-order rate constants ($k_{obsd}$) directly depended on 5,8-dihydroxy-1,4-naphthoquinone concentration (15–75 μM) at both wavelengths used (Fig. 3B,C), yielding bimolecular rate constant of reoxidation, $9.5 \pm 0.9 \cdot 10^5\ M^{-1}\ s^{-1}$. Thus, it appears that during oxidation of two-electron reduced *Anabaena* FNR by quinones, the oxidation of $FADH^{\cdot}$ to FAD proceeds slower than oxidation $FADH^-$ to $FADH^{\cdot}$. This is analogous to the data of reoxidation of dithionite-reduced spinach FNR by other single-electron oxidant, ferricyanide [5]. We have considered the possibility that in conditions

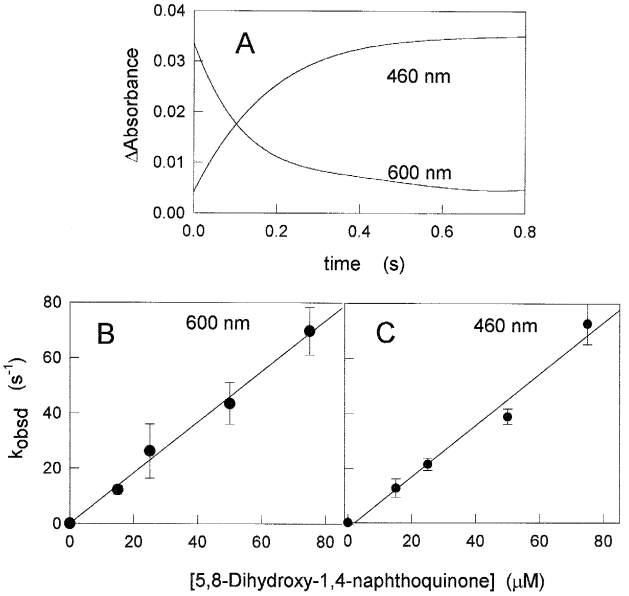


Fig. 3. Reoxidation of photoreduced FNR by 5,8-dihydroxy-1,4-naphthoquinone in stopped-flow experiments. A: kinetic traces of absorbance changes at 460 nm and 600 nm during oxidation of 7.5 μM photoreduced FNR by 15 μM quinone (concentrations after mixing). Dependence of first-order oxidation rate constants on oxidant concentration, determined at 600 nm (B) and 460 nm (C). 0.1 M phosphate, pH 7.0.

analogous to steady-state experiments, i.e., in the presence of excess NADPH, a complex of two-electron reduced FNR and NADPH ($K_d \approx 1$ μM [4,6]) and, probably, a complex of NADPH and FNR semiquinone, could act as electron donors. However, the stopped-flow experiments of reoxidation of reduced FNR-NADPH complex would result in multiple turnover. Besides, in our understanding, the thermodynamic parameters of FNR should not be markedly altered in the presence of NADPH or $NADP^+$, since the redox titrations of spinach or *Anabaena* FNR by redox mediators or by $NADP^+$/NADPH give almost identical values of two-electron reduction potentials, and do not change noticeably the stability of semiquinone [3,4,6,28,30].

In order to elucidate the role of electrostatic interactions in reoxidation of FNR by electron acceptors, we have investigated effects of solution ionic strength on kinetics of reduction of positively charged methylviologen and negatively charged ferricyanide and $Fe(EDTA)^-$. In 0.1 M phosphate, pH 7.0, the latter compounds are reduced with $k_{cat}/K_m$ equal to $1.2 \cdot$

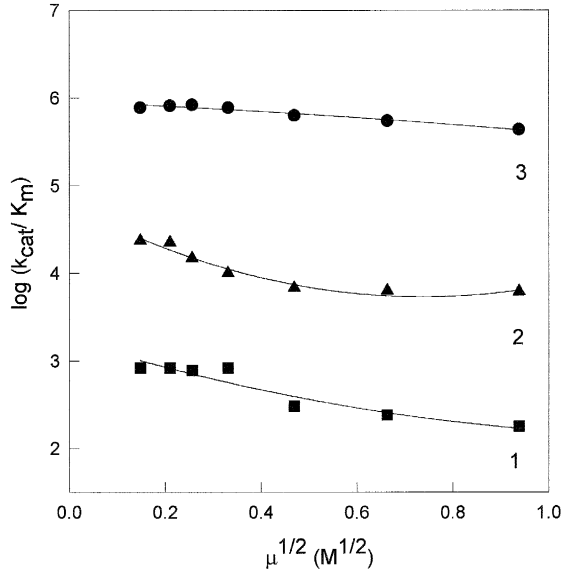


Fig. 4. Dependence of $k_{cat}/K_m$ of Fe(EDTA)$^-$ (1), methylviologen (2) and ferricyanide (3) reduction on solution ionic strength, pH 7.0.

$10^6$ $M^{-1} \cdot s^{-1}$ and $1.3 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ (on the one-electron base), respectively. In accordance with previous observations [21], $k_{cat}/K_m$ of methylviologen decreased with an increase of ionic strength (Fig. 4). However, a decrease has been also observed for $k_{cat}/K_m$ of Fe(EDTA)$^-$, and reactivity of ferricyanide almost did not depend on ionic strength.

## 4. Discussion

By analogy with previous findings [21,22], the data of this work indicate that single-electron reduction of quinones and aromatic nitrocompounds by *Anabaena* ferredoxin:NADP$^+$ reductase proceeds with insignificant structural specificity for oxidants (Tables 1 and 2), and that the main factor governing the reduction rate is the energetics of single-electron transfer. For analysis of oxidative half-reaction, we should identify the redox state of enzyme, responsible for observed steady-state $k_{cat}/K_m$ of quinone reduction. It follows from presteady-state experiments (Fig. 3) that oxidation of two-electron reduced FNR to the semiquinone by quinones is a very fast process, and that the oxidation of FADH$^\cdot$ occurs at measurable rate. Thus, observed $k_{cat}/K_m$ of acceptors could reflect the oxidation of semiquinone form of FNR. Another point of evidence is that the steady-state $k_{cat}/K_m$ of 5,8-dihydroxy-1,4-naphthoquinone (Table 1) is close to a bimolecular rate constant of semiquinone oxidation obtained in presteady-state conditions. The character of inhibition by reaction product NADP$^+$ also provides indirect support for involvement of FNR semiquinone in a rate-limiting step of oxidative half-reaction. The competitive vs. NADPH and uncompetitive vs. quinone acceptor inhibition by NADP$^+$ indicates that NADP$^+$ competes for a NADPH-binding site in the oxidized enzyme, and does not compete with electron acceptor in the reoxidation of reduced enzyme form. If two-electron reduced FNR is reoxidized in a rate-limiting step, we should observe a decrease of $k_{cat}/K_m$ of oxidant in the presence of NADP$^+$, since the rates of reoxidation of two-electron reduced spinach or *Anabaena* FNR by NADP$^+$ are fast, exceeding 100 $s^{-1}$ [5,6,21]. In our understanding, the decrease of $k_{cat}/K_m$ of oxidant in the presence of NADP$^+$ should be well pronounced, since, under conditions of our experiments, the variation of [NADP$^+$]/[NADPH] ratio from 1:1 to 4:1 imposes the redox potential of media between $-0.32$ V and $-0.302$ V, that is more positive than $E_7^0$ of FNR, $-0.344$ V [3]. However, this was not observed, evidently, due to low rate of reoxidation of single-electron donor, FNR semiquinone, by NADP$^+$. (The more detailed analysis of possible reaction product inhibition patterns, including the reversal of reaction equilibrium, is given in Refs. [31,32]).

In a next step, dependences of log $k_{cat}/K_m$ of quinones and aromatic nitrocompounds vs. their $E_7^1$ values have been fitted by a nonlinear least-squares regression to the 'outer-sphere' electron transfer equation [25]:

$$\log \frac{k_{cat}}{K_m} = \log \nu_{ET} - \frac{\{4\Delta G^{\neq}(0) - nF\Delta E_7^1\}^2}{2.303 \cdot RT \cdot 4 \cdot \Delta G^{\neq}(0)} \qquad (1)$$

where $\nu_{ET}$ is the limiting rate constant of electron transfer, $4\Delta G^{\neq}(0)$ is the reorganization energy, $n$ is the number of electrons transferred, and $\Delta E_7^1$ is the difference in redox potentials of oxidants and of FAD/FADH$^\cdot$, $-0.376$ V [3]. For quinone reduction (Fig. 5A), the calculated values of $\Delta G^{\neq}(0)$ (0.17

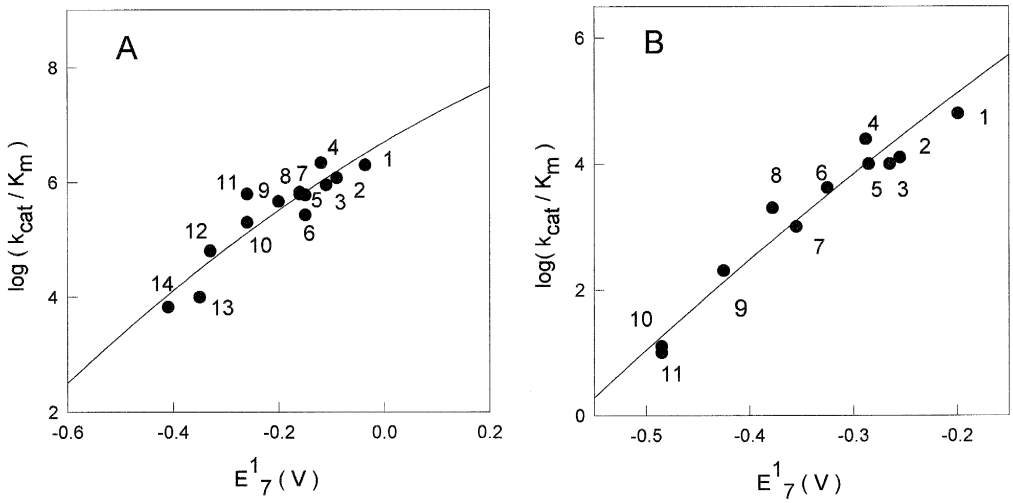


Fig. 5. Dependence of $k_{cat}/K_m$ of quinones (A) and aromatic nitrocompounds (B) on their single-electron reduction potentials, fitted to Eqn. 1. A: quinones: 2,3-dichloro-1,4-naphthoquinone (1), 5-hydroxy-1,4-naphthoquinone (2), 5,8-dihydroxy-1,4-naphthoquinone (3), 9,10-phenanthrene quinone (4), 1,4-naphthoquinone (5), 2,3-diglutathionyl-1,4-naphthoquinone (6), 5-hydroxy-2-methyl-1,4-naphthoquinone (7), 2-methyl-3-glutathionyl-1,4-naphthoquinone (8), 2-methyl-1,4-naphthoquinone (9), tetramethyl-1,4-benzoquinone (10), fusarubin (11), adriamycin (12), 2,5-dihydroxy-1,4-naphthoquinone (13), 2-hydroxy-1,4-naphthoquinone. B: aromatic nitrocompounds: chinifur (1), nifuroxim (2), nitrofurantoin (3), nifurtimox (4), 4-nitrobenzaldehyde (5), 4-nitroacetophenone (6), *N*-[(4-nitroimidazol-5-yl)carbonyl]-acetaldehyde (7), megazole (8), 4-nitrobenzoic acid (9), nitrobenzene (10), metronidazole (11).

$\text{eV} \cdot \text{mol}^{-1}$ or 16.6 $\text{kJ} \cdot \text{mol}^{-1}$) and $\nu_{\text{ET}}$ ($4 \cdot 10^9$ $\text{M}^{-1} \cdot \text{s}^{-1}$) are in the range of corresponding parameters of reactions of another quinoidal compound, lumiflavin semiquinone, with series of redox proteins [25]. For aromatic nitrocompounds (Fig. 5B), $\Delta G^{\neq}(0) = 0.24$ $\text{eV} \cdot \text{mol}^{-1}$ (23.2 $\text{kJ} \cdot \text{mol}^{-1}$). Alternatively, the rate constant of electron transfer between reagents ($k_{12}$) may be expressed as a function of electron self-exchange constants of redox protein ($k_{11}$), electron acceptor ($k_{22}$), and equilibrium constant of reaction ($K$) ($\log K = n\Delta E_7^1/0.059$) [24]:

$$k_{12} = (k_{11} \cdot k_{22} \cdot K \cdot f)^{1/2} \quad (2)$$

and

$$\log f = \frac{(\log K)^2}{4 \cdot \log(k_{11} \cdot k_{22}/Z^2)} \quad (3)$$

where $Z$ is a frequency factor ($10^{11}$ $\text{M}^{-1}$ $\text{s}^{-1}$). The self-exchange rate constant of redox protein may be calculated using the Eqn. 4 [33]:

$$\log k_{11} = (\log k_{12} - 0.5 \log K + \log Z) - \log k_{22} - \left\{(\log Z - \log k_{12})^2 + \log K (\log Z - \log k_{12})\right\}^{1/2}$$

For quinoidal compounds, $k_{22}$ is $10^8$ $\text{M}^{-1} \cdot \text{s}^{-1}$ [34], whereas for nitroaromatics it is markedly lower, $10^4$ $\text{M}^{-1} \cdot \text{s}^{-1}$ [35]. Thus, the lower reactivity and higher reorganization energy of FNR towards nitroaromatics (Fig. 5A,B) could be at least partly explained by differences in $k_{22}$ for quinones and nitroaromatics. The $k_{11}$ value of FNR may be approximately estimated from the data of Fig. 5A,B, corresponding to $\Delta E_7^1 = 0$, i.e., $E_7^1$ of oxidant being equal to $-0.376$ V. These data are presented in Table 3, together with

Table 3
Electron self-exchange rate constants ($k_{22}$), and redox potentials ($E_7^1$) of oxidants and estimated electron self-exchange rate constants ($k_{11}$) of ferredoxin:$\text{NADP}^+$ reductase and NADPH:cytochrome P-450 reductase (P-450R) for reactions with quinones, aromatic nitrocompounds, ferricyanide and $\text{Fe(EDTA)}^-$

| Oxidant | $k_{22}$($\text{M}^{-1} \cdot \text{s}^{-1}$) | $E_7^1$ [a] (V) | $k_{11}$($\text{M}^{-1} \cdot \text{s}^{-1}$) | |
|---|---|---|---|---|
| | | | FNR | P-450 R [b] |
| Quinones | $1 \cdot 10^8$ | – | 2.5 | $1 \cdot 10^4$ |
| Nitroaromatics | $1 \cdot 10^4$ | – | 10 | $4 \cdot 10^4$ |
| Ferricyanide | $4.6 \cdot 10^5$ [a] | 0.41 | $2 \cdot 10^{-5}$ | $6 \cdot 10^{-3}$ |
| $\text{Fe(EDTA)}^-$ | $6.9 \cdot 10^4$ [a] | 0.12 | $5.1 \cdot 10^{-7}$ | $4 \cdot 10^{-2}$ |

[a] Data from [36].
[b] Data from [27].

the values of $k_{11}$ of FNR for reduction of ferricyanide and $Fe(EDTA)^-$ calculated according to Eqn. 4. It is evident that $k_{11}$ for reduction of quinones and nitrocompounds are markedly higher than those for reduction of ferricyanide and $Fe(EDTA)^-$ (Table 3). This is most probably caused by an increased ability of aromatic compounds to penetrate into the protein globule and, consequently, to decrease the distance of electron transfer [24,36]. For comparison, Table 3 also contains electron self-exchange constants of FMN of another dehydrogenase-electrontransferase, NADPH:cytochrome P-450 reductase, determined in our previous work [27]. It is evident that for all types of nonphysiological oxidants investigated, $k_{11}$ of FNR are considerably lower than those of cytochrome P-450 reductase (Table 3).

According to the model of Mauk et al. [36], at an infinite solution ionic strength where electrostatic interactions are absent, the $k_{11}$ value of protein ($k_{11}$) for the reaction with the inorganic complex is related to the distance (in nm) of electron transfer ($R_p$):

$$R_p = 0.63 - 0.035 \cdot \ln k_{11} \quad (5)$$

For a hydrophilic oxidant, e.g., $Fe(EDTA)^-$, which is incapable of entering the protein globule, $R_p$ obtained from this equation and decreased by Van der Waals' radius of aromatic carbon (0.18 nm) is supposed to be equal to the shortest distance of the active center from the surface of protein globule. With respect to the interaction of FNR with negatively charged ferredoxin, it is known that the affinity of FNR for ferredoxin decreases on the increase of solution ionic strength [15], and that positively charged groups (Lys 53, Arg 77 and Lys 294) of *Anabaena* FNR are involved in the formation of electrostatic complex [7]. However, $k_{cat}/K_m$ of both positively and negatively charged low-molecular weight oxidants decrease on the increase of ionic strength (Fig. 4), besides, the effects of ferredoxin upon quinone reductase kinetics (Fig. 2) argue against involvement of ferredoxin binding site in acceptor reduction. It implies that ionic strength effects observed (Fig. 4) more probably reflect other factors differing from electrostatic interactions, e.g., conformational changes of protein globule. Thus, the site-to-surface distances of FAD of FNR were calculated according to rate constants in 0.1 M phosphate (Table 3). Their values (0.95 nm for $Fe(EDTA)^-$ and 0.82 nm for ferricyanide) markedly exceed the site-to-surface distance of FMN of cytochrome P-450 reductase (0.63 nm–0.55 nm) and flavodoxin (0.34 nm), calculated in our previous work according the same method [27]. It seems that calculations according to this model give overestimated distances, since, according to the X-ray data [7,8,37], dimethylbenzene ring of FAD of FNR, and FMN of flavodoxin are partly exposed to solvent. One could argue that *re*-side of isoalloxazine ring of FAD of *Anabaena* FNR, interacting with pyridine nucleotides, is shielded from solvent by tyrosine-303, and *si*-side — by tyrosine-79 [7], and that these interactions could decrease the rates of electron transfer. However, our data indicate that the oxidation of $FADH^-$ to $FADH^\cdot$ of FNR proceeds much faster than oxidation of $FADH^\cdot$, irrespective of a less negative redox potential of $FADH^-/FADH^\cdot$ couple, $-0.312$ V. This points out to a higher electron self-exchange constant of $FADH^-/FADH^\cdot$ couple and, possibly, to a lower electron transfer distance or kinetic stabilization of $FAD/FADH^\cdot$ couple. Thus, other factors determining reaction rates of $FADH^-/FADH^\cdot$ and $FADH^\cdot/FAD$ couples should be considered.

The data of this paper and previous investigations [5,23,38] indicate that oxidation of two-electron reduced FNR by quinones and ferricyanide proceed by analogy with corresponding reactions of NADH:cytochrome $b_5$ reductase, where a transient formation of neutral FAD semiquinone has been also observed. In both enzymes, the redox potential of $FAD/FADH^\cdot$ couple is more negative than redox potential of $FADH^\cdot/FADH^-$, and significant homology of three-dimensional structure exists in the surroundings of FAD [39,40]. The data of pulse-radiolysis of cytochrome $b_5$ reductase indicate that the initial formation of anionic $FAD^-$ semiquinone resulting from reaction of FAD with hydrated electron, is accompanied by much slower protonization with formation of neutral $FADH^\cdot$ [39]. Thus, among possible explanations, one could suggest that the oxidation of $FADH^-$ to $FADH^\cdot$ in FNR represents a 'pure' single-electron transfer, whereas the oxidation of $FADH^\cdot$ to FAD is an electron transfer coupled to a partly rate limiting proton exchange. In spinach FNR, N-5 atom of FAD is not exposed to solvent [8]. It has been suggested that during the net two-electron reduction of spinach FNR by two ferredoxin molecules, the formation of

$FADH^-$ can involve proton donation from side-chain hydroxyl group of serine-96 to N-5 atom of FAD, coupled to a conformational change [8]. The latter residue corresponding to serine-80 of *Anabaena* FNR [7], forms a hydrogen bond with N-5 of FAD [7,8], and plays an important role in stabilization of semiquinone of FNR [41]. Thus, it is possible to suggest, that reverse reaction of *Anabaena* FNR, namely, oxidation of $FADH^{\cdot}$ to FAD by quinones or other types of nonphysiological oxidants, may involve a partly rate-limiting proton exchange step with serine-80, that could decrease an intrinsic reactivity of $FAD/FADH^{\cdot}$ couple.

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