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FAD semiquinone stability regulates single- and two-electron reduction of quinones by *Anabaena* PCC7119 ferredoxin:NADP$^+$ reductase and its Glu301Ala mutant

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Abstract

Flavoenzymes may reduce quinones in a single-electron, mixed single- and two-electron, and two-electron way. The mechanisms of two-electron reduction of quinones are insufficiently understood. To get an insight into the role of flavin semiquinone stability in the regulation of single- vs. two-electron reduction of quinones, we studied the reactions of wild type *Anabaena* ferredoxin:NADP$^+$reductase (FNR) with 48% FAD semiquinone (FADH$^\cdot$) stabilized at the equilibrium (pH 7.0), and its Glu301Ala mutant (8% FADH$^\cdot$ at the equilibrium). We found that Glu301Ala substitution does not change the quinone substrate specificity of FNR. However, it confers the mixed single- and two-electron mechanism of quinone reduction (50% single-electron flux), whereas the wild type FNR reduces quinones in a single-electron way. During the oxidation of fully reduced wild type FNR by tetramethyl-1,4-benzoquinone, the first electron transfer (formation of FADH$^\cdot$) is about 40 times faster than the second one (oxidation of FADH$^\cdot$). In contrast, the first and second electron transfer proceeded at similar rates in Glu301Ala FNR. Thus, the change in the quinone reduction mechanism may be explained by the relative increase in the rate of second electron transfer. This enabled us to propose the unified scheme of single-, two- and mixed single- and two-electron reduction of quinones by flavoenzymes with the central role of the stability of flavin/quinone ion–radical pair.

Keywords: Ferredoxin:NADP$^+$ reductase; Glu301Ala mutant of; Quinones; Electron transfer mechanism

Quinones represent an important group of natural and synthetic compounds widely used in medicine and agriculture. Their cytotoxic and/or therapeutic activity is frequently related to their reduction by flavoenzymes ([1–4], and references therein). Quinones may be reduced in a single-, two- and mixed single- and two-electron way [2,5]. Single-electron reduction of quinones to semiquinones contributes to their oxidative stress-type cytotoxicity. Two-electron reduction of quinones may either decrease their cytotoxicity due to hydroquinone conjugation and excretion ([6] and references therein), either potentiate it in case of anticancer aziridinyl-substituted quinones [7].

The mechanism of single-electron reduction of quinones is relatively well understood. These reactions are performed by flavoenzymes dehydrogenases–electron-transferases, e.g., NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4),[1] nitric oxide synthase (NOS, EC

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[1] *Abbreviations used:* P-450R, NADPH:cytochrome P-450 reductase; NOS, nitric oxide synthase; FNR, ferredoxin:NADP$^+$ reductase; NQO1, DT-diaphorase; NR, nitroreductase; Q, quinone; E_7^1, single-electron reduction potential at pH 7.0; $E_{m,7}$, midpoint potential at pH 7.0; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant in the steady-state kinetics.

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1.14.13.39), ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2), and NADH:cytochrome b_5 reductase (EC 1.6.2.2), which possess single-electron physiological oxidants and contain flavins with the stabilized neutral (blue) semiquinone state ([2,3,5,8–11] and references therein). Their presteady-state kinetics studies identified the redox states of flavins taking part in quinone reduction [2,3,5,10]. Typically, the steady-state reaction bimolecular rate constants (k_{cat}/K_m) of quinones are insensitive to their particular structure, and show a linear or parabolic dependence of $\log k_{cat}/K_m$ on their single-electron reduction potential, E_7^1 [8–10]. In general, these reactions follow an 'outer-sphere' electron transfer mechanism with the weak electronic coupling of reactants [12].

In contrast, the mechanisms of two-electron reduction of quinones by flavoenzymes are less understood. Typically, flavoenzymes performing two-electron reduction possess destabilized anionic (red) flavin semiquinone. In mammalian NAD(P)H:quinone reductase (NQO1, DT-diaphorase, EC 1.6.99.2), only 8% $FAD^{-\cdot}$ is stabilized at the equilibrium [13]. The formation of FMN semiquinone in *Enterobacter cloacae* nitroreductase (NR, EC 1.6.99.7) is even undetectable [14]. The formation of $FAD^{-\cdot}$ intermediate was not detected during the oxidation of reduced NQO1 by quinones [13]. This is equally well consistent with two mechanisms of two-electron reduction, a single-step hydride (H^-) transfer without the formation of ion–radical intermediates, and with a three-step (e^-, H^+, e^-) hydride transfer with the rate-limiting first electron transfer [13,15–19]. However, it is unclear whether the instability of flavin semiquinone is a sufficient condition for a two-electron reduction of quinones by flavoenzymes or whether it may require specific quinone binding in the active centre. For example, quinones or other aromatic compounds bind in the active centers of NQO1 and NR at 0.3–0.4 nm distance from the flavin izoalloxazine ring, the stacking efficiency depending on their structure [15,16,20]. For this reason, the reactivity of NQO1 and NR is strongly influenced by the structure of quinone substrates [17–19].

To understand the mechanism of two-electron reduction of quinones by flavoenzymes, it is important to find out whether the changes in the flavin semiquinone stability may change the reaction mechanism. One of the possible approaches is to destabilize flavin semiquinone in flavoenzymes dehydrogenase-electron-transferases which perform the obligatory single-electron quinone reduction. The Glu301Ala substitution in *Anabaena* ferredoxin:$NADP^+$ reductase results in a significant destabilization of $FADH^{\cdot}$ redox state as compared to the wild type FNR [21]. In this work, we examined the possible differences in quinone reduction mechanism between wild type and Glu301Ala FNR.

Materials and methods

Materials

Wild type and Glu301Ala mutant of *Anabaena* FNR were prepared as described [22]. The enzyme concentrations were determined spectrophotometrically using $\Delta\varepsilon_{458} = 9.4$ and 9.15 $mM^{-1}\,cm^{-1}$, respectively. 2-Methyl-3-glutathionyl-5-hydroxy-1,4-naphthoquinone and 2,3-diglutathionyl-1,4-naphthoquinone were a generous gifts from Dr. K. Ollinger (Linkoping University, Sweden). Daunorubicin was obtained from Minmedprom (Russia). Other reagents were obtained from Sigma or Serva and used as received.

Steady-state enzyme kinetic measurements

Kinetic measurements were carried out using Hitachi-557 UV–VIS spectrophotometer in 0.1 M K-phosphate (pH 7.0) containing 1 mM EDTA at 25 °C, unless specified otherwise. For experiments with the varied ionic strength, the 0.01–0.4 M phosphate buffer solutions (pH 7.0) were used. The pH-dependence of reaction rates was studied using 0.1 M K-phosphate solutions with pH 5.5–8.0. The reaction rates were monitored spectrophotometrically following the oxidation of 200 μM NADPH ($\Delta\varepsilon_{340} = 6.2\ mM^{-1}\,cm^{-1}$) at 6–7 electron acceptor concentrations. The reduction rates of cytochrome *c*, added to the reaction mixture for separate experiments, were monitored according to the increase in absorbance at 550 nm ($\Delta\varepsilon_{550} = 20\ mM^{-1}\,cm^{-1}$). The enzyme catalytic constant (k_{cat}) and bimolecular rate constants (k_{cat}/K_m) correspond to the reciprocal intercepts and slopes of the Lineweaver–Burk plots, [E]/*v* vs. 1/[quinone], where [E] is the enzyme concentration and *v* is the reaction rate. k_{cat} is the number of NADPH molecules oxidized by the single active center of the enzyme per second. The rates obtained were corrected for the intrinsic NADPH-oxidase activity of FNR. Nonlinear estimations of kinetic data were performed by Quasi-Newton or Simplex-Quasi-Newton estimation methods using Statistica (version 4.30, StatSoft, 1993).

Presteady-state kinetic measurements

Wild type and Glu031Ala FNR were photoreduced under anaerobic conditions in the presence of 1–2 μM 5-diazariboflavin and 10 mM EDTA. The anaerobic conditions were established by the repeated cycles of evacuation and flushing with argon purified by the passage over a heated BASF catalyst [10]. The anaerobic reoxidation of reduced enzymes (concentration after the mixing, 10 μM) by tetramethyl-1,4-benzoquinone (concentration after mixing, 30–150 μM) was performed using a stopped-flow SX.17 MV spectrophotometer (Applied Photophysics) equipped with Acorn 5000

computer. The measurements were performed at pH 7.0, and 25 °C. The kinetics of absorbance increase at 460 nm was analyzed according to a single- or two-exponent fit using the software supplied by Applied Photophysics. The absorbance changes at 600 nm corresponding to the transient formation and decay of FADH· were analyzed according to the two-step mechanism:

$$\mathrm{FADH^-} \xrightarrow{k_1} \mathrm{FADH^\bullet} \xrightarrow{k_2} \mathrm{FAD} \quad (1)$$

$$A_{600} = \frac{k_1}{k_2 - k_1} C(\mathrm{e}^{-k_1 t} - \mathrm{e}^{-k_2 t}) + b, \quad (2)$$

using Eq. (2): where C is an amplitude term and b is an off-set value. The calculations were performed using Statistica (version 4.30, StatSoft, 1993).

Results

The potentials of single-electron reduction of wild type and Glu301Ala *Anabaena* FNR at pH 7.0 (E_7^1), i.e., the potentials of FAD/FADH· and FADH·/FADH$^-$ redox couples, and their midpoint potentials ($E_{m,7}$) are given in Table 1. It is evident that the Glu301Ala substitution significantly destabilizes FAD semiquinone (Table 1).

Quinone reduction by wild type *Anabaena* FNR is characterized by a parabolic $\log k_{cat}/K_m$ dependence on their E_7^1 (potential of quinone/semiquinone couple) [10]. It shows that quinone reactivity depends on their single-electron reduction energetics, but not on their particular structure. The Glu301Ala substitution in FNR results in a loss of Glu301-Ser80 hydrogen bond and a negative electrostatic charge, but almost does not influence the binding of $NADP^+$ [22,24]. To assess whether these changes may affect the quinone substrate specificity of Glu301Ala FNR, a number of structurally diverse quinones possessing a broad range of E_7^1 values and several single-electron acceptors were used (Table 2). In the presence of 200 μM NADPH as an electron donor, the k_{cat} values of electron acceptors varied in the range 55–75 s^{-1}, being independent of their E_7^1 values (data not shown). On the other hand, $\log k_{cat}/K_m$ of quinones exhibited parabolic dependence on their E_7^1 (Fig. 1A). There also exists a linear relationship between the reactivities of quinones towards Glu301Ala and wild type FNR ($r^2 = 0.9028$, Fig. 1B). Further, the k_{cat}/K_m of the charged single-electron oxidants, ferricyanide, Fe(EDTA)$^-$, and methylviologen almost did not depend on the solution ionic strength (data not shown). These data are analogous to those previously reported for the wild type FNR [10], and point to the absence of important electrostatic interactions in the oxidation of both enzyme types. Thus, the mutation does not induce FNR selectivity for particular structures of oxidizing substrates.

Quantitatively, the percentage of a single-electron flux during the reduction of quinones by NAD(P)H oxidizing flavoenzymes is obtained using 1,4-benzoquinone-mediated reduction of cytochrome *c*, since 1, 4-benzohydroquinone reduces cytochrome *c* at a negligibly low rate at pH < 7.2, whereas 1,4-benzosemiquinone rapidly reduces it ($k = 10^6\ M^{-1}\ s^{-1}$ [2,5]). The percentage of a single electron flux is expressed as the ratio of a cytochrome *c* reduction rate and the doubled rate of NAD(P)H oxidation in the presence of 1,4-benzoquinone [2,5]. At pH ⩽ 7.0, the percentage of a single-electron flux in the quinone reduction by Glu301Ala FNR is around 50% (Fig. 2A), whereas for the wild type FNR it is close to 100% at pH 5.5–8.0. The use of low-potential quinones in this assay is problematic, since their hydroquinones also reduce cytochrome *c*, although at a slower rate than semiquinones [2,5,25]. Nevertheless, the efficiency of the 2-methyl- and 2,5-dimethyl-1,4-benzoquinone-mediated reduction of cytochrome *c* by Glu301Ala FNR was lower than that of the wild type enzyme (Fig. 2B). This also points to the increased two-electron character of their reduction by Glu301Ala FNR, i.e., formation of a higher amount of hydroquinones which reduce cytochrome *c* at slower rate.

Previously we found that during the oxidation of fully reduced wild type FNR by 5,8-dihydroxy-1,4-naphthoquinone, the rate limiting step was the oxidation of FADH· to FAD, and that the formation of FADH· was too fast to be measured [10]. In this work, using less active oxidant tetramethyl-1,4-benzoquinone (Table 2), we were able to measure the rates of FADH· formation from FADH$^-$ and FAD formation from FADH·. Dur-

Table 1
Single-electron reduction potentials of wild type and Glu301Ala ferredoxin:$NADP^+$ reductase at pH 7.0 (E_7^1), their midpoint potentials ($E_{m,7}$), and the percentage of FAD semiquinone stabilized at the equilibrium

Enzyme	E_7^1(V)[a]		$E_{m,7}$ (V)[a]	FADH· stabilized at equilibrium (%)[b]
	FAD/FADH·	FADH·/FADH$^-$		
Wild type FNR	−0.280	−0.312	−0.296	48
Glu301AlaFNR	−0.299	−0.210	−0.254	8

[a] Calculated from data at pH 8.0 [19] assuming that the potential of FAD/FADH· decreases by −0.059 V/U pH, and the potential of FADH·/FADH$^-$ is pH-independent.
[b] The percentage of FADH· form of enzyme at $E = E_{m,7}$ calculated according to the Nernst equation as in [13,21].

Table 2
The single-electron reduction potentials (E_7^1) of quinones and single-electron acceptors and the bimolecular rate constants (k_{cat}/K_m) of their reduction by Glu301Ala and wild type *Anabaena* ferredoxin:NADP$^+$ reductase

No.	Compound	E_7^1(V)[a]	k_{cat}/K_m (M^{-1} s^{-1})	
			Glu301Ala FNR	Wild type FNR[b]
Quinones				
1.	1,4-Benzoquinone	0.09	$(2.6 \pm 0.6) \times 10^6$	$(4.0 \pm 0.3) \times 10^5$ [c]
2.	2-Methyl-1,4-benzoquinone	0.01	$(4.3 \pm 0.7) \times 10^6$	$(2.4 \pm 0.2) \times 10^5$
3.	2,3-Dichloro-1,4-naphthoquinone	−0.035	$(8.8 \pm 0.7) \times 10^6$	$(2.0 \pm 0.2) \times 10^6$
4.	2,5-Dimethyl-1,4-benzoquinone	−0.07	$(2.3 \pm 0.7) \times 10^6$	$(2.2 \pm 0.2) \times 10^5$
5.	5-Hydroxy-1,4-naphthoquinone	−0.09	$(7.4 \pm 1.0) \times 10^6$	$(1.2 \pm 0.1) \times 10^6$
6.	5,8-Dihydroxy-1,4-naphthoquinone	−0.11	$(6.1 \pm 1.9) \times 10^6$	$(9.0 \pm 1.0) \times 10^5$
7.	9,10-Phenanthrenequinone	−0.12	$(5.8 \pm 0.4) \times 10^6$	$(2.2 \pm 0.4) \times 10^6$
8.	1,4-Naphthoquinone	−0.15	$(9.2 \pm 1.0) \times 10^6$	$(6.0 \pm 0.5) \times 10^5$
9.	2,3-Diglutathionyl-1,4-naphthoquinone	−0.15	$(1.2 \pm 0.4) \times 10^6$	$(2.7 \pm 0.3) \times 10^5$
10.	2-Methyl-5-hydroxy-1,4-naphthoquinone	−0.16	$(9.2 \pm 1.2) \times 10^6$	$(6.7 \pm 0.7) \times 10^5$
11.	2-Methyl-3-glutathionyl-1,4-naphthoquinone	−0.16	$(4.0 \pm 0.3) \times 10^6$	$(6.2 \pm 0.6) \times 10^5$
12.	2-Methyl-1,4-naphthoquinone	−0.20	$(3.2 \pm 0.2) \times 10^6$	$(4.6 \pm 0.5) \times 10^5$
13.	Tetramethyl-1,4-benzoquinone	−0.26	$(1.6 \pm 0.4) \times 10^6$	$(2.0 \pm 0.3) \times 10^5$
14.	1,8-Dihydroxy-9,10-anthraquinone	−0.325	$(2.7 \pm 0.3) \times 10^5$	$(7.2 \pm 0.6) \times 10^4$ [c]
15.	Daunorubicin	−0.34	$(4.5 \pm 0.3) \times 10^5$	$(1.1 \pm 0.4) \times 10^5$
16.	2-Hydroxy-1,4-naphthoquinone	−0.41	$(1.4 \pm 0.2) \times 10^4$	$(6.7 \pm 0.5) \times 10^3$
17.	2-Methyl-3-hydroxy-1,4-naphthoquinone	−0.46	$(2.6 \pm 0.1) \times 10^4$	$(2.1 \pm 0.2) \times 10^3$ [c]
Single-electron acceptors				
18.	Ferricyanide	0.41	$(1.2 \pm 0.2) \times 10^7$	$(1.3 \pm 0.4) \times 10^7$ [d]
19.	Fe(EDTA)$^-$	0.12	$(1.3 \pm 0.1) \times 10^3$	$(2.2 \pm 0.2) \times 10^3$ [d]
20.	Methylviologen	−0.44	$(1.6 \pm 0.1) \times 10^4$	$(1.1 \pm 0.1) \times 10^4$ [d]

[a] Taken from [19,23].
[b] Taken from our previous work [10].
[c] Determined in the present study.
[d] Calculated on the single-electron basis.

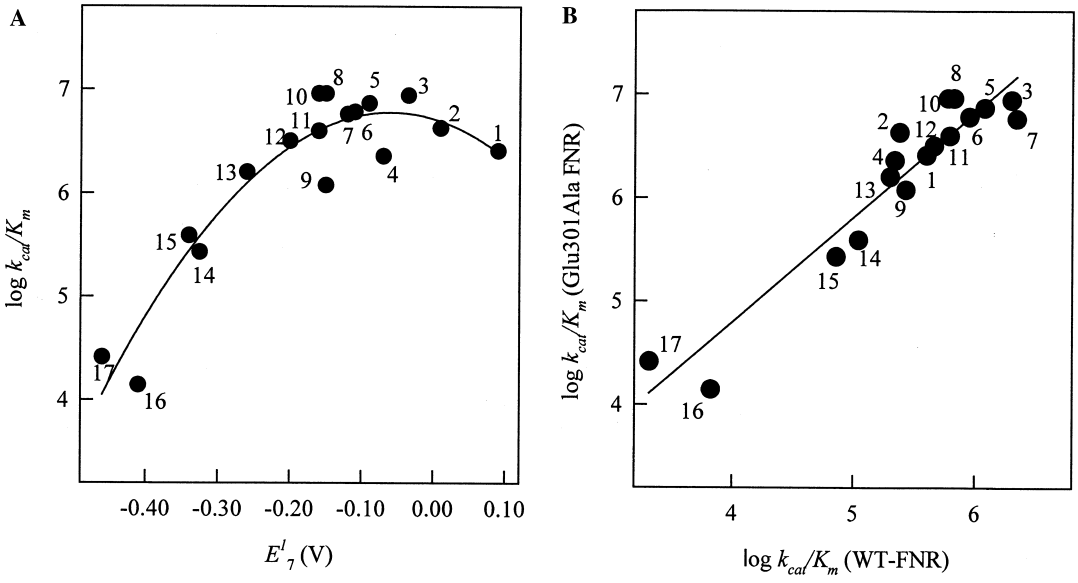


Fig. 1. (A) The dependence of quinone reactivity towards Glu301Ala FNR on the potential of their single-electron reduction at pH 7.0, (E_7^1), and (B) the interrelationship between quinone reactivities towards E301A and wild type FNR. The numbers of compounds are those as in Table 1.

ing the oxidation of wild type FNR, the maximal absorbance increase at 600 nm corresponded to ca. 55% stoi- chiometric FADH$^\cdot$ formation ($\Delta\varepsilon_{600} = 5\ mM^{-1}\ cm^{-1}$ [21,22]) (Fig. 3A). The obtained pseudofirst order rate constants showed linear dependence on the quinone concentration up to the spectrophotometer sensitivity limit, $>400\ s^{-1}$, giving the bimolecular rate constants of $4.4 \pm 0.5 \times 10^6\ M^{-1}\ s^{-1}$

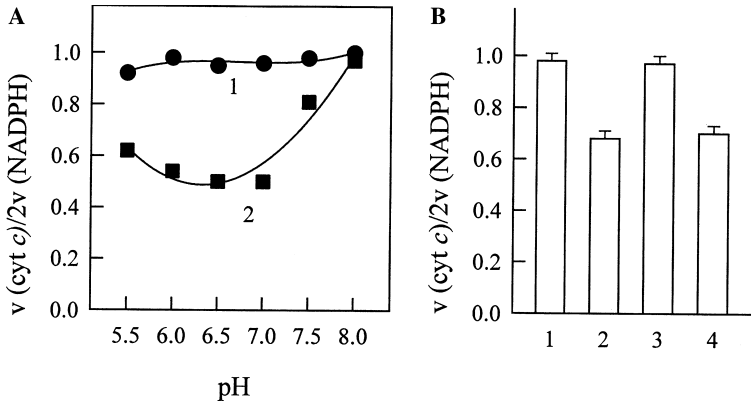


Fig. 2. (A) The pH-dependence of the single-electron flux in 1,4-benzoquinone reduction by wild type (1) and Glu301Ala FNR (2), expressed as the ratio of coupled cytochrome *c* reduction rate and the doubled rate of enzymatic NADPH oxidation. (B) The efficiency of quinone-mediated cytochrome *c* reduction in reactions of wild type FNR (1,3) and Glu301Ala FNR (2,4), expressed as the ratio of coupled cytochrome *c* reduction rate and the doubled rate of enzymatic NADPH oxidation. Electron acceptors used: 2,5-dimethyl-1,4-benzoquinone (1,2) and 2-methyl-1,4-benzoquinone (3,4). In all the experiments, concentration of NADPH, 200 μM, concentration of quinone and cytochrome *c*, 50 μM, and the rate of enzymatic reaction, 12–15 μM NADPH/min.

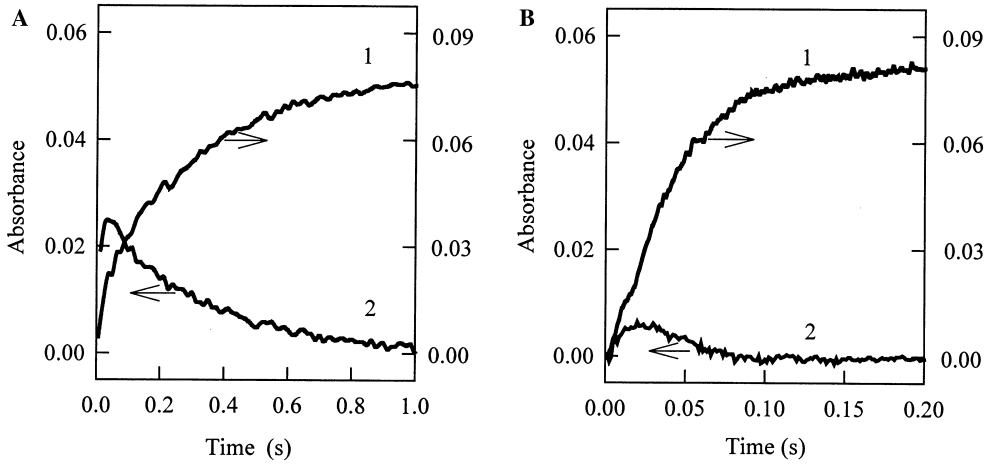


Fig. 3. The presteady-state kinetics of oxidation of 10 μM reduced wild type FNR (A) or Glu301Ala FNR (B) monitored at 460 nm (1, right-hand scale) and 600 nm (2, left-hand scale). Concentration of tetramethyl-1,4-benzoquinone, 50 μM (A) or 30 μM (B).

($FADH^- \rightarrow FADH^{\cdot}$), and $1.2 \pm 0.3 \times 10^5\ M^{-1}\ s^{-1}$ ($FADH^{\cdot} \rightarrow FAD$). Analogously, the analysis of absorbance traces at 460 nm gives the bimolecular rate constant of the net FAD formation from $FADH^-$, $1.1 \pm 0.2 \times 10^5\ M^{-1}\ s^{-1}$ (determined according to a single-exponent fit), and the rate constants of $3.7 \pm 0.8 \times 10^6\ M^{-1}\ s^{-1}$ ($FADH^- \rightarrow FADH^{\cdot}$), and $1.1 \pm 0.1 \times 10^5\ M^{-1}\ s^{-1}$ ($FADH^{\cdot} \rightarrow FAD$), determined according to a two-exponent fit. In this case, the obtained ratio between the amplitudes of the first and second phases, ca. 1:4, was lower than the ratio of extinction coefficients, $\Delta\varepsilon_{460}$ ($FADH^{\cdot}$–$FADH^-$) = $2.5\ mM^{-1}\ cm^{-1}$, and $\Delta\varepsilon_{460}$ (FAD–$FADH^{\cdot}$) = $6.0\ mM^{-1}\ cm^{-1}$ [21], evidently due to a rapid first phase of reaction. Thus, tetramethyl-1,4-benzoquinone oxidizes $FADH^-$ 40 times faster than $FADH^{\cdot}$. During the oxidation of Glu301Ala FNR, the bimolecular rate constant determined at 460 nm according to a single-exponent fit, $1.3 \pm 0.3 \times 10^6\ M^{-1}\ s^{-1}$, was close to k_{cat}/K_m of tetramethyl-1,4-benzoquinone in the steady-state reaction (Table 2). Using a two-exponent fit, the obtained bimolecular rate constants were equal to $8.9 \pm 1.9 \times 10^5\ M^{-1}\ s^{-1}$ ($FADH^- \rightarrow FADH^{\cdot}$) and $1.4 \pm 0.4 \times 10^6\ M^{-1}\ s^{-1}$ ($FADH^{\cdot} \rightarrow FAD$). However, in this case, the ratio of amplitudes of first and second phases varied from 1:1.5 to 2:1. The absorbance traces at 600 nm show that only ca. 10% $FADH^{\cdot}$ was transiently formed (Fig. 3B). As compared to Fig. 3A, it shows the relatively increased $FADH^{\cdot}$ decay rate over the rate of its formation [26]. Using 30–50 μM tetramethyl-1,4-benzoquinone, the maximal $FADH^{\cdot}$ concentration was observed 10–20 ms after the mixing (Fig. 3B). An increase in the oxidant concentration led to the shorter times of the maximal $FADH^{\cdot}$ accumulation (t_{max}), tending to approach the dead-time of theinstrument. This is in accordance with the Eq. (3) [26]:

$$t_{max} = \frac{\ln k_2/k_1}{k_2 - k_1}, \quad (3)$$

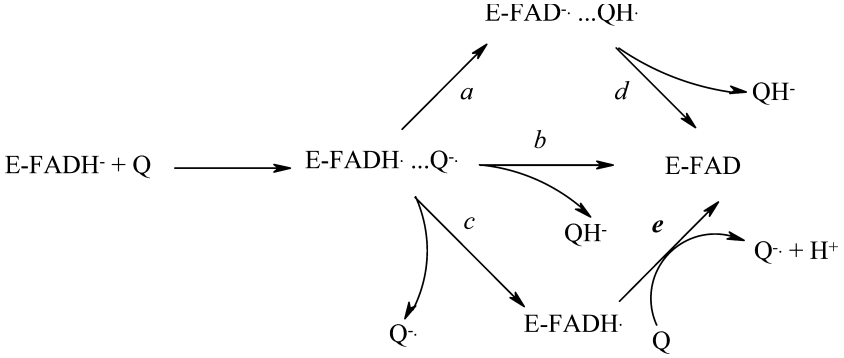
where k_1 and k_2 are the pseudofirst order rate constants of FADH$^{\cdot}$ formation and decay (Eq. (2)), increasing with an increase in oxidant concentration. For this reason, only the data at the low oxidant concentrations were analyzed according to Eq. (2). We obtained $k_1 = 41 \pm 5.0\ s^{-1}$ and $k_2 = 60 \pm 7.0\ s^{-1}$ (30 μM tetramethyl-1,4-benzoquinone), and $k_1 = 65 \pm 8.0\ s^{-1}$ and $k_2 = 82 \pm 10\ s^{-1}$ (50 μM tetramethyl-1,4-benzoquinone). These rate constants agree with the bimolecular rate constants determined at 460 nm, and show that the rate of a second electron transfer is similar to or even higher than that of the first electron transfer.

Discussion

Our data show that Glu301Ala substitution does not affect quinone substrate specificity of FNR (Figs. 1A and B), and its interaction with electrostatically charged single-electron oxidants. However, the FAD semiquinone destabilization (Table 1) confers partial two-electron character of quinone reduction by Glu301Ala FNR (Figs. 2A and B), which is most easily explained by the alteration of single-electron transfer rates (Figs. 3A and B). Thus, the 40-fold higher rate of the oxidation of FADH$^-$ by quinone in wild type FNR as compared to the oxidation of FADH$^{\cdot}$ (Fig. 3A) may be at least partly explained by the more negative potential of FADH$^{\cdot}$/FADH$^-$ couple (Table 1). In turn, the potential of FADH$^{\cdot}$/FADH$^-$ in Glu301Ala FNR is higher than that of FAD/FADH$^{\cdot}$ (Table 1). It may confer the similar rates of the first and second electron transfer in the oxidation of FADH$^-$ (Fig. 3B), thus increasing the possibility of a net two-electron transfer.

The data obtained support the hypothesis on the involvement of single-electron transfer steps in both mixed- and two-electron reduction of quinones [2,3,17–19], and enable us to propose a more thorough and unified scheme of the single-, two-, and mixed single- and two-electron reduction with the central role of the stability of flavin/quinone ion–radical pair, E-FADH$^{\cdot}$...Q$^{-\cdot}$ formed after the first electron transfer (Scheme 1). Subsequently, the ion–radical pair may undergo a sequential (steps a and d) or a concerted proton and second electron transfer (step b), or dissociate (step c) (Scheme 1). The E-FADH$^{\cdot}$ formed may be oxidized by a second molecule of quinone (step e). The ratio between the single-electron (steps c and e) and two-electron flux (steps a, d, and b) should depend on the rates of steps c and a or b.

For a more thorough analysis of the quinone reduction mechanism, the comparison of the properties of Glu301Ala FNR with mammalian NQO1 is useful, since both enzymes contain the same amount of FAD semiquinone stabilized at pH 7.0, at equilibrium, 8% [13]. However, in contrast to the two-electron transferring NQO1, Glu301Ala FNR still performs partly single-electron reduction of quinones (Figs. 2A and B). There may be several reasons for the easier transfer of the second electron in NQO1 as compared to GluE301Ala FNR: (i) efficient electronic coupling and a short electron transfer distance between the izoalloxazine and quinone rings in NQO1 [15,16]. Indeed, the comparison of quinone reduction rates shows that the intrinsic reactivity of NQO1 is higher than that of Glu301Ala FNR. For example, in the reduction of tetramethyl-1,4-benzoquinone ($E_7^1 = -0.26$ V) by FADH$^-$ of Glu301Ala FNR, the driving force of the reaction, −0.05 V, corresponds to the rate constant around $10^6\ M^{-1}\ s^{-1}$ (Table 2). During the two-electron reduction of quinones by NQO1, the oxidation of FADH$^-$ to FAD$^{-\cdot}$ (E_7^1 of FAD$^{-\cdot}$/FADH$^-$ couple, −0.118 V) is the rate-limiting step [13]. At the same as above driving force of the reaction, i.e., in the oxidation of NQO1 by quinones with E_7^1 ca. −0.17 V, the reaction rate constants are in the range of 10^7–$10^8\ M^{-1}\ s^{-1}$ [18]; and (ii) the formation of anionic FAD semiquinone in NQO1 [13] instead of neutral E-FADH$^{\cdot}$ in Glu301Ala FNR [21]. This may accelerate the proton transfer in the ion–radical pair E-FADH$^{\cdot}$...Q$^{-\cdot}$ (step a), thus contributing to the two-electron character of the reaction of NQO1. One may also



Scheme 1. The unified scheme of single- and two-electron reduction of quinones by flavoenzymes.

suppose that the neutral E-FADH$^{\cdot}$ more easily dissociates from the ion–radical pair than E-FAD (step c, Scheme 1), thus contributing to the partly single-electron character of the reaction of Glu301Ala FNR.

In conclusion, we have shown for the first time that the two-electron character of quinone reduction is not an intrinsic property of flavoenzymes possessing their specific binding sites, e.g., NQO1 [15,16,18], but that it may be imposed by the destabilization of flavin semiquinone in dehydrogenases–electrontransferases. These enzymes reduce quinones according to an outer-sphere electron transfer model with the weak electronic coupling of reactants [12]. Our data also support the multistep (e^-, H^+, e^-) model of two-electron reduction of quinones by flavoenzymes [2,3,17–19].

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