

Quantitative Structure–Activity Relationships in Two-Electron Reduction of Nitroaromatic Compounds by *Enterobacter cloacae* NAD(P)H:Nitroreductase

Henrikas Nivinskas,* Ronald L. Koder,† Žilvinas Anusevičius,* Jonas Šarlauskas,* Anne-Frances Miller,† and Narimantas Čėnas*¹

*Institute of Biochemistry, Mokslininku 12, Vilnius 2600, Lithuania; and †Department of Chemistry, University of Kentucky, 106 Chemistry-Physics Building, Lexington, KY 40506-0055

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***Enterobacter cloacae* NAD(P)H:nitroreductase (NR; EC 1.6.99.7) catalyzes the reduction of a series of nitroaromatic compounds with steady-state bimolecular rate constants (k_{cat}/K_m) ranging from 10^4 to 10^7 $\text{M}^{-1} \text{s}^{-1}$. In agreement with a previously proposed scheme of two-step four-electron reduction of nitroaromatics by NR (Koder, R. L., and Miller, A. F. (1998) *Biochim. Biophys. Acta* 1387, 395–405), 2 mol NADH per mole mononitrocompound were oxidized. An oxidation of excess NADH by polinitrobenzenes, including explosives 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenyl-*N*-methylnitramine (tetryl), has been observed as a slower secondary process, accompanied by O_2 consumption. This type of “redox cycling” was not related to reactions of nitroaromatic anion-radicals, but was caused by the autoxidation of relatively stable reaction products. The initial reduction of tetryl and other polinitrophenyl-*N*-nitramines by *E. cloacae* NR was analogous to a two-step four-electron reduction mechanism of TNT and other nitroaromatics. The logs k_{cat}/K_m of all the compounds examined exhibited parabolic dependence on their enthalpies of single-electron or two-electron (hydride) reduction, obtained by quantum mechanical calculations. This type of quantitative structure–activity relationship shows that the reactivity of nitroaromatics towards *E. cloacae* nitroreductase depends mainly on their hydride accepting properties, but not on their particular structure, and does not exclude the possibility of multistep hydride transfer.**

Key Words: nitroreductase; tetryl; pentryl; TNT; explosive; electron transfer.

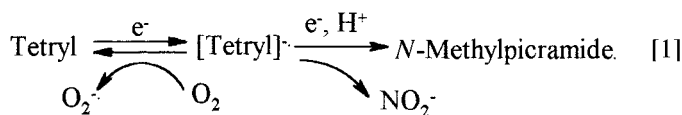
Nitroaromatic compounds are widely used as pharmaceuticals, pesticides, explosives, and comprise an important group of environmental pollutants (1–3). For manifestation of their therapeutic and/or cytotoxic properties, most nitroaromatics should undergo single- or two-electron enzymatic reduction in organism. Single-electron reduction of nitroaromatics is most frequently catalyzed by flavoenzymes dehydrogenases-electrontransferases, e.g., NADPH: cytochrome P-450 reductase (EC 1.6.2.4) (4–6), ferredoxin:NADP⁺ reductase (EC 1.18.1.2) (5, 7), NADH:ubiquinone reductase (EC 1.6.99.3) (8), bacterial oxygen-sensitive nitroreductases (9). Under aerobic conditions, single-electron reduction of nitroaromatics to their anion-radicals results in their reoxidation by oxygen with the formation of superoxide and, subsequently, hydrogen peroxide and hydroxyl radical, that damage proteins, nucleic acids, and lipids. Two-electron reduction of nitroaromatics to nitroso compounds and, subsequently, to hydroxylamines, is catalyzed by mammalian DT-diaphorase (NAD(P)H: quinone reductase; EC 1.6.99.2) (10, 11) and bacterial oxygen-insensitive nitroreductases (9, 12).

The reactivity of nitroaromatics in single-electron enzymatic reduction increases with an increase in their single-electron reduction potential (E_1^{\downarrow})² and is relatively insensitive to their structure (4–8). This is consistent with an “outer-sphere” electron transfer mechanism (13). In contrast, the mechanism of two-electron reduction of nitroaromatics is less well under-

² Abbreviations used: NR, *E. cloacae* nitroreductase; k_{cat} , catalytic constant; k_{cat}/K_m , bimolecular rate constant; E_1^{\downarrow} , single-electron reduction potential; ΔH_f , enthalpy of reaction; TLC, thin layer chromatography.

¹ To whom correspondence and reprint requests should be addressed. Fax: (370)-2-729196. E-mail: nčenās@bchi.lt.

stood. The reactivity of nitrobenzenes and nitrofurans towards the most thoroughly studied two-electron transferring enzyme, DT-diaphorase, did not depend on their redox potential (14), and sometimes was strongly influenced by compound structure (derivatives of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB-1954) (11)). However, certain data point to a possible involvement of single-electron transfer steps in two-electron (hydride) transfer by DT-diaphorase and other oxygen-insensitive nitroreductases: (i) the reactivity of nitrobenzimidazoles increased upon an increase in their electron accepting properties (15); (ii) the reactivity of several nitroaromatics toward *Enterobacter cloacae* nitroreductase (EC 1.9.99.7) increased upon an increase in their E_7^1 value (12); (iii) DT-diaphorase catalyzed N-denitration of high explosive tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine) in a single-electron way, the reaction being accompanied by redox cycling (16):



Enterobacter cloacae nitroreductase (NR) was originally characterized by Bryant and DeLuca (12). It is a homodimeric 24.5-kDa protein containing an FMN cofactor, which reduces nitrofurans, TNT, and other nitroaromatics. NR follows “ping-pong” mechanism, and reduces nitrobenzene to phenylhydroxylamine in two successive two-electron transfers at the expense of two molecules of NADH, the nitroso intermediate being reduced much faster than nitrobenzene (17). Thus, NR shares the properties of other oxygen-insensitive nitroreductases, such as *Escherichia coli* nitroreductase (18) and DT-diaphorase (19). However, the structure–activity relationships and the mechanism of two-electron (hydride) transfer by *E. cloacae* NR have been insufficiently studied. These studies may be of certain interest, since various strains of *E. cloacae* are currently being used in biodegradation of nitroaromatic or nitrate ester explosives (20, 21). In particular, the two-electron (hydride) enzymatic reduction of high explosives like TNT (2,4,6-trinitrotoluene) to hydroxylamine or amine derivatives (3, 22, 23), or to hydride-Meisenheimer complexes (3, 24), is the initial step of their biodegradation.

In the present work, we have studied the reactions of *E. cloacae* NR with a series of nitroaromatic compounds including high explosives TNT, tetryl, and pentryl (Fig. 1). Furthermore, we have established quantitative structure–activity relationships linking the reaction rate with the enthalpies of single- and two-electron reduction of nitroaromatic compounds, obtained by quantum chemical calculations.

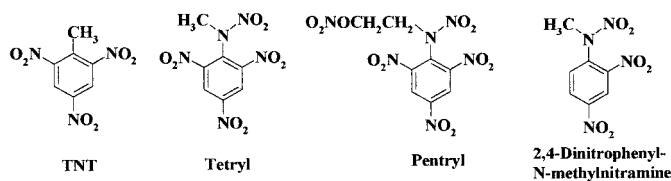


FIG. 1. The formulae of nitroaromatic explosives studied in this work.

MATERIALS AND METHODS

Materials. *E. cloacae* NR has been expressed in *E. coli*, purified, and stored as previously described (25). The enzyme concentration was determined spectrophotometrically using $\epsilon_{454} = 14.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for the bound FMN cofactor (25). Nitroaromatic compounds were synthesized according to published methods: TNT (26); tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine) and *N*-methylpicramide (27); pentryl (2,4,6-trinitrophenyl-*N*-nitraminoethyl-nitrate) (28); and 2,4-dinitrophenyl-*N*-methylnitramine (29). The purity of nitroaromatic compounds was determined using melting points, TLC, NMR, IR, and elemental analysis. All other compounds were obtained from Sigma or Aldrich and used as received.

Enzymatic assays and analytical procedures. Kinetic measurements were carried out in 0.1 M Tris–Cl (pH 7.0), containing 0.5 mM desferrioxamine at 25°C. The rate of NR-catalyzed oxidation of NADH by various nitroaromatics was determined by monitoring NADH oxidation ($\Delta\epsilon_{340} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) using a Hitachi-557 spectrophotometer. Corrections were introduced when necessary for the formation of reaction products absorbing at 340 nm. The catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of nitrocompound reduction correspond to the reciprocal intercepts and slopes of plots $[E]/v$ vs $1/[\text{ArNO}_2]$, where $[E]$ is enzyme concentration, and $[\text{ArNO}_2]$ is concentration of nitrocompound. k_{cat} is the number of NADH molecules oxidized by a single active center of the enzyme per second. The reduction of cytochrome *c*, added into the reaction mixture in separate experiments, was monitored spectrophotometrically using $\Delta\epsilon_{550} = 20 \text{ mM}^{-1} \text{ cm}^{-1}$. The rate of oxygen consumption during enzymatic reactions was monitored using a Clark electrode. The concentrations of nitrite were determined spectrophotometrically at 540 nm, monitoring the formation of azo dye in the presence of sulfanilamide, naphthylethylene diamine dihydrochloride, and four-fold diluted reaction mixture, as described (30). NaNO_2 (10–150 μM) solutions were used for the calibration curve. During thin-layer chromatography (TLC) analysis of tetryl enzymatic reduction products, tetryl (100 μM), NADH (600 μM), and NTR (20 nM) were incubated in 0.1 M Tris–Cl, pH 7.0, for 3 min or 1 h. At the end of reaction, 2 ml of reaction mixture was extracted by 0.2 ml ethylacetate. The organic fraction was applied on Silufol UV254 plates (Kavalier, Czech Republic), and eluted with ethylacetate.

Quantum-mechanical calculations. In semiempirical calculations of compound heat formation (Hf) by AM1 and PM3 methods, PC Spartan Pro (version 1.0.1, Wavefunction, Inc.) was used. The calculations were performed on nitrocompounds and their single- and two-electron reduced forms specified below. For all calculations, geometries were fully optimized. The enthalpies of reactions (ΔHf) were calculated from Eqs. [2–5], where ArNO_2 denotes nitroaromatic compound, $\text{ArNO}_2^{\cdot-}$ denotes its anion-radical, ArNO denotes its nitroso derivative, ArN(OH)_2 denotes *N,N*-dihydroxylamine precursor of nitroso derivative (31), and ArN(OH)O^- —its deprotonized form:

(a) $1e^-$ transfer:

$$\Delta\text{Hf}(\text{ArNO}_2^{\cdot-}) = \text{Hf}(\text{ArNO}_2^{\cdot-}) - \text{Hf}(\text{ArNO}_2), \quad [2]$$

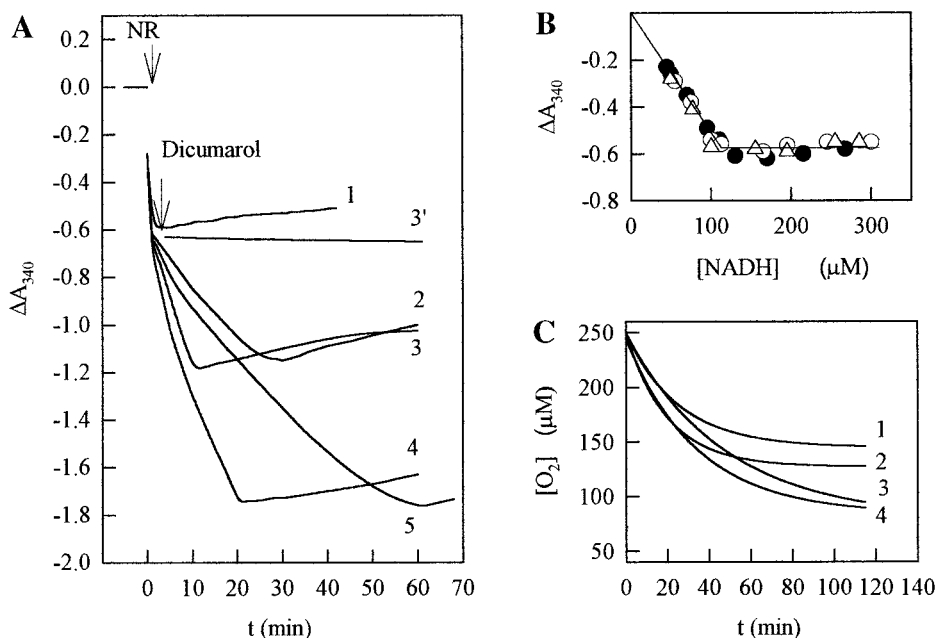


FIG. 2. (A) Kinetics of oxidation of NADH by 50 μM tetryl (curves 1, 3, 3', 5) or by 50 μM TNT (curves 2, 4) in the presence 20 nM *E. cloacae* NR, monitored at 340 nm. Concentration of NADH, 100 μM (1), 200 μM (2, 3, 3'), and 300 μM (4, 5). The time of addition of 20 μM dicumarol (curve 3') indicated by arrow. The rise in absorbance at the end of reaction is due to formation of reaction products, absorbing at 340 nm. (B) The changes in absorbance at 340 nm during the first phase of NADH oxidation showing that 2 mol NADH were oxidized per mole of oxidant. Oxidant: 50 μM tetryl (triangles), 50 μM TNT (filled circles), 50 μM *m*-dinitrobenzene (open circles), concentration of NR, 5 nM. (C) Kinetics of oxygen consumption during *E. cloacae* NR-catalyzed oxidation of 200 μM NADH (1, 2) or 300 μM NADH (3, 4) by 50 μM tetryl (1, 3) or 50 μM TNT (2, 4). NR concentration, 20 nM.

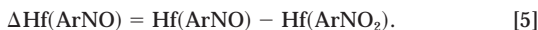
(b) $2e^-$ (hydride)transfer:



or:



or:



RESULTS

The two-electron transferring properties of *E. cloacae* NR have been demonstrated in the reduction of nitrobenzenes and nitrofurans insofar (12, 17). In view of the similarity of the nitrobenzene reduction mechanism of *E. cloacae* NR and DT-diaphorase (17–19), and the unusual single-electron reductive N-denitration of tetryl by DT-diaphorase (Eq. [1]) (16), the detailed study of tetryl reduction by NR may be useful in the evaluation of a possibility of single-electron transfer steps in catalysis of NR.

First, we have examined the possibility of redox cycling during enzymatic reduction of tetryl and other nitroaromatics. We have found that 2 mol NADH were oxidized per mole of mononitrobenzenes (data not

shown), whereas di- and trinitrobenzenes, tetryl, and pentryl oxidized significant excess NADH in several phases (Fig. 2A). During the first phase, 2 NADH equivalents were oxidized (Fig. 2B). NR participated in all phases of reaction, since both fast and slow phases were inhibited by dicumarol (Fig. 2A). The further increase in NADH/oxidant ratio above 6 lead to the low NADH oxidation rates at the end of reaction, approaching intrinsic NADH oxidase activity of NR, 0.2–0.25 s^{-1} . The first rapid phase of NR-catalyzed NADH oxidation by tetryl, pentryl, TNT, or dinitrobenzenes was not accompanied by O_2 uptake, while O_2 has been consumed on a time scale of subsequent phase(s) (Figs. 2A and 2C), with rates and amounts nonstoichiometric to NADH oxidation. The addition of catalase caused return of oxygen, indicating that H_2O_2 was formed as final reaction product (data not shown).

The NR-catalyzed NADH oxidation by tetryl was accompanied by the reduction of added cytochrome *c*. The reduction was much slower than the fast phase of NADH oxidation, and was not inhibited by superoxide dismutase (60 $\mu\text{g}/\text{ml}$). In separate experiments 60 μM cytochrome *c* was introduced into the reaction mixture (200 μM NADH, 50 μM tetryl, 20 nM NR) at different reaction times: (a) before starting the reaction by NR; (b) at the end of fast phase of NADH oxidation (3 min); (c) after terminating the reaction by 20 μM dicumarol,

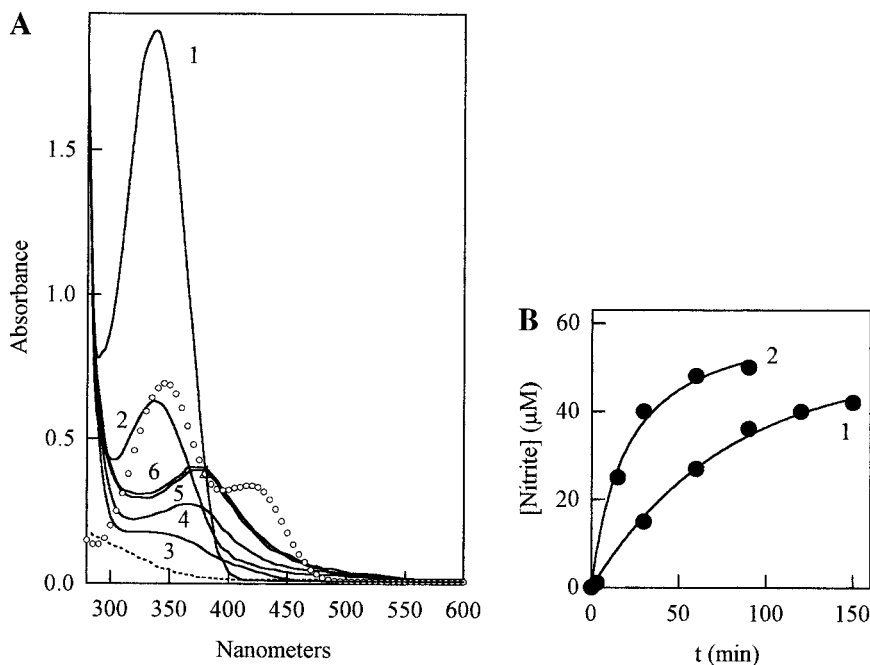


FIG. 3. (A) Oxidation of 300 μM NADH by 50 μM tetryl in the presence of 20 nM *E. cloacae* NR. Spectrum before enzyme addition (1) and after 10 min (2), 20 min (3), 30 min (4), 50 min (5), and 60 min (6) reaction. Dashed line shows absorbance of tetryl in the absence of NADH; absorbance spectrum of 50 μM *N*-methylpicramide shown in circles. (B) Kinetics of nitrite formation during NR-catalyzed reduction of 50 μM tetryl (1) or 50 μM pentryl (2) by 300 μM NADH. Enzyme concentration, 20 nM.

added at the 3rd min of reaction; (d) after 20 min. In all cases, cytochrome *c* was reduced at similar rates, with $t_{1/2} = 1.0$ – 1.5 min for complete reduction. The reactions were not inhibited by superoxide dismutase. The same was observed for cytochrome *c* reduction during NR-catalyzed reduction of TNT and dinitrobenzenes. This indicates that cytochrome *c* is reduced not by nitroaromatic radicals being in redox equilibrium with oxygen/superoxide, but by relatively stable reduction products, which evidently were oxidized also by O_2 (Fig. 2C).

The spectral characteristics (Fig. 3A) of tetryl reduction products rule out *N*-methylpicramide formation (Eq. [1]). During the NR-catalyzed reduction of tetryl by 6 equivalents of NADH, products weakly absorbing at 340–420 nm were formed which slowly transformed into secondary products with $\lambda_{max} = 375$ nm (Fig. 3A). The later process is accompanied by the production of close to stoichiometric amount of nitrite (Fig. 3B). The same 375 nm absorbing species were obtained using 2 equivalents of NADH. Thus, the 375 nm absorbing species are formed during slow denitration of products of four-electron reduction of tetryl. In TLC analysis, we have failed to observe yellow spots of *N*-methylpicramide ($R_f = 0.825$) or UV-fluorescent spots of tetryl ($R_f = 0.81$) after either 3 min or 1 h of reaction. Instead, after 3 min we have observed two brown spots with R_f of 0.67 and 0.72, and the appearance of a third brown spot with $R_f = 0.63$ after 1 h. Analogously,

pentryl reduction was accompanied by slow secondary processes with formation of 400 nm absorbing species and parallel nitrite production (Fig. 3B). During the reduction of TNT, we have failed to observe an increase in absorbance at 450–600 nm, which is characteristic for hydride–Meisenheimer complex (24); instead, TNT was reduced to products weakly absorbing at 320–420 nm, the reaction not being accompanied by nitrite formation.

Our next aim was to compare the reactivity of nitrophenyl-*N*-nitramines and TNT with model nitroaromatic compounds. In agreement with the “ping-pong” scheme for the steady-state kinetics of *E. cloacae* NR (17), we have obtained a series of parallel plots in Lineweaver–Burk coordinates at varied concentrations of tetryl, pentryl or 2,4-dinitrophenyl-*N*-methylnitramine as electron acceptor (10–100 μM) and fixed concentrations of NADH (50–250 μM) (data not shown). The values of k_{cat} obtained by extrapolation to an infinite concentration of NADH and the above electron acceptors and k_{cat}/K_m values of all the electron acceptors investigated are given in Table I. The k_{cat}/K_m for NADH was almost identical for several electron acceptors used (tetryl, pentryl, and 2,4-dinitro-*N*-methylnitramine), being equal to $(5.9 \pm 0.77) \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

Table I also contains the values of single-electron reduction potentials (E_1^{\cdot}) of nitroaromatic compounds used in this work. Similar to earlier data (12), the reactivity of *E. cloacae* NR increased upon increase in

TABLE I
Kinetic Characteristics of Nitrocompound Reduction by *Enterobacter cloacae* Nitroreductase and Single-Electron Reduction Potentials (E_7^1) of Nitrocompounds

No. Compound	k_{cat} (s ⁻¹) ^a	k_{cat}/K_m (M ⁻¹ s ⁻¹)	E_7^1 (V) ^b
1. Tetryl	800 ± 92 (1820 ± 120)	$(7.3 ± 0.51) × 10^6$	—
2. Pentryl	620 ± 75 (1250 ± 115)	$(6.3 ± 0.52) × 10^6$	—
3. 2,4-Dinitrophenyl-N-methylnitramine	410 ± 52 (1020 ± 80)	$(1.0 ± 0.15) × 10^7$	—
4. 2,4,6-Trinitrotoluene	143 ± 22	$(9.8 ± 1.5) × 10^6$	—
5. 2,4-Dinitrotoluene	$(660 ± 60)^c$	$(2.0 ± 0.06) × 10^{5c}$	—
6. Nifuroxim	47 ± 4.2	$(7.8 ± 0.5) × 10^5$	-0.255
7. Nitrofurantoin	102 ± 9.2	$(5.0 ± 0.2) × 10^5$	-0.255
8. <i>p</i> -Dinitrobenzene	305 ± 15	$(3.1 ± 0.2) × 10^6$	-0.257
9. <i>o</i> -Dinitrobenzene	91 ± 7.0	$(1.46 ± 0.07) × 10^6$	-0.287
10. <i>p</i> -Nitrobenzaldehyde	104 ± 8.7	$(8.02 ± 0.80) × 10^5$	-0.315
11. <i>m</i> -Dinitrobenzene	167 ± 9.0	$(5.0 ± 0.23) × 10^5$	-0.345
12. 3,5-Dinitrobenzamide	370 ± 21	$(4.1 ± 0.18) × 10^6$	-0.350
13. <i>p</i> -Nitroacetophenone	100 ± 12	$(8.05 ± 0.95) × 10^5$	-0.355
14. <i>o</i> -Nitrobenzaldehyde	83 ± 7.5	$(3.9 ± 0.25) × 10^5$	-0.355
15. <i>p</i> -Nitrobenzyl alcohol	20 ± 4.2	$(1.0 ± 0.1) × 10^4$	-0.477
16. Nitrobenzene	10 ± 1.3	$(3.0 ± 0.33) × 10^4$	-0.485

^a NADH concentration, 150 μM. The k_{cat} values in parentheses obtained at infinite concentrations of both substrates.

^b From Ref. (32).

^c From Ref. (17).

E_7^1 of several nitroaromatic compounds (Table I). However, the linear correlation between $\log k_{cat}/K_m$ and E_7^1 values was poor, $r^2 = 0.6480$ (data not shown), and the data were better described by parabolic approximation ($r^2 = 0.8066$) (Fig. 4). It indicates that nitrocompound reactivities are determined mainly by their electron accepting properties and not by their particular structure. This served as a starting point for the de-

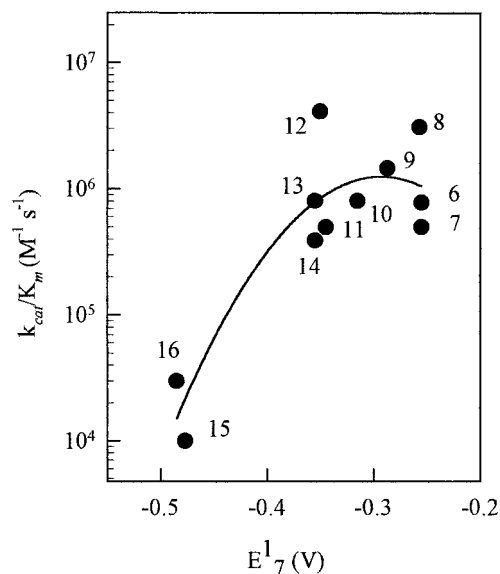
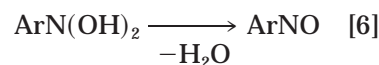


FIG. 4. The dependence of $\log k_{cat}/K_m$ of nitroaromatic compounds on their single-electron reduction potentials (E_7^1). The numbers of compounds are taken from Table I.

scription of quantitative structure–activity relationships involving compounds with unknown E_7^1 values, like TNT, tetryl, and other nitrophenyl-*N*-nitramines. It is known that the enthalpies of reactions (ΔH_f) obtained by means of quantum-mechanical calculation, exhibit a correlation with single-electron transfer redox potentials (33). The calculated ΔH_f values of nitrocompound single-electron reduction ($\Delta H_f(\text{ArNO}_2^{\cdot-})$) are given in Table II. The linear correlation between $\log k_{cat}/K_m$ and $\Delta H_f(\text{ArNO}_2^{\cdot-})$ was characterized by $r^2 = 0.6753$ (PM3), and 0.6615 (AM1); however, the data were better described by a parabolic correlation ($r^2 = 0.7731$ (PM3), and 0.8253 (AM1, Fig. 5A)). Further, we have extended this approach for the description of two-electron (hydride) transfer. During reduction of nitro group to nitroso, a *N,N*-dihydroxylamine intermediate ($\text{ArN}(\text{OH})_2$) is formed (31) (Eq. [6]). It seems unlikely that the formation of $\text{ArN}(\text{OH})_2$ with a net $2e^-$, $2H^+$ (or H^- , H^+) transfer, proceeds in a single step. We propose that the initial step in ArNO_2 reduction is a hydride transfer, yielding anionic *N,N*-dihydroxylamine form ($\text{ArN}(\text{OH})\text{O}^-$) (Eq. [6]):



An analogous mechanism has been postulated for the nonenzymatic NADH reduction of quinones, where the

TABLE II
Enthalpies of Single- and Two-Electron Reduction of Nitroaromatics Calculated by Eqs. [2–5]
Using PM3 and AM1 Methods

No. Compound	ΔH_f (kcal/mol)							
	$\Delta H_f(\text{ArNO}_2^-)$		$\Delta H_f(\text{ArN}(\text{OH})\text{O}^-)$		$\Delta H_f(\text{ArN}(\text{OH})_2)$		$\Delta H_f(\text{ArNO})$	
	PM3	AM1	PM3	AM1	PM3	AM1	PM3	AM1
1. Tetryl	-87.90	-91.21	-67.07 ^a	-86.64 ^a	-14.83 ^a	-38.89 ^a	21.19 ^a	-2.91 ^a
2. Pentryl	-89.98	-94.33	-70.09 ^a	-88.96 ^a	-14.03 ^a	-37.64 ^a	21.04 ^a	-1.02 ^a
3. 2,4-Dinitrophenyl- <i>N</i> -methylnitramine	-71.04	-72.60	-54.89 ^a	-78.68 ^a	-12.30 ^a	-34.09 ^a	22.64 ^a	2.82 ^a
4. 2,4,6-Trinitrotoluene	-75.63	-74.26	-58.29 ^a	-75.71 ^a	-12.75 ^a	-34.59 ^a	21.73 ^a	0.27 ^a
5. 2,4-Dinitrotoluene	-60.74	-59.89	-43.71 ^a	-60.88 ^a	-10.74 ^a	-32.25 ^a	23.15 ^a	2.84 ^a
6. Nifuroxim	-54.48	-53.08	-41.48	-59.84	-13.58	-31.50	21.78	-0.53
7. Nitrofurantoin	-59.74	-57.86	-46.71	-66.67	-13.49	-34.57	22.19	-0.13
8. <i>p</i> -Dinitrobenzene	-67.17	-65.32	-53.81	-65.83	-12.80	-33.11	22.63	3.04
9. <i>o</i> -Dinitrobenzene	-62.38	-61.50	-60.08	-67.17	-22.98	-41.29	7.27	-7.34
10. <i>p</i> -Nitrobenzaldehyde	-53.34	-53.09	-40.74	-55.68	-10.38	-31.12	23.85	4.30
11. <i>m</i> -Dinitrobenzene	-62.65	-60.85	-44.49	-62.36	-11.14	-32.39	22.86	2.65
12. 3,5-Dinitrobenzamide	-69.11	-67.90	-50.80	-66.98	-12.00	-33.14	22.28	2.05
13. <i>p</i> -Nitroacetophenone	-52.12	-51.88	-38.97	-54.64	-9.85	-30.91	24.01	4.44
14. <i>o</i> -Nitrobenzaldehyde	-49.53	-52.05	-41.16	-57.50	-11.44	-31.72	21.89	3.75
15. <i>p</i> -Nitrobenzyl alcohol	-46.04	-44.54	-33.60	-45.18	-8.99	-29.64	24.47	4.90
16. Nitrobenzene	-41.13	-40.07	-29.49	-45.68	-8.72	-29.39	24.61	5.09

^a ΔH_f for reduction of 4-nitro group. The ΔH_f values for reduction of 2-nitro group are more negative by 0.5–1.5 kcal/mol.

rate-limiting net hydride transfer with formation of anionic hydroquinone (QH^-) is followed by fast protonation (formation of QH_2) (34). The calculated ΔH_f values for all the possible steps in the two-electron reduction pathway (Eq. [6]) are given in Table II. The correlations between $\log k_{\text{cat}}/K_m$ and ΔH_f for a net

reaction ($\Delta H_f(\text{ArNO})$) were poor: $r^2 = 0.0695$ (PM3) and 0.2256 (AM1) for a linear approximation, and $r^2 = 0.5597$ (PM3) and 0.4559 (AM1) for a parabolic approximation (data not shown). The use of ΔH_f for hydride transfer and protonation ($\Delta H_f(\text{ArN}(\text{OH})_2)$) modestly improved correlations ($r^2 = 0.2139$ (PM3) and

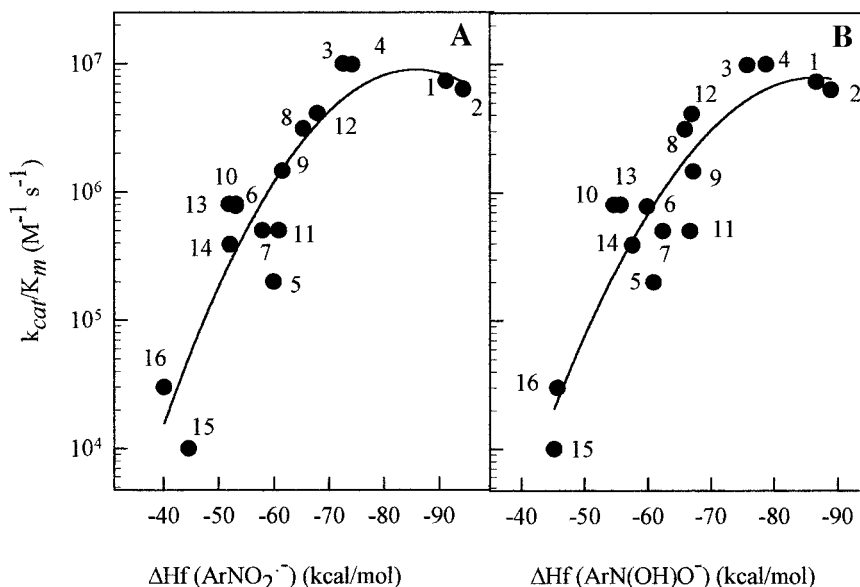
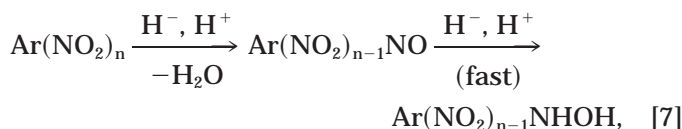


FIG. 5. The dependence of $\log k_{\text{cat}}/K_m$ of nitroaromatic compounds on their enthalpies of single-electron reduction ($\Delta H_f(\text{ArNO}_2^-)$) (A), or reduction by hydride ion ($\Delta H_f(\text{ArN}(\text{OH})\text{O}^-)$) (B), calculated according to Eqs. [2, 3] using AM1 method. The numbers of compounds are taken from Table I, $\Delta H_f(\text{ArN}(\text{OH})\text{O}^-)$ for trinitrobenzenes correspond to reduction of 4-nitro group.

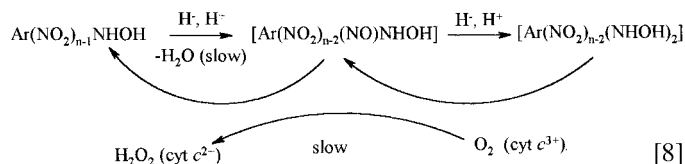
0.3934 (AM1) for a linear approximation, and $r^2 = 0.6390$ (PM3) and 0.7361 (AM1) for a parabolic approximation, data not shown). On the other hand, the use of ΔH_f for hydride transfer alone ($\Delta H_f(\text{Ar-N}(\text{OH})\text{O}^-)$) resulted in linear correlations with $r^2 = 0.6971$ (PM3) and 0.7410 (AM1), and in even better parabolic correlations ($r^2 = 0.7992$ (PM3) and 0.8445 (AM1, Fig. 5B).

DISCUSSION

The data of our work enabled us to make certain conclusions on the mechanism of reduction of nitroaromatics by *E. cloacae* NR: (i) NR does not catalyze the single-electron reductive N-denitration of tetryl which is characteristic of DT-diaphorase (Eq. [1]) (16). The oxygen consumption during the enzymatic reaction (Fig. 2C) and the reduction of added cytochrome *c* are due to the oxidation of relatively stable reduction products and not of free radicals. These reactions are characteristic of hydroxylamine compounds: 4-hydroxylamine reduction product of CB-1954 and 2,4-dihydroxylamine reduction product of TNT can react with O_2 , yielding H_2O_2 (19, 22), the products of four-electron reduction of dinitropyrenes by DT-diaphorase can reduce cytochrome *c* (35); (ii) the formation of nitrite does not proceed simultaneously with 4-electron reduction of tetryl and pentryl (oxidation of 2 NADH equivalents), but takes place in a secondary slower process (Fig. 3B). It is known that nitrite may be formed during slow C-denitration of hydride-Meisenheimer complexes of TNT aromatic ring (3, 24). However, we did not observe the characteristic absorbance of the Meisenheimer complex during TNT reduction by *E. cloacae* NR. Thus, the formation of nitrite during the reduction of *N*-nitramines (Fig. 3B) is most probably due to N-denitration; (iii) the oxidation of over 2 mol NADH per mole of oxidant (Fig. 2A) indicates that NR may reduce polinitroaromatics by more than 4 electrons to some currently unidentified products which are then slowly reoxidized by O_2 (Figs. 2C and 3A), e.g., dihydroxylamines (22, 23). However, the identification of reaction products was beyond the scope of the present work; (iv) the same rapid oxidation of 2 mol NADH per mole of oxidant was observed with nitrobenzenes and nitrophenyl-*N*-nitramines (Figs. 2A and 2B). Thus, all the groups of nitrocompounds examined share the same initial four-electron nitroreduction step, which, according to previous studies (17), involves a rate limiting formation of nitroso derivative. Although in the present work we did not attempt to identify the reaction products, one may propose the tentative scheme of reduction of polinitroaromatic compounds ($\text{Ar}(\text{NO}_2)_n$) by excess NADH in *E. cloacae* NR-catalyzed reactions (Eq. [7, 8]): (a) the initial fast phase of NADH oxidation:



(b) the subsequent slower phases of NADH oxidation accompanied by O_2 consumption or cytochrome *c* reduction:



Among the calculated ΔH_f values for all the possible steps in the two-electron reduction pathway (Eq. [6], Table II), the use of $\Delta H_f(\text{ArN}(\text{OH})\text{O}^-)$ resulted in the best approximation of a quantitative structure-activity relationship (Fig. 5B). This may indicate that the two-electron reduction of nitroaromatics by *E. cloacae* NR proceeds with a rate-limiting hydride transfer and that subsequent steps (i.e., protonation and dehydration, see Eq. [6]) are faster.

One should note that the rates of nitroaromatics reduction by NR depend equally well on both ΔH_f of hydride and electron transfer (Figs. 5A and 5B) and increase upon an increase in their E_1^+ values (Fig. 4). This closely resembles the regularities observed in the reduction of quinones by 1,4-dihydronicotinamides: (i) the parabolic reactivity vs E^1 relationship and the transient formation of an ion-radical pair or charge-transfer complex (36) points to multistep (e.g., e^- , H^+ , e^-) hydride transfer (36); (ii) the reaction rate increases upon an increase in redox potential of the quinone/anionic hydroquinone (Q/QH^-) couple as well, which is formally consistent with the single-step hydride transfer model (34). Thus, our data do not exclude a possibility of a multistep hydride transfer during the reduction of nitroaromatics by NR. In this aspect, it is of some interest to discuss the differences in the mechanisms of tetryl reduction by *E. cloacae* NR and DT-diaphorase with regard to the single-electron reduction potentials of their flavin cofactors. At pH 7.0, the redox potentials of $\text{FAD}/\text{FADH}^\cdot$ and $\text{FADH}^\cdot/\text{FADH}_2$ couples of DT-diaphorase are equal to -200 mV and -118 mV, respectively (37), which corresponds to 8% of the radical produced at equilibrium. On the other hand, less than 1% FMN semiquinone was produced at the midpoint potential of *E. cloacae* NR (-190 mV, pH 7.0) (38). An upper limit of -290 mV can be calculated for FMN/FMN semiquinone couple, and a lower limit of -90 mV for FMN semiquinone/ FMNH^- couple respectively (38). Thus, the transfer of the first electron from FADH_2 of DT-diaphorase to electron ac-

ceptor is thermodynamically more favourable than from FMNH⁻ of *E. cloacae* NR. Irrespective of this, DT-diaphorase catalyzes single-electron tetryl reduction with $k_{\text{cat}}/K_m = (2.6 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (16), which is much lower than in the net two-electron (hydride) reduction by NR ($k_{\text{cat}}/K_m = (7.3 \pm 0.51) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, (Table I)). This may point to the different mechanisms of reactions, e.g., multistep hydride transfer in catalysis of DT-diaphorase, and a single-step transfer in NR catalysis. However, the tetryl anion-radical formed after the first electron transfer, may either dissociate from the active center or accept a second electron. The transfer of a second electron from NR semiquinone is thermodynamically more favourable than from FADH[•] of DT-diaphorase. This may explain why DT-diaphorase catalyzes single-electron reduction of tetryl, which is not characteristic for *E. cloacae* NR. Thus, the possibility of multistep hydride transfer in reduction of nitroaromatics by *E. cloacae* is not excluded. However, one may suppose that if transient ion-radical pairs or charge-transfer complexes were formed, they would be even less stable than in reactions of DT-diaphorase.

CONCLUSIONS

In contrast to the well-documented reactivity vs. E¹ relationships in single-electron reduction of nitroaromatics by flavoenzymes (5–8), the quantitative descriptions of two-electron enzymatic reduction are almost absent. The enthalpies of two-electron (hydride) reduction obtained by quantum chemical calculations in the present work may serve as useful tool for analysis of the mechanisms of two-electron reduction, especially nitroaromatic compounds with presently unknown redox potentials. Although the quantitative structure–activity relationships obtained in this work are specific for a particular enzyme, *E. cloacae* NR is a member of a larger family of proteins including the *E. coli* and *Salmomella typhimurium* nitroreductases, with which it shares over 80% amino acid sequence identity (39, 40). Thus, these results may be extended to related nitroreductases.

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