FEBS Letters 450 (1999) 44–48 FEBS 21929

Quantitative structure activity relationships for the electron transfer reactions of *Anabaena* PCC 7119 ferredoxin-NADP$^+$ oxidoreductase with nitrobenzene and nitrobenzimidazolone derivatives: mechanistic implications

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Received 13 March 1999

Abstract The steady state single electron reduction of polynitroaromatics by ferredoxin-NADP$^+$ oxidoreductase (EC 1.18.1.2) from cyanobacterium *Anabaena PCC 7119* has been studied and quantitative structure activity relationships are described. The solubility of the polynitroaromatics as well as their reactivity towards ferredoxin-NADP$^+$ oxidoreductase are markedly higher than those for previously studied mononitroaromatics and this enabled the independent measurement of the kinetic parameters k_{cat} and K_m. Interestingly, the natural logarithm of the bimolecular rate constant, k_{cat}/K_m, and also the natural logarithm of k_{cat} correlate with the calculated energy of the lowest unoccupied molecular orbital of the polynitroaromatic substrates. The minimal kinetic model in line with these quantitative structure activity relationships is a ping-pong mechanism which includes substrate binding equilibria in the second half reaction.

Key words: Nitrobenzene; Nitrobenzimidazolone; Electron transfer; Quantitative structure activity relationship; Ferredoxin reductase; Lowest unoccupied molecular orbital; Kinetic model

1. Introduction

Ferredoxin-NADP$^+$ oxidoreductase (EC 1.18.1.2) is a single subunit FAD containing enzyme that participates in the reductive part of the photosynthetic chain of higher plants and cyanobacteria, transferring electrons from reduced ferredoxin or flavodoxin to NADP$^+$ [1]. The primary structure of ferredoxin-NADP$^+$ oxidoreductase from the cyanobacterium *Anabaena* PCC 7119 is partly homologous to enzymes from higher plants [2]. A three-dimensional structure of *Anabaena* ferredoxin-NADP$^+$ oxidoreductase has been obtained at a 0.18 nm resolution [3] and closely resembles, for example, the structure of the spinach enzyme [4].

Although extensive literature exists on the mechanism of electron transfer between ferredoxin-NADP$^+$ oxidoreductase and ferredoxin [5–12], relatively little is known about the mechanism of the single electron reduction of low-molecular weight oxidants and redox active xenobiotics by ferredoxin-NADP$^+$ oxidoreductase. The single electron reduction of quinones [13,14], viologen derivatives [15,16] and heteropentalenes [17] by ferredoxin-NADP$^+$ oxidoreductase is supposed to be partly responsible for the herbicidal action of these compounds [16,17]. In addition to these compounds, nitroaromatics can undergo a single electron reduction by ferredoxin-NADP$^+$ oxidoreductase [14,18]. Nitroaromatic compounds are widely used as industrial intermediate reagents in the production of dyes, rubbers, explosives and pharmaceuticals or are used as agrochemicals such as pesticides or veterinary drugs [19–21]. Attempts to correlate and quantitatively understand the role of ferredoxin-NADP$^+$ oxidoreductase in the reduction and bioactivation of nitroaromatics were hampered by the relatively low reactivity of the mononitroaromatic substrates studied so far. Recently, however, a series of newly synthesised nitrobenzimidazolones proved to be more efficient substrates for some of the single or two electron reducing flavin-dependent enzymes such as ferredoxin-NADP$^+$ oxidoreductase or DT-diaphorase [22,23].

Up to now, attempts to define quantitative structure activity relationships (QSARs) for k_{cat} as well as k_{cat}/K_m values for the reduction of various nitroaromatic compounds by flavoenzymes were unsuccessful [14,18,22,24]. Recently, however, we have described a QSAR for the two electron reduction of a series of nitrobenzimidazol(on)es by DT-diaphorase based on calculated energy of the lowest unoccupied molecular orbital (E(LUMO)) [23]. The calculated E(LUMO) is a parameter characterising the reactivity of compounds in single or two electron reduction reactions.

The objective of the present work was to investigate whether the use of quantum mechanical computer calculations and the use of the series of more reactive and better water soluble nitroaromatics, enabling measurement of independent K_m and k_{cat} values, provide a basis for the investigation of the possible existence of QSARs for the single electron reduction of polynitroaromatic substrates by ferredoxin-NADP$^+$ oxidoreductase.

2. Materials and methods

2.1. Chemicals

Fig. 1 presents the structural formulas of the compounds used in the present study. Compounds 1–8 were synthesised as previously described [22,26,27,28]. All compounds were characterised by melting points, ^{1}H NMR and IR spectroscopy as described [26,27].

NADPH, cytochrome *c*, bovine serum albumin, Tween-20, glucose-

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PII: S0014-5793(99)00464-0

6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma.

2.2. Enzymatic assays

Ferredoxin-NADP$^+$ oxidoreductase was purified from the cyanobacterium *Anabaena* PCC 7119 and its concentration determined spectrophotometrically using an extinction coefficient $\Delta\varepsilon_{459} = 9.7$ mM^{-1}/cm as described [25]. All kinetic measurements were carried out in 0.1 M potassium phosphate pH 7.0 containing 1 mM EDTA at 25°C. Tween-20 (final concentration 0.01%) and bovine serum albumin (final concentration 0.25 mg/ml) were used as enzyme activators [18]. The reaction rates were monitored spectrophotometrically following NADPH oxidation ($\Delta\varepsilon_{340} = 6.2$ mM^{-1}/cm) or cytochrome *c* reduction ($\Delta\varepsilon_{550} = 19.2$ mM^{-1}/cm). The latter measurements were carried out in the presence of a NADPH regenerating system (2 mM glucose-6-phosphate and 30 μg/ml glucose-6-phosphate dehydrogenase). To a 1 ml reaction mixture, 10–20 μl of a desired stock solution of the compound, dissolved in DMSO, was added to start the reaction. The presence of 1–2% of DMSO in the enzyme reaction mixture has no influence on the activity of the enzyme [14]. All experiments were run at least in triplicate.

The kinetic parameters, the apparent catalytic constant (k_{cat}) and Michaelis constant (K_m) were determined by fitting the data to the Michaelis-Menten equation. The k_{cat} value corresponds to the amount of NADPH oxidised per active center of enzyme per second. Typically, five or six concentrations of the nitroaromatic compounds were used for determination of the kinetic parameters. Assuming ping-pong kinetics, the bimolecular rate constant for the reduction of the polynitroaromatic substrates by reduced ferredoxin-NADP$^+$ oxidoreductase can be calculated as k_{cat}/K_m. The rates obtained were corrected for the intrinsic NADPH-oxidase activity of the enzyme.

In analogy to previous observations [14], the reduction of added cytochrome *c* can be used instead of the determination of NADPH oxidation to quantify ferredoxin-NADP$^+$ oxidoreductase reaction rates. The rates of one electron cytochrome *c* reduction correspond to twice the rates of two electron NADPH oxidation. Taking this into account, the k_{cat}/K_m and k_{cat} values determined from the rates of cytochrome *c* reduction equal those determined by measuring the rates of NADPH oxidation.

2.3. Quantum mechanical calculations

Quantum mechanical molecular orbital calculations were carried out on a Silicon Graphics Indigo using Spartan (version 5.0) (Wavefunction). Different levels of theory and methods were used. For semiempirical molecular orbital calculations, the Hartree-Fock method and the AM1 and PM3 Hamiltonian were used. Ab initio calculations were performed using a Hartree-Fock method with the 3-21G(*) basis set and density functional calculations were performed with the pBP method using the DN** basis set. For all calculations, geometries were fully optimised.

In this study, the outcomes of quantum mechanical calculations on molecules in vacuum are related to the electronic characteristics of the substrates in the active site of ferredoxin-NADP$^+$ oxidoreductase. Due to solvation effects and a different dielectric constant, the intrinsic properties of the nitroaromatics might be influenced upon binding to this active site. Nevertheless, it can be assumed that this phenomenon will not influence the relative differences between parameters of a series of closely related compounds to a significant extent. The outcomes of computer calculations in vacuum can thus be used as an approach to study relative differences within a series of homologous compounds [29–32] or within one molecule [33].

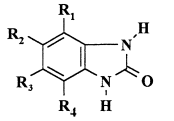
2.4. Linear and multiple regression analysis

Linear and multiple regression analysis was performed using Statistica for windows release 4.3.

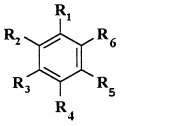
3. Results

3.1. Kinetic parameters for reduction of polynitroaromatics by ferredoxin-NADP$^+$ oxidoreductase

The reduction of the polynitroaromatic substrates by ferredoxin-NADP$^+$ oxidoreductase appeared to follow a classical ping-pong reaction scheme, resulting in a series of parallel lines in Lineweaver-Burk plots at increasing concentrations of the aromatic substrates and fixed concentrations of NADPH. The series of parallel lines was also observed at fixed concentrations of polynitroaromatic substrates and an increasing concentration of NADPH. Table 1 presents the catalytic (k_{cat}) and bimolecular (k_{cat}/K_m) rate constants obtained for the reduction of the various nitroaromatic model compounds by ferredoxin-NADP$^+$ oxidoreductase. Since in this paper we focus on the single electron reduction of nitroaromatic substrates by ferredoxin-NADP$^+$ oxidoreductase, k_{cat} values are presented on the basis of one electron-reduced substrate molecules, which actually equals two times the amount of oxidised NADPH.



Compound no	R_1	R_2	R_3	R_4
1	NO_2	Cl	NO_2	NO_2
2	NO_2	CH_3	NO_2	NO_2
3	NO_2	NO_2	NO_2	H
4	NO_2	$N(CH_3)NO_2$	NO_2	H



	R_1	R_2	R_3	R_4	R_5	R_6
5	NO_2	H	NO_2	H	NO_2	H
6	NO_2	H	H	NO_2	H	H
7	NO_2	H	NO_2	H	H	H
8	NO_2	NO_2	H	H	H	H

Fig. 1. Structural formulas of the polynitrobenzimidazolone (1–4) and polynitrobenzene (5–8) model compounds used in the present study.

3.2. Quantum mechanical calculations

Table 2 presents the calculated E(LUMO) values of the polynitroaromatic substrates of the present study. Although the absolute outcomes of E(LUMO) values vary from one method to another, the relative orders are consistent going from one method to another. This is corroborated by the fact that the data calculated by one method correlate with those calculated by another method with r^2 values varying between 0.908 and 0.980.

3.3. QSARs

In Fig. 2a, the natural logarithm of the $k_{cat}/K_{m(polynitroaromate)}$ values for the reduction of polynitroaromatic substrates by ferredoxin-NADP$^+$ oxidoreductase is plotted against the calculated E(LUMO) values of the compounds. Fig. 2a presents the data obtained with the 3-21G(*) method ($r^2 = 0.832$). Similar correlations are found with the other methods, namely $r^2 = 0.837$ for PM3, $r^2 = 0.802$ for AM1 and $r^2 = 0.708$ for the DN** method. The natural logarithm of the k_{cat} values plotted against the calculated E(LUMO) values of the compounds is shown in Fig. 2b. Again, a clear correlation is observed for all methods used, i.e. $r^2 = 0.890$ for AM1, $r^2 = 0.877$ for PM3, $r^2 = 0.944$ for 3-21G(*) and $r^2 = 0.888$ for the DN** method.

The quantum chemical calculations also provide log *P* values for the polynitroaromatic substrates. In a multilinear regression analysis, this hydrophobicity parameter log *P* was used as a second parameter in addition to E(LUMO) to describe the QSARs. The results obtained did not show a substantial improvement of the correlations (data not shown) and

therefore point out the negligible influence of this parameter in addition to the E(LUMO). Thus, it can be concluded that the main characteristic determining the single electron reduction of the polynitroaromatic substrates is their ease of reduction, reflected by their calculated E(LUMO) value.

3.4. Re-evaluation of the kinetical model

The single electron oxidation of the two electron-reduced ferredoxin-NADP$^+$ oxidoreductase (FADH$^-$ form) should proceed in two steps, namely the oxidation of FADH$^-$ to the single electron-reduced form, FADH$^\bullet$ and the subsequent oxidation of FADH$^\bullet$ to the oxidised enzyme form containing FAD [5,14]. Fig. 3a presents the kinetic scheme for the single electron reduction of polynitroaromatic substrates by ferredoxin-NADP$^+$ oxidoreductase, on which the idea of using k_{cat}/K_m for QSAR studies is based. In this model, $k_{cat}/K_{m(polynitroaromate)}$ is dependent on the bimolecular rate constants, k_7 and k_8, for the two single electron transfer reactions between reduced enzyme and the polynitroaromatic substrate (see below). This mechanism can be characterised as an incomplete ping-pong kinetic mechanism. For this model, the steady state rate equation for the formation of the single electron-reduced nitroaromatic product can be derived as:

$$v = \frac{2 \cdot k_2[e_{total}]}{1 + \frac{(k_{-1}+k_2)}{k_1[NADPH]} + \frac{k_2(k_7+k_8)}{k_7k_8[polynitroaromate]}} \quad (1)$$

From this equation, it follows that k_{cat} equals k_2, the apparent $K_{m(NADPH)}$ for the first half reaction equals $(k_{-1}+k_2)/k_1$ and the $K_{m(polynitroaromate)}$ for the single electron reduction of the polynitroaromatic substrate equals $k_2(k_7+k_8)/k_7k_8$. At saturating concentrations of NADPH, $[NADPH] \gg K_{m(NADPH)}$, the ratio $k_{cat}/K_{m(polynitroaromate)}$ equals $k_7k_8/(k_7+k_8)$, which implies that $k_{cat}/K_{m(polynitroaromate)}$ becomes a function of the bimolecular rate constants k_7 and k_8 for the two consecutive substrate reduction steps. As determined in our previous studies by transient kinetics of oxidation of photo-reduced *Anabaena* ferredoxin-NADP$^+$ oxidoreductase by 5,8-dihydroxy-1,4-naphthoquinone [14], the oxidation of the single electron-reduced form of ferredoxin-NADP$^+$ oxidoreductase containing the FADH$^\bullet$ cofactor proceeds much slower than the oxidation of the two electron-reduced enzyme form. The patterns of the steady state reaction inhibition by NADP$^+$ and its redox inactive analogue 2′,5′-ADP also suggested that the rate limiting step in the enzyme re-oxidation is the oxidation of the single electron-reduced form of the enzyme [14]. A similar result has been obtained by transient kinetics for the oxidation of the dithionite-reduced spinach ferredoxin-NADP$^+$ oxidoreductase by another single electron oxidant, ferricyanide [5]. Thus, based on the results obtained by transient kinetics, it can be assumed that $k_7 \gg k_8$, which leads to the situation that the ratio $k_{cat}/K_{m(polynitroaromate)}$ can be presented as k_8. This explains the correlation observed between the natural logarithm of $k_{cat}/K_{m(polynitroaromate)}$ and the calculated E(LUMO) of the polynitroaromatic substrates.

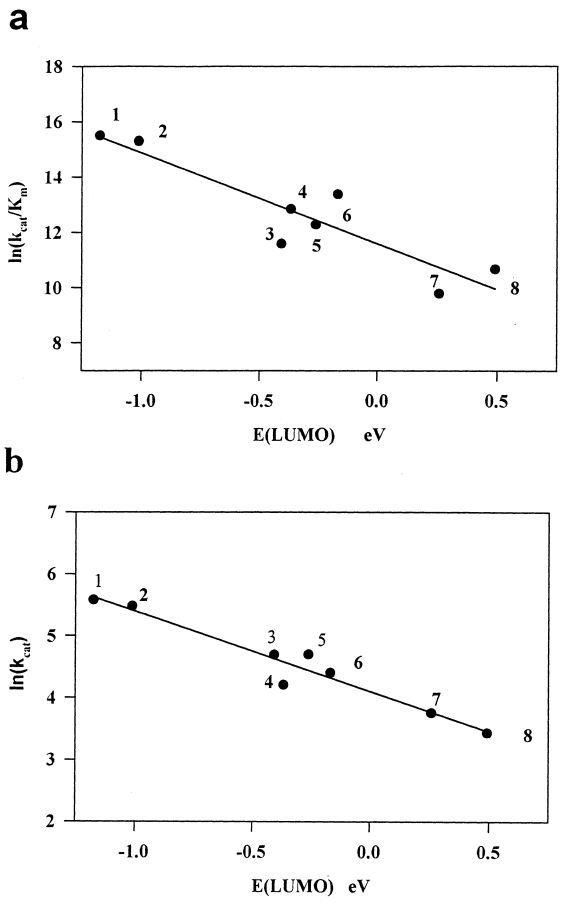


Fig. 2. Quantitative structure activity relationships describing the correlation between (a) the natural logarithm of $k_{cat}/K_{m(polynitroaromate)}$ and E(LUMO) ($r^2 = 0.832$), as well as between (b) the natural logarithm of k_{cat} and E(LUMO) ($r^2 = 0.944$), for the reduction of a series of polynitrobenzimidazolones and polynitrobenzenes by ferredoxin-NADP$^+$ oxidoreductase. E(LUMO) data used are those calculated by the 3-21G(*) basis set.

However, if the kinetic model presented in Fig. 3a would apply, a correlation between the natural logarithm of k_{cat} and the parameter describing the ease of reduction of the polynitroaromatic substrates, E(LUMO), cannot be explained. This can be derived from the fact that the rate equation for this model predicts that $k_{cat} = k_2$. Therefore, the observation of a correlation between the natural logarithm of k_{cat} and E(LUMO) of the polynitroaromatic substrates requires the re-evaluation of the kinetical model often used to describe the oxidation of flavine-dependent reductases [14,16,18,24,34,35].

In order to find a rationale for the observed correlation of the calculated E(LUMO) values with the natural logarithm of both apparent k_{cat} and $k_{cat}/K_{m(polynitroaromate)}$, Fig. 3b presents the complete minimal ping-pong kinetic scheme. The rate equation that can be derived for this model is as follows:

$$v = \frac{\frac{2\,k_2k_4k_6}{(k_2+k_4+k_6)}[e_{total}]}{1 + \frac{k_4k_6(k_{-1}+k_2)}{k_1(k_2+k_4+k_6)[NADPH]} + \frac{k_2(k_5k_6(k_{-3}+k_4)+k_3k_4(k_{-5}+k_6))}{k_3k_5(k_2+k_4+k_6)[polynitroaromate]}} \quad (2)$$

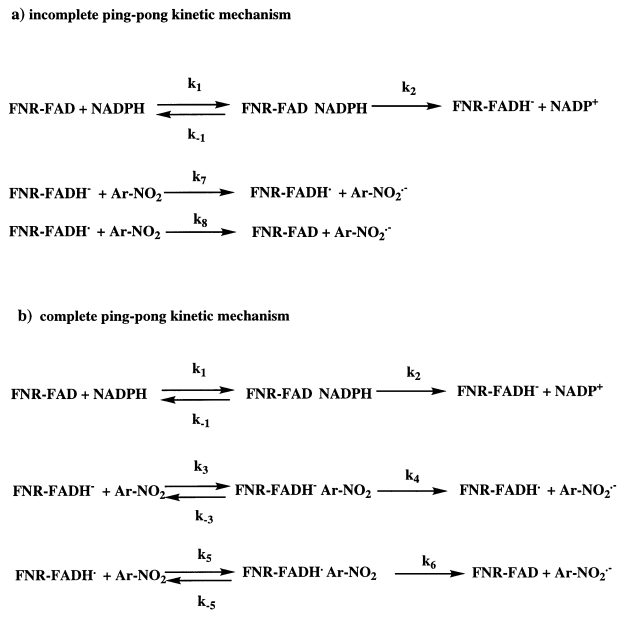


Fig. 3. Kinetic schemes for (a) an incomplete ping-pong kinetic mechanism, i.e. without substrate binding equilibria in the substrate reduction steps, and (b) a complete ping-pong kinetic mechanism, i.e. with substrate binding equilibria in the substrate reduction steps. Ar-NO_2 and Ar-$NO_2^{\bullet-}$ represent the oxidised and single electron-reduced forms of the polynitroaromatic substrates. FNR-FAD, FNR-$FADH^-$ and FNR-$FADH^\bullet$ represent oxidised, two electron and single electron-reduced forms of ferredoxin-$NADP^+$ oxidoreductase.

In this rate equation, the k_{cat} is a complex function of the first order rate constants k_2, k_4 and k_6, $k_2k_4k_6/(k_2+k_4+k_6)$. The apparent $K_{m(NADPH)}$ and $K_{m(polynitroaromate)}$ equal $k_4k_6(k_{-1}+k_2)/k_1(k_2+k_4+k_6)$ and $k_2(k_5k_6(k_{-3}+k_4)+k_3k_4(k_{-5}+k_6))/k_3k_5(k_2+k_4+k_6)$, respectively. Thus, $k_{cat}/K_{m(polynitroaromate)}$ now equals $k_3k_4k_5k_6/(k_5k_6(k_{-3}+k_4)+k_3k_4(k_{-5}+k_6))$ and contains the kinetic constants k_3, k_{-3}, k_5 and k_{-5} for substrate binding in the second half reaction with the $FADH^-$ and $FADH^\bullet$ forms of ferredoxin-$NADP^+$ oxidoreductase whereas it is also governed by the first order rate constants k_4 and k_6 for the consecutive single electron substrate reduction steps by the two electron and single electron-reduced ferredoxin-$NADP^+$ oxidoreductase forms. So, when $k_{-3} \gg k_4$ and $k_{-5} \gg k_6$ and the association constants k_3/k_{-3} and k_5/k_{-5} for the different polynitroaromatic substrates do not vary substantially, the $k_{cat}/K_{m(polynitroaromate)}$ parameter can be simplified to $K_i(k_4k_6/(k_4+k_6))$, where $K_i \approx k_3/k_{-3} \approx k_5/k_{-5}$. Since, as has been outlined above, the second substrate reduction step by the one electron-reduced enzyme is the rate limiting step, it can be derived that $k_{cat}/K_{m(polynitroaromate)}$ now equals K_ik_6 and this can explain the correlation between the natural logarithm of $k_{cat}/K_{m(polynitroaromate)}$ and E(LUMO).

Furthermore, in contrast to the model presented in Fig. 3a, for which k_{cat} equals k_2, the model of Fig. 3b results in a $k_{cat} = k_2k_4k_6/(k_2+k_4+k_6)$. Taking into account that transient kinetics have demonstrated k_4 to be much higher than k_6 [5,14], the expression for the apparent k_{cat} becomes $k_{cat} = k_2k_6/(k_2+k_6)$ and, thus, can also explain the observation of a correlation between the calculated E(LUMO) and the natural logarithm of k_{cat}. Finally, it is of importance to stress that the model of Fig. 3b represents a minimal kinetic model able to explain our QSAR observations. More detailed kinetic studies may lead to a more extended kinetic scheme. This is, however, beyond the scope of the present studies.

Table 1
Catalytic, k_{cat}, and bimolecular rate, k_{cat}/K_m, constants for the single electron reduction of polynitroaromatic substrates by ferredoxin-$NADP^+$ oxidoreductase

Compound	$k_{cat}(s^{-1})$	$k_{cat}/K_m(M^{-1}s^{-1})$
1	266 ± 87	$5.4 \pm 0.9 \times 10^6$
2	241 ± 17	$4.4 \pm 0.9 \times 10^6$
3	67 ± 10	$3.8 \pm 2.1 \times 10^5$
4	82 ± 10	$6.5 \pm 1.3 \times 10^5$
5	109 ± 20	$1.1 \pm 0.4 \times 10^5$
6	110 ± 27	$2.2 \pm 1.0 \times 10^5$
7	43 ± 3	$1.8 \pm 0.3 \times 10^4$
8	31 ± 3	$4.3 \pm 0.9 \times 10^4$

Structural formulas are presented in Fig. 1. The K_m refers to $K_{m(polynitroaromate)}$. The k_{cat} values are presented on a single electron basis.

4. Discussion

The results obtained in the present study show the correlations between a parameter describing the relative ease of a single electron reduction of a series of polynitroaromatic substrates and experimental data for their single electron reduction by ferredoxin-$NADP^+$ oxidoreductase (EC 1.18.1.2). The parameter used for description of the quantitative structure-activity relationships (QSARs) was the E(LUMO) of the polynitroaromatic substrates calculated by quantum mechanical calculations. This E(LUMO) is representative for the relative ease of single or two electron reduction of the nitroaromatic substrates. Especially for the polynitrobenzimidazolones for

Table 2
Calculated E(LUMO) values for the polynitroaromatic substrates, as taken from the literature or calculated in the present study

Compound	E(LUMO) in eV				Reference
Method:	HF/AM1	HF/PM3	HF/3-21 G(*)	pBP/DN**	
1	−3.002	−2.897	−1.175	−5.388	[23]
2	−2.875	−2.834	−1.013	−5.187	[23]
3	−2.469	−2.493	−0.367	−4.911	Present study
4	−2.245	−2.426	−0.168	−4.791	Present study
5	−2.527	−2.527	−0.407	−5.095	Present study
6	−2.245	−2.273	−0.261	−4.871	Present study
7	−1.911	−1.953	0.258	−4.574	Present study
8	−1.843	−1.847	0.491	−4.574	Present study

Structural formulas of compounds are presented in Fig. 1. All energies are presented in eV.

which redox potentials for their single and two electron reduction are still not known, the use of the computer calculation-based approach proved to be valid.

A correlation of E(LUMO) values with the natural logarithm of $k_{cat}/K_{m(polynitroaromate)}$ was observed (Fig. 2a). This k_{cat}/K_m is a parameter previously often used to define QSARs for the single electron reduction of quinones and nitroaromatics by various flavoenzymes [14,16,18,22,24,34,35]. However, due to the relatively high reactivity and solubility of polynitroaromatic substrates as compared to mononitroaromatic substrates, determination of independent k_{cat} and K_m values by Michaelis-Menten type saturation kinetics became feasible. Using the k_{cat} and k_{cat}/K_m values thus obtained, quantitative structure activity relationships (QSARs) could be described between the natural logarithm of $k_{cat}/K_{m(polynitroaromate)}$ and E(LUMO), but also between the natural logarithm of the apparent k_{cat} and E(LUMO). Kinetic analysis shows that a possible minimal kinetic scheme able to explain the observed QSARs includes reversible enzyme substrate binding equilibria preceding substrate reduction steps (Fig. 3b). Thus, it can be concluded that the QSARs and the kinetic analyses of the present study corroborate the existence of a binding equilibrium between the single and two electron-reduced enzyme forms of ferredoxin-$NADP^+$ oxidoreductase and polynitroaromatic substrates.

Correlations reported by Orna et al. [18] and O'Connor et al. [24] between the logarithm of V_{max} and V_{max}/K_m and single or two electron reduction potential values for the reduction of aromatics by other flavoenzymes, such as cytochrome P450 reductase, spinach ferredoxin reductase, or xanthine oxidase, might as well be explained in the framework of the complete ping-pong kinetic schemes discussed here.

Finally, one may conclude that the existence of quantitative structure activity relationships for the conversion of nitroaromatic substrates by ferredoxin-$NADP^+$ oxidoreductase may prove to be of interest for the prediction of the activity of the enzyme with newly synthesised nitroaromatic compounds.

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