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Reduction of aziridyl-substituted anticancer benzoquinones by lipoamide dehydrogenase

Henrikas Nivinskas,

Benjaminas Valiauga,

Jonas Šarlauskas,

Narimantas Čėnas*

Institute of Biochemistry of Vilnius University, Mokslininkų 12, LT-08662 Vilnius, Lithuania

Among NAD(P)H-dependent disulfide-reducing flavoenzymes, mammalian lipoamide dehydrogenase (LipDH, EC 1.8.1.4) possesses the highest quinone reductase activity. The mixed single- and two-electron reduction of quinones is performed via the FAD cofactor, with the participation of both $4e^-$- and $2e^-$-reduced forms of LipDH. We found that LipDH reduced the anticancer aziridinyl-substituted quinones AZQ, DZQ, MeDZQ, RH1, and BZQ, whose reactivity (k_{cat}/K_m) increased with an increase in their single-electron reduction potential (E^1_7). At [NAD^+]/[NADH] = 4.7 which corresponds to the LipDH turnover under the physiological conditions, i.e. its cycling between the oxidized and $2e^-$-reduced forms, the k_{cat}/K_m values for quinones were decreased by 8–20 times. We also found that the physiological substrate of LipDH, lipoamide, accelerated the reduction of aziridinyl-benzoquinones because of their parallel reduction by the reduction product, dihydrolipoamide ($Lip(SH)_2$). These reactions may be partly responsible for the cytotoxicity of aziridinyl-substituted benzoquinones, which arise both from their $1e^-$-reduction (oxidative stress) and from their $2e^-$-reduction (formation of DNA-alkylating aziridinyl-hydroquinones).

Key words: quinone, reduction potential, lipoamide dehydrogenase, oxidative stress, bioreductive activation

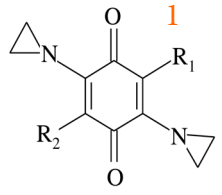
Abbreviations: E^0_7 – potential of two-electron redox couple at pH 7.0; flavin E^1_7 – potential of quinone / semiquinone redox couple at pH 7.0; E_o – oxidized lipoamide dehydrogenase; EH_2 – two-electron reduced lipoamide dehydrogenase; EH_4 – four-electron reduced lipoamide dehydrogenase; GR – glutathione reductase; FAD – flavin adenine dinucleotide; k_{cat} – the catalytic constant (maximal turnover rate) in enzymatic steady-state reactions; k_{cat}/K_m – the bimolecular reaction rate constant in enzymatic steady-state reactions; LipDH – lipoamide dehydrogenase; $LipS_2$ – lipoamide; Q – quinone; TrxR – thioredoxin reductase.

INTRODUCTION

Aziridinyl-substituted 1,4-benzoquinones (Fig. 1) comprise an important group of potential antitumour agents ([1, 2], and references therein). Recently, their new representative, 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1) (Fig. 1), underwent the successful preclinical and phase-I clinical trials [3, 4]. Their cytotoxic / antitumour activities arise mainly from their net two-electron reduction into aziridinyl-hydroquinones, which alkylate DNA more rapidly than the parent quinones. This reaction is performed mainly by flavoenzyme NAD(P)H : quinone oxidoreductase (NQO1, EC 1.6.99.2) [1, 2, 5]. The additional mode of their cytotoxicity is the oxidative stress, being exerted through their single-electron enzymatic reduction with the subsequent redox cycling of their free radicals. The most characterized representative of flavoenzymes reducing quinones in a single-electron way is NADPH: cytochrome P-450 reductase (P-450R, EC 1.6.2.4) [5, 6].

The cytotoxicity of aziridinyl-benzoquinones in several tumour cell lines correlated with the amount of NQO1 [7], however, this rule is not universal [6, 8]. Therefore, their additional action mechanisms, and / or their additional enzyme targets should be considered. In the latter case, flavoenzymes *C-S* transhydrogenases, e. g. glutathione reductase (GR), lipoamide dehydrogenase (LipDH), and thioredoxin reductase (TrxR) deserve certain interest ([9–11], and references therein). These enzymes contain flavin adenine dinucleotide (FAD) cofactor and a catalytic disulfide group, and, in the case of mammalian TrxR, the additional catalytic selenocysteine.

* Corresponding author. E-mail: narimantas.cenas@bchi.vu.lt



$R_1 = R_2 = –H$ (DZQ)
$R_1 = R_2 = –NHCOOC_2H_5$ (AZQ)
$R_1 = R_2 = –CH_3$ (MeDZQ)
$R_1 = –CH_3$, $R_2 = –CH_2OH$ (RH1)
$R_1 = R_2 = –NHC_2H_4OH$ (BZQ)

Fig. 1. The structures of anticancer aziridinyl-benzoquinones studied in this work

During the catalytic cycle, FAD accepts two redox equivalents from NAD(P)H, and subsequently reduces the catalytic disulfide. The latter reduces their physiological disulfide oxidants, glutathione or lipoamide ($LipS_2$), or, in the case of TrxR, reduces catalytic selenocysteine, which subsequently reduces thioredoxin. Among these enzymes, mammalian LipDH possesses the highest quinone reductase activity, which may reach 70 s^{-1} ([10], and references therein). These reactions proceed via the FAD cofactor in a mixed single- and two-electron way.

In this study we examined the reduction of anticancer aziridinyl-substituted antitumour quinones (Fig. 1) by pig heart LipDH. We found that their reactivity increased with an increase in their single-electron reduction potential (E^1_7) and correlated with the previously determined reactivity of aziridinyl-unsubstituted (model) quinones [10]. In addition, we found that the physiological substrate for LipDH and $LipS_2$ significantly stimulated the reduction of some aziridinyl-substituted benzoquinones.

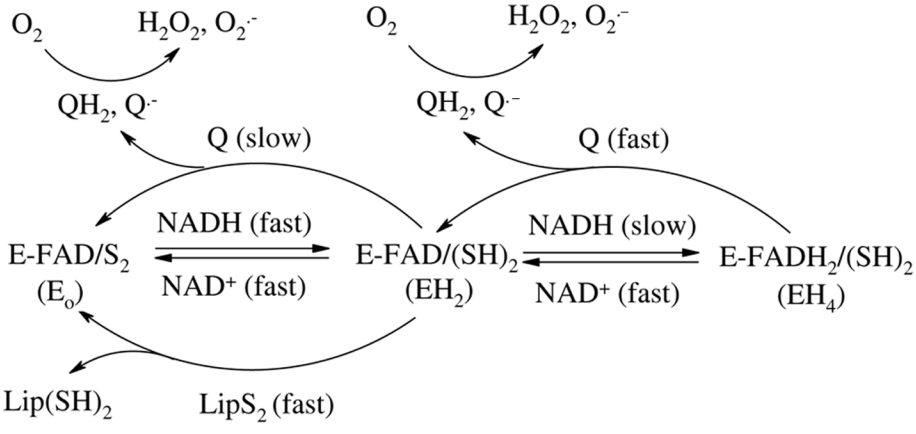
EXPERIMENTAL

Pig heart lipoamide dehydrogenase (LipDH, EC 1.8.1.4), NAD^+, NADH, cytochrome *c*, superoxide dismutase, sodium formate, formate dehydrogenase (EC 1.2.1.2), lipoamide, and model quinones were obtained from Sigma-Aldrich and used as received. The synthesis of aziridinyl-substituted quinones (Fig. 1) was performed according to the established methods [12–15]. All synthesized compounds were characterized by a melting point and ^{1}H-NMR, UV, and IR spectroscopy.

All the spectrophotometric measurements were performed using a Hitachi-557 spectrophotometer at 25 °C in 0.1 M K-phosphate (pH 7.0) containing 1.0 mM EDTA. The concentration of LipDH was determined with the use of $\varepsilon_{455} = 11.3\ mM^{-1}cm^{-1}$. The rate of the oxidation of NADH (100 μM) by LipDH in the presence of 1.0 mM lipoamide or in the presence of quinones (20–200 μM) was determined according to $\Delta\varepsilon_{340} = 6.2\ mM^{-1}cm^{-1}$. In several cases, the rates of NADH oxidation were corrected for the changes in quinone absorbance at 340 nm, using the NADH regeneration system, 100 mM formate and 0.4 mg/ml formate dehydrogenase. Kinetic parameters of the reactions were determined at a fixed concentration of NADPH (100 μM) and varied concentrations of quinones. Typically, 6–8 concentrations of the compounds were used. The catalytic (k_{cat}) and apparent second-order rate (k_{cat}/K_m) constants of reduction of the compounds were obtained in a nonlinear way by a standard hyperbolic expression using SigmaPlot 2000 (version 6.1) or Statistica (version 4.30, Statsoft, 1993). The k_{cat} corresponds to a number of molecules of NADH oxidized by the active center of LipDH per second. In separate experiments, cytochrome *c* (50 μM) was added into the reaction mixture, and its reduction was monitored according to $\Delta\varepsilon_{550} = 20\ mM^{-1}cm^{-1}$. Some experiments were performed in a sealed spectrophotometer cell thus preventing the access of external oxygen. The parallel rates of oxygen consumption were monitored at 25 °C in 0.1 M K-phosphate (pH 7.0) containing 1 mM EDTA, using the Digital Model 10 Clark electrode (Rank Brothers Ltd.) and assuming that the initial O_2 concentration is 250 μM.

RESULTS AND DISCUSSION

LipDH catalyzes the rapid oxidation of NADH at the expense of $LipS_2$. In the presence of 1.0 mM $LipS_2$ and varied concentrations of NADH, 100–15 μM, the k_{cat} of reaction is equal to 290 ± 10s^{-1}, and the k_{cat}/K_m for NADH is equal to $1.3 \pm 0.1 \times 10^7\ M^{-1}s^{-1}$, which are close to the previously reported values ([10], and references therein). In this reaction, LipDH cycles between the oxidized form (E_o), which contains the oxidized FAD and the oxidized catalytic disulfide, and the two-electron reduced form (EH_2), which contains a charge-transfer complex between the oxidized FAD and reduced disulfide ([16], Scheme).



Scheme. The simplified scheme of the reactions of quinones (Q) with two-electron (EH_2)- and four-electron (EH_4)-reduced forms of LipDH according to [10, 19]

The steady-state reactions of quinones with LipDH are much slower than the reduction of $LipS_2$ (Table, Scheme, [10]). It has been shown that the quinone reductase activity of LipDH is mainly attributed to its $4e^-$-reduced form (EH_4), which is partly formed under the reducing conditions in the absence of $LipS_2$ [10] and contains both reduced FAD and reduced catalytic disulfide [10, 16] (Scheme). In this case, the log k_{cat}/K_m of quinones follow a second-order (parabolic) dependence on their single-electron reduction potential (E^1_7) [17], which reflects the ease of their single-electron reduction (Fig. 2, curve A). Importantly, both aziridinyl-substituted and -unsubstituted quinones follow this dependence except the decreased activity of AZQ, which may be attributed to the sterical hindrances. The EH_2 form of LipDH plays a minor role in quinone reduction (Scheme) because of a low electron density on the FAD cofactor in the FAD-thiolate charge-transfer complex [16]. There also exists a rapid redox equilibrium between the NAD^+/NADH and the E_o/EH_2 (E^0_7 = –0.285 V), and EH_2/EH_4 (E^0_7 = –0.345 V) redox couples of LipDH [16, 18], which is attained much faster than the rate of quinone reduction. Thus, the role of EH_2 in the quinone reduction rate by LipDH may be estimated at $[NAD^+]/[NADH]$ = 4.7, which, according to the Nernst equation, corresponds to the redox potential of the medium of –0.300 V (Table). Under these conditions, EH_4 is not formed, and EH_2 is present at the amount of ca. 75% [19]. The data of the Table show that the rates of LipDH-catalyzed reduction of quinones under the physiological conditions are by 8–20 times lower than in the steady-state assays of LipDH at $[NAD^+]$ = 0 (Table). These reactions are also characterized by a parabolic dependence of log k_{cat}/K_m on E^1_7 (Fig. 2, curve 2). Nevertheless, in terms of k_{cat}/K_m, the reactivity of LipDH towards quinones comprises ≤1% activity of P-450R or NQO1, which are the most important mammalian flavoenzymes performing the single- or two-electron electron reduction of quinones, respectively [5].

The ratio between 1,4-benzoquinone-mediated reduction of cytochrome *c* added into the reaction mixture at pH < 7.2, and the doubled rate of NADH enzymatic oxidation in the presence of 1,4-benzoquinone is the percentage of a single-electron flux in quinone reduction [20]. This assay is based on the rapid 1,4-benzosemiquinone-mediated reduction of cytochrome *c* (k ~10^6 $M^{-1}s^{-1}$), and its slow reduction by 1,4-hydroquinone (~10 $M^{-1}s^{-1}$). In the case of LipDH, the single-electron flux is equal to 20%, i. e. to 40% LipDH-catalyzed 1,4-benzoquinone-mediated cytochrome *c* reduction rate vs the NADH oxidation rate in the same reaction [10, 20]. For aziridinyl-benzoquinones, the ratios between the quinone-mediated reduction of cytochrome *c* and NADH oxidation rates are higher, making up 122 ± 2% (AZQ, DZQ), 170 ± 5% (MeDZQ, RH1), and 195 ± 4% (BZQ), respectively.

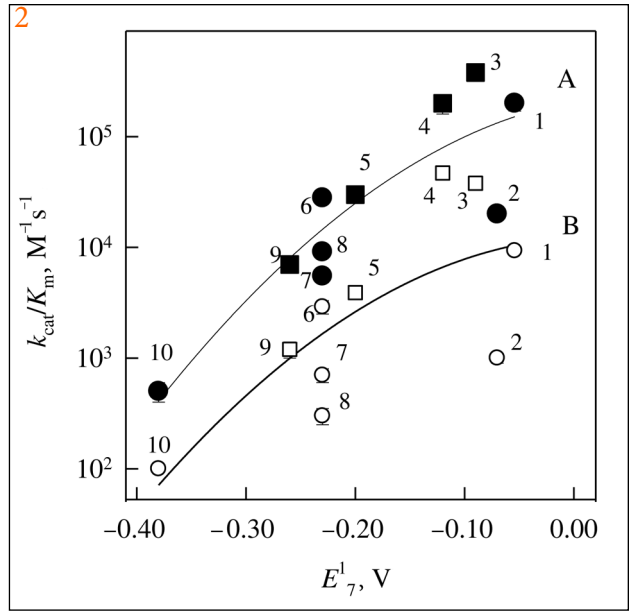


Fig. 2. Quinone reactivity in LipDH-catalyzed reactions. The correlations between the bimolecular rate constants (k_{cat}/K_m) of quinone reduction by LipDH and quinone E^1_7 values: A – $[NAD^+]$ = 0; B – $[NAD^+]/[NADH]$ = 4.7. The curves represent the second order regressions, the numbers of compounds are taken from the Table. The squares represent the aziridinyl-unsubstituted quinones whereas the circles represent aziridinyl-substituted ones

Table. **The steady-state reduction rate constants of aziridinyl-substituted and model quinones (k_{cat} and k_{cat}/K_m) by pig heart LipDH at pH 7.0 and 25 °C in the absence of NAD^+, and at $[NAD^+]/[NADH]$ = 4.7. Concentration of NADH, 100 μM**

No.	Quinone	E^1_7, V [17]	a) $[NAD^+]$ = 0		b) $[NAD^+]/[NADH]$ = 4.7	
			k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}s^{-1}$	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}s^{-1}$
1.	DZQ	–0.054	25 ± 3.0	$2.0 \pm 0.3 \times 10^5$	0.60 ± 0.05	$9.3 \pm 0.3 \times 10^3$
2.	AZQ	–0.07	1.0 ± 0.08	$2.0 \pm 0.2 \times 10^4$	0.05 ± 0.01	$1.0 \pm 0.1 \times 10^3$
3.	5-Hydroxy-1.4-naphthoquinone	–0.09	23 ± 3.0	$3.8 \pm 0.3 \times 10^5$	0.52 ± 0.03	$3.8 \pm 0.2 \times 10^4$ [19]
4.	9,10-Phenanthrene quinone	–0.12	20 ± 2.0	$2.3 \pm 0.4 \times 10^5$ [10], $2.0 \pm 0.4 \times 10^5$	0.50 ± 0.04	$4.7 \pm 0.4 \times 10^4$ [19]
5.	2-Methyl-1,4-naphthoquinone	–0.20	22 ± 3.0	$3.0 \pm 0.5 \times 10^4$ [10], $3.0 \pm 0.4 \times 10^4$	0.45 ± 0.07	$3.9 \pm 0.5 \times 10^3$ [19]
6.	MeDZQ	–0.23	2.0 ± 0.3	$2.8 \pm 0.3 \times 10^4$	2.0 ± 0.3	$2.9 \pm 0.4 \times 10^3$
7.	RH1	–0.23	0.5 ± 0.1	$5.5 \pm 0.7 \times 10^3$	0.15 ± 0.05	$0.7 \pm 0.1 \times 10^3$
8.	2-Aziridinyl-trimethyl-1,4-benzoquinone	–0.23	0.7 ± 0.1	$9.0 \pm 0.1 \times 10^3$	–	$0.3 \pm 0.05 \times 10^3$
9.	Tetramethyl-1,4-benzoquinone	–0.26	–	$7.0 \pm 0.5 \times 10^3$ [10]	–	$1.2 \pm 0.2 \times 10^3$ [19]
10.	BZQ	–0.38	–	$0.5 \pm 0.1 \times 10^3$	–	$<10^2$

It is caused by a parallel rapid reduction of cytochrome *c* by the low-potential aziridinyl-hydroquinones, which are formed during the two-electron enzymatic reduction [5, 17]. The occurrence of the single-electron reduction of aziridinyl-substituted quinones by LipDH is evidenced by the partial inhibition of cytochrome *c* reduction by superoxide dismutase (100 U/mg), e. g. 15 ± 3% (DZQ and AZQ), 20 ± 4% (MeDZQ and RH1), and 30 ± 4% (BZQ). It points to a mixed single- and two-electron reduction of aziridinyl-substituted anticancer benzoquinones by the EH_2 and EH_4 forms of LipDH (Scheme).

Next, we attempted to clarify the role of dihydrolipoamide ($Lip(SH)_2$) in the reduction of aziridinyl-substituted quinones. $Lip(SH)_2$ is an obligatory reducing substrate for LipDH in mitochondrial pyruvate- and ketoglutarate dehydrogenase complexes, which contain several covalently bound molecules of $LipS_2/Lip(SH_2)$ per the active center of LipDH. $Lip(SH)_2$ reduces fully-substituted 9,10-phenanthrene quinone (FQ, $E^1_7 = -0.12$ V) in a two-electron way with a rate constant of $10^4\ M^{-1}s^{-1}$ (Eq.):

$$Lip(SH)_2 + Q \rightarrow LipS_2 + QH_2,$$

whose hydroquinone form (QH_2) undergoes autoxidation [21]. Thus, $Lip(SH)_2$ may participate in the reduction of quinones under the physiological conditions. In this context, we investigated the $Lip(SH)_2$-mediated reduction of aziridinyl-benzoquinone AZQ (Fig. 1), which, being a fully-substituted quinone, does not form quinone-thiolate adducts [21]. Their formation may complicate the reaction analysis. The data of Fig. 3A show that 1.0 mM $LipS_2$ significantly stimulated the consumption of O_2 in the reaction mixture (NADH + LipDH + AZQ + NADH regeneration system). It may be attributed to an additional reaction pathway, $Lip(SH)_2$-mediated reduction of AZQ, where $Lip(SH)_2$ is formed under the action of LipDH (Scheme) and the subsequent oxidation of reduced AZQ. In this case, the demonstration of reduction of AZQ is complicated because both AZQ and NADH absorb at 340 nm. To eliminate the 340 nm absorbance changes caused by the oxidation of NADH, we used the NADH regeneration system. The data of Fig. 3B show that in spite of a rapid LipDH-catalyzed oxidation of NADH by $LipS_2$ (curve 1) the regeneration system ensures the long lasting constant absorbance of NADH (curve 2). The introduction of AZQ increases an absorbance at 340 nm (Fig. 3B), whose subsequent decline is attributed to a LipDH-catalyzed reduction of AZQ. The significant delay in this reaction is caused by the reoxidation of the reduced forms of AZQ by O_2 and their redox cycling. However, after the partial exhaustion of O_2 (Fig. 3A), the reduction of AZQ to its hydroquinone takes place (Fig. 3B, curve 3). Subsequently, curves 4–6 in Fig. 3B show that $LipS_2$ accelerates the reduction of AZQ by LipDH via its direct reduction by $Lip(SH)_2$ (Eq. 1). The analogous phenomena have been also observed in the case of another fully substituted aziridinyl-benzoquinone, RH1 (Fig. 1), although at slower rates (data not shown). The studies of a direct reduction of

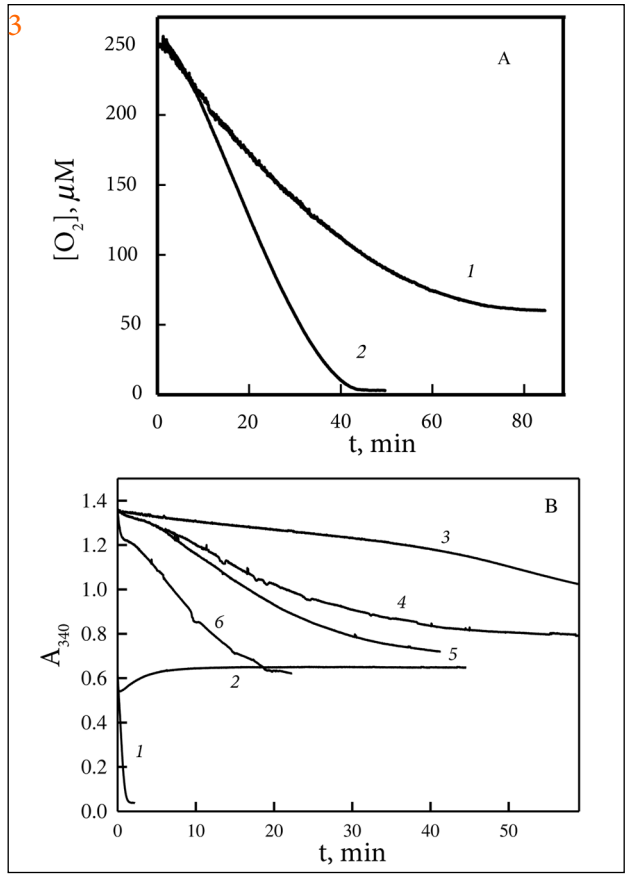


Fig. 3. $LipS_2$ stimulation of the reduction of AZQ by LipDH. A – the oxygen consumption during the LipDH-catalyzed reduction of AZQ: *1* – the oxygen consumption in the presence of 100 μM NADH, 50 μM AZQ, 200 nM LipDH, and NADH regeneration system; *2* – the same as in (*1*), but in the presence of 1.0 mM $LipS_2$. B – the absorbance changes at 340 nm in the sealed spectrophotometer cell (the restricted acess of external oxygen) during the oxidation of 100 μM NADH by 25 nM LipDH: *1* – NADH oxidation in the presence of 1.0 mM $LipS_2$; *2* – the regeneration of NADH (100 μM) in the presence of 1.0 mM $LipS_2$ and NADH regeneration system; *3* – the reduction of added 50 μM AZQ, absorbing at 340 nm, in the presence of 100 μM NADH, 25 nM LipDH, and NADH regeneration system; *4* – the same as in (*3*) in the presence of 0.33 mM $LipS_2$; *5* – the same as in (*3*) in the presence of 0.66 mM $LipS_2$, and *6* – the same as in (*3*) in the presence of 1.0 mM $LipS_2$

AZQ and other fully-dubstituted quinones by $Lip(SH)_2$ are currently underway.

CONCLUSIONS

Lipoamide dehydrogenase catalyzes the mixed single- and two-electron reduction of anticancer aziridinyl-substituted benzoquinones. Thus, it may contribute to the two parallel modes of their cytotoxicity, i. e. the oxidative stress, and the bioreductive alkylation, exerted by the two-electron reduced forms of aziridinyl-benzoquinones. Although the quinone reduction rates of LipDH are relatively low, making up close to 1% of P-450R or NQO1-catalyzed rates, their cytotoxicity may be enhanced by their parallel reduction by $Lip(SH)_2$, the physiological substrate for LipDH.

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AZIRIDINIL-PAVADUOTŲ PRIEŠNAVIKINIŲ CHINONŲ REDUKCIJA LIPOAMIDDEHIDROGENAZE

S a n t r a u k a

Iš NAD(P)H-priklausomų flavininių disulfidreduktazių žinduolių lipoamiddehidrogenazė (LipDH, FK 1.8.1.4) pasižymi didžiausiu chinonreduktaziniu aktyvumu. Šią reakciją atlieka fermento FAD kofaktorius, kuris redukuoja chinonus mišriu vien- ir dvielektroniniu keliu dalyvaujant tiek $4e^-$, tiek $2e^-$-redukuotosioms LipDH būsenoms. Nustatėme, kad LipDH redukuoja ir priešnavikinius aziridinil-pavaduotus chinonus AZQ, DZQ, MeDZQ, RH1 ir BZQ. Jų reaktingumas (k_{cat}/K_m) didėjo kylant vienelektroninės redukcijos potencialui (E^1_7). LipDH funkcionuojant fiziologinėmis sąlygomis, t. y. virsmuose dalyvaujant tik oksiduotajai ir $2e^-$-redukuotajai būsenoms ([NAD^+]/[NADH] = 4,7), chinonų k_{cat}/K_m sumažėdavo 8–20 kartus. Taip pat paaiškėjo, kad fiziologinis LipDH substratas lipoamidas ($LipS_2$) pagreitina aziridinil-pavaduotų chinonų redukciją dėl juos lygiagrečiai redukuojančio fermentinės reakcijos produkto dihidrolipoamido ($Lip(SH_2)$ susidarymo. Šios reakcijos gali būti iš dalies atsakingos už aziridinil-pavaduotų benzochinonų citotoksiškumą, sukeltą tiek jų $1e^-$-redukcijos (oksidacinis stresas), tiek $2e^-$-redukcijos (DNR alkilinančių aziridinil-hidrochinonų susidarymas).