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THE BIOACTIVATION OF 5-(AZIRIDIN-1-YL)-2,4-DINITROBENZAMIDE (CB1954)—I

PURIFICATION AND PROPERTIES OF A NITROREDUCTASE ENZYME FROM *ESCHERICHIA COLI*—A POTENTIAL ENZYME FOR ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY (ADEPT)

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Abstract—A nitroreductase enzyme has been isolated from *Escherichia coli* B. This enzyme is an FMN-containing flavoprotein with a molecular mass of 24 kDa and requires either NADH or NADPH as a co-factor. Partial protein sequence analysis showed extensive homology with the "classical nitroreductase" of *Salmonella typhimurium* and a nitroreductase induced in *Enterobacter cloacae*. In common with the *Salmonella* enzyme, the *E. coli* B enzyme is capable of reducing nitrofurazone. The *E. coli* nitroreductase is also capable of reducing the anti-tumour agent CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide], a property shared with the mammalian enzyme DT diaphorase [NAD(P)H dehydrogenase (quinone)] as isolated from Walker cells. The reduction of CB1954 by the *E. coli* enzyme results in the generation of cytotoxic species. Both enzymes also share the properties of being able to reduce quinones and are both inhibited by dicoumarol. The nitroreductase is a more active enzyme against CB1954 (k_{cat} = 360 min^{-1}) than Walker DT diaphorase (k_{cat} = 4 min^{-1}) and also has a lower K_m for NADH (6 vs 75 μM).

Prodrugs are agents which are not toxic in their own right but can be activated to produce cytotoxic species. If the activation mechanism is specifically associated with tumour cells then a selective antitumour effect can be obtained. CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide‖] (Fig. 1) was initially studied because it exhibited a highly specific toxicity towards the Walker 256 tumour when grown either in the rat [1] or in cell culture [2, 3]. This was not expected from a monofunctional alkylating agent such as CB1954 and, in fact, the basis of its selective action is the formation of DNA interstrand crosslinks in toxically affected cells, but not in non-sensitive cells [3]. Thus, in Walker cells, CB1954 is acting as a difunctional, not a monofunctional, alkylating agent. The observed crosslink formation is a result of the bio-activation of the drug, by the specific reduction of the 4-nitro group, to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide [4]. This compound is capable of reacting difunctionally and is toxic to those cells which are resistant to CB1954. The enzyme which performs this aerobic reduction of CB1954 in rat cells has been isolated, purified and identified as a form of DT diaphorase [NAD(P)H dehydrogenase (quinone), EC 1.6.99.2] [5], an enzyme involved in the metabolism of other chemotherapeutic agents [6].

Apart from Walker cells, sensitivity towards CB1954 has also been reported in bacteria. Thus, it has been reported that the differential response of DNA-repair proficient and defective strains of *Escherichia coli* to the cytotoxic effects of CB1954 resembled that of a difunctional crosslinking agent rather than a monofunctional alkylating agent [7]. Subsequently it was reported that the toxicity and mutagenicity of CB1954 was greatly reduced in a nitroreductase-deficient strain of *E. coli* [8]. It would therefore appear that CB1954 can also be activated by nitro reduction in *E. coli*, in an analogous manner to its bioactivation in Walker cells.

We now report the purification of a nitroreductase from *E. coli* B capable of reducing CB1954 under aerobic conditions and compare some of the properties of this enzyme with the mammalian enzyme DT diaphorase, as purified from Walker cells.

MATERIALS AND METHODS

Materials. All chemicals and reagents were supplied by the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. CB1954 was supplied by

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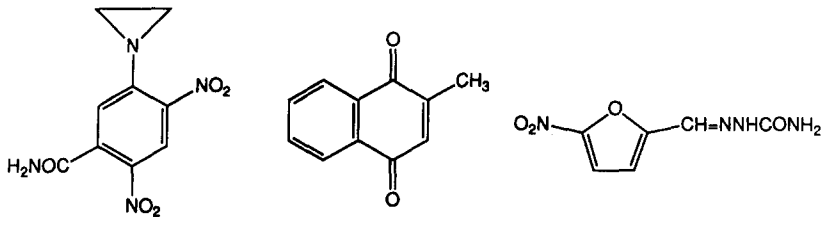
‖ Abbreviations: CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; BCA, bicinchoninic acid; BSA, bovine serum albumin; ADEPT, antibody-directed enzyme prodrug therapy; PBS, phosphate-buffered saline.



CB 1954 menadione nitrofurazone

Fig. 1. The structures of some of the substrates for the *E. coli* nitroreductase.

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Purified DT diaphorase from Walker 256 tumour cells was prepared as previously described [4]. *E. coli* B cell paste was produced by the Division of Biotechnology, PHLS-CAMR.

Isolation and purification of a nitroreductase enzyme from E. coli *B.* Two hundred grams of *E. coli* B cell paste were resuspended to a total volume of 1 L in 20 mM potassium phosphate buffer, pH 7, containing 0.3 M ammonium sulphate. The cells were broken ultrasonically, whilst being cooled in an ice bath, using an MSE Soniprep 150 disintegrator (3 × 30 sec on full power with 60 sec intervals to allow heat to dissipate). To aid clarification of the extract, DNase (23,000 Kunitz units/L) and RNase (2400 Kunitz units/L) were added prior to cell debris removal by centrifugation at 8000 *g* for 30 min. The clear yellowish supernatant was passed through a 0.45 μm filter prior to chromatography.

The filtered extract was applied to a column (25 × 5 cm) of Phenyl-Sepharose CL-6B (Pharmacia) in 20 mM potassium phosphate buffer, pH 7, containing 0.3 M ammonium sulphate. After washing with two column volumes of starting buffer, the column was eluted with 10 mM Tris–HCl buffer, pH 7.6. Active fractions were pooled and dialysed for 18 hr against 20 mM Tris–HCl, pH 7.6, to remove traces of ammonium sulphate. The dialysed fractions were applied in 50 mL aliquots to Q-Sepharose High Performance (Hi-Load 26/10 column, Pharmacia) in 20 mM Tris–HCl at a flow rate of 4 mL/min. Elution was by a 0–0.2 M gradient of KCl, the nitroreductase eluting at 0.1–0.12 M KCl. Active fractions were pooled and desalted into 20 mM Bis–Tris propane, pH 7, using a column (32 × 6 cm) of Sephadex G25 medium. These fractions were applied to Q-Sepharose High Performance equilibrated in 20 mM Bis–Tris propane, pH 7. Elution was by a 0–0.1 M gradient of KCl. Nitroreductase eluted as the first major peak at 0.07–0.09 M KCl.

Homogeneity of the final product was ascertained using precast 8–25% gradient gels for SDS–PAGE (Pharmacia Phastsystem). Electrophoresis was performed for 75 vh.

The nitroreductase in crude and partially purified fractions was routinely assayed by its quinone reductase activity using menadione as substrate, NADH as cofactor and cytochrome *c* as terminal electron acceptor [5].

Determination of isoelectric point. The isoelectric point of nitroreductase was determined by isoelectric focusing (Pharmacia Phastsystem, IEF 3-9 gels, focusing for 400 vh) and chromatofocusing using a Mono P column (Pharmacia Mono P HR5/20, 20 mM Bis–Tris pH 6.3 and polybuffer 74, pH 4.0).

*Enzymatic reduction of CB*1954. CB1954 (100 μM), and NADH or NADPH (500 μM) were incubated with enzyme (2 μg/mL *E. coli* nitroreductase or 50 μg/mL Walker DT diaphorase) in 100 mM sodium phosphate buffer (pH 7) under either air or helium [4]. At various times aliquots (10 μL) were injected onto a Partisphere SCX (100 × 4.7 mm) HPLC column and eluted isocratically (2 mL/min) with 100 mM NaH_2PO_4. The eluate was continuously monitored for absorption at 310, 260 and 360 nm and the spectra of eluting components recorded using a diode-array detector.

Enzyme kinetic and inhibition studies. Quinone reductase activities were assayed by a spectrophotometric method using menadione as a substrate and cytochrome *c* as a terminal electron acceptor as detailed previously [5] ([menadione] = 10 μM, [NADH] = 50 μM, enzyme 1 μg/mL) or by using CB1954 as substrate as described earlier. Initial rates of reaction were determined by linear regression analysis ($r > 0.995$) and kinetic parameters determined from the resulting plots as described previously [9]. Protein concentration was determined using the commercial protein assay (bicinchoninic acid, BCA; Pierce Chemical Co.) calibrated against bovine serum albumin (BSA).

The reduction of nitrofurazone was determined by HPLC by following the formation of NAD^+ by the oxidation of NADH. Experiments were carried out as for the reduction of CB1954 except that the aliquots were injected onto a Partisphere SAX (110 × 4.7 mm) HPLC column and eluted isocratically (1.5 mL/min) with 0.1 M NaH_2PO_4.

Amino acid sequence analysis. Sequence analysis was performed by automated Edman degradation using an Applied Biosystems 470A gas phase protein sequencer (Kelvin Close, Warrington, U.K.).

Initial sequencing of the N-terminus was performed and fragments suitable for sequence analysis were subsequently produced by digestion of the enzyme with cyanogen bromide. The peptides which resulted from these digests were purified by reverse-phase HPLC, using a RP 300 column (25 × 4.6 mm) (Brownlee) and a solvent gradient of 10–60%

acetonitrile in water with 0.06% trifluoroacetic acid in each solvent.

Electrophoresis and western blotting. SDS–PAGE was carried out on 15% gels as described by Laemmli [10]. Duplicate gels were blotted onto Hybond-C Extra membranes. Membranes were blocked in a solution containing 50 mM Tris, 80 mM NaCl, 2% BSA and 5% dry milk (Marvel) pH 8.0 at 37° and then washed three times after each incubation with phosphate-buffered saline (PBS) containing 0.1% Tween 20. All incubations were at 37° for 2 hr. A polyclonal antibody was raised against Walker DT diaphorase in the rabbit. Purified primary antibody was routinely used at a concentration of 2–4 μg/mL in PBS containing 5% BSA. ^{125}I-Labelled sheep anti-rabbit F(ab)$_2$ fragment was employed as second antibody at 10^5 cpm in PBS containing 0.5% BSA. Autoradiograms were exposed at −70° for 1–7 days.

RESULTS

Properties of the E. coli *nitroreductase*

A pure protein with a molecular mass of 24 kDa was isolated from *E. coli* B cell paste (Fig. 1, Table 1). A second protein which had quinone reductase activity but was inactive as a nitroreductase against CB1954 was also identified. It partially co-eluted from Phenyl Sepharose but could be fully separated from the nitroreductase enzyme by the ion exchange chromatography step on Q-Sepharose High Performance at pH 7.6 (Fig. 2, Table 1). The active *E. coli* nitroreductase was estimated to have a molecular mass of 24 kDa compared with 33.5 kDa for the Walker NADP(H) dehydrogenase (quinone) (Fig. 3A) and an isoelectric point of 5.1 (Fig. 3B). The active protein has a yellow coloration suggesting the presence of a flavin coenzyme. After heating at 70° for 20 min this flavin can be separated from the apoenzyme by ultrafiltration and shown to be FMN, rather than FAD, using HPLC as previously described [5].

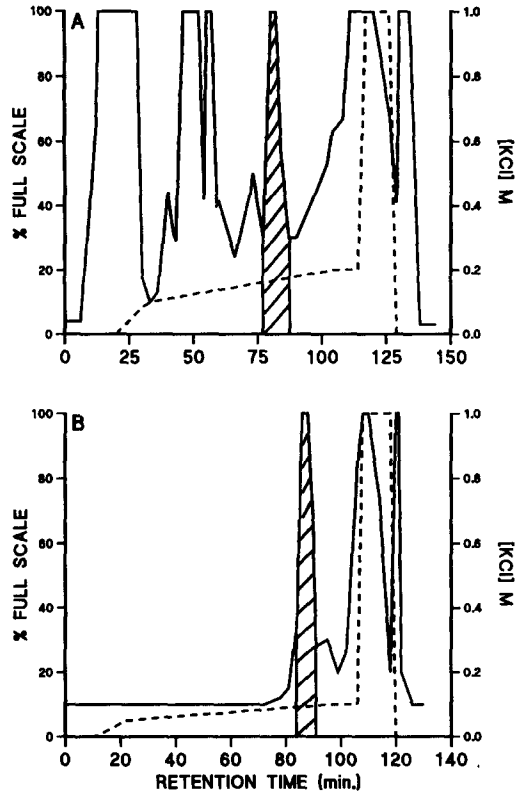

Fig. 2. Elution profiles of *E. coli* B nitroreductase from; (A) Q-Sepharose High Performance in 20 mM Tris–HCl pH 7.6. Column dimensions: 12 × 5.2 cm. Flow rate: 4 mL/min. Sample: 50 mL of dialysate of active Phenyl Sepharose fractions. AU/full scale: 0.5. (B) Q-Sepharose High Performance in 20 mM Bis–Tris propane pH 7.0. Column and flow rate as for (A). Sample 50 mL desalted active fractions from (A). AU/full scale: 0.2. KCl gradients are indicated by dotted lines and fractions active with CB1954 are shown by cross-hatching.

Kinetic parameters

Kinetic parameters for the nitroreductase and Walker NAD(P)H dehydrogenase (quinone) are presented in Table 2. Both enzymes have comparable K_m values for CB1954. The absolute rates of reduction of CB1954 (i.e. under saturating conditions) by the two enzymes is their k_{cat} values and this is 90-fold higher for the *E. coli* nitroreductase. Menadione was also a substrate for both enzymes with little difference in their respective k_{cat} although the K_m of

Table 1. The purification of a nitroreductase from *E. coli*

Fraction	Total activity (units)		Specific activity (units/mg protein)	Yield (%)	
Crude	3784*		0.35	100	
Phenyl Sepharose	2371*		1.6	63	
	CB1954 active	CB1954 inactive		CB1954 active	CB1954 inactive
	1109	1262		30	33
Q-Sepharose (Tris, pH 7.6)	666	—	79	18	—
Q-Sepharose (Bis–Tris propane, pH 7)	310	—	130	8	—

The enzyme activity was assayed, at 37°, by its quinone reductase activity using menadione (10 μM) as substrate, NADH (500 μM) as cofactor and cytochrome *c* (70 μM) as terminal electron acceptor. A unit was defined as 1 μmole of cytochrome *c* reduced per minute.

* This includes activity from enzymes not active against CB1954.

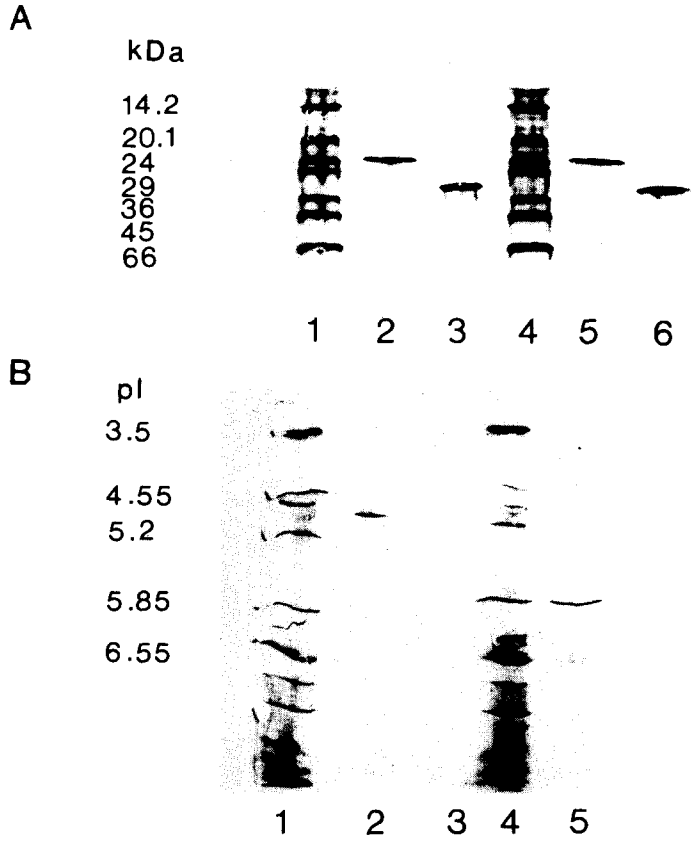

Fig. 3. (A) Homogeneity of final product and estimation of molecular mass of *E. coli* B nitroreductase by SDS–PAGE performed under reducing conditions using Coomassie blue-stained precast 8–25% gradient gels. Lanes 1 and 4, standards; 2 and 5 nitroreductase; 3 and 6 Walker DT diaphorase. One microgram of enzyme was applied in each case and electrophoresis performed for 65 vh. (B) Determination of pI by isoelectric focusing using Coomassie blue-stained precast IEF 3–9 gels. One microgram of the enzymes was applied in two positions (lane 2 nitroreductase, lane 5 Walker DT diaphorase) and focusing performed for 400 vh. Isoelectric points were determined graphically using calibration standards (lanes 1 and 4, pI calibration standards 3–10).

the *E. coli* nitroreductase for this substrate was 60-fold higher. Nitrofurazone was also shown to be a substrate for the *E. coli* enzyme ($K_m = 64 \pm 10\ \mu M$, $k_{cat} = 612\ min^{-1}$); this compound is not a substrate for DT diaphorase. *E. coli* nitroreductase can also use NADPH as cofactor, giving identical kinetic

Table 2. Kinetic parameters for the *E. coli* nitroreductase and Walker DT diaphorase

Compound		Nitroreductase	Walker
NADH	K_m	6 μM	75 μM
Nitrofurazone	K_m	64 ± 10 μM	NS
	k_{cat}	612 min^{-1}	NS
CB1954	K_m	862 ± 145 μM	826 ± 46 μM
	k_{cat}	360 min^{-1}	4 min^{-1}
Menadione	K_m	80 μM	1.3 μM*
	k_{cat}	4.2×10^4 min^{-1}	6.5×10^4 min^{-1}

* Data from Knox *et al.* [5].
NS, not substrate.
± Indicates mean ± SEM.

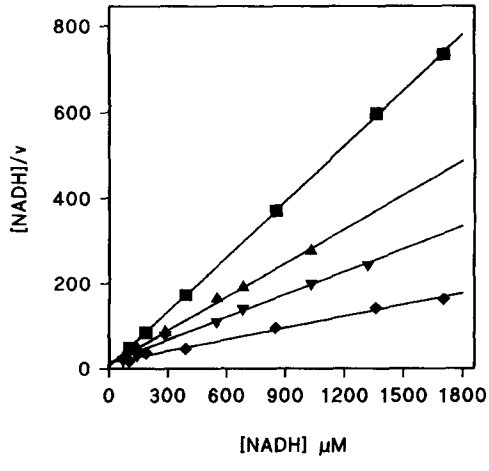

Fig. 4. Primary plots of [NADH]/rate against [NADH] for *E. coli* B nitroreductase at different CB1954 concentrations. Enzyme activity was determined by HPLC after incubation at 37° for 15 min. (■) 200 μM CB1954, (▲) 400 μM, (▼) 800 μM, (◆) 1000 μM.

parameters with the above substrates (data not shown). The calculated K_m of NADH for the nitroreductase decreases with decreasing CB1954 concentration, as shown in Fig. 4, suggesting that the kinetics conform to that of a substituted enzyme mechanism with respect to CB1954, a similar mechanism being proposed for menadione reduction by a quinone reductase enzyme also isolated from *E. coli* [11].

Dicoumarol was an inhibitor of the *E. coli* nitroreductase. No kinetic parameters could be measured with respect to NADH because of the difficulties of accurately measuring reduction rates at non-saturating concentrations of NADH. With respect to menadione, dicoumarol was an uncompetitive inhibitor with a K_i' of $1.98 \pm 0.38\,\mu$M.

Cross-reactivity with anti-NAD(P)H dehydrogenase (quinone) antibodies

Western blotting of the *E. coli* nitroreductase showed no cross-reaction with a polyclonal antibody raised against the Walker enzyme (data not shown).

Amino acid sequence analysis

The protein was subjected to amino acid sequence analysis. In contrast to the Walker enzyme the nitroreductase was not blocked at the N-terminus and gave a clear N-terminal sequence of 31 amino acid residues and a peptide, generated by digestion with cyanogen bromide, of a further 41 residues. These partial sequences are compared with those reported for *Salmonella typhimurium* [12] and *Enterobacter cloacae* [13] in Table 3.

DISCUSSION

Sensitivity towards the monofunctional alkylating agent CB1954 is a consequence of its ability to be metabolized to a difunctional compound which can induce DNA interstrand crosslinks. In mammalian cells this activation can be mediated by the rat form of DT diaphorase, which can reduce the 4-nitro group of CB1954 to the corresponding hydroxylamine thus forming a cytotoxic compound [4, 5].

Previous studies in bacteria have demonstrated that CB1954 can react with their DNA [7] and that nitroreductase-deficient strains of *E. coli* are not sensitive to CB1954 cytotoxicity and much less sensitive to its mutagenic effects [8]. It was suggested that bacteria may also contain an enzyme(s) capable of activating CB1954 in an analogous manner to DT diaphorase. We report the isolation of a nitroreductase enzyme from *E. coli* that is similar to DT diaphorase in that it can also reduce CB1954 but at a rate 60-fold greater than that of DT diaphorase.

The purified *E. coli* nitroreductase enzyme is a monomeric FMN containing flavoprotein with a molecular mass of 24 kDa. The protein was subjected to sequence analysis which generated the N-terminal and an internal sequence, both over 30 amino acid residues in length. A search of a protein sequence data base showed no match or significant homology of these sequences with any other protein but comparison with the recently reported sequences of nitroreductases from *Salmonella typhimurium* [12] and *Enterobacter cloacae* [13] shows good homology between all three enzymes. The *Salmonella typhi-*

Table 3. Amino acid sequences of the N-terminal of the *E. coli* nitroreductase and of a peptide obtained after digestion of the nitroreductase with cyanogen bromide (bold type) and a comparison with the deduced protein sequence of the nitroreductases from *Salmonella typhimurium* [12] and *Enterobacter cloacae* [13] (italic type)

1									10									20									30							
M	D	I	V	S	V	A	L	Q	R	Y	S	T	K	A	F	D	P	S	K	K	L	T	A	E	E	A	D	K	I	K	T	L		
M	**D**	**I**	**I**	**S**	**V**	**A**	**L**	**K**	**R**	**H**	**S**	**T**	**K**	**A**	**F**	**D**	**A**	**S**	**K**	**—**	**L**	**T**	**(P)**	**E**	**Q**	**A**	**(D)**	**Q**	**I**	**K**				
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L	Q	Y	S	P	S	S	T	N	S	Q	P	W	H	F	I	V	A	S	T	E	E	G	K	A	R	V	A	K	S	A	A	G	N	Y
L	*Q*	*Y*	*S*	*P*	*S*	*S*	*T*	*N*	*S*	*Q*	*P*	*W*	*H*	*F*	*I*	*V*	*A*	*S*	*T*	*E*	*E*	*G*	*K*	*A*	*R*	*V*	*A*	*K*	*S*	*A*	*A*	*G*	*T*	*Y*
70												80									90												100		
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V	*F*	*N*	*E*	*R*	*K*	*M*	*L*	*D*	*A*	*S*	*H*	*V*	*V*	*V*	*P*	*C*	*A*	*K*	*T*	*A*	*M*	*D*	*D*	*A*	*W*	*L*	*E*	*R*	*V*	*V*	*D*	*Q*	*E*	*E*
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A	D	G	R	F	A	T	P	E	A	K	A	A	N	D	K	G	R	R	F	F	A	D	M	H	R	V	S	L	K	D	D	H	Q	W
A	*D*	*G*	*R*	*F*	*N*	*T*	*P*	*E*	*A*	*K*	*A*	*A*	*N*	*H*	*K*	*G*	*R*	*T*	*Y*	*F*	*A*	*D*	*M*	*H*	*R*	*V*	*D*	*L*	*K*	*D*	*D*	*D*	*Q*	*W*
140											150									160									170						
M	A	K	V	V	Y	L	N	V	G	N	F	L	L	G	V	A	A	M	G	L	D	A	V	P	I	E	G	F	D	A	E	V	L	
(M)	**A**	**K**	**Q**	**V**	**Y**	**L**	**N**	**V**	**G**	**N**	**F**	**L**	**L**	**G**	**V**	**A**	**A**	**L**	**G**	**L**	**D**	**A**	**V**	**P**	**I**	**E**	**G**	**F**	**D**	**A**	**A**	**I**	**L**		
M	*A*	*K*	*Q*	*V*	*Y*	*L*	*N*	*V*	*G*	*N*	*F*	*L*	*L*	*G*	*V*	*G*	*A*	*M*	*G*	*L*	*D*	*A*	*V*	*P*	*I*	*E*	*G*	*F*	*D*	*A*	*A*	*I*	*L*		
									180									190									200							
D	A	E	F	G	L	K	E	K	G	Y	T	S	L	V	V	V	P	V	G	H	H	S	V	E	D	F	N	A	G	L	P	K	S	R
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murium enzyme, commonly called "classical nitroreductase", is involved in the activation of mutagenic nitroarenes, particularly nitrofurans such as nitrofurazone, niridazole, 2-nitronaphthalene, 1-nitropyrene and 2-nitroflurine [13, 14] but does not activate other compounds such as 4-nitroquinoline *n*-oxide [15, 16] or 1,8-dinitropyrene [15, 17]. Similarly the *Enterobacter cloacae* enzyme reduces a range of nitroarenes, e.g. 2,4,6-trinitrotoluene, 2,4-dinitrotoluene and nitrofurazone [18]. Given the high degree of sequence homology between these enzymes and the partial protein sequences obtained from the *E. coli* nitroreductase, it would appear that we have isolated the *E. coli* form of "classical nitroreductase" and this was further confirmed by the fact that the *E. coli* enzyme can reduce nitrofurazone. Although both the *E. coli* nitroreductase and DT diaphorase can metabolize menadione and CB1954 and are inhibited by dicoumarol, there is no sequence homology between these two enzymes. A polyclonal antibody raised against DT diaphorase does not cross react with the nitroreductase, indicating a lack of structural homology. The differences between the enzymes are emphasized by the fact that whilst both DT diaphorase and the *E. coli* nitroreductase are flavoproteins, DT diaphorase uses FAD as a coenzyme, whilst the nitroreductase uses FMN. With both enzymes the flavin group can be separated from the enzyme by heating, showing that it is tightly but not covalently bound. The name for DT diaphorase arose from the enzyme's, then unique, ability to utilize both NADH and NADPH as electron donors (these cofactors then abbreviated by convention as DPNH and TPNH, respectively) [19]. Whilst other enzymes are now known which also possess this property, most enzymes can usually only use one of these cofactors. The *E. coli* nitroreductase also has the ability to use both cofactors as electron donors, and this shared property with DT diaphorase may explain why both enzymes can be inhibited by dicoumarol. Dicoumarol has been described as a specific inhibitor of DT diaphorase and is a potent inhibitor of this enzyme (for a review see Ref. 20). Kinetic constants (K_i 50 pM–200 nM) for this inhibitor are normally quoted with respect to NAD(P)H because, as we have shown, it is a non-competitive inhibitor ($K_i = K_i' = 10$ nM) with respect to other substrates such as menadione [8]. For the *E. coli* nitroreductase it was not feasible to measure kinetic parameters with respect to NADH or NADPH because of the extremely low K_m the enzyme has for these cofactors, but dicoumarol was shown to be an uncompetitive inhibitor ($K_i' = 2\ \mu$M) of the enzyme with respect to menadione.

Menadione is a better substrate for either enzyme than CB1954. The initial rates of reduction (under saturating conditions), of menadione compared to CB1954, are 1.2×10^4 faster for nitroreductase and 1.6×10^4 faster for the Walker DT diaphorase. However, the *E. coli* enzyme can intrinsically reduce CB1954 92-fold faster than DT diaphorase (k_{cat} 360 min^{-1} compared to 4 min^{-1}). The actual reduction products formed from CB1954 are described fully elsewhere, but whilst DT diaphorase generates only the 4-hydroxylamino reduction product, the *E. coli* nitroreductase generates equimolar amounts of the 2- and 4-hydroxylamino products, but does not reduce both nitrogroups [21].

In summary, we have isolated and characterized a nitroreductase enzyme from *E. coli* which appears to be analogous with the "classical reductase" of *Salmonella typhimurium*. In common with DT diaphorase isolated from mammalian Walker cells, this protein can also reduce the prodrug, CB1954 to its cytotoxic form. Compared to the Walker cell enzyme this nitroreductase can perform this reduction much more rapidly and may be less limited by the NAD(P)H concentration which it requires as a cofactor.

It is possible that this bacterial enzyme may have a role in the therapy of human tumours. The concept of targeting enzymes towards tumours with antibodies (ADEPT) has been discussed and demonstrated [22]. CB1954 is a monofunctional agent capable of being activated to a difunctionally reacting compound which is 10,000–100,000-fold (on a dose basis) more cytotoxic than the pro-drug [3]. Thus, if a conjugate is made between the nitroreductase and a tumour-specific antibody it may be possible to activate CB1954, or similar pro-drugs, selectively at the site of the tumour using this bacterial enzyme.

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