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IDENTIFICATION OF NOVEL REDUCED PYRIDINIUM DERIVATIVES AS SYNTHETIC CO-FACTORS FOR THE ENZYME DT DIAPHORASE (NAD(P)H DEHYDROGENASE (QUINONE), EC 1.6.99.2)

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Abstract—The enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2) is unusual in that it can utilize either NADH or NADPH as a co-factor for the reduction of its substrates. We have shown that the intact NAD(P)H molecule is not required and that other reduced pyridinium compounds can also act as co-factors for DT diaphorase. The entire adenine dinucleotide portion of NAD(P)H can be dispensed with entirely and the simplest quaternary (and therefore reducible) derivative of nicotinamide, 1-methylnicotinamide, was as effective as NAD(P)H as a co-factor for the reduction of the quinone, menadione. Nicotinamide 5′-*O*-benzoyl riboside was also as effective a co-factor as NAD(P)H, whilst nicotinamide ribotide and riboside have a higher K_m, and decreased the k_{cat} of DT diaphorase. Nicotinic acid derivatives had little activity. Kinetic analysis indicated that both nicotinamide ribotide and riboside may be interacting with the menadione binding site rather than the NAD(P)H site.

Irrespective of the differences between the various reduced pyridinium derivatives in their ability to act as co-factors for the reduction of menadione by DT diaphorase, all the compounds that showed activity in this assay were equally effective co-factors for the reduction of the nitrobenzamide, CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). The apparent K_m of DT diaphorase for all these co-factors approached zero. It was concluded that co-factor binding is not a rate-limiting step in the nitroreductase activity of DT diaphorase.

The enzyme NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2) is commonly known as DT diaphorase. This name arose because of its then unique ability to use either NADH or NADPH as co-factors in its reduction of quinone substrates. At the time of its discovery these two co-factors were abbreviated as DPNH and TPNH, respectively. As well as its ability to use both of these co-factors, this enzyme is also remarkable in that it can transfer two electrons directly to its substrate. The primary cellular role of this enzyme appears to be the detoxification of quinones by directly catalysing a two-electron reduction of these compounds to form the hydroquinone. This avoids the cytotoxicity resulting from the redox cycling produced by a one-electron reduction of quinones and the resulting generation of superoxide radicals (for a review see Ref. 1).

As well as reducing quinones, DT diaphorase has activity against other types of substrates such as azo compounds [2, 3], chromium VI compounds [4], benzotriazine di-*N*-oxides [5] and nitro compounds [6, 7]. In contrast to the enzyme's role in the protection of cells from the cytotoxic effects of quinones, it may also have a role in the activation of bioreductive quinone drugs such as diaziquone [8]. It has also been implicated in the activation of certain nitro-compounds to their cytotoxic forms. In particular, DT diaphorase is responsible for the bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954), a compound that is a potent but selective antitumour agent against certain rat tumours [7, 9]. The enzyme can reduce the 4-nitro group of CB 1954, in air, to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, which is the active, cytotoxic form of the parent compound [9].

The ability of DT diaphorase to utilize either NADH or NADPH as co-factors prompted us to investigate the ability of this enzyme to use other reduced pyridinium derivatives as co-factors for the reduction of either the quinone, menadione or CB 1954.

MATERIALS AND METHODS

Synthesis of pyridinium derivatives. NADH (**1**, Fig. 1) was supplied by the Sigma Chemical Co. (Poole, U.K.), as was NADPH (**2**, Fig. 1), α-NADH (**3**, Fig. 1), α-NADPH (**4**, Fig. 1), 3-acetylpyridineadenine dinucleotide (reduced form) (**5**, Fig. 1), nicotinamide ribotide (nicotinamide mononucleotide) (reduced form) (**8**, Fig. 1), nicotinic acid ribotide (nicotinic acid mononucleotide) (**10**, Fig. 1) and 1-methylnicotinamide (**13**, Fig. 1).

Nicotinamide riboside (**9**, Fig. 1) and nicotinic acid riboside (**11**, Fig. 1) were prepared from their respective ribotides by the action of the enzyme alkaline phosphatase. To a 10 mM solution of

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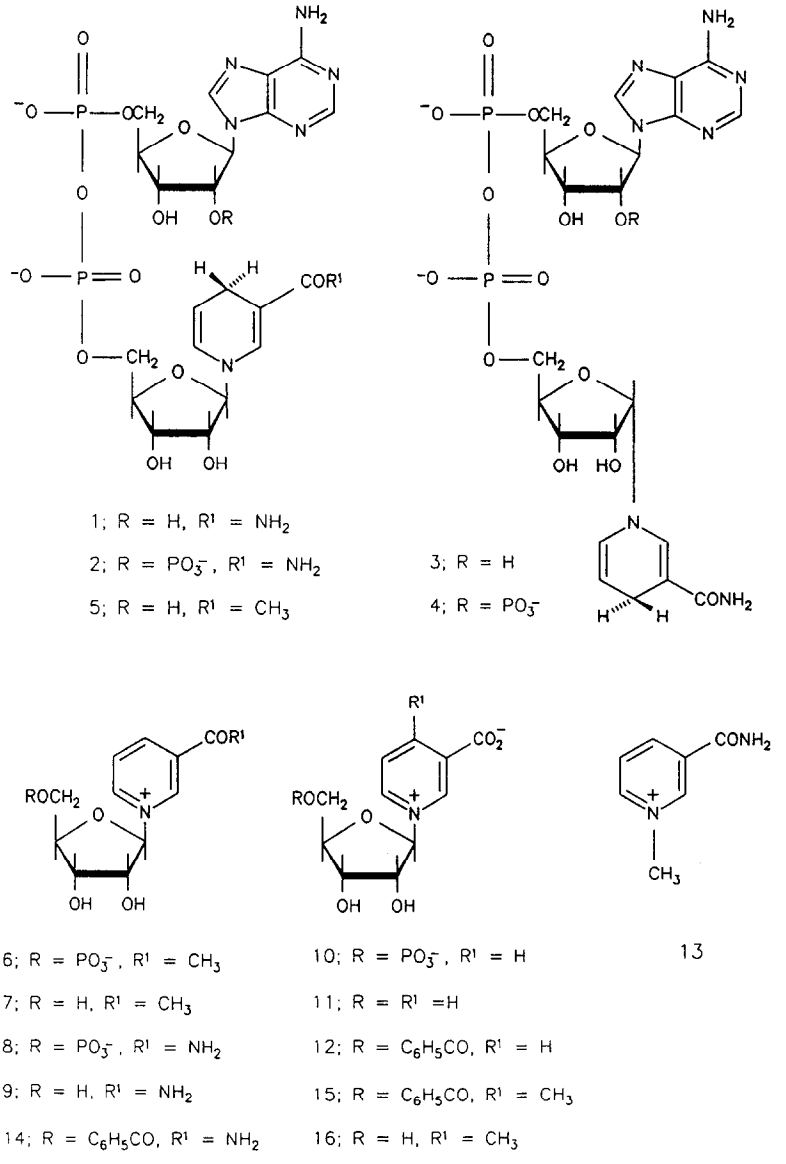

Fig. 1. The structures of the pyridinium compounds used in this study. The numbers refer to the compounds as listed in Table 1. Compounds **1–5** are illustrated in the reduced form, the others are illustrated in the oxidized state. It is only in the reduced form that these compounds can act as co-factors for the enzyme DT diaphorase.

ribotide in phosphate-buffered saline, was added alkaline phosphatase (Sigma, type VII-S) to a final concentration of 1000 U/mL. After 15 min the enzyme was removed by ultrafiltration (Centricon 10, Amicon) and the filtrate used without further modification. The total conversion of the ribotide to the riboside was confirmed by HPLC [Partisphere SAX (110 × 4.7 mm) column (Whatman) eluted isocratically (1 mL/min) with 100 mM NaH_2PO_4].

3-Acetylpyridine ribotide (reduced form) (**6**, Fig. 1) was prepared by cleaving 3-acetylpyridineadenine dinucleotide (reduced form) (**5**, Fig. 1) with phosphodiesterase. To a solution of 3-acetylpyridineadenine dinucleotide (reduced form) (10 mM in 0.1 M Tris, 2 mM $MgCl_2$) was added phosphodiesterase 1 (Sigma, type II) to a final concentration of 0.5 U/mL. The enzyme was removed after 30 min by ultrafiltration and the product assayed for purity as described above.

3-Acetylpyridine riboside (reduced form) (**7**, Fig. 1) was prepared from the ribotide as described for nicotinamide riboside above.

Nicotinamide 5′-*O*-benzoyl riboside (**14**, Fig. 1) was synthesized as described previously [11]. 4-Methylnicotinic acid 5′-*O*-benzoyl riboside (**15**, Fig. 1) and 4-methylnicotinic acid riboside (**16**, Fig. 1) were both synthesized from 1-(3′,5′-di-*O*-benzoyl-D-ribofuranosyl)-4-methylpyridinium-3-carboxylate

[10] by the action of methanolic ammonia as described previously [10]. The previously unreported compound, nicotinic acid 5′-*O*-benzoyl riboside [1-(5′-*O*-benzoyl-D-ribofuranosyl)pyridinium-3-carboxylate] (**12**, Fig. 1) was prepared from 1-(3′(2′),5′-di-*O*-benzoyl-D-ribofuranosyl)pyridinium-3-carboxylate [10] as described below.

A 1:3 mixture of 1-(2′,5′-di-*O*-benzoyl-D-ribo-furanosyl)- and 1-(3′,5′-di-*O*-benzoyl-D-ribofu-ranosyl)pyridinium-3-carboxylates (70 mg, 0.15 mmol) in methanol (5 mL), was cooled to −20°. Methanolic ammonia (2 M, 0.25 mL) was added. After 16 hr no starting material remained (as assessed by TLC on fluorescent cellulose (Merck 5574), using butan-1-ol:acetic acid:water (5:2:3) as solvent). The solution was filtered, evaporated to dryness and the residue partitioned between water (20 mL) and ether (10 mL). The aqueous solution was evaporated to dryness and the residue dissolved in hot methanol. This solution was concentrated to a few millilitres and the product allowed to crystallize at −20°, yielding 14 mg (26%) of cream coloured crystals, m.p. 160° (decomp.). ^{1}H NMR (Bruker AC spectrometer, 250 MHz, solution in d_6-DMSO): δ4.20 (1H, q, ribose H-3), 4.30 (1H, t, ribose H-2), 4.57 (1H, d, ribose H-4), 4.65 (1H, d, ribose H-5), 5.66 (1H, t, ribose 3-OH), 6.18 (1H, d, ribose 2-OH), 6.21 (1H, d, ribose H-1), 7.51 (2H, t, benzoyl H-3), 7.67 (1H, t, benzoyl H-4), 7.94 (2H, d, benzoyl H-2), 8.01 (1H, t, pyridine H-5), 8.83 (1H, d) and 8.97 (1H, D) (pyridine H-4 + H-6), 9.29 (1H, s, pyridine H-2). Mass spectrometry was carried out on a TSQ 700 triple-quadrupole system (Finnigan Mat) equipped with an electrospray ion source (Analytica): m/z 360 $[M+H]^+$, 237 [M-nicotinic acid]$^+$, 124 [nicotinic acid + H]$^+$. Anal. Calc. for $C_{18}H_{17}NO_7$, $0.2H_2O$: C 59.57; H 4.83; N 3.86. Found: C 59.53; H 4.73; N 3.75.

Chemical reduction of synthetic cofactors. Chemical reduction was as reported previously for the production of 4-methyl NADH from 4-methyl NAD$^+$ [12]. To an aqueous solution of oxidized compound (4 mg/mL), was added 10 mg/mL of anhydrous sodium carbonate, 10 mg/mL of sodium bicarbonate and 10 mg/mL of sodium hydrosulphite. The stoppered solution was allowed to stand at 37° and aliquots assayed, at intervals, by reverse phase HPLC for the formation of a fluorescent product. When the optimum reduction time had been established the reduced compound was purified from the reaction mixture by preparative HPLC. Up to 5 mL of reaction mixture was injected onto a Microsorb 5 μM C18 (10 × 250 mm) reverse-phase column (Rainin) and eluted by a gradient of methanol in water (0–100% over 30 min) at 4.0 mL/min. The eluate was continually monitored at 340 nm and a fraction corresponding to a peak of absorbance collected. That this peak also corresponded to a fluorescent product was confirmed by injection of an aliquot of the peak onto an analytical HPLC system equipped with a fluorescence detector (Gilson 121 equipped with wide band glass filters with the excitation wavelength centred at 350 nm and the emission on 450 nm). The concentration of the purified reduced product was calculated assuming a ε_{340} of 6200 $M^{-1}cm^{-1}$ (i.e. the same as NAD(P)H).

Purification of DT diaphorase. DT diaphorase was purified to homogeneity from rat Walker 256 tumour cells as described previously [7].

Enzyme activity and kinetic studies. DT diaphorase activity was assayed as previously described employing either menadione as the substrate and cytochrome *c* as terminal electron acceptor [7] or CB 1954 as substrate. CB 1954 reduction was monitored directly by HPLC [13]. K_m values were determined from Eadie–Hofstee plots and inhibition constants (K_i and K_i' values) and the types of inhibition determined from Dixon plots, also as described previously [14]. Standard deviations of these determinations were <10%.

RESULTS

The co-factor specificity of DT diaphorase as a quinone reductase

Using menadione as a substrate, the ability of various reduced quaternary pyridinium derivatives to act as co-factors for DT diaphorase was measured. The limiting reaction rate per unit concentration of enzyme (k_{cat}) and the K_m value for these compounds are shown in Table 1. None of the oxidized forms of these compounds had any activity in this assay and no cytochrome *c* reduction occurred in the absence of the enzyme. Only approximate data could be obtained for nicotinic acid ribotide (**10**, Fig. 1) and 1-methylnicotinamide (**13**, Fig. 1) due to the rapid spontaneous oxidation of the reduced forms at neutral pH. However, 1-methylnicotinamide was as good a co-factor as NAD(P)H (**1** and **2**, Fig. 1) for the reduction of menadione. Addition of a ribose moiety to nicotinamide (**8** and **9**, Fig. 1) decreased the k_{cat} and increased the K_m of the enzyme when compared with 1-methylnicotinamide or NAD(P)H. The 5′-phosphate group (**8**, Fig. 1), as found in the natural product, appeared to make little difference to the K_m or k_{cat} values. However, both these kinetic parameters could be fully restored by the addition of a 5′-*O*-benzoyl group (**14**, Fig. 1). The alpha configuration of the sugar (**3** and **4**, Fig. 1) appeared to increase slightly the affinity of the enzyme for the co-factor, whilst substitution of the 3-carboxamide group by an acetyl group (**5–7**, Fig. 1) had little effect, when compared to the natural forms. However, substitution at this latter position by a carboxylic acid group seemed to destroy most of the compound's ability to act as a cofactor (**10–12**, Fig. 1). A 5′-*O*-benzoyl substitution (**12**, Fig. 1) on the riboside again increased both the activity and the affinity of the enzyme for the co-factor in comparison to both the corresponding ribotide and riboside. The reduced forms of 4-methylnicotinic acid 5′-*O*-benzoyl riboside (**15**, Fig. 1) and 4-methylnicotinic acid riboside (**16**, Fig. 1) are optically active. Whilst the two isomers of 4-methylnicotinic acid 5′-*O*-benzoyl riboside could not be resolved, those of 4-methylnicotinic acid riboside could. In this latter case neither isomer was active as a cofactor for DT diaphorase.

Inhibition studies

In comparison to NADH, both the ribotide and riboside of nicotinamide were poor cofactors for DT

Table 1. The ability of various quaternary pyridinium analogues to act as co-factors for the reduction of menadione by DT diaphorase isolated from Walker 256 cells

Compound	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
1. NADH	6.5×10^4	78	8.33×10^2
2. NADPH	6.5×10^4	71	9.15×10^2
3. α-NADH	3.5×10^4	26	1.35×10^3
4. α-NADPH	4.9×10^4	22	2.23×10^3
5. 3-Acetylpyridineadenine dinucleotide	6.4×10^4	151	4.24×10^2
6. 3-Acetylpyridine ribotide	5.0×10^2	1383	0.362
7. 3-Acetylpyridine riboside	4.5×10^2	1528	0.295
8. Nicotinamide ribotide	1.4×10^3	1667	0.84
9. Nicotinamide riboside	4.6×10^3	2268	2.03
10. Nicotinic acid ribotide	~10	ND	—
11. Nicotinic acid riboside	<1.0	—	—
12. Nicotinic acid 5′-*O*-benzoyl riboside	7.1×10^2	62	11.5
13. 1-Methylnicotinamide	$\sim 6 \times 10^4$	~200	$\sim 3 \times 10^2$
14. Nicotinamide 5′-*O*-benzoyl riboside	4.3×10^4	79	5.44×10^2
15. 4-Methylnicotinic acid 5′-*O*-benzoyl riboside	<1.0	—	—
16. 4-Methylnicotinic acid riboside	<1.0	—	—

All compounds were used in their reduced forms, the oxidized forms having no activity. Measurements were carried out at 37° at a menadione concentration of 10 μM.

diaphorase when reducing menadione as a substrate. Therefore, it was possible that they could also act as inhibitors of the enzyme in the presence of NADH. The results obtained with nicotinamide ribotide are shown in Fig. 2. DT diaphorase activities were assayed by the spectrophotometric method using menadione as substrate and cytochrome *c* as the terminal electron acceptor. Values for K_i were derived from Dixon plots of $1/v$ against nicotinamide ribotide concentration at various concentrations of NADH or menadione and K_i, values from plots of [NADH]/v or [menadione]/v against nicotinamide ribotide concentration at various concentrations of NADH or menadione, where v is the initial reaction rate. The point of intersection on these plots gave the appropriate constant and the pattern of the plots the type of inhibition.

From such plots both nicotinamide ribotide and nicotinamide riboside (data not shown) were shown to be uncompetitive inhibitors of DT diaphorase with respect to NADH (K_i' = 1.12 and 2.46 mM, respectively) but competitive inhibitors with respect to menadione (K_i = 1.13 and 2.39 mM, respectively).

The co-factor specificity of DT diaphorase as a nitroreductase

All the compounds shown in Table 1 were also tested for their ability to act as a co-factor in the reduction of CB 1954 by DT diaphorase. Only nicotinic acid riboside, 4-methylnicotinic acid riboside and 4-methylnicotinic acid 5′-*O*-benzoyl riboside could not act as cofactors in this reduction. All the other compounds gave the same rate of CB 1954 reduction (k_{cat} = 4.1 min^{-1}) and the apparent K_m of the enzyme for these cofactors were <10 μM and appeared to be approaching zero. This is illustrated in Fig. 3 with four of the compounds that, when acting as cofactors in the menadione reduction assay, gave great variation in both enzyme activity and K_m values (Table 1).

In all cases, the product of the reduction was identical and identified as 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide as had been described previously [7, 13] (data not shown).

DISCUSSION

In most biochemical reductions a fundamental distinction is drawn between NADH and NADPH. Thus, NADPH is used as an electron donor in reductive biosynthesis while NADH is used in respiratory catabolism to generate ATP. Most enzymes can therefore distinguish between these two co-factors (presumably using the phosphoryl group) and thus use only one or the other. An exception to this rule is the enzyme DT diaphorase which can use either NADPH or NADH with equal activity. Neither why its physiological role should require it to be able to use both NADH and NADPH as cofactors, nor the mechanism by which the enzyme can directly transfer two electrons to its substrates, is known.

We have now shown that the actual structural requirements in a co-factor of DT diaphorase are fairly lax and, indeed, it would appear that there is little requirement for the adenine nucleotide portion of NAD(P)H at all. Thus, the simplest quaternary (and therefore reducible) derivative of nicotinamide, 1-methylnicotinamide (**13**, Fig. 1), was as good a co-factor as NAD(P)H for the reduction of menadione by this enzyme. Whilst 1-methylnicotinamide was chemically unstable, nicotinamide 5′-*O*-benzoyl riboside (**14**, Fig. 1) was stable in its reduced form, and this compound is thus a synthetic and novel co-factor for DT diaphorase, equal to NAD(P)H in both its activity and affinity for the enzyme. Little activity was seen with any nicotinic acid derivatives (**10–12**, Fig. 1) (in comparison to equivalent nicotinamide and 3-acetylpyridine compounds). This would suggest that a negative charge, at the 3-

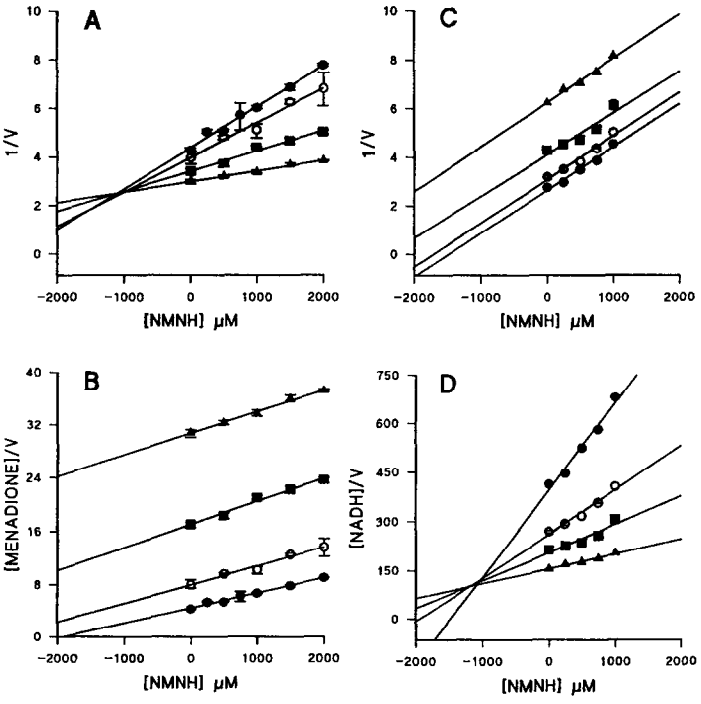

Fig. 2. Inhibition by nicotinamide ribotide (reduced) of menadione reduction by Walker cell DT diaphorase. Determination of (A) K_i from plots of $1/v$ against nicotinamide ribotide concentration at various concentrations of menadione and (B) K_i' from plots of [menadione]/v against nicotinamide ribotide (NMNH) concentration at various concentrations of menadione, where v is the initial reaction rate, (●, 1 μM; ○, 2 μM; ■, 5 μM; ▲, 10 μM menadione). The initial concentration of NADH was 500 μM. Determination of (C) K_i and (D) K_i' at various concentrations of NADH (▲, 25 μM; ■, 50 μM; ○, 75 μM; ●, 100 μM NADH). The concentration of menadione was constant at 10 μM. All determinations were made at 37°, with cytochrome c as terminal electron acceptor. The inhibition was competitive with respect to menadione and uncompetitive with respect to NADH with K_i and K_i' values of 1.13 and 1.12 mM, respectively. The error bars represent the standard error of the mean of triplicate measurements.

position on the pyridine ring, is poorly tolerated in the enzyme's binding site.

A pyrophosphate binding region in DT diaphorase for NAD(P)H has been proposed [15]. However, judging from the results shown above, it is probable that this site is not the major determinant for cofactor binding. This would explain why, although the region is fully conserved between the rat [16] and human [17] forms of DT diaphorase, these two forms of the enzyme have significant differences in their K_m values for NAD(P)H [13, 18].

It would be predicted that all these cofactors would share a common binding site. However, this does not appear to be the case. Both nicotinamide ribotide (**8**, Fig. 1) and riboside (**9**, Fig. 1) were uncompetitive inhibitors of DT diaphorase with respect to NADH, suggesting that they do not interact directly with the NADH binding site. This notion was supported by the fact that these two compounds were competitive inhibitors with respect to menadione. Thus, it would appear that nicotinamide ribotide and riboside can bind to the menadione binding site and presumably act as (poor) cofactors through this site. The similarity of the values of the inhibition constants of nicotinamide ribotide and riboside and the K_m values of DT diaphorase for both these compounds (when used as cofactors), supports this view. DT diaphorase is a flavoprotein (FAD) and the role of the cofactor is to reduce the flavin group that can then transfer the electrons to the substrate. It is quite conceivable that an oxidized flavin group could be reduced from the substrate rather than the NAD(P)H binding site. With the more active cofactors rates of reduction of menadione were similar to those measured with NADH and such a kinetic analysis was not possible. Thus, it could not be ascertained whether such cofactors as nicotinamide 5′-*O*-benzoyl riboside act through the NADH or menadione binding sites.

The cofactor specificity of the reduction of CB 1954 by DT diaphorase was also examined. In contrast to the results obtained with menadione (where a range of activities were observed), all the compounds that showed a measurable activity when assayed by menadione reduction, showed identical activities with CB 1954. Further, the apparent K_m values of all these cofactors were unmeasurably small and appeared to be approaching zero. For example, nicotinamide ribotide was just as good a cofactor for the reduction of CB 1954 as NADH or

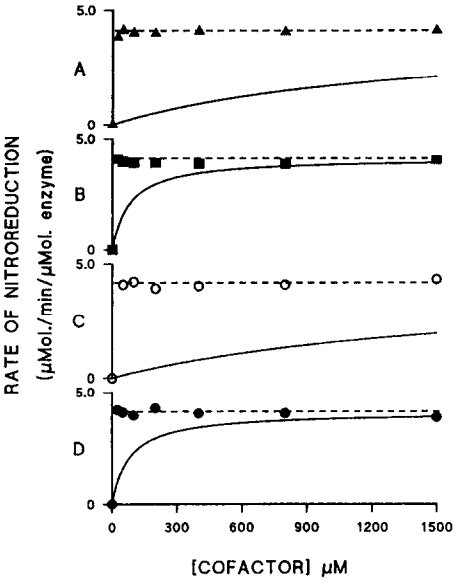

Fig. 3. The effect of concentration of various cofactors on the initial rate of reduction of CB 1954 by Walker cell DT diaphorase. (A) 3-Acetylpyridine ribotide, (B) nicotinamide 5′-*O*-benzoyl riboside, (C) nicotinamide ribotide, (D) NADH. The rate of reduction of CB 1954 was measured at 37° by HPLC as described in the text. In each case the solid line indicates the predicted rate of reduction given the enzyme's differing K_m values for these cofactors when assayed by menadione reduction (see Table 1) and the dashed line the k_{cat} value of 4.1 min^{-1}.

nicotinamide 5′-*O*-benzoyl riboside. However, as a cofactor for menadione reduction (by the same enzyme), nicotinamide ribotide is intrinsically about 45 times less active and the enzyme has a 20-fold higher K_m towards it, when compared with these other two cofactors. The K_m is commonly taken as a binding constant (although, even at the simplest interpretation of the Michaelis–Menten equation, this assumes that the rate constant for formation of product is negligible in comparison to that for the dissociation of the enzyme–substrate complex). For the enzyme, DT diaphorase, the situation is more complicated because there are two substrates (including the cofactor) and two products (including the oxidized cofactor). However, the enzyme cannot reduce its substrate until its FAD group has been reduced by the cofactor. Therefore, a simple explanation as to why the apparent K_m values of the cofactors are very low when assayed by CB 1954 reduction, is that, in this reaction, transfer of reducing equivalents from the enzyme (cofactor-reduced) to this substrate is slow and very rate limiting. Compared to menadione reduction, the reduction of CB 1954 by DT diaphorase was intrinsically 1.6×10^4 times slower. That the K_m, as measured by CB 1954 reduction, for the cofactors was an artefact, was emphasized by the observation that in a mixture of NADH and nicotinamide ribotide as cofactors, NADH was oxidized in almost total preference to nicotinamide ribotide during the reduction of CB 1954. Nicotinamide ribotide inhibited this NADH oxidation with a similar K_i' to that determined with the menadione assay (data not shown). Similar results were also obtained with nicotinamide riboside.

At first sight, it would seem that the ability to utilize cofactors without the AMP moiety of NADH or NADPH (by an enzyme that possesses the rare property of being able to use either NADH or NADPH as a cofactor) would suggest that DT diaphorase lacks the requirement for the adenine nucleotide component of these compounds for the binding of these cofactors. Thus, it can bind molecules in which this moiety is entirely absent. In support of this view, we have preliminary data that another NAD(P)H utilizing enzyme, NAD(P)H: FMN oxidoreductase (EC 1.6.8.1) can also utilize some of these novel cofactors (although the actual pattern of activities is different) whilst an obligate NADH utilizing enzyme, namely NADH dehydrogenase (EC 1.6.99.3), cannot. However, our data also show that both nicotinamide ribotide and riboside appear not to interact with DT diaphorase directly through a NAD(P)H binding site. If this was the case with all the other cofactors, then the ability of the enzyme to utilize them may be coincidental to its ability to utilize both NADH and NADPH. Therefore, it is possible that some of the novel cofactors we have identified could be utilized by other dehydrogenases that are obligate for NADH or NADPH.

In summary, we have demonstrated for the first time that some simple, synthetic, reduced pyridinium compounds can act as efficient cofactors for the enzyme DT diaphorase. There is a good possibility that some of our synthetic cofactors (such as nicotinamide 5′-*O*-benzoyl riboside) may be highly enzyme selective. Such selective cofactors would be of great use in the biochemical study of DT diaphorase and allow the role of the enzyme in crude cell preparations to be more unequivocally identified. As a further example, the histochemical staining and location of DT diaphorase has recently been reported [19]. However, identification of the enzyme had to be made on a comparison of staining, on separate sections, in the presence or absence of dicoumarol (an inhibitor of DT diaphorase). A specific cofactor would eliminate this problem and allow the enzyme to be identified directly on a single section.

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