The stereospecificity of the reduction of nitrate by reduced nicotinamide–adenine dinucleotides catalysed by *Candida utilis* preparations

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The reduction of nitrate by reduced nicotinamide–adenine dinucleotides, catalysed by extracts of *Candida utilis*, exhibits an apparent high degree of stereospecificity for the 'B' methylene hydrogen atom of NADPH and mixed stereospecificity for the methylene hydrogen atoms of NADH. Purified nitrate reductase, on the other hand, exhibits 'A' stereospecificity for NADH and NADPH. The apparent switch of stereospecificity from the 'B' to the 'A' side of NADPH, which occurs after purification of the enzyme, is partly explained by the fact that in crude extracts nitrate is reduced completely to ammonia. Nitrite does not accumulate but is reduced to ammonia by nitrite dehydrogenase, which is 'B'-specific, so that up to 75% of hydrogen removed from NADPH during the reduction of nitrate could occur from the 'B' side. A further increase in the removal of hydrogen from the 'B' side of NADPH could be the kinetic isotope effect that is observed when [A' - 3H]NADPH is the reductant, the H–C bond being cleaved 2.3 times faster than the 3H –C bond. The mixed stereospecificity observed with NADH has been traced to an uncharacterized enzyme that catalyses a 'B'-specific exchange between NAD^+ and NADH. This reaction is discussed in relation to the possibility that it may explain other cases of apparent mixed stereospecificity that have been reported.

A number of generalizations concerning the stereospecificity of nicotinamide–adenine dinucleotide-linked dehydrogenases have been proposed (Bentley, 1970; You *et al.*, 1978; Jeffery, 1980). One of these generalizations states that 'when a metabolic sequence involves consecutive nicotinamide adenine dinucleotide dependent reactions, the dehydrogenases have the same stereospecificity'. Nine examples have been reported (Jeffery, 1980), including the reduction of nitrate to ammonia by *Candida utilis*, which involves consecutive 'B'-specific dehydrogenases (Davies *et al.*, 1972). However, Guerrero *et al.* (1977) reported that crude preparations of nitrate reductase from *Chlorella* showed mixed stereospecificity whereas the purified enzyme was 'A'-specific.

We have confirmed that in crude extracts of *Candida utilis* the reduction of NO_3^- appears to be 'B'-specific with NADPH, but with NADH mixed stereospecificity was observed. However, when the nitrate reductase was purified, 'A'-stereospecificity was observed with NADH and NADPH.

In the present paper we seek to explain why apparent changes of stereospecificity occur as a consequence of the purification of nitrate reductase.

Experimental

Materials

Chemicals and labelled compounds were obtained as previously reported (Davies *et al.*, 1972), except that Blue Dextran and CNBr-activated Sepharose 4B were obtained from Pharmacia, and Szechrome reagents from Yedatek (Rimon 10, Omer, Israel).

Labelled compounds

These were prepared essentially as described by Davies *et al.* (1972) and Do Nascimento & Davies (1975).

Enzymes

Nitrate reductase was purified (104-fold) on Blue Dextran–Sepharose by adapting the method of Solomonson (1975) for use with *Candida utilis*. Nitrite reductase was purified (53-fold) by $(NH_4)_2SO_4$ fractionation, and separation from nitrate reductase was achieved by passing the active fraction through a column of Blue Dextran–Sepharose.

Assays

Nitrate and nitrite were determined by the Szechrome method (details supplied with the reagents). Ammonia was determined by the method of McCullough (1967). Nitrate reductase was assayed as previously described (Davies *et al.*, 1972), except that FAD (3.3 µg/ml) was added. For the determination of nitrite reductase, FAD was omitted and KNO₃ was replaced with KNO₂.

Results

Stereospecificity of crude and purified nitrate reductase preparations

The stereospecificity of hydrogen transfer from NADH and NADPH during the reduction of nitrate was examined with a number of preparations. The results presented in Table 1 indicate: (1) that purified nitrate reductase is 'A'-specific; (2) that fresh extracts of *Candida* show 'B'-specificity for NADPH during the reduction of nitrate but that with aging there is an apparent switch from 'B'- to 'A'-stereospecificity; (3) that the degree of stereospecificity appears to depend on which side of the nicotinamide ring is tritiated; (4) that extracts show mixed specificity for NADH during the reduction of nitrate.

Starting from the assumption that the true stereospecificity of nitrate reductase is that of the purified enzyme, i.e. 'A', it is necessary to explain why in crude extracts there is apparent 'B'-stereospecificity with NADPH and mixed stereospecificity with NADH.

Interaction of nitrate reductase and nitrite reductase

One partial explanation of the apparent switch in stereospecificity is that the extract contains a highly

active and 'B'-stereospecific nitrite reductase, and that the reduction of nitrate proceeds to ammonia rather than nitrite.

Stereospecificity of nitrite reductase

Purified nitrite reductase (0.1 ml) was incubated with [³B'-³H]NADPH (160 nmol), KNO₂ (1 µmol) and potassium phosphate buffer, pH 7.3 (80 mM), in a total volume of 1 ml. When the oxidation of [³H]NADPH was complete, NADP⁺ was isolated and found to contain no ³H. Consequently, nitrite reductase is 'B'-specific (Table 2). With freshly prepared extracts the reduction of nitrate to ammonia appears to proceed with the oxidation of 4 equivalents of NADPH and with little or no accumulation of nitrite (results not shown). Furthermore, the interaction of purified nitrate reductase and nitrite reductase produces an apparent change of stereospecificity (Table 2). However, the maximum difference between crude and purified preparations that can be due to the presence of nitrite reductase is 75%. Furthermore, since nitrite reductase is inactive with NADH, the presence of nitrite reductase cannot explain the mixed stereospecificity observed with NADH. Additional and/or alternative factors must be involved.

Kinetic isotope effects

The loss of ³H from [³A'-³H]- and [³B'-³H]-NADH was measured during the course of nitrate reduction catalysed by purified nitrate reductase. The results show that with [³B'-³H]NADH there is little or no isotope effect (Fig. 1b). However, with [³A'-³H]-NADH the ¹H is removed approximately 2.3 times faster than ³H (Fig. 1a). The isotope kinetic effect observed with [³A'-³H]NADH is consistent with the 'A'-specificity of nitrate reductase, and can be invoked to partly explain differences in measured

Table 1. *Stereospecificity of the oxidation of NADH and NADPH by nitrate catalysed by various preparations of nitrate reductase*

For experimental details see the text.

Preparation	NADH or NADPH	³ H on 'A' or 'B' side	Measured stereospecificity
<i>Candida</i> fresh extract	NADPH	'B'	88% 'B'
	NADPH	'A'	92% 'B'
	NADH	'B'	70% 'A'
	NADH	'A'	53% 'A'
<i>Candida</i> extract stored for 6 days at -18°C	NADPH	'B'	96% 'A'
	NADPH	'A'	60% 'B'
Purified nitrate reductase from <i>Candida</i>	NADPH	'B'	94% 'A'
	NADPH	'A'	89% 'A'
	NADH	'B'	93% 'A'
	NADH	'A'	86% 'A'
Purified nitrate reductase from spinach	NADPH	'B'	98% 'A'

Table 2. *Stereospecificity of nitrate reductase and nitrite reductase*

Nitrate reductase and nitrite reductase were purified from *Candida utilis*. For experimental details see the text.

Enzyme	Substrate	Radioactivity in 'B' side of [^3H]NADPH (c.p.m.)	Recovery of radioactivity in NADP $^+$ (c.p.m.)	Stereospecificity
Nitrite reductase	NO_2^-	35 300	250	100% 'B'
Nitrate reductase	NO_3^-	29 900	27 750	93% 'A'
Nitrite reductase + nitrate reductase	NO_3^-	28 700	19 100	62% 'A'

Fig. 1. Transfer of ^3H from [$\text{A}'\text{-}^3\text{H}$]- and [$\text{B}'\text{-}^3\text{H}$]-NADH into NAD $^+$ and product during the reduction of nitrate catalysed by a purified preparation of nitrate reductase from *Candida*

(a) Transfer of ^3H from [$\text{A}'\text{-}^3\text{H}$]NADH; (b) transfer of ^3H from [$\text{B}'\text{-}^3\text{H}$]NADH. ●, ^3H in NAD $^+$; ○, ^3H in water. For experimental details see the text.

stereospecificity when [$\text{A}'\text{-}^3\text{H}$]- and [$\text{B}'\text{-}^3\text{H}$]-NADPH are compared. However, the kinetic isotope effect cannot be invoked to explain the near absence of stereospecificity that is apparent when NADH is used as the reductant for nitrate. Consequently, additional factors must be considered.

Enzyme-catalysed exchange of hydrogen between NAD $^+$ and NADH

An exchange of hydrogen between NAD $^+$ and NADH would account for the anomalous results observed with extracts of *Candida*, and accordingly we have examined extracts for this exchange reaction. [^3H]NAD $^+$ (37 kBq, 0.13 μmol) was incubated for 20 min with 0.25 μmol of NADH (or NADPH) in Tris/HCl buffer, pH 8 (0.1 M) and *Candida* extract (0.1 ml) in a final volume of 1 ml. [^3H]NADH (or [^3H]NADPH) was isolated by chromatography on DEAE-cellulose. [^3H]NADH contained 46% of the ^3H supplied and [^3H]NADPH 14.5%. These results establish an exchange between NAD $^+$ and NADH and a slower exchange between NAD $^+$ and NADPH.

Stereospecificity of the enzyme-catalysed exchange between [^3H]NAD $^+$ and NADH

[^3H]NADH formed from [^3H]NAD $^+$ in the exchange reaction was incubated with acetaldehyde and alcohol dehydrogenase and also with α -oxoglutarate, NH_4^+ and glutamate dehydrogenase. The NAD $^+$ isolated by chromatography on DEAE-cellulose was found not to have significant amounts of ^3H (2–5%) when formed by the alcohol dehydrogenase reaction, whereas an almost quantitative recovery of ^3H in NAD $^+$ (92–96%) formed in the glutamate dehydrogenase reaction was observed. Consequently, the NADH–NAD $^+$ exchange must be 'B'-specific.

Discussion

It is clear that our earlier designation of the *Candida* nitrate reductase as a 'B'-specific enzyme (Davies *et al.*, 1972) was in error. We have now shown that purified nitrate reductases from *Candida* and spinach are 'A'-specific, in agreement with the findings of 'A'-specificity for the enzyme from

Chlorella (Guerrero *et al.*, 1977). Accordingly, nitrate reductase should be removed from the compilations of 'B'-specific enzymes (You *et al.*, 1978; Jeffery, 1980), and it no longer appears as an exception to Bentley's (1970) first generalization that the stereospecificity of a reaction is independent of the source of the enzyme.

In the present study we have established purified nitrite reductase as a 'B'-specific enzyme. Consequently, nitrate reductase and nitrite reductase form an exception to the generalization that consecutive dehydrogenases have the same stereospecificity (Jeffery, 1980). The observation of apparent mixed stereospecificity in the oxidation of NADH by nitrate in the presence of an extract of *Candida* adds to the few examples of mixed stereospecificity recorded in the literature. The demonstration of an enzyme-catalysed 'B'-specific exchange between NADH and NAD⁺ explains this apparent lack of stereospecificity. Two points should be noted: (1) it follows from the 'B'-specificity of the exchange reaction that apparent mixed stereospecificity will only be observed when the dehydrogenase is 'A'-specific; (2) the degree of mixed stereospecificity will depend on the relative activities of the dehydrogenase and the exchange reaction.

These considerations lead us to suggest that two of the other three reported cases of mixed stereospecificity may also be due to the presence of the 'B'-specific NADH–NAD⁺ exchange in the enzyme preparations. Thus orcinol mono-oxygenase has been reported to show mixed stereospecificity (Ribbons *et al.*, 1971), but subsequent studies with more active preparations indicated 'A'-specificity (Ryerson & Walsh, 1979). This is consistent with the presence of a 'B'-specific exchange between NADH and NAD⁺ in the preparations examined by Ribbons *et al.* (1971). The transfer of hydrogen from NADH to

5-deazariboflavin in a relatively crude extract was shown to have mixed stereospecificity (Fisher & Walsh, 1972). However, NADH:FMN oxidoreductase is 'A'-specific, and it appears likely that the combination of the exchange reaction and the relatively slow reaction with 5-deazariboflavin would produce mixed stereospecificity. The third case, the reduction of CDP-6-deoxy-4-keto-D-glucose, appears not to involve a direct transfer of hydrogen from NAD(P)H (Rubenstein & Strominger, 1974), and the lack of stereospecificity in this reaction cannot be explained by the exchange reaction.

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