Inhibition of the Nitrate Reductase Complex by Dibromothymoquinone

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The plastoquinone antagonist 2,5-dibromothymoquinone was found to inhibit NO_3^- reduction from NADH by the nitrate reductase complex from wheat. It accepts electrons from NADH through the NADH dehydrogenase activity of the nitrate reductase. However, it does not inhibit the reduction of 2,6-dichlorophenol-indophenol by the enzyme. This suggests that the two compounds may be accepting electrons at different places from the enzyme. Further it was observed that reduced DCIP could be oxidized by DBMIB in the absence of NADH indicating that the electron flow in the nitrate reductase complex may take place in a unidirectional way.

Introduction

Nitrate reductase is a high molecular weight complex enzyme. It possesses two partial activities in addition to the reduction of NO_3^- from NAD(P)H [1]. The NADH dehydrogenase activity is usually measured by the oxidation of NADH in the presence of electron acceptors such as DCIP or cytochrome c. Alternately it could be assayed by the reduction of these electron acceptors. The terminal nitrate reductase ($FMNH_2$-NR) is assayed by following NO_2^- formation in the presence of reducing substrates such as reduced FMN. Recent evidence suggests that these two partial activities (NADH dehydrogenase and $FMNH_2$-NR) may be physically separated and located on two different polypeptides. It has been proposed that in higher plants the NADH dehydrogenase and the terminal nitrate reductase is linked by cytochrome b-557. In our earlier work [2] we have shown that NH_2OH – a potent inhibitor of nitrate reductase may interfere with the functioning of the heme part of the NR complex and therefore does not affect either of the partial activities. In this communication we show that DBMIB, an antagonist of plastoquinone and widely used in photosynthesis research [3] at very low concentrations inhibits nitrate reductase as it acts as an electron acceptor from NADH prior to the site of NH_2OH interaction. We further show that DBMIB may accept electrons at a site different than the one at which DCIP is reduced by the NADH dehydrogenase moiety of NR.

Abbreviations: DBMIB, 1,5-dibromothymoquinone; DCIP, 2,6-dichlorophenol-indophenol; EDTA, ethylenediaminetetraacetate; $FMNH_2$, reduced flavin mononucleotide; NR, nitrate reductase.

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Materials and Methods

Ten-day old field grown seedlings of wheat provided with NO_3^- were used for the extraction of the enzyme. The leaves were washed and homogenized in 0.1 M phosphate buffer, pH 7.5 containing 1 mM EDTA, 10 mM NO_3^- and 1 mM cysteine. After passing through cheese cloth, 0.1 volume of a protamine sulphate solution (1.1%) containing 0.6% of tris was added. After centrifugation at 16,000 × *g* for 10 min the supernatent was brought to 50% ammonium sulphate saturation by adding solid ammonium sulphate. The pH of the supernatent was maintained at 7.5 by the addition of 5 N NH_4OH during ammonium sulphate addition. After 20 min of stirring it was centrifuged at 15,000 × *g* for 10 min. The precipitate was dissolved in the 10 mM phosphate buffer, pH 7.5 containing 1 mM EDTA, 1 mM cysteine and 10 mM NO_3^-. The protein dialysed against the same buffer for an hour was chromatographed on DEAE cellulose. The fractions showing NR activity were pooled and concentrated by 50% ammonium sulphate precipitation. This fraction was further purified on blue sepharose according to Cambell and Smarrelli [4].

Nitrate reductase, $FMNH_2$-NR and NADH dehydrogenase were assayed according to Jawali *et al.* [2]. The pH of the assay was 7.5 unless mentioned other-

wise. DBMIB reduction was measured by the oxidation of NADH at 340 nm or polarographically by monitoring O_2 consumption. A 10 mM DBMIB solution and its dilutions were prepared in ethanol. Appropriate amounts of ethanol were added to the control for comparison with the treatments involving DBMIB additions.

Results

The effect of different concentrations of DBMIB on the activities of nitrate reductase complex is shown in Table I. The NO_3^- reduction from NADH is severely affected by as low as 0.07 mM DBMIB concentration. However, neither the nitrate reduction from $FMNH_2$ nor the DCIP reduction (NADH-dehydrogenase) is affected to any significant extent by even higher concentrations. The DCIP reduction was unaffected even at 0.5 mM concentration of DBMIB (data not shown). Since the NO_3^- reduction from $FMNH_2$ was not affected it is apparent that the site of inhibition must be before the molybdenum but after the site at which DCIP is reduced.

One of the possibilities we considered was that DBMIB may oxidize NADH through the NADH dehydrogenase activity of the nitrate reductase complex and thus inhibit the NADH → NO_3^- activity. Table II shows that NADH is indeed oxidized by DBMIB and that this reaction is dependent on both DBMIB and NADH concentration (see Tables II and III). The NADH oxidation is already saturated at 0.1 mM NADH and 0.2 mM DBMIB concentration.

Table I. Effect of DBMIB on NR complex and its partial activities.

DBMIB [mM]	NADH-NR	$FMNH_2$-NR	NADH dehydrogenase
	Percent activity		
0.00	100	100	100
0.05	26	91	100
0.07	7	93	100
0.10	3	93	100

The $FMNH_2$-NR and NADH dehydrogenase activity was not affected any more upto 0.5 mM concentration of DBMIB.

The enzyme preparation used reduced about 10 nmol of nitrate/mg protein/minute.

Table II. Effect of increasing DBMIB concentration on NADH (0.2 mM) oxidation.

DBMIB [mM]	Δ OD 340 nm/min
0.1	0.29
0.2	0.46
0.5	0.43

Table III. Effect of increasing concentration of NADH on DBMIB reduction.

NADH [mM]	Δ OD 340 nm/min
0.05	0.14
0.10	0.20
0.20	0.20

The DBMIB concentration used was 0.2 mM.

Table IV. The effect of NH_2OH on the reduction of DBMIB by the NR complex. The reaction medium contained phosphate buffer pH 7.5, 50 mM, FAD 10 μM, NADH 0.2 mM, and DBMIB 0.2 mM. a: The assay was carried out in the presence of 1 mM NH_2OH. This inhibits NO_3^- reduction from NADH by about 85% [2]. b: The enzyme was preincubated with NADH and NH_2OH (1 mM) for 5 min before the assay. Such a preincubation inhibits NO_3^- reduction from NADH totally.

Treatment	Δ OD 340 nm/min
Control	0.46
NH_2OH [a]	0.40
NH_2OH [b] preincubated	0.40

The reduction of DCIP was not inhibited by DBMIB even at higher concentrations although it could accept electrons from NADH in a dehydrogenase type reaction. We, therefore, wondered if DBMIB was accepting electrons beyond the cytochrome b-557 of the nitrate reductase complex or prior to it. Earlier report from our laboratory had shown that NH_2OH may interfere with the cytochrome functioning of the nitrate reductase complex [2]. If DBMIB was accepting electrons before cytochrome b-557 then its reduction (as monitored by NADH oxidation) would not be stopped by NH_2OH, on the other hand if DBMIB was accepting electrons at or beyond cytochrome b-557, DBMIB would not be able to oxidize NADH in the presence of NH_2OH. Table IV shows that the addition of NH_2OH during the reaction or preincubation of the enzyme with NH_2OH did not affect much the oxidation of NADH by DBMIB. These data indicate that

DBMIB accepts electrons prior to cytochrome b-557. It therefore may be considered as an electron acceptor of the NADH dehydrogenase moiety of the nitrate reductase complex.

The data presented in Table I had shown that DCIP reduction was unaffected by DBMIB but DBMIB could oxidize NADH through NADH dehydrogenase activity. It was, therefore, interesting to find out if DBMIB could also oxidize reduced DCIP in the absence of NADH.

DBMIB reduction can be studied by a polarographic procedure by monitoring O_2 consumption. This procedure is based on the auto-oxidizable property of the reduced DBMIB. Using this method and studying DCIP reduction we have observed that the presence of DBMIB does not have any effect on DCIP reduction (curve D, Fig. 1). However, in the presence of DCIP, the DBMIB reduction does not take place until all of the DCIP is reduced (compare curve D and curve B). In the absence of DCIP the DBMIB reduction starts immediately and the rate of reduction is faster than in the presence of DCIP (compare curve C and B). This is not unexpected because in the presence of DCIP some of the NADH would have been oxidized by it. In these experiments we had observed that after some time the reduction of DBMIB is associated with the oxidation of DCIP previously reduced from NADH.

In order to confirm whether the oxidation of reduced DCIP in the presence of DBMIB is a mere chemical reaction or is enzyme catalyzed reaction we studied the oxidation of chemically reduced DCIP by DBMIB. The oxidation of DCIP could not take place either in the absence of enzyme or in the presence of enzyme inactivated by boiling. The reduction of DBMIB from reduced DCIP is strictly dependent on the active enzyme and the concentration of reduced DCIP (Table V). These observations suggest that in the absence of NADH, reduced DCIP could feed electrons into the enzyme complex and reduce DBMIB. However, DCIP could not be reduced from reduced DBMIB even in the presence of enzyme and under anaerobic conditions (to prevent auto-oxidation of DBMIB). This could be interpreted to mean that in the NADH dehydrogenase moiety of nitrate reductase there may be a unidirectional flow of electrons. The fact that DBMIB can oxidize reduced DCIP in the presence of the enzyme suggests that possibly the two acceptors drain off electrons from the enzyme from different sites.

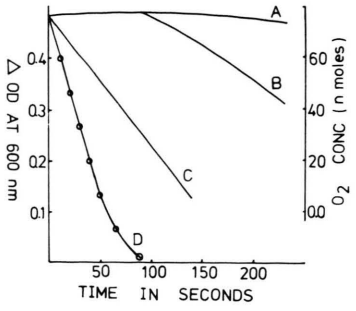

Fig. 1. Reduction of DCIP and DBMIB from NADH by the nitrate reductase complex. —— O_2 consumption; o—o ΔOD at 600 nm.
A: O_2 consumption during DCIP reduction;
B: O_2 consumption during DBMIB reduction in the presence of DCIP;
C: O_2 consumption during DBMIB reduction in the absence of DCIP;
D: DCIP reduction as assayed by Δ OD at 600 nm in the absence of DBMIB. The curve is identical in the presence of DBMIB.

Table V. The reduction of DBMIB by chemically reduced DCIP.

DCIP [μM]	Δ OD 600 nm/min
30	0.035
60	0.105
120	0.190

The reaction medium contained phosphate buffer pH 8.0, 50 mM, DBMIB 0.1 mM and reduced DCIP obtained by titrating it with the required amount of ascorbic acid.

Discussion

DBMIB has proved very useful in probing the proton translocation and vectorial electron transport in the chloroplast membranes [5]. This plastoquinone antagonist has redox properties due to its quinone character and was found to inhibit electron transport from NADH to NO_3^- by the nitrate reductase complex. However, it had no effect on the $FMNH_2$-NR activity nor did it inhibit NADH dehydrogenase activity of the nitrate reductase complex. In these respects its action was similar to the action of NH_2OH on the nitrate reductase complex. Further studies showed that DBMIB acts as an electron acceptor during NADH dehydrogenase function of the nitrate reductase complex (Tables II and III) and its site of action is prior to NH_2OH interaction (Table IV). An

interesting observation made was that DCIP which is also an electron acceptor of the NADH dehydrogenase moiety of NR could be reduced in the presence of DBMIB. The presence of DBMIB had no effect on the reduction of DCIP. This indicates that either the DCIP is a better electron acceptor than DBMIB or that the two accept electrons at different sites. Another interesting point brought out by the present studies is that the reduced DCIP can feed electrons into the NR complex and can reduce DBMIB but not vice versa. This shows that within the NADH dehydrogenase part of the NR complex there is a unidirectional flow of electrons and that electrons from the enzyme can be drained off at different sites. A further proof for this will be presented in the subsequent communication.

The observations reported here indicate that although the primary effect of DBMIB in an intact cell may be the inhibition of photosynthetic electron transport it could also affect the cell metabolism by the oxidation of NADH through NR and possibly through other diaphorases. Because of its auto-oxidizable property it can perform this function even if present in catalytic amounts and could deprive the cell of reducing power needed so vitally for its biosynthetic functions.

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