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DIAPHORASE ACTIVITY OF FERREDOXIN: NADP OXIDOREDUCTASE IN THE PRESENCE OF DIBROMOTHYMOQUINONE

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Abstract—In the presence of dibromothymoquinone (10 μM) NADPH- and ferredoxin:NADP oxidoreductase-dependent oxygen uptake was observed in buffered (40 mM Tris–HCl buffer) medium. The rate of O_2 uptake depended on the pH of the reaction mixture and was about two orders of magnitude faster at pH 8.7 than at pH 6.7. In alkaline medium the rate was about 0.2 μmol O_2 min^{-1} cm^{-3} (or 9.3 nmol O_2 min^{-1} mg^{-1} FNR) at the time of tracing, whereas at a lower pH (7.7 or 6.7) oxygen consumption was 1.5–2.0-fold faster during the first minute of monitoring than during the second minute. Ferredoxin was not an obligatory component involved in this process. However, at a lower pH (7.7, 6.7) oxygen consumption by the reaction mixture was stimulated 2–8-fold by adding ferredoxin (0.6 μM). The v_2 value was also enhanced by *ca* 36% in the presence of 0.2 M NaCl, whereas inorganic pyrophosphate caused reduction of v_1 and v_2 to about 62 and 3%, respectively, of the control. Under anaerobic conditions the Michaelis constant (K_m) and the pseudo-first-order rate constant (k) for the FNR-catalysed DBMIB reduction was about 16 μM and 0.34 s^{-1}, respectively. The results indicate that DBMIB can accept electrons from NADPH-ferredoxin:NADP oxidoreductase, and the reduced DBMIB (presumably in the unprotonated form) can be reoxidized by molecular oxygen.

INTRODUCTION

Ferredoxin:NADP oxidoreductase (EC 1.18.1.2) is an essential enzyme that normally catalyses the terminal step in photosynthetic NADP reduction [1–3]. There is now substantial evidence to indicate that the isolated enzyme exhibits NADPH-specific diaphorase mediated reduction of ferricyanide and various dyes [4, 5] or trans-hydrogenase activity involving the reduction of NAD or its analogues by NADPH [6]. Ferrodoxin:NADP oxidoreductase (FNR) and ferrodoxin (Fd) can also be involved in the reduction of methyl viologen [7] or cytochrome *c* [5] by reducing equivalents derived from NADPH. Despite numerous studies it is still an open question whether diaphorase activity of FNR plays any significant role *in vivo*. Answering this question one should take into account data which indicate that in darkened chloroplast preparations the reducing power of NADPH could be utilized for the reduction of plastoquinone [8–10], the primary electron acceptor from PS II [11], P700 [12–14], cytochrome *f* [15], cytochrome *b* [16], the Rieske iron centre [12] and plastocyanin [12]. FNR has been found to be implicated in all of these reactions. It has also been proposed that FNR and Fd are involved, in addition to other NADPH-dependent oxidoreductases [17, 18], in the oxidation of NADPH generated in chloroplastic glycolytic and oxidative pentose phosphate pathways [17, 19, 20]. Reverse electron flow through a portion of the normal photosynthetic carrier sequences seems to be more pronounced at the earlier stages of chloroplast biogenesis [14, 21], although this pathway(s) in both developed and undeveloped chloroplasts has not been fully characterized. Thus, further studies are needed in order to gain an understanding the physiological function of diaphorase activity of FNR and the pathway(s) of the electron flow in the reverse direction at the reducing site of PSI assembly. In this communication we report the results of studies on the diaphorase activity of FNR in the presence of dibromothymoquinone (DBMIB).

RESULTS

The rate of oxygen consumption (v) by an aqueous mixture of FNR, Fd and NADPH was low ($< 1.55 \times 10^{-2}$ nmol O_2 min^{-1} mg^{-1} Fd). However, it was a little higher at pH values of 7.7–7.8 than at pH 6.7. Adding DBMIB (10 μM) to the assay mixture substantially stimulated oxygen uptake and this stimulation depended on the pH of the medium (Fig. 1A). The v_1 values (first minute) were 0.155, 0.051 or 0.030 at pH 8.7, 7.7 or 6.7,

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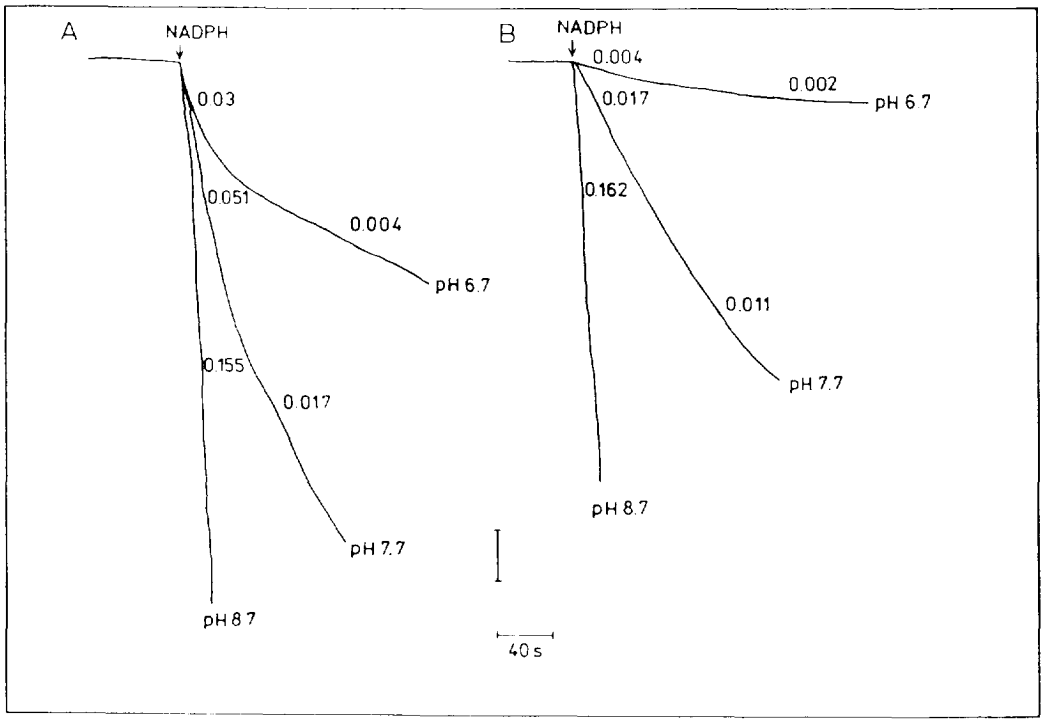

Fig. 1. Effect of pH on the oxygen uptake by the buffered (Tris–HCl buffer, 40 mM) reaction mixture composed of 0.6 μM FNR, 10 μM DBMIB and 1 mM NADPH in (A) the presence, or (B) the absence of 0.6 μM ferredoxin. DBMIB was added in ethanol solution. The final concentration of ethanol in the assay medium did not exceed 1%. Vertical line corresponds to 6 nmol O_2 cm^{-3} or 0.28 μmol O_2 mg^{-1} FNR. The rates of oxygen uptake, in μmol O_2 cm^{-3} min^{-1}, are also marked.

respectively. Thus, lowering pH from 8.7 to 7.7 or from 8.7 to 6.7 caused respectively a three- or five-fold decrease in the rate of molecular oxygen utilization. These differences were enhanced during the course of the reaction as v_2 (second minute) increased nine- and 40-fold in response to change of pH from 8.7 to 7.7 and from 8.7 to 6.7, respectively. It is also apparent that at pH 8.7 a monophasic pattern of oxygen uptake were observed, whereas at a lower pH (7.7 or 6.7) one could see an apparently biphasic pattern.

The next set of experiments revealed that Fd was not an obligatory component involved in the transfer of reducing equivalents from NADPH to DBMIB. At pH 8.7 v_1 reached 0.16 in the assay mixture deprived of Fd, about equal to that in the presence of Fd (Fig. 1A, B). However, at lower pH, v_1 were found to be 0.017 (at pH 7.7) or 0.004 (at pH 6.7) and was three- to eight-fold lower than those measured in the reaction mixture supplemented with Fd. v_2 was also 1.5–2.0-fold lower in the absence of Fd from those in the presence of Fd. Thus, Fd was not implicated in the NADPH- and FNR-mediated reduction and reoxidation of DBMIB, although it could influence the rate of the process at lower pH values. These results also indicated that in the absence of Fd there occurs a significant difference in the v_1 values measured at different pH values: v_1 expressed in relative units were 1.0:0.1:0.02 at pH 8.7, 7.7 and 6.7, respectively, whereas in the presence of Fd the v_1 values were almost equal. Thus, at lower pHs (7.7, 6.7) Fd accelerated oxygen uptake (particularly v_1 values) by DBMIB.

Table 1 shows the v_1 and v_2 values to be greater by *ca* 13–15% in the presence of chemically modified Fd, whereas 0.2 M NaCl increased v_2 by about 36%. It is known that under high ionic strength, dissociation of ferredoxin from the Fd-FNR complex takes place [22], and this separation clearly facilitated oxygen consumption. On the other hand, inhibition of the FNR activity by 20 μM pyrophosphate caused depression of v_1 or v_2 by *ca* 40 or 97%, respectively (Table 1), implying interaction of pyrophosphate with both Fd and DBMIB reduction site(s) on FNR. The v_1 value at pH 6.7 did not depend on the DBMIB concentration and was *ca* 0.075 (Fig. 2) whereas v_2 increased from *ca* 0.004 in the presence of 5 μM DBMIB to *ca* 0.01 in the presence of 30 μM DBMIB.

Kinetics of NADPH oxidation in the presence of various concentrations of DBMIB and under anaerobic conditions were also measured (Fig. 3). We assumed that in the presence of 1 mM NADPH and 0.6 μM FNR the initial rate of NADPH oxidation corresponded to the initial rate of DBMIB reduction. Under this assumption the calculated pseudo-first-order rate constant (k) was *ca* 0.40 s^{-1} and the Michaelis constant (K_m) for DBMIB reduction was about 16 μM.

Table 1. Effect of inorganic pyrophosphate (PPi), modified ferredoxin (mFd) or high ionic strength on the rate of oxygen uptake by the buffered (Tris–HCl buffer) reaction mixture composed of FNR, Fd(or mFd), DBMIB and NADPH

Reaction mixture		Oxygen uptake, $v_1(v_2)$, rel. units
1. 0.6 μM FNR, 0.6 μM Fd (or mFd), 10 μM DBMIB, 1 mM NADPH, Tris–HCl buffer (40 mM, pH 7.7)		100(100)
2. 0.6 μM FNR, 0.6 μM Fd (or mFd), 10 μM DBMIB, 1 mM NADPH, Tris–HCl buffer (40 mM, pH 7.7)	+ 20 μM PPi*	62(3)
3. 0.6 μM FNR, 0.6 μM Fd (or mFd), 10 μM DBMIB, 1 mM NADPH, Tris–HCl buffer (40 mM, pH 7.7)	+ mFd†	113(115)
4. 0.6 μM FNR, 0.6 μM Fd (or mFd), 10 μM DBMIB, 1 mM NADPH, Tris–HCl buffer (40 mM, pH 7.7)	+ 0.2 M NaCl	96(136)

*The pH of the sodium pyrophosphate (PPi) solution was brought up to 7.7 prior to adding to the reaction mixture.

† Modified ferredoxin (mFd), added instead of native Fd, was dialysed against 0.6 M NaCl in order to removal carbodiimide and ethyl glycine ester. Thus, our preparation of mFd contained also some of NaCl. However, its final concentration in the reaction mixture did not exceed 1.2 mM.

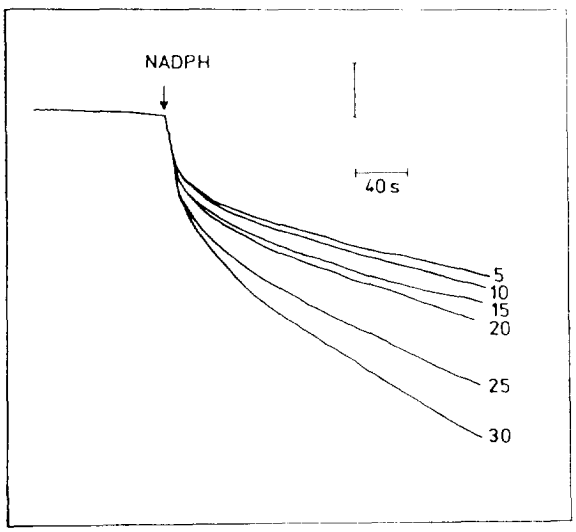

Fig. 2. Effect of DBMIB concentration (μM, as indicated) on the oxygen uptake by the buffered (Tris–HCl buffer, 40 mM, pH 6.8) reaction mixture composed of 0.6 μM FNR, 0.6 μM Fd, and 1 mM NADPH. Vertical line corresponds to 10 nmol O_2 cm^{-3} or 0.46 μmol O_2 mg^{-1} FNR. Other details as in Fig. 1.

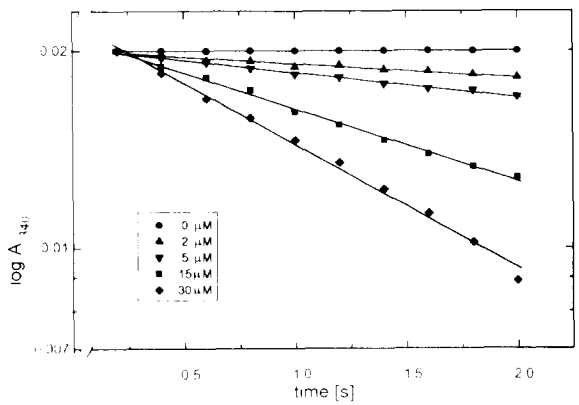

Fig. 3. Kinetics of FNR dependent NADPH oxidation (monitored at 340 nm and at room temperature) in presence of various concentrations of DBMIB and under anaerobic conditions. The buffered (Tris–HCl buffer, 40 mM, pH 7.7) reaction mixture contained: 0.6 μM FNR, 1 mM NADPH, 100 U cm^{-3} glucose oxidase, 10 mM glucose, *ca* 1100 U cm^{-3} calatase and DBMIB in concentration as indicated.

DISCUSSION

DBMIB in low concentration (< 5 μM) inhibits electron flow in chloroplasts at the site of plastoquinol oxidation [23] whereas at higher concentration (> 5 μM) it may also acts as an efficient electron acceptor at the reducing site of photosystem II [24–26]. The data reported here clearly suggest that DBMIB could also play the role of an electron acceptor in the diaphorase activity of ferredoxin: NADP oxidoreductase and in this way reduced DBMIB may be reoxidized by molecular oxygen. To our knowledge, this is the first report showing DBMIB to accept electrons at the site of FNR activity. The reduction of O_2 by $DBMIBH_2$ proceeded presumably via H_2O_2 [27, 28]. Reduced DBMIB might also interact with O_2 as had been postulated for other quinones [29, 30]. Presumably only unprotonated mol-

ecules ($DBMIB^{-(2-)}$) could be oxidized effectively by molecular oxygen [31].

In this study net oxygen uptake was mainly monitored and therefore no detailed pathway and steady-state kinetics will be discussed. However, we found that Fd was not required for the DBMIB reduction and its reoxidation. We may assume that the mechanism of the FNR-mediated DBMIB reduction is comparable with that of FNR-mediated reduction of ferricyanide or some dyes by NADPH. Fd appeared, however, to stimulate oxygen consumption by the system investigated and this is in accordance with many other published data, for example, ref. [2]. We suppose that Fd induced conformational changes of the FNR molecules and this favored DBMIB reduction. This might also suggests, as has been proposed for 2,6-dichlorophenol-indophenol reduction [32], that the Fd binding site differs from that of the DBMIB binding site and no competition for binding sites on FNR molecules occurred. The rate of O_2 uptake in the presence of modified Fd [33] was greater than in the presence of native form. One possible explanation for this is that the modified Fd also altered the conformational state of FNR. A similar interpretation may concern the data on the effect of high ionic strength of the medium that impairs interaction of Fd with FNR [22, 34, 35]. It is also possible that Fd and/or high ionic strength influenced the redox properties of FNR and DBMIB in our reaction mixture. However, pyrophosphate, an inhibitor acting at the Fd binding site on FNR [36], effectively blocked O_2 uptake, presumably due to the inhibition of DBMIB reduction. This suggests that pyrophosphate inhibited electron flow at Fd and DBMIB accepting sites on FNR molecules.

The O_2 uptake by the system investigated depended apparently on the pH of the reaction medium. In general, this process proceeded faster in alkaline medium (pH 8.7) than at an acidic pH (pH 6.7). Presumably, it could not be attributed exclusively to the effect of pH on the FNR activity as the optimal pH has been found to range between 6.5 and 8.0, for example, ref. [22]. It is proposed that the high activity of the oxygen consumption at the initial step of DBMIB reduction as well as in alkaline pH was associated with the predomination of the unprotonated form of DBMIB molecules. Shifting the equilibrium $DBMIB^{-(2-)} \rightleftharpoons DBMIBH_2$ towards the $DBMIBH_2$ in an acidic medium was presumably responsible for lowering the rate of oxygen uptake. However, ionization of sulphydryl groups in the active site of FNR [37] and/or two-electron reduction of the FNR flavin-cofactor [38] at higher pH might also contribute to faster DBMIB reduction and indirectly to its reoxidation.

The pseudo-first-order rate constant for DBMIB reduction under anaerobic conditions ($k = 0.40\ s^{-1}$) is much lower from those calculated for the electron transport from FNR to, for example, high potential redox proteins [39], but higher than k_{obs} for Fd_{red} oxidation by O_2 [40]. The K_m for DBMIB reduction in our reaction mixture was found to be rather high but comparable with that calculated for FNR catalysed cytochrome c reduction in the presence of ferredoxin [41].

The results reported in this work extend information available on the diaphorase activity of FNR. Although DBMIB is a non-physiological agent, it resembles in many respects some of the natural quinones. Our preliminary data show that oxygen uptake was also detectable in the reaction mixture of NADPH, FNR and plastoquinone or α tocopherol quinone. In conclusion, the results presented here demonstrated that the plastoquinone antagonist, DBMIB, can be an appropriate electron acceptor in diaphorase activity of FNR, and that reduced DBMIB, presumably in unprotonated form, could be oxidized by molecular oxygen, and that this oxidation is favored in alkaline pH.

EXPERIMENTAL

Reagents. Glycine ethyl ester, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, NADPH, glucose oxidase and Tricine were purchased from Sigma, DBMIB was from Aldrich; Tris was obtained from Fluka; DEAE cellulose 23SH, DEAE cellulose 52 and Sephadex G-50 fine were purchased from Serva, Whatman and Pharmacia, respectively. Catalase was from Koch Light Lab. Other reagents of the analytical grade came from the Polskie Odczynniki Chemiczne, Poland.

Ferredoxin and a crude prepn of ferredoxin:NADP oxidoreductase were isolated from spinach by repeated chromatography on DEAE cellulose 23SH, DEAE cellulose 52 and Sephadex G-50 fine columns, as described in ref. [42]. The isolated ferredoxin formed a single protein band in Na dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and the A ratio (A_{420}/A_{276}) was 0.20. An extinction coefficient of 9.7 $mM^{-1}\ cm^{-1}$ at 420 nm was used to determine Fd_{ox} concn. The crude prepn of FNR showed several protein bands on SDS–PAGE with the major one having M_r of about 35 000. The sp. act. of this prepn in our assay medium was comparable with the activity of FNR purified to homogeneity according to ref. [43] (data not shown). An extinction coefficient of 9.8 $mM^{-1}\ cm^{-1}$ at 459 nm was used to determine the FNR concn. Concd suspension of Fd or FNR in Tris–HCl buffer (40 mM, pH 8.0) were stored at − 30° prior to use. Modified ferredoxin (Fd treated with 0.5 M glycine ethyl ester in the presence of 0.3 mM 1-ethyl-3(3-dimethylamine propyl) carbodiimide was prepared according to ref. [24]. O_2 uptake was monitored at 25° by a Clark-type oxygen electrode (Hansatech, U.K.) connected with the TZ 4100 Line Recorder (Praha, The Czech Republic).

Kinetics of NADPH oxidation (changes of A at 340 nm) were monitored by the Stopped-flow spectrophotometer (SX-17 MV Applied Photophysics, U.K.). Other spectrophotometric measurements were carried out by the LSM-Aminco DW 2000 Spectrophotometer.

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