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2,3-DIMERCAPTOPROPAN-1-OL (BAL)

AN AEROBIC ELECTRON-TRANSPORT INHIBITOR, BUT AN ANAEROBIC PHOTOSYNTHETIC ELECTRON DONOR

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The effect of 2,3-dimercaptopropan-1-ol (BAL) on photosynthetic electron transport was studied in *Oscillatoria limnetica* cells as well as in spinach chloroplasts. Preincubation of the cyanobacterial cells with BAL under aerobic conditions caused inhibition of the sulfide-dependent H_2 evolution. The inhibited site was bypassed by *N,N,N′,N′*-tetramethyl-*p*-phenylene diamine. Similarly, NADP photoreduction by spinach chloroplasts was inhibited by BAL pretreatment and restored by the addition of reduced 2,6-dichlorophenolindophenol. These results accord previous data demonstrating damage of mitochondrial Rieske iron-sulfur protein after BAL treatment (Slater, E.C. and De Vries, S. (1980) Nature 288, 717–718). However, in chloroplasts there was no change in the Rieske center ESR spectrum after BAL treatment. When added anaerobically, BAL served as an electron donor for light-dependent, H_2 evolution in cyanobacteria or for NADP reduction in spinach chloroplasts. These reactions were insensitive to 3-(3′,4′-dichlorophenyl)-1,1 dimethylurea (DCMU), but sensitive to 2,5-dibromothymoquinone or to aerobic pretreatment with BAL. The Rieske protein ESR signal of chloroplasts ($g = 1.89$) which disappeared upon illumination in the presence of DCMU and methyl viologen, was restored by BAL. It is suggested that as opposed to its aerobic inhibitory effect, BAL donates electrons to the Rieske protein under anaerobic conditions.

Introduction

The mercaptane BAL (British Antilewisite, 2,3-dimercaptopropan-1-ol) was shown as early as 1948 1948 to inactivate the succinate oxidase system of mitochondria completely [1]. Inactivation was obtained by an aerobic incubation in the presence of BAL. The BAL-sensitive factor in mitochondria was recently identified [2] as the Rieske iron-sulfur protein [3]. This protein is now believed to play an important role in respiratory and photosynthetic electron transport, as well as in proton transfer across biological membranes [4–6]. It comprises a part of the cytochrome b/c complex in the electron-transport chain of mitochondria and photosynthetic bacteria, as well as the b_6/f complex in chloroplasts and cyanobacteria [4]. It was, there-

Abbreviations: Chl, chlorophyll; PS I, Photosystem I; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; BAL, British Antilewisite, 2,3-dimercaptopropan-1-ol; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; DBMIB, 2,5-dibromothymoquinone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

fore, of interest to extend the mitochondrial studies with BAL to photosynthetic systems.

The b_6/f complex is localized in the segment of electron carriers connecting the two photosystems in chloroplasts and in cyanobacteria, accepting its electrons from plastoquinol. In cyanobacteria this segment also accepts electrons from sulfide [7–10]. Thus, in the cyanobacterium *Oscillatoria limnetica,* PS-I-driven, sulfide-dependent CO_2 photoassimilation and hydrogen evolution are insensitive to DCMU but inhibited by plastoquinone antagonists [8,10]. This system, as well as spinach chloroplasts were used in the present work to study the effect of BAL on photosynthetic electron transport.

It is shown that, when added aerobically, BAL completely inhibits electron flow at or near the Rieske protein site. However, under anaerobic conditions, BAL serves as an electron donor to this protein in both *O. limnetica* and spinach chloroplasts.

Materials and Methods

Oscillatoria limnetica [11] was grown aerobically [12] or anaerobically [13], and chlorophyll was determined [14] as previously described.

Spinach plants were grown [15], broken (type C) chloroplasts prepared [16] and chlorophyll determined [17] as previously described.

Light-dependent H_2 evolution was determined in intact *O. limnetica* cells. 5 days old, aerobically grown cells were washed and resuspended at a concentration of 10–20 μg Chl *a* per ml in an anaerobic growth medium [13] lacking Na_2CO_3 and containing 10 μM DCMU, unless otherwise stated. In order to avoid precipitate formation at the high pH range, phosphate concentration was reduced to 0.3 mM. The medium was buffered with 25 mM Hepes for the pH range 7.0–8.2. For lower and higher pH values, 25 mM Mes or 25 mM Taps were used, respectively. Serum bottles of 15 ml, containing 2 ml of the cell suspension, were sealed with serum stoppers, flushed with N_2 for 20 min to attain anaerobic conditions, and incubated at 35 °C with shaking, with or without illumination (cool white fluorescent lamps, $2.5 \cdot 10$ J $\cdot$ m^{-2} $\cdot$ s^{-1}). All additions and withdrawals were made anaerobically with syringes after the N_2 flushing. The evolution of H_2 was determined by gas chromatography in 0.5 ml gas samples as previously described [9].

Hydrogenase activity was determined in cell free preparations of *O. limnetica* [8], in the dark, at 35 °C, with 2.5 mM methyl viologen and 10 mM $Na_2S_2O_4$. The H_2 evolved was determined as above.

CO_2 photoassimilation was determined in intact *O. limnetica* cells under conditions identical to the H_2 evolution assay, except for the injection of [^{14}C]$NaHCO_3$ after the N_2 flushing, to a final concentration of 14 mM and a specific activity 0.06 Ci/mol. At different time intervals gas samples were withdrawn for H_2 measurements. The bottles were then opened, and 1.0 ml of the cell suspension was assayed for the CO_2 fixed as previously described [12].

NADP photoreduction was determined in an Aminco dual wavelength spectrophotometer model DW-2, at 340–400 nm. Actinic light was provided by a 250 W halogen lamp filtered through a Schott RG 645 filter. The photomultiplier was protected by a 1.5 cm width of saturated $CoSO_4$ solution. The cuvette holder was thermostated at 23 °C. Standard reaction mixture contained in 3 ml, 40 mM sodium Tricine (pH 8), 30 mM NaCl, 25 μM ferredoxin, 1.2 mM NADP and chloroplasts containing 8 μg Chl/ml. The mixture was constantly stirred during the measurement. Anaerobiosis was obtained by N_2 flushing through the reaction mixture for 1 h before the experiments. The cuvette was tightly closed by a rubber stopper, through which additions were made.

ESR measurements were carried out in a Varian E-12 spectrometer equipped with a cryostat (Air Products) suitable for liquid helium temperature.

Methyl viologen, NADP, Hepes, Mes, Taps and TMPD were purchased from Sigma, and [^{14}C]$NaHCO_3$ from Amersham. DCMU was obtained from Du Pont. Ferredoxin (isolated from *Spirulina platensis* as described in Ref. 18) was kindly provided by Mr. M. Sheffer, and DBMIB by Prof. A. Trebst.

Results

BAL as a photosynthetic inhibitor

Aerobic preincubation of *O. limnetica* cells in the presence of BAL led to an inhibition of the

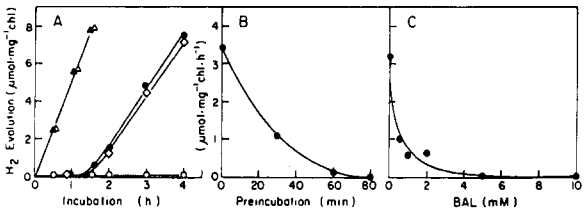

Fig. 1. BAL-inhibition of sulfide-dependent H_2 evolution. *O. limnetica* cells were transferred to anaerobic conditions at pH 7.5 as described under Materials and Methods. Before illumination, 10 μM DCMU, 3.5 mM Na_2S and 10 mM $Na_2S_2O_4$ were added. (A) BAL inhibition requires oxygen: No incubation before assay of hydrogen evolution (●); 70 min preincubation with 10 mM BAL in air prior to the N_2 flushing (○); preincubation with 10 mM BAL under N_2 (◇); hydrogenase activity in the presence of 2.5 mM methyl viologen and 10 mM $Na_2S_2O_4$ in cell-free preparations (▲); as the latter, but following aerobic pretreatment for 30 min with 10 mM BAL (△). (B) BAL-inhibition as a function of preincubation time: cell aliquots were incubated under air in the presence of 10 mM BAL. After various incubation periods the samples were flushed with N_2 for 20 min, 3.5 mM Na_2S and 10 mM $Na_2S_2O_4$ were introduced and the H_2 evolved was determined over 2.5 h of illumination. (C) BAL-inhibition as a function of BAL concentration: cell aliquots were preincubated as in B, under air and in the presence of various BAL concentrations. After 70 min the systems were flushed with N_2 for 20 min, 3.5 mM Na_2S and 10 mM $Na_2S_2O_4$ were added, and the H_2 evolved was determined.

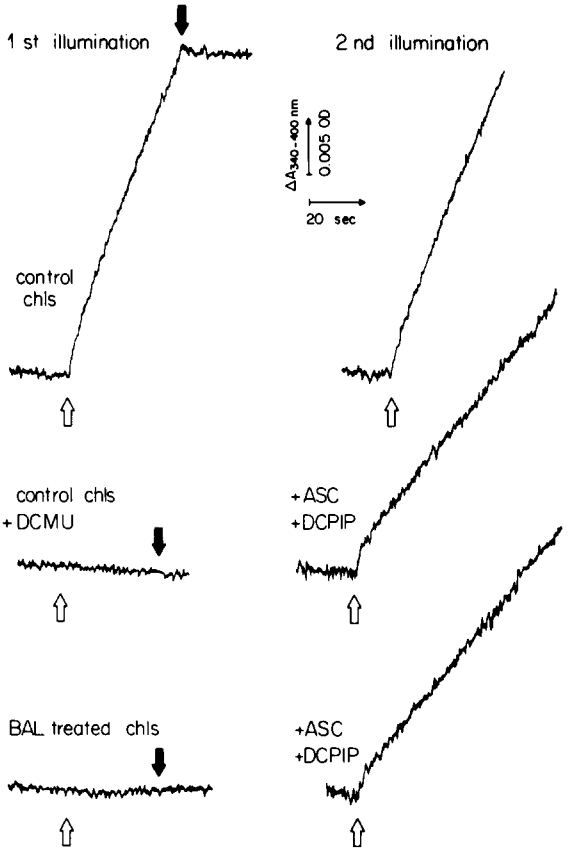

Fig. 2. Effect of aerobic BAL treatment on NADP photoreduction in spinach chloroplasts. Reaction conditions were as described under Materials and Methods. Where indicated, DCMU, DCIP and ascorbate (Asc) were added at final concentrations of 5 μM, 0.1 mM and 2 mM, respectively. Chloroplasts containing 0.12 mg Chl/ml were preincubated under air in the dark for 90 min on ice in the absence (control) or presence (BAL-treated chloroplasts) of 3.3 mM BAL, then centrifuged and washed once in the resuspension medium. Final chlorophyll concentration in the assay was 8 μg/ml. Empty and full arrows indicate turning the light on and off, respectively.

photosynthetic sulfide-dependent H_2 evolution which was assayed under anaerobic conditions (Fig. 1A). Inhibition was complete after 80 min of aerobic BAL-pretreatment (50% inhibition obtained within 20 min, Fig 1B), with a full effect at 4 mM BAL ($C_{50} = 0.3$ mM, Fig. 1C). Inactivation was dependent on the presence of O_2 during the preincubation. Pretreatment of the cells with BAL under N_2 had no effect on the reaction (Fig. 1A). Note that the onset of the photosynthetic H_2 evolution in the cells has a time lag (Fig. 1A) as has been described before in detail [7–10].

Photosynthetic electron transport from sulfide to H_2 starts at plastoquinone and culminates at the hydrogenase which is reduced by ferredoxin [9]. Hydrogenase activity from reduced methyl viologen in cell-free extracts of *O. limnetica* was not inhibited by aerobic BAL-pretreatment (Fig. 1A), suggesting that BAL inhibition of H_2 evolution in the illuminated cells was not due to an effect on this enzyme. Indeed, when electrons are supplied to plastocyanin via TMPD [19], BAL inhibition is bypassed and the rate of H_2 evolution resumes to 50% of the control. The results indicate that the BAL-sensitive factor is located between plastoquinone and plastocyanin.

To generalize BAL inhibition in photosynthetic systems, we have also studied its effect on spinach chloroplasts. Fig. 2 demonstrates the effect of BAL on NADP photoreduction. Preincubation of isolated chloroplasts with 3.3 mM BAL for 60–90

min under aerobic conditions on ice, resulted in a full inhibition of NADP photoreduction by electrons coming from water, but not from $DCIPH_2$. This indicates that the site of inhibition precedes the plastocyanin site, and that the electron carriers between plastocyanin and NADP, are not affected. It is worth mentioning that during preincubation with BAL, a low chlorophyll concentration (0.1–0.2 mg Chl/ml) was required for full inhibition. NADP photoreduction was only 50% inhibited after 90 min incubation of 3 mg Chl/ml chloroplast suspension with 15 mM BAL.

The inhibition is probably due to covalent binding of BAL in the presence of oxygen, since washing the chloroplasts or flushing the cyanobacteria with N_2, which was routinely done before the assays, did not remove the inhibition.

BAL as a photosynthetic electron donor

When added to anaerobic *O. limnetica* cell preparations, in the absence of any other electron donor, BAL supported, rather than inhibited, H_2 evolution (Fig. 3). The activity was dependent on light and on the presence of cells (Fig. 3A), and was maximal at 10 mM BAL (Fig. 3B). The rate of the BAL-supported H_2 evolution was pH-independent (Fig. 3C). In addition, no adaptation was needed and H_2 evolution set on immediately upon the introduction of BAL (Fig. 3A).

Upon the anaerobic addition of BAL to CO_2-photoassimilating *O. limnetica* cells, linear H_2 evolution started immediately, concomitant with complete arrest of the CO_2 fixation (Fig. 4). Hence,

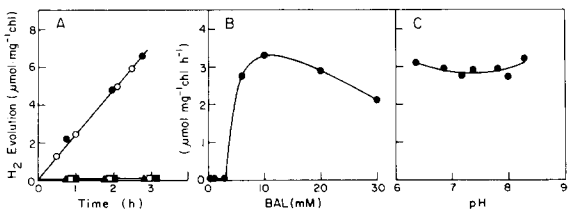

Fig. 3. BAL-supported H_2 evolution in *O. limnetica*. Experimental conditions as in Fig. 1, but with 10 mM BAL added to the N_2-flushed reaction mixtures instead of Na_2S and $Na_2S_2O_4$. (A) Time course: (●), control; (○), 10 μM DCMU; (■), dark; (□), without cells; (▲), without BAL; (B) BAL concentration dependence: conditions as in control (A) but with different BAL concentrations. (C) pH dependence: conditions as in control (A) but with 25 mM Mes (for pH lower than 7) or Taps (for pH higher than 8) substituting for Hepes.

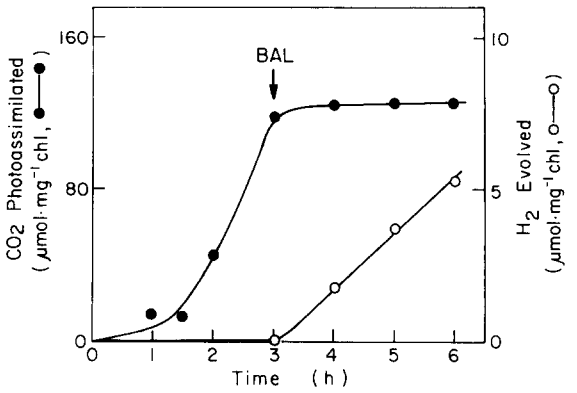

Fig. 4. BAL inhibition of sulfide-dependent CO_2 photoassimilation. Experimental conditions as in Fig. 1, but with 5 μg Chl/ml. After the N_2 flushing, 3.5 mM Na_2S and [^{14}C]$NaHCO_3$ (14 mM, specific activity 0.06 Ci/mol) were added, and the samples incubated as in Fig. 1. 10 mM BAL was added anaerobically after 3 h. The CO_2 photoassimilated (●) was determined in 1 ml suspension samples at 1 h intervals, and the H_2 evolved (○) in gas samples as in Fig. 1.

unlike sulfide-dependent H_2 evolution, which occurs only in the absence of CO_2 [9], the BAL-dependent reaction is insensitive to CO_2.

The anaerobic BAL-dependent, like the sulfide-dependent H_2 evolution, was insensitive to DCMU (Fig. 3A) while inhibited by DBMIB or by aerobic BAL pretreatment (Table I). Both BAL and DBMIB inhibitions could be bypassed by reduced TMPD (Table I).

The anaerobic electron donation by BAL is not unique to *O. limnetica*. As summarized in Table II, BAL restored NADP photoreduction in DCMU-poisoned spinach chloroplasts. The rate of electron transport from BAL to NADP ranged between 30 and 50% as compared with water to NADP. Both reactions were inhibited by an aerobic preincubation of the chloroplasts with BAL, whereas electron flow from $DCIPH_2$ to NADP was unaffected (Table II).

As opposed to *O. limnetica* in which both BAL and Na_2S serve as efficient electron donors to PS I, in chloroplasts Na_2S was found to be a very poor donor (Table II). Nevertheless, it did not inhibit the $DCIPH_2$ to NADP reaction.

Low temperature ESR spectroscopy of the Rieske Fe-S protein

Slater and De Vries have clearly shown that

TABLE I

DONATION SITE OF BAL ELECTRONS IN *O. LIMNETICA* UNDER ANAEROBIC CONDITIONS

BAL-supported H_2 evolution was measured as in Fig. 3. BAL treatment consisted of a 70 min preincubation with 10 mM BAL under air, followed by N_2-flushing for 20 min, and then the addition of Na_2S (3.5 mM) and $Na_2S_2O_4$ (10 mM).

O. limnetica cells	Additions to assay	H_2 evolution rate (μmol/mg Chl per h)	(%)
Control	–	4.4	100
	DBMIB, 25 μM	1.5	34
	DBMIB and TMPD, 0.1 mM	3.4	77
BAL-treated	–	0.6	14
	TMPD	2.3	52

aerobic BAL treatment of submitochondrial particles results in the disappearance of the ESR signals originating in the Rieske Fe-S center [2]. Although the biochemical data brought above suggests that the BAL-sensitive factor in the photosynthetic electron-transport chain is indeed the Rieske protein, we did not observe significant effects of aerobic BAL treatment on the Rieske specific ESR signals. Both in control and in BAL-treated chloroplasts (which were fully blocked in their H_2O to NADP reaction) the typical signal at $g = 1.89$ [20] had about the same extent in the reduced form, and disappeared after incubation with ferricyanide (Fig. 5A).

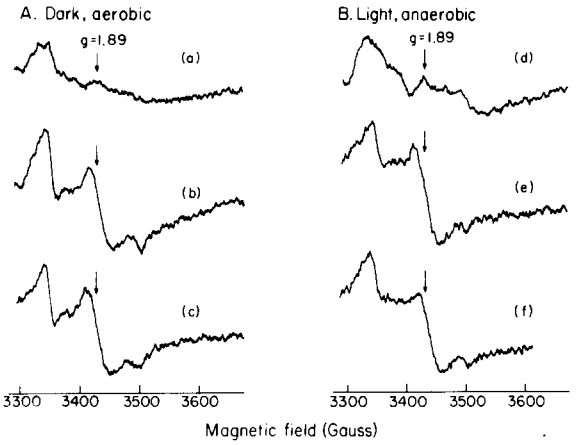

Fig. 5. BAL effect on ESR spectra of spinach chloroplasts. Chloroplasts were treated as in Fig. 2, namely preincubated without or with 3.3 mM BAL at 0.12 mg Chl/ml for 1.5 h, then centrifuged, washed once and resuspended to yield 3 mg Chl/ml. Samples of these chloroplasts were put in quartz ESR tubes (3 mm diameter) and treated as indicated below, then rapidly frozen in liquid N_2. (A) Chloroplasts frozen in the dark under aerobic conditions. (a) Control chloroplasts to which 1 mM ferricyanide was added at the washing step, before the final resuspension. (b) Control chloroplasts with 10 mM Na ascorbate added 10 min before freezing. (c) BAL-treated chloroplasts with ascorbate. (B) Chloroplasts mixtures (control or BAL-treated) were made anaerobic by flushing N_2 gas, and illuminated with white light by a 250 W halogen lamp for 3 min under N_2 atmosphere in the presence of 20 μM DCMU and 0.1 mM methyl viologen, and frozen under illumination; (d) control chloroplasts; (e) control chloroplasts. 10 mM BAL was added 30 s before the end of illumination; (f) BAL-treated chloroplasts. ESR spectra were taken at 10 K. Microwave power, 10 mW; modulation amplitude, 10 G.

TABLE II

ANAEROBIC PHOTOREDUCTION OF NADP BY BAL IN SPINACH CHLOROPLASTS

NADP photoreduction was measured under N_2 atmosphere, as described under Materials and Methods. BAL pretreatment was done as described for Fig. 2, followed by bubbling of N_2 for 30 min to remove BAL residues. Control chloroplasts were treated the same way, except for the absence of BAL. Control rates without or with reduced DCIP were 145 and 90 μmol NADP reduced/mg Chl per h, respectively.

Chloroplasts	NADP reduction rate (% of control)	
	$-DCIPH_2$	$+DCIPH_2$
(A) control	100	–
+ DCMU, 10 μM	0	100
+ DCMU + BAL, 10 mM	45	102
+ DCMU + Na_2S, 10 mM	9	96
(B) BAL-treated	0	95
+ BAL, 10 mM	10	105

On the other hand, electron donation by BAL to the Rieske protein could be seen easily by ESR spectroscopy, as demonstrated in Fig. 5B. During illumination of chloroplasts in the presence of DCMU and methyl viologen under anaerobic conditions, the Rieske center became fully oxidized (Fig. 5 trace d). The addition of BAL induced a pronounced increase of the signal amplitude at $g = 1.89$ (Fig. 5 trace e), indicating a reduction of the center. It was also observed that in chloroplasts which had been pretreated aerobically with BAL and then washed and flushed with N_2, the signal did not disappear during illumination in the

presence of DCMU and methyl viologen (Fig. 5 trace f).

Discussion

The results demonstrate that in the presence of O_2, BAL is an effective inhibitor of the photosynthetic electron-transport system. As in mitochondria [2], the aerobic inhibitory effect of BAL seems to involve the Rieske Fe-S protein. In the cyanobacterium, sulfide-dependent H_2 evolution was inhibited by BAL (Fig. 1), but inhibition was alleviated when the inhibited site was bypassed via plastocyanin by TMPD. Similarly, in chloroplasts, electron transport from water to NADP was BAL-sensitive, whereas that from $DCPIPH_2$ was not (Fig. 2).

BAL was reported to destroy the Rieske Fe-S center of mitochondria, as indicated by ESR spectra at low temperature [2]. However, we did not detect significant differences between ESR spectra of control and of BAL treated chloroplasts, at the *g* values typical to the Rieske center (Fig. 5A). This might suggest that in chloroplasts BAL binds to the protein and inhibits the transfer of electrons to or from the Fe-S center through the protein moiety. The observation that in BAL-treated chloroplasts the Rieske center does not undergo photooxidation by PS I (Fig. 5B) may indicate that BAL inhibits electron transfer from the Rieske protein to cytochrome *f*. Indeed, preliminary results show that in BAL-treated chloroplasts cytochrome *f* is fully oxidized by red as well as far red light (not shown).

It should be noted that in addition to the Rieske protein, there is another BAL sensitive site. Anoxygenic CO_2 fixation in *O. limnetica* was inhibited by BAL. This inhibition required neither prolonged incubation nor oxygen (Fig. 4). Since BAL serves as an anoxygenic electron donor to H_2 evolution (as further discussed below), the inhibition does not involve photosynthetic electron transport up to the ferredoxin. It is therefore suggested, that the inhibition of sulfide dependent CO_2 fixation in *O. limnetica* is due to an inhibition of one or more of the carbon cycle enzymes.

In spite of its inhibitory effects, BAL acts as an anaerobic electron donor to PS I both in chloroplasts and cyanobacterial cells. In DCMU poisoned spinach chloroplasts BAL restored NADP photoreduction up to 50% of the maximal electron transport rate. In *O. limnetica* it proved to be an efficient donor, as good as sulfide and water, the natural electron donors. Sulfide donates its electrons to plastoquinone in *O. limnetica* [7,8,10]. Electron transport from sulfide to H_2 is DCMU-insensitive, and inhibited by DBMIB. Since the BAL-dependent reaction is also sensitive to DBMIB and to aerobic BAL pretreatment (Tables I and II), the BAL donation site has to be located very close to that of sulfide. However, the donation sites of these two donors appear different. (i) As opposed to the sulfide reaction, which requires an adaptation [7], BAL-dependent H_2 evolution by *O. limnetica* is not preceded by any lag period (Fig. 3). (ii) In DCMU-poisoned spinach chloroplasts sulfide is a poor electron donor for NADP photoreduction, as compared to BAL (Table II). As indicated by ESR spectra, BAL reduces the Rieske iron-sulfur protein, under anaerobic conditions (Fig. 5). It is suggested that the Rieske protein and/or the plastoquinone is the immediate electron acceptor for BAL electrons. In the former case BAL differs from other donors of the Rieske protein in that it is a non-quinone compound. It might therefore be a useful tool to study the Q cycle and related reactions [4].

In many strains of cyanobacteria, sulfide has been shown to be an efficient electron donor for anoxygenic photoassimilation of CO_2 [21]. However, excluding very inefficient thiosulfate utilization by *Anacystis nidulans* [22], no other reduced sulfur-containing compound was shown to serve as a photosynthetic electron donor in these organisms [23]. Cyanobacterial evolutionary history dates as far back as $3 \cdot 10^9$ years [24], when reducing conditions were prevalent [25]. Many of the contemporary cyanobacteria grow in highly reducing environments. The ability of *O. limnetica* to utilize BAL, an organic sulfur-containing compound, may thus be of both evolutionary and ecological significance.

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