A Radioactive 1,4-Benzoquinone as Inhibitor of the DBMIB-Type in Photosynthetic Electron Transport

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An ^{125}iodine labelled 1,4-benzoquinone, an analogue of DBMIB, was studied for its interaction with the photosynthetic system of chloroplasts. It is not covalently bound to the thylakoid membrane. Instead, chloroplasts slowly decompose it under release of ^{125}iodine and formation of a quinone of only small inhibitory potency, *i. e.* photosynthetic activity is restored after a few minutes. The inhibition in photosynthetic electron transport caused by the ^{125}iodine labelled 1,4-benzoquinone can also be neutralized by bovine serum albumin to which it binds covalently.

Introduction

Among the inhibitors of photosynthetic electron transport 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) [1] is unique in that it allows each photosystem to function separately, but does not allow electron flow between the photosystems. Though the functional site of DBMIB inhibition is localized in the region between plastoquinone and plastocyanin, its precise binding site in the membrane is not known. Haehnel [2] reported that two or less molecules of DBMIB per electron transport chain are sufficient for inhibitory activity.

In order to identify the binding site of DBMIB at the thylakoid membrane, to estimate the binding constant and number of binding sites, the synthesis of a radioactive DBMIB was desirable. Since the synthesis of a [^{14}C]labelled DBMIB is not easily accomplished, an appropriate analogue was chosen.

We have recently analyzed the quantitative relationship of chemical structure and inhibitory activity on photosynthetic electron transport of various 1,4-benzoquinones [3]. In course of these investigations 2,3-diiodo-5-*tert*-butyl-1,4-benzoquinone (DIBB) turned out to be an as potent inhibitor as DBMIB with an identical pI_{50}-value of 7.52 [3]. For both DBMIB and DIBB an identical site of inhibition in the photosynthetic electron transport chain was suggested [3]. For the DBMIB analog DIBB, however, we recently reported a convenient one-step radioactive synthesis by exchange with ^{125}iodine [4]. We wish to report here on our studies with this radioactive inhibitor. They indicate an inactivation of the inhibitor by chloroplasts. The reversal of inhibition by BSA is due to a similar inactivation mechanism.

Abbreviations: BSA, bovine serum albumin; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DIBB, 2,3-diiodo-5-*tert*.butyl-1,4-benzoquinone; TMPD, N,N,N′N′-tetramethylphenylenediamine.

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Materials and Methods

2,3-^{125}diiodo-5-*tert*-butyl-1,4-benzoquinone ([^{125}I]-DIBB) has been synthesized by reaction of Na^{125}I with 2,3-dibromo-5-*tert*-butyl-1,4-benzoquinone as described recently [4]. For purification from autoradiolysis products [^{125}I]DIBB was chromatographed freshly every day on silica gel pre-coated plastic sheets with benzene as the solvent. The zone corresponding to [^{125}I]DIBB was cut out, eluted with methanol and the concentration determined by estimation of the optical density at 282 nm ($\varepsilon = 10\,250\ \mathrm{mM^{-1}\,cm^{-1}}$).

Radioactivity was counted in a Packard Tri Carb Liquid Scintillation Spectrometer, Model 3385, in γ-vials (Zinsser, Frankfurt/Main) or in a W+W Electronic Automatic γ-Counting System, Model MR 252. Thin layer chromatograms were assayed for radioactivity in a Dünnschichtscanner II (Berthold, Wildbad).

Chloroplasts from spinach were prepared according to Nelson *et al.* [5].

For incubation experiments with [^{125}I]DIBB, chloroplasts at a concentration of 50 – 200 μg chlorophyll/ml in 2 ml 20 mM tricine, pH 8.0, and 40 mM sucrose at various concentrations of [^{125}I]DIBB

were allowed to stand at room temperature for 10 to 15 min. The chloroplasts were removed by centrifugation at 12 000 × *g* for 2 min and the supernatant assayed for radioactivity and used for further experiments. A sample without chloroplasts added served as the control.

For extraction experiments, both supernatant and control were shaken with 1 ml of ether for 1 min on a whirlmix and the phase separated by centrifugation. To the ether phases were added small amounts of inactive DIBB and aliquots chromatographed.

In the AgI precipitation studies, supernatant and control were acidified by addition of 0.2 ml conc. HNO_3. 4 μmol NaI were added and inorganic iodide precipitated by addition of 20 μmol of $AgNO_3$. AgI was removed by centrifugation and the supernatants tested for radioactivity.

In the reaction with BSA, 40 mg of BSA (purity 99.4%; Serva) in 1 ml 20 mM tricine buffer, pH 8.0, were allowed to react with [^{125}I]DIBB at a concentration of 10^{-5} M for 10 min. The reaction mixture was chromatographed on a Sephadex G-50 column (2 × 20 cm) under elution with 20 mM tricine buffer, pH 8.0. Fractions of about 3 ml were collected. Each fraction was assayed for radioactivity and the optical density at 280 nm was determined as estimate of the protein content.

Photosynthetic $NADP^+$ reduction was measured at 340 nm in a Zeiss PMQ III spectrophotometer modified for illumination with red light (645 nm) at an intensity of 2.5×10^5 erg cm^{-2} sec^{-1}. The reaction mixture contained in a volume of 2 ml in μmol: tricine-NaOH, pH 8.0, 40; $MgCl_2$ 10; NH_4Cl 10; $NADP^+$ 3; ferredoxin from spinach, 0.01; and chloroplasts with 14 μg chlorophyll.

Results

DIBB like DBMIB inhibits photosynthetic electron transport between the two photosystems [3]. In order to develop its inhibitory activity, DIBB has to be bound to the thylakoid membrane. Its binding properties, therefore, are of special interest.

1. Inhibitor binding studies of DIBB by broken chloroplasts

Convenient for binding studies a radioactively labelled DIBB has been synthesized [4]. A typical experiment for [^{125}I]DIBB binding to the thylakoid membrane at a concentration of chloroplasts with

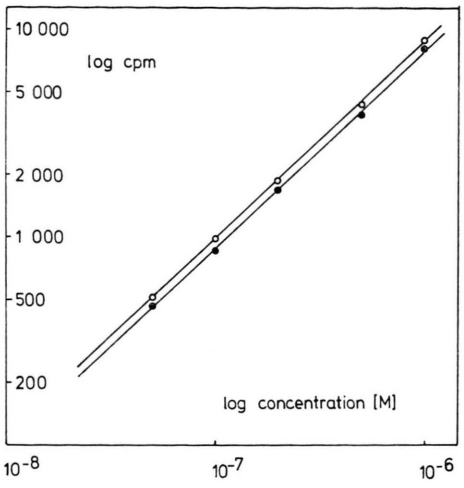


Fig. 1. Absorption of [^{125}I]DIBB by chloroplasts (●—●). The concentration of chloroplasts corresponded to 50 μg chlorophyll/ml. Control without chloroplasts (○—○).

50 μg chlorophyll/ml is shown in Fig. 1. A double-logarithmic plot of concentration of DIBB versus cpm in the supernatant after removal of chloroplasts by centrifugation shows a straight line parallel to the control without chloroplasts added. Independent of the concentration of DIBB, the percentage of DIBB bound to the chloroplasts amounts to only about 10%. It can be increased to about 20% if the concentration of chloroplasts is increased to 200 μg chlorophyll/ml. This constant ratio between bound and free inhibitor seems to be exceptional and does not indicate any specific binding constant or binding site. The reason for this is the chemical instability of DIBB in the presence of chloroplasts.

The binding of the inhibitor to the membrane is reversibel. Bound inhibitor can be removed by washing, as is demonstrated in Fig. 2. As can be seen, photosynthetic $NADP^+$ reduction by chloroplasts after addition of 1.75×10^{-7} M DIBB is inhibited 97% as compared to the control (Fig. 2; left side a)).

The chloroplasts are then centrifuged down, washed once in 20 mM tricine buffer, pH 8.0, centrifuged, and resuspended again in the complete reaction mixture for $NADP^+$ reduction. By this procedure inhibitor not covalently bound should be removed. The rate of photosynthetic $NADP^+$ reduction is restored to a rate which is only 32% inhibited as compared to the control (Fig. 2; left side b)). This remaining inhibition is probably not due to inhibitor still bound, but due to a loss of activity of

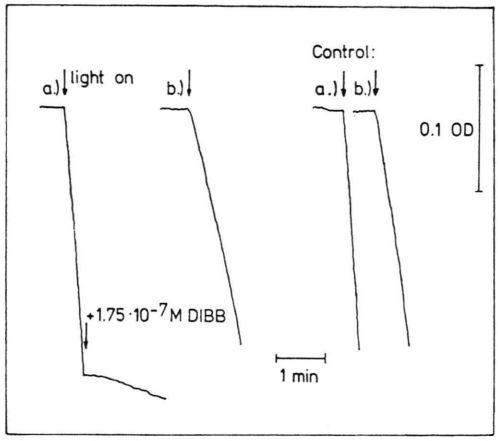


Fig. 2. Reversal of DIBB inhibition by chloroplast washing. a) Basal rate of photosynthetic $NADP^+$ reduction and after addition of DIBB. b) Rate after chloroplasts have been washed once with buffer. Control: same procedure as before but no DIBB added. Photosynthetic $NADP^+$ reduction was measured at 340 nm; a downward slope means increase in optical density. Further conditions as described in Materials and Methods.

chloroplasts during the washing and centrifugation procedure. A control treated in the same way but without DIBB added, shows the same diminished rate in photosynthetic $NADP^+$ reduction due to inactivation (Fig. 2; right side).

This would mean that in the supernatant a concentration of inhibitor is still present, which should lead to high inhibition in photosynthetic $NADP^+$ reduction, if fresh chloroplasts are added. This experiment is described in Fig. 3. Addition of 10^{-7} M DIBB to the assay system leads to 91% inhibition (Fig. 3 a)). The chloroplasts are then removed by centrifugation and fresh ones at the same concentration of chlorophyll are added. The inhibited rate of photosynthetic $NADP^+$ reduction amounts to about 11% (Fig. 3 b)). The binding of only 10% of the inhibitor to the membrane seems not to be sufficient to explain an inhibition of more than 90%. This would also be in contrast to the experiments by Haehnel [2], who has found two or less molecules of inhibitor per electron transport chain. Furthermore, the concentration of inhibitor present in the supernatant should lead to a higher inhibition then the one observed. This indicates that a (chemical) reaction must take place, which will now be analyzed in more detail.

The supernatant of chloroplasts treated with [^{125}I]DIBB and the control are both extracted with ether. To the ether phases inactive DIBB is added and their composition examined in thin layer chromatography. Whereas in the spot corresponding to DIBB a high amount of radioactivity is found in the control, there is almost none in the spot from the sample treated with chloroplasts (Fig. 4). The fact that the supernatant of [^{125}I]DIBB treated chloroplasts contains 90% of the original radioactivity which now is no longer extractable with an organic solvent will lead to the conclusion that the labelled iodine is no longer at the quinone moiety.

The results in Table I indicate that the originally organic bound iodine has been converted into inorganic iodide anion. Inactive NaI is added to chlo-

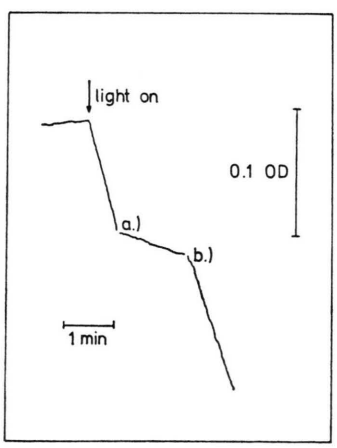


Fig. 3. Inactivation of DIBB in the presence of chloroplasts. a) 10^{-7} M DIBB added, b) chloroplasts centrifuged down and fresh chloroplasts added to supernatant. Further conditions like in Fig. 2.

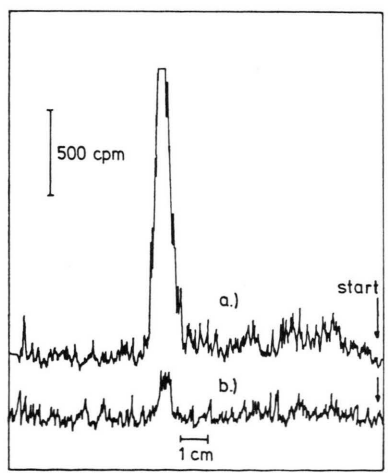


Fig. 4. Thin layer chromatographic comparison of ether extracts of a supernatant from [^{125}I]DIBB treated chloroplasts b) and a control without chloroplasts a). Silica gel; solvent benzene.

Table I. Precipitation of inorganic iodide by $AgNO_3$ in a supernatant from [^{125}I]DIBB treated chloroplasts as compared to a control without chloroplasts added. For conditions see Materials and Methods.

	Conc. (M) [^{125}I]DIBB	cpm
	2×10^{-7}	365
control		1269
	5×10^{-7}	891
control		3350
	10^{-6}	1786
control		6384

roplast sample and control, followed by silver nitrate. After removal of the silver iodide formed, the radioactivity in the chloroplast samples is found to be about 3.5 fold less than compared to the control. The final proof for the formation of inorganic iodide anion from DIBB was made by comparison of chloroplast supernatant after treatment with [^{125}I]DIBB and authentical Na^{125}I in thin layer chromatography (Fig. 5).

2. Inhibitor binding studies of DIBB by BSA

It has been shown recently [6 – 8] that the inhibition of photosynthetic electron transport by DBMIB can be overcome by BSA, *i. e.* the inhibited rate of photosynthetic electron transport after addition of DBMIB can be restored to its original rate by addition of BSA. The same is true for DIBB [6]. In order to understand the mechanism of inactivation of DBMIB and DIBB as well, BSA was treated with

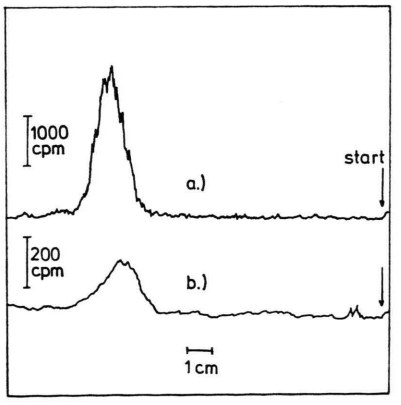


Fig. 5. Thin layer chromatographic comparison of a supernatant from [^{125}I]DIBB treated chloroplasts b) and a sample of Na^{125}I a). Silica gel; solvent: acetone, 65; *n*-butanol, 20; conc. NH_3, 10; H_2O, 5; by volume.

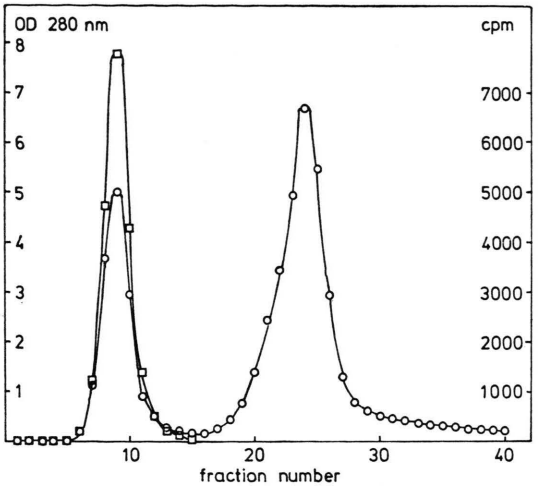


Fig. 6. Elution profile of [^{125}I]DIBB treated BSA from a Sephadex G-50 column. □—□ optical density at 280 nm, ○—○ cpm.

[^{125}I]DIBB. The reaction mixture was chromatographed on a Sephadex G-50 column (Fig. 6). As judged from the optical density at 280 nm as measure for the protein content, BSA was eluted in fractions 7 to 11. In addition, these fractions were highly radioactively labelled (Fig. 6). Another peak with high radioactivity was eluted in fractions 19 to 28. The radioactivity in these fractions was due to inorganic ^{125}iodide, as was proved by the same methods as described in the binding studies of [^{125}I]DIBB with chloroplasts. Most of the radioactivity in the protein peak was tightly bound. This is jugded from the following observations. After treatment of the protein containing fraction with 6 M urea and rechromatography on Sephadex G-50, most of the radioactivity still remained in the protein peak with some minor amount of radioactivity found in the low molecular weight fractions.

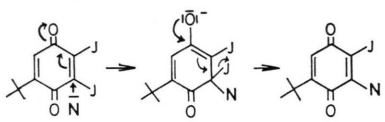
Discussion

Inhibitors of photosynthetic electron transport are useful tools in studies of partial reactions of the photosynthetic electron transport chain. In addition, many of them are important herbicides. In case of the inhibitors of the DCMU and DBMIB type, which act before or after the plastoquinone pool, respectively, the binding site of DCMU type inhibitors has recently been extensively examined. By use of radioactively labelled inhibitors binding constants and

number of specific binding sites could be determined [9]. Furthermore, it could be demonstrated by a replacement technique that compounds of quite different chemical structure share an identical binding site [6, 9]. In contrast binding of DBMIB has been studied to a far lesser extent except for some recent work by Haehnel [2, 10]. For this reason we have synthesized a radioactively labelled inhibitor of the DBMIB type, [^{125}I]DIBB [4]. The results of the paper show that the quinone inhibition may be quickly inactivated by chloroplasts. Due to the high chemical reactivity of the quinone toward nucleophiles, [^{125}I]DIBB in contact with chloroplasts is decomposed under release of inorganic ^{125}iodide. For this reason the determination of binding constants and number of binding sites by use of [^{125}I]-DIBB has been impossible. However, a detailed knowledge about the mechanisms of inactivation and reversal of inhibition of inhibitors of the DBMIB type was possible.

DIBB and halogen substituted 1,4-benzoquinones in general can be considered as vinylogous acid halides. In a Michael type addition a nucleophile N can add to the quinone under release of iodide (Scheme I). The iodine in the 2-position of the

Scheme 1:



quinone can be replaced by a second molecule of nucleophile in the same way. As nucleophiles can serve molecules with sulfhydryl, hydroxyl or amino groups. We have recently reported that the inhibitory activity of a quinone in photosynthetic electron transport is mainly determined by its lipophilicity [3]. Especially halogen substituted quinones stand out by their high lipophilicity. Substitution of the halogen in the quinone moiety by one of the nucleophiles mentioned above leads to a decrease in lipophilicity of the quinone and in consequence to less inhibitory activity, *i. e.* the inhibitor is inactivated. For example, exchange of two bromines in tetrabromo-1,4-benzoquinone against amine or alcohol lowers pI_{50} from 6.34 to around 5. Similarly, replacement of one idoine in DIBB by a hydroxyl group causes a drop in pI_{50} from 7.52 to 5.02 [3]. Thus, an originally highly inhibited rate of photosynthetic electron transport can be restored almost to its control rate after the inactivation has taken place.

As shown in results, DIBB is not covalently bound to the thylakoid membrane, because it can be removed by washing. The inactivation process will completely remove any inhibitor located at the binding site. The chemical reaction with the nucleophile will probably not take place at the binding site but in the aqueous phase outside the membrane. By this reaction the equilibrium between inhibitor molecules at the binding site and in the aqueous phase gets unbalanced and inhibitor from the binding site will dissociate into the aqueous phase to restore equilibrium and subsequently will undergo inactivation. The latter is true for the inactivation by BSA, as can be concluded from the results. DIBB is not mereley absorbed to BSA, but it is covalently bond. It is known that BSA besides the nucleophilic thiol group has two more nucleophilic groups [11]. Consequently, up to three molecules of [^{125}I]DIBB can bind to one molecule of BSA and split off inorganic iodide. The bound quinone has still one iodide attached, labelling the BSA molecule radioactive.

A surprising result, however, is that DIBB even without any inactivating compounds added gets slowly decomposed in the presence of chloroplasts. For this decomposition a binding of DIBB to nucleophilic proteins in the thylakoid membrane, a chloroplast catalysed hydrolysis, and a combination of both are feasible. This inactivation caused by chloroplasts is different from that of BSA.

We have reported recently that DIBB can be hydrolyzed to 2-iodo-3-hydroxy-5-*tert*-butyl-1,4-benzoquinone by mild alkaline treatment [3]. As already stressed, this compound is much less active than DIBB. Unknown compounds within the thylakoid membrane or released from it into the medium may catalyze a similar hydrolysis.

On the other hand, if a binding of DIBB to the thylakoid membrane occurs, different from BSA also the remaining iodine in the quinone molecule must be split off by hydrolysis. This is concluded from the fact that the radioactivity left in the chloroplasts after incubation with [^{125}I]DIBB is much lower as compared to the radioactivity connected with BSA after the same treatment.

DBMIB and DIBB are quite similar in their chemical nature. A reversal of DBMIB inhibition

by thiols and BSA has been well documented [6 – 8, 12]. It can be assumed, that a chloroplast-catalyzed inactivation as described for DIBB will also occur with the widely used DBMIB.

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