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Interaction of 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide with Photosystem II in Chloroplasts and Subchloroplast Particles

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Abstract

The effects of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide on electron transport in thylakoids and oxygen-evolving photosystem II particles has been examined. Kinetic fluorescence studies reveal that the site of inhibition for alkyl derivatives of hydroxyquinoline-*N*-oxide ($I_{50} \simeq 2$ μM) is located between Q and plastoquinone. Studies with thylakoids isolated from atrazine-resistant pigweed plants indicate that the modification in the Q/B membrane complex that confers increased resistance to inhibition by atrazine also results in decreased sensitivity to inhibition by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (resistant/sensitive ratio = 11). From the results of tetramethylphenylenediamine by-pass experiments, determinations of inhibitor sensitivity in trypsin-treated thylakoids and competitive displacement experiments made with [^{14}C]metribuzin in thylakoids and photosystem II particles, it is suggested that 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide binds in a region of the Q/B complex that is distinct from the 3-(3,4-dichloro)-1,1-dimethyl urea and atrazine binding sites.

Key Words: Electron transport; thylakoids; photosystem II particles; alkylhydroxyquinoline-*N*-oxide; herbicide binding; Q/B complex.

Introduction

The mitochondrial electron transport inhibitor 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO)[3] was first introduced for use with chloroplasts by

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[3]Abbreviations used: B, secondary acceptor of photosystem II—a protein-bound quinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichloro)-1,1-dimethylurea; DMBQ, 2,5-dimethyl-*p*-benzoquinone; FeCy, potassium ferricyanide; FoCy, potassium ferrocyanide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; MDBQ, 2,3-dimethyl-5,6-methylenedioxy-*p*-benzoquinone; MV, methylviologen; Q, first stable acceptor of photosystem II; TMPD, N,N,N′,N′-tetramethylphenylenediamine.

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Avron in 1961. Since then various reports have appeared concerning its site of action in the photosynthetic electron transfer chain (Avron and Shavit, 1965; Izawa *et al.*, 1967; Hind and Olson, 1967; Gromet-Elhanan, 1969; Satoh and Takamiya, 1971; Rienits *et al.*, 1974; Schreiber and Avron, 1979; Droppa *et al.*, 1981a,b). A number of workers have concluded that HQNO is DCMU-like in its action, but that its inhibition site is somewhat different (Izawa *et al.*, 1967; Hind and Olson, 1967; Rienits *et al.*, 1974; Schreiber and Avron, 1979; Droppa *et al.*, 1981a,b). The aim of the present study was to compare and contrast the inhibitory action of HQNO with other inhibitors, e.g., DCMU and metribuzin, that act on the reducing side of photosystem II (PS II) in both thylakoids and subchloroplast particles.

Materials and Methods

Isolation of Intact Chloroplasts, Thylakoids, and Subchloroplast Particles

Intact chloroplasts were isolated from 10–14-day-old pea seedlings (*Pisum sativum* var. Progress #9) using the Percoll gradient method of Mills and Joy (1980). Thylakoids were isolated from the same age plants and stored at −70°C as described by Cohen (1978). Thylakoids were isolated from greenhouse-grown pigweed plants (*Amaranthus retroflexus*) using a modification of the procedures described by Arntzen *et al.* (1979). Subchloroplast particles, enriched in photosystem II, were isolated from field-grown spinach as described (Berthold *et al.*, 1981). Chlorophyll concentration was estimated spectrophotometrically using acetone extracts (Arnon, 1949).

Electron Transport and Fluorescence Assays

Electron transport was monitored by following oxygen concentration changes polarographically (Cohen, 1978). Changes in fluorescence yield that occur after a series of laser flashes were measured as described by Bowes and Crofts (1981). Thylakoids and PS II particles were incubated for 2 min in the dark with inhibitors prior to all measurements of photo-induced activity unless otherwise indicated. The concentration of HQNO in assay mixtures was determined using an extinction coefficient of 9.45 mM^{-1} cm^{-1} (Cornforth and James, 1956).

Herbicide Binding Assays

Thylakoids equivalent to 50 μg Chl were incubated for 3 min at room temperature in dim light in 1 ml of medium containing 100 mM sorbitol, 10 mM Hepes buffer (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, and 0.8–1.0 $\times$ 10^4 cpm of [^{14}C]metribuzin (specific activity = 26.8 mCi/mmol). After the 3-min

incubation period, the thylakoids were removed by centrifugation at top speed in a Beckman microfuge B, and radioactivity was determined with 0.5 ml aliquots of the supernatant using liquid scintillation spectrometry. Binding assays with subchloroplast particles were performed in a similar manner. Particles equivalent to 60 μg Chl/ml were incubated in 0.2 ml of a medium containing 50 mM sorbitol, 25 mM MOPS buffer (pH 7.0), 10 mM KCl, 5 mM $MgCl_2$, and [^{14}C]metribuzin. After 3 min of incubation, the particles were removed by centrifugation at 178,000 $\times$ g in a Beckman airfuge and radioactivity determined in 0.1 ml aliquots of the supernatant.

Results

Analysis of Electron Transport Inhibition

It has been reported that there are acceptor-dependent differences in the inhibition pattern when electron transport is assayed in thylakoids treated with HQNO (Avron, 1961; Gromet-Elhanan, 1969; Droppa *et al.*, 1981a]. Figure 1 shows the concentration-dependent inhibition of both methylviologen and 2,5-dimethyl-*p*-benzoquinone (DMBQ) reduction in HQNO-treated pea thylakoids. The inhibition patterns are the same both in terms of the I_{50} values (1.75 μM) and the percent inhibition at the highest concentrations employed (25 μM). From Hill plot analysis of the electron transport inhibition, a Hill coefficient of 1.12 was determined for the interaction of low concentrations of HQNO with pea thylakoid membranes. We have also examined the effect of high concentrations of HQNO on the photosystem I-dependent oxidation of duroquinol in thylakoids and on the oxidation of plastoquinol by isolated cytochrome b_6/f complexes. Electron transport was inhibited to the 50% level at 50 μM HQNO in both preparations (Barton and Cohen, 1982). These findings are consistent with the idea that there is a high-affinity site for HQNO in photosystem II and a low-affinity site located at the cytochrome b_6/f complex.

In Table I is shown a summary of the I_{50} values for inhibition of photosystem II electron transport by HQNO with a variety of acceptors in different types of preparations. With the exception of the ferricyanide Hill reaction in thylakoids and the water to 2,3-dimethyl-5.6-methylenedioxy-*p*-benzoquinone (MDBQ) reaction in PS II particles, the I_{50} values all fall into the range of 1–5 μM. With ferricyanide as the acceptor, we routinely observed that when pea thylakoids were incubated for 2 min in the dark together with 1.5 mM ferricyanide and HQNO, electron transport was more severely inhibited at the 0.5 μM level than with other acceptors (Table II). When ferricyanide was added to the thylakoids after a 2-min preincubation with HQNO, the level of inhibition was comparable to that observed with other

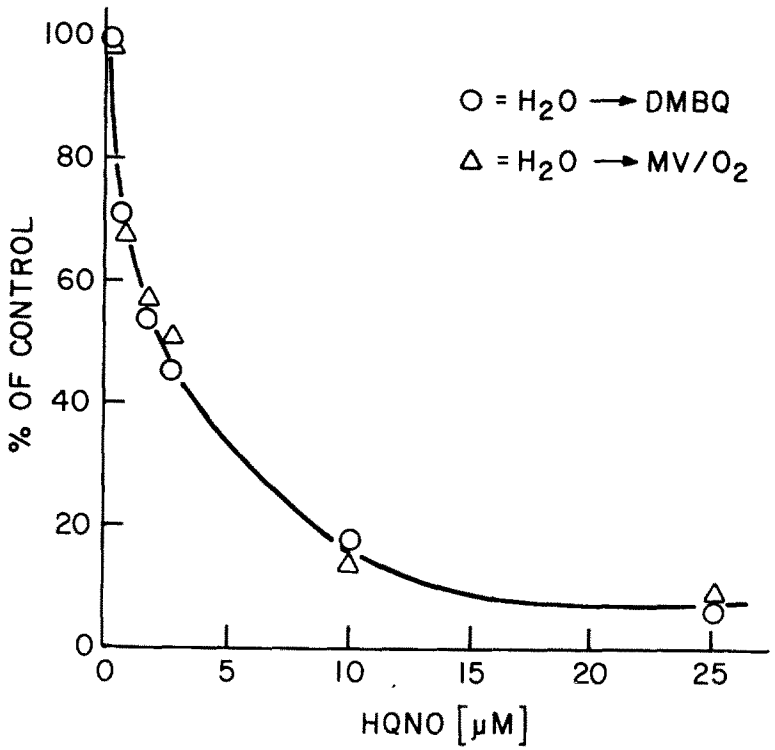

Fig. 1. Effect of HQNO on methylviologen and 2,5-dimethylbenzoquinone reduction. The reaction mixture (in 1.2 ml) contained 100 mM sorbitol, 25 mM Tricine buffer (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 25 mM methylamine, either 0.5 mM DMBQ or 0.1 mM methylviologen plus 0.5 mM NaN_3, and pea thylakoids equivalent to 20 μg/ml Chl. The control rates (μmol O_2/mg Chl · h) were: $H_2O \rightarrow MV/O_2$, 369; $H_2O \rightarrow$ DMBQ, 398.

Table I. I_{50} Values for the HQNO Inhibition with Different Acceptors

Electron transport pathway	I_{50} (μM)[a] thylakoids	PS II particles
$H_2O \rightarrow MV/O_2$	1.8	
$H_2O \rightarrow$ FeCy	0.4	
$H_2O \rightarrow$ DMBQ	1.8	
$H_2O \rightarrow$ DCBQ	2.5	1.8
$H_2O \rightarrow$ MDBQ	4.5	30.0
$H_2O \rightarrow$ phenylBQ	2.2	
$H_2O \rightarrow HCO_3^-$	1.4[b]	

[a] I_{50} values were obtained by taking the mean of the half inhibitions from 2–4 separate experiments.
[b] Determined in intact pea chloroplasts.

Table II. Effect of Ferricyanide Pretreatment in the Dark on the HQNO Inhibition[a]

Pretreatment	Percent inhibition
1. Incubated 2 min with 1.25 mM FeCy plus 0.5 μM HQNO	77
2. Incubated 2 min with HQNO, then add FeCy	20
3. Incubated 2 min with FoCy/FeCy (1:1) plus HQNO	59
4. Incubated 2 min with 1.25 mM DMBQ plus HQNO	28
5. Incubated 2 min with 0.1 mM MV plus HQNO	21

[a]The reaction mixture (in 1.2 ml) contained 100 mM sorbitol, 25 mM Tricine-NaOH (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 5 μg/ml gramicidin D, 2.5 mM NH_4Cl, and pea thylakoids equivalent to 15 μg/ml Chl. The control rates (μmol O_2/mg Chl · h) were: $H_2O \rightarrow$ FeCy, 203; $H_2O \rightarrow$ FoCy/FeCy, 203; $H_2O \rightarrow$ DMBQ, 345; $H_2O \rightarrow$ MV/O_2, 280.

acceptors. Shifting the redox level in the assay mixture to a more reducing one during the dark pretreatment period by adding ferrocyanide together with ferricyanide also lessened the severity of inhibition (Satoh and Takamiya, 1971). In PS II particles which have a very limited capacity for ferricyanide reduction, inclusion of ferricyanide together with HQNO increased the inhibition of $H_2O \rightarrow$ DCBQ reduction by 0.6 μM HQNO from 24% with no ferricyanide present to 60% with 0.5 mM ferricyanide present (data not shown).

MDBQ reduction in photosystem II particles, which lack the cyt b_6/f complex (Berthold *et al.*, 1981) is considerably less sensitive to inhibition by HQNO than is MDBQ reduction in thylakoids (Fig. 2). The portion of MDBQ reduction in particles resistant to 25 μM HQNO was sensitive to inhibition by DCMU, atrazine, dinoseb, and ioxynil. Kinetic analysis (Guikema and Yocum, 1979) did not reveal any alteration in the affinity for MDBQ in HQNO-treated particles compared with control particles.

Electron transport from water to DCBQ in thylakoids isolated from atrazine-sensitive plants is severely inhibited by 2 μM DCMU, 10 μM atrazine, and 25 μM HQNO (Table III). In contrast, DCBQ reduction in thylakoids isolated from atrazine-resistant pigweed is totally insensitive to 10 μM atrazine, only partially sensitive to HQNO, but still totally sensitive to 2 μM DCMU. The resistant/sensitive ratio (I_{50} values for inhibition of electron transport) for HQNO in pigweed thylakoids is approximately 11 (Pfister and Arntzen, 1979).

The Site of Inhibition for HQNO Compared to DCMU and Atrazine

A number of reports have indicated that HQNO is DCMU-like in its action, but somehow different. To determine whether the two inhibitors act at

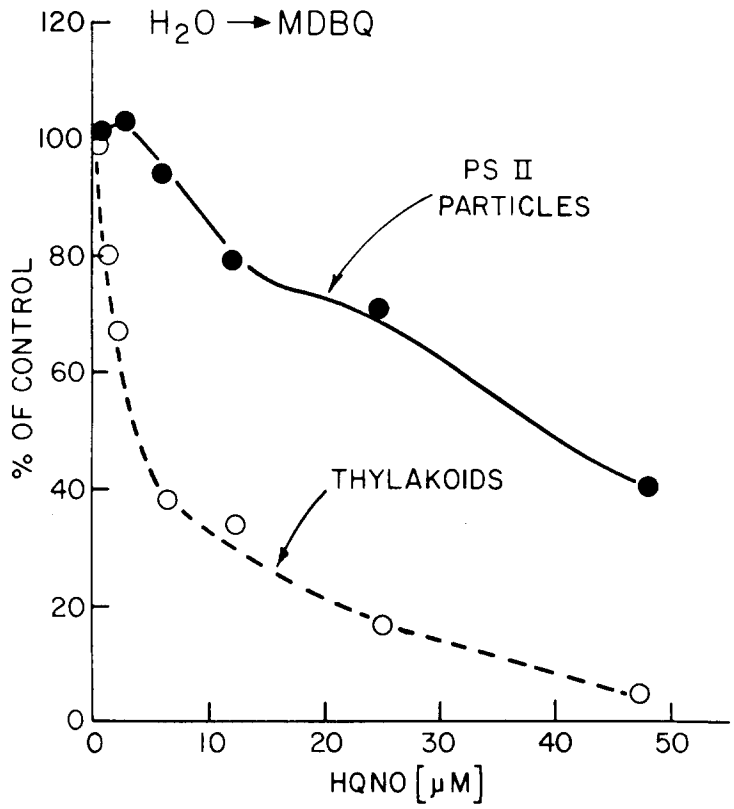

Fig. 2. Effect of HQNO on MDBQ reduction in thylakoids and photosystem II particles. The reaction mixture (in 1.2 ml) contained 50 mM sorbitol, 25 mM Tricine buffer (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$ 0.25 mM $MnCl_2$, 0.25 mM MDBQ, 5 μg/ml gramicidin D for thylakoid assays, and chloroplasts or PS II particles equivalent to 12 and 13 μg/ml Chl, respectively. The control rates (μmol O_2 consumed/mg Chl · h) were: thylakoids, 291; PS II particles, 183.

Table III. Effect of Various Inhibitors on Electron Transport in Pigweed Thylakoids[a]

Inhibitor added	Atrazine sensitive	Atrazine resistant
	(Percent of control)	
2 μM DCMU	1	11
10 μM Atrazine	0	106
25 μM HQNO	11 (5.3)[b]	55 (58.0)

[a]The reaction mixture (in 1.2 ml) contained 50 mM sorbitol, 25 mM MOPS-NaOH (pH 7.0), 10 mM KCl, 5 mM $MgCl_2$, 5 μg/ml gramicidin D, 0.25 mM DCBQ, and thylakoids equivalent to 15 μg/ml Chl. The control rates (μmol O_2/mg Chl · h) were: atrazine sensitive, 152; atrazine resistant, 81.

[b]The numbers in parentheses represent the I_{50} (μM) values for inhibition of DCBQ reduction.

the same locus, we have analyzed their relative activities in a number of different assays which are indicative of inhibition in the Q-B segment of the electron transfer chain (Trebst, 1980). Using fluorescence yield changes in flashing light to monitor the oxidation of Q^- by the plastoquinone pool, we observed that HQNO inhibits the re-oxidation of Q^- in the same concentration range required to inhibit steady-state electron flow (Fig. 3).

TMPD, in the absence of ascorbate, has been used to distinguish between DCMU- and DBMIB-like inhibitors (Trebst *et al.*, 1979; Sane *et al.*, 1979; Draber, *et al.*, 1981). Table IV shows that electron transfer from water to methylviologen is restored upon addition of 0.1 mM TMPD to HQNO-poisoned thylakoids, but is not restored in thylakoids treated with DCMU or atrazine. The TMPD-restored electron transport in HQNO-treated thylakoids was sensitive to inhibition by DCMU and Hg^{2+} treatment (Kimimura and Katoh, 1972). The DCMU and atrazine inhibitions of the ferricyanide Hill reaction were reversed to some extent after trypsin treatment (Renger, 1976; Boger and Kunert, 1979; Steinback *et al.*, 1981) of the thylakoids, whereas the HQNO inhibition was not affected (data not shown).

An approach which has been used to determine whether two inhibitors share a common binding site on the thylakoid membrane has been to examine

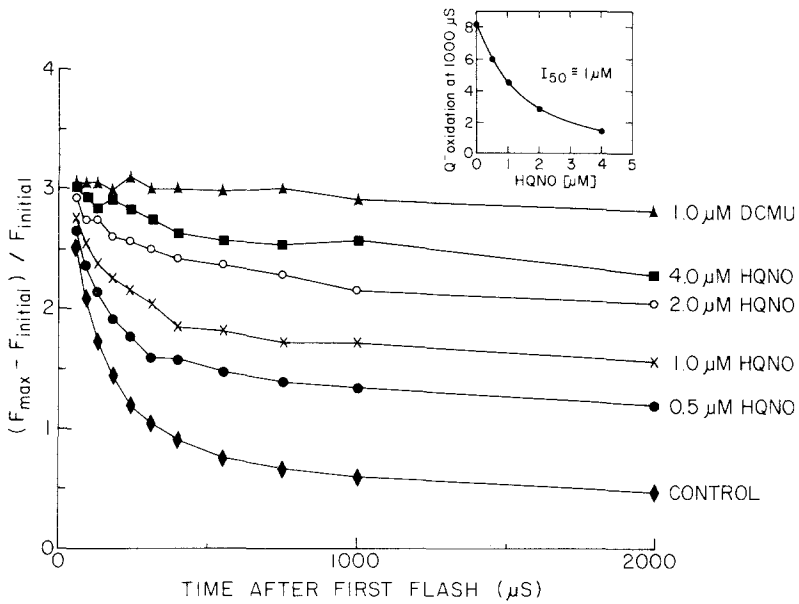

Fig. 3. Effect of DCMU and HQNO on Q^- re-oxidation. The reaction mixture (in 0.6 ml) contained 100 mM sucrose, 20 mM Hepes buffer (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ 0.05 mM methylviologen, and spinach thylakoids equivalent to 5 μg/ml Chl.

Table IV. Effect of TMPD on Inhibition of Photosynthetic Electron Transport[a]

Inhibitor added	$H_2O \rightarrow MV/O_2$	$H_2O \rightarrow MV/O_2$ + 0.1 mM TMPD
	(μmol O_2/mg Chl · h)	
None	225	291
4 μM DCMU	18	20
4 μM Atrazine	20	14
15 μM HQNO	36	225

[a]The reaction mixture employed was similar to the one described in the legend to Table II. The chlorophyll concentration was 20 μg/ml.

the ability of an unlabeled competitor to displace a radiolabeled inhibitor from the membrane (Tischer and Strotmann, 1977; Pfister *et al.*, 1979; Trebst, 1979; Oettmeier *et al.*, 1981; Oettmeier *et al.*, 1982). Figure 4 illustrates data from a series of experiments in which the binding of [^{14}C]metribuzin was measured in the presence of increasing concentrations of either DCMU or HQNO. As noted by other workers, DCMU displaces an appreciable amount of the bound metribuzin, whereas the amount of metribuzin bound is only slightly affected at concentrations of HQNO that are 25–50-fold higher than the I_{50} value for inhibition of electron transport. In Table V is presented a comparison of binding data for [^{14}C]metribuzin in thylakoids and PS II particles measured in the absence and presence of HQNO.

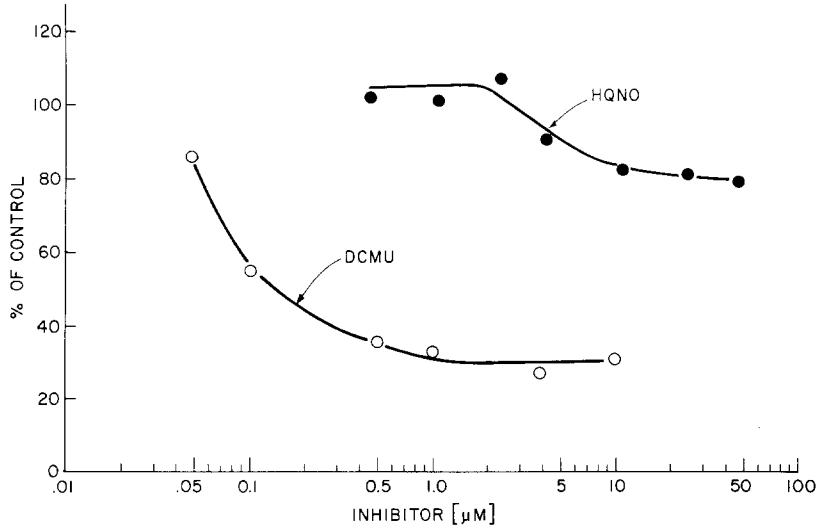

Fig. 4. Effect of DCMU and HQNO on [^{14}C]metribuzin binding to pea thylakoid membranes. Binding assays were performed as described in the Materials and Methods section. The DCMU and HQNO experiments were performed on different days. The control levels of binding (nmol bound/mg Chl) were: DCMU, 2.07; HQNO, 2.54.

Table V. Effect on HQNO on [^{14}C]Metribuzin Binding in Thylakoids and Subchloroplast Particles

Additions	nmol [^{14}C]Metribuzin bound per mg Chl	
	Thylakoids	PS II particles
None	2.54	1.29
25 μM HQNO	2.07 (81)[a]	1.15 (89)
50 μM HQNO	1.97 (78)	0.99 (77)

[a]The numbers in parentheses represent the percentage of labeled metribuzin bound compared to controls.

Binding of HQNO to Thylakoid Membranes

Van Ark and Berden (1977) have shown that when submitochondrial particles specifically bind HQNO, there is considerable quenching of the inhibitor's fluorescence. The quenching of HQNO flourescence has been used to determine the number of HQNO binding sites and to study interactions between the HQNO- and antimycin A-binding sites in complex III in both mitochondria and submitochondrial particles (Van Ark and Berden, 1977; Burger, 1980). Following the procedures outlined by Van Ark and Berden, we attempted to study the interaction of HQNO with pea thylakoid membranes by examining the fluorescence of the inhibitor after incubation with thylakoids either in the light or in the dark. In all instances we failed to observe any fluorescence quenching in the concentration range 0.25–10 μM HQNO.

Discussion

Previous studies have provided evidence for the interaction of inhibitors which act on the reducing side of photosystem II with a membrane complex consisting of at least two polypeptides (Oettmeier *et al.*, 1982; Pfister and Arntzen, 1979; Mullett and Arntzen, 1981). The major binding site for herbicides of the DCMU/atrazine type appears to be a 32–34-kD polypeptide, whereas phenolic inhibitors, e.g., dinoseb, bind primarily to a 41-kD polypeptide. Based on the studies presented in this report, we conclude that HQNO ($I_{50} \cong 2$ μM) also interacts with the reducing side of photosystem II, but at a site that is different from the DCMU–atrazine binding site. Our conclusions are based on the following pieces of evidence: (1) both DCMU and HQNO block Q^- re-oxidation, (2) the DCMU and atrazine inhibitions can be overcome partially by trypsin treatment of thylakoid membranes, whereas the HQNO inhibition cannot, (3) TMPD (in the absence of ascorbate) partially relieves the HQNO inhibition, but has no effect on the DCMU and atrazine inhibitions, (4) the DCMU inhibition is enhanced under rate-limiting light conditions, whereas the HQNO inhibition is not affected

(Izawa *et al.*, 1967; Cohen, unpublished), (5) MDBQ reduction in photosystem II particles is totally sensitive to DCMU, atrazine, etc., whereas it is only partially sensitive to HQNO, and (6) DCMU displaces radiolabeled metribuzin from prelabeled thylakoid membranes and photosystem II particles, whereas HQNO only slightly displaces [^{14}C]metribuzin at concentrations that are 25- to 50-fold higher than the I_{50} value for inhibition of steady-state electron transport. Only one other inhibitor has been reported to inhibit on the reducing side of photosystem II without displacing a radiolabeled atrazine-like inhibitor from thylakoid membranes, that is, the halogenated naphthoquinone 2-bromo-3-isopropyl-1,4-naphthoquinone (Pfister *et al.*, 1981). In contrast to the situation with bromoisopropylnaphthoquinone, we have observed that the halogenated naphthoquinone dichloroallyl lawsone [$I_{50} \simeq 0.8$ μM] also inhibits on the reducing side of PS II, but it is capable of displacing [^{14}C]metribuzin from pea thylakoid membranes (MacPeek, 1981).

One approach which has been used to ascertain whether PS II inhibitors interact with the Q/B membrane complex has been to determine the I_{50} values for inhibition of electron transport in weed species that have an altered sensitivity to the herbicide atrazine (Pfister and Arntzen, 1979). Such a determination in which HQNO was employed indicated that the resistant/sensitive ratio was approximately 11. When the effect of HQNO was tested in wild type and DCMU-resistant mutants of the blue-green alga *Anacystis nidulans*, electron transport in the DCMU-resistant mutant was also highly resistant to inhibition by HQNO (Louis Sherman, personal communication). Such results may be interpreted to indicate that the mutations which confer atrazine resistance in higher plants or DCMU resistance in blue-green algae also alter the sensitivity to inhibition by HQNO. Based on the models presented by Arntzen and co-workers (Pfister and Arntzen, 1979), the site of interaction for HQNO in the Q/B complex would be in a domain not occupied by either DCMU- or atrazine-like inhibitors. Data are presently unavailable in the literature which would allow us to reach any conclusions regarding the relationship between the binding sites for HQNO and phenolic herbicides. Our data do not support the conclusion of Droppa *et al.* [1981a] that the site of action of HQNO is closer to the PS II reaction center than the DCMU inhibition site, but they do appear to be consistent with their suggestion that HQNO and DCMU interact differently with the Q/B complex.

Enhancement of the HQNO inhibition by dark pretreatment of thylakoids (or PS II particles) with ferricyanide would seem to suggest a direct effect of ferricyanide on the redox state of a membrane component. Satoh and Takamiya [1971] have suggested that dark pretreatment of chloroplasts with ferricyanide and HQNO results in the destruction of chlorophyll a_{683}. Our results which indicate that the ferricyanide enhancement of the HQNO inhibition is observable in a preparation (Photosystem II particle) that lacks

chlorophyll–protein complex I would tend to rule out an effect of ferricyanide on a photosystem I chlorophyll. C_{400}, a secondary acceptor associated with the reducing side of PS II, can be oxidized by dark pretreatment of thylakoids with ferricyanide (Vermaas and Govindjee, 1981). Perhaps the oxidation of C_{400} enhances the binding of HQNO to the Q/B complex, thus increasing the level of inhibition. Burger (1980) has reported that HQNO binding to yeast mitochondria is sensitive to the redox state of the electron-transfer chain; oxidized mitochondria have an increased affinity for HQNO compared to reduced mitochondria. An alternative explanation might be that the oxidation of C_{400} coupled with the presence of HQNO leads to a shift in the redox potential of the Q/B couple (Velthuys and Amesz, 1974). Experiments are currently in progress to allow us to decide between these two alternative hypotheses.

Acknowledgments

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