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Halogenated 1,4-benzoquinones as irreversibly binding inhibitors of photosynthetic electron transport

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Halogenated 1,4-benzoquinones are effective inhibitors of photosynthetic electron transport through Photosystem II and the cytochrome b_6/f-complex as well. Due to their properties as vinylogous acid halides they can react with nucleophilic groups of soluble molecules or membrane-bound proteins under formation of a covalent linkage. Despite its high inhibitory properties a ^{14}C-labeled tetrabromo-1,4-benzoquinone (bromanil) shows no Michaelis–Menten type binding behaviour in isolated thylakoids. As detected by its covalent binding, [^{14}C]bromanil in isolated spinach cytochrome b_6/f-complex binds to the 20 kDa Rieske iron sulfur protein and the 33–34 kDa cytochrome f. Upon [^{14}C]bromanil treatment in spinach thylakoids the highest amount of radioactivity is found in a 20 kDa protein, which may be the Rieske protein, and a 41 kDa protein.

Introduction

In the photosynthetic electron-transport systems of photosynthetic bacteria, algae and higher plants quinones play an important role as electron and proton carriers. Inhibitors of quinone redox reactions have served as efficient tools for the elucidation of partial sequences of the electron-transport chain. Many of these inhibitors play an important role in pest control as herbicides. Inhibitors of quinone redox reactions are of special interest if they have themselves a 1,4-quinone moiety and consequently can undergo reduction and subsequent reoxidation. In DBMIB, Trebst et al. [1] have found an efficient inhibitor of plastohydroquinone oxidation at the Cyt b_6/f-complex. In the following years several other 1,4-quinones were found to interfere with photosynthetic electron transport though their site of inhibition is not necessarily identical to that of DBMIB (for reviews, see Refs. 2 and 3). Depending on their substitution pattern they may block either plastoquinone reduction or plastohydroquinone oxidation, or both.

In previous studies we have assayed a variety of 1,4-benzoquinons for their inhibitory activity on NADP-photoreduction [4] or Photosystem II electron transport [5]. It turned out that 1,4-quinones with halogen substitution are always better inhibi-

Abbreviations: atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DCIP, dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2-iodo-2′,4,4′-trinitro-3-methyl-6-isopropyl-diphenylether; DQH_2, durohydroquinone; Chl, chlorophyll; QSAR, quantitative structure-activity relationship; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; Cyt, cytochrome.

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tors than those without halogen. Moreover, inhibitory potency depends on the type of halogen and increased in the order from chlorine to bromine to iodine [4,5]. Halogen-substituted 1,4-benzoquinones can be considered as vinylogous acid halides. This property allows for reaction with nucleophiles in an addition/elimination reaction under formation of a covalent linkage.

In order to perform binding studies and identify quinone binding proteins in the photosynthetic electron transport system a radioactive [^{14}C]tetrabromo-1,4-benzoquinone (bromanil) has been synthesized. In a model reaction β-mercaptoethanol can react with bromanil and consequently lower its inhibitor activity. One bromanil molecule binds covalently to the sulfhydryl group of bovine serum albumin. Furthermore, in isolated spinach Cyt b_6/f-complex [^{14}C]bromanil tags preferentially the 20 kDa Rieske Fe-S protein and cyt f. If isolated spinach thylakoids are treated with [^{14}C]bromanil the highest amounts of radioactivity are found within a 20 kDa protein, which may be the Rieske protein, and a 41 kDa protein.

Materials and Methods

Source and synthesis of Chemicals

2,6-Diiodo-1,4-benzoquinone was purchased from Ventron, Karlsruhe. 2,5-Dichloro-3,6-dimethoxy-1,4-benzoquinone was synthesized according to Volter and Rogers [6], and 2,5-dinitro-3-methyl-6-isopropyl-1,4-benzoquinone analogous to a synthesis as described by Schill [7]. The synthesis of the other 1,4-benzoquinones used in this study was reported in [4].

2-Iodo-3-amino-5-tert-butyl-1,4-benzoquinone. 0.416 g (1 mmol) 2,3-diiodo-5-*tert*-butyl-1,4-benzoquinone [4] in 8 ml CH_3OH and 1 ml conc. NH_3 were stirred at room temperature for 4 h. The deep-red precipitate was removed by filtration, yield 0.22 g (72%), m.p. 178–181°C. $C_{10}H_{12}INO_2$ (305.12), Calc. C 39.36%, H 3.97%, N 4.59%, I 41.59%; found C 39.29%, H 3.92%, N4.46%, I 41.05%. The compound may also be the isomeric 2-iodo-3-amino-6-*tert*-butyl-1,4-benzoquinone or a mixture of both.

[^{14}C]Bromanil. 46.1 μg (0.49 μmol) phenol and 48.0 μg (0.51 μmol [^{14}C]phenol (50 μCi, spec. act., 98 mCi/mmol; Amersham-Buchler, Braunschweig), 1.97 mg (24 μmol) sodium acetate and 10 μl (194 μmol) bromine in 200 μl of acetic acid were gently shaken in a small glass vial immersed into an oil bath of 80–85°C for 90 min. The acetic acid was evaporated in the vacuum, the residue dissolved in 70 μl of acetone and applied to silica gel pre-coated plastic sheets (Alugram Nano-Sil G/UV$_{254}$, Macherey-Nagel, Düren). They were pre-washed twice with methanol and dried at 70°C for 10 min immediately before use. The chromatogram was developed with benzene/*n*-hexane 6 : 4 (v/v) and the zone corresponding to bromanil ($R_f = 0.38$) cut out and eluted with methanol. The concentration of bromanil was determined from its absorption maximum at 306 nm ($\varepsilon = 13940$ $M^{-1} \cdot cm^{-1}$). The radiochemical yield was 280 nmol (28%).

[^{14}C]atrazine (spec. act., 5.9 mCi/mmol) and [^{3}H]DCMU (spec. act., 171 mCi/mmol) were generous gifts from Ciba-Geigy, Basel, Switzerland, and Prof. Dr. I. Ohad, Department of Biological Chemistry, The Hebrew University, Jerusalem, Israel, respectively.

Biochemical methods

Chloroplasts from spinach were prepared according to Ref. 8 and stored in liquid nitrogen in the presence of 10% glycerol. Spinach chloroplast Cyt b_6/f-complex was isolated according to Hurt and Hauska [9]. Photosynthetic NADP reduction, DCIP-reduction in the presence of DNP-INT and methyl viologen reduction at the expense of duroquinol in the presence of DCMU were estimated as described previously [4,10]. First, the control rates were determined. Then the quinone was added and its inhibitory activity assayed immediately after addition. pI_{50}-values (negative log of concentration giving 50% inhibition) were extrapolated from series of experiments with varying concentrations of inhibitors. Binding experiments were performed according to method B in Ref. 11. Radioactivity distribution in gels after binding of [^{14}C]bromanil was determined as described in Ref. 12.

Results

Inhibition of electron transport through Photosystem II and the cytochrome b_6/f-complex by 1,4-benzoquinones

Table I lists pI_{50}-values of 17 halogen- and one

TABLE I

pI_{50} VALUES FOUND (H_2O-DCIP AND DQH_2-METHYL VIOLOGEN) AND CALCULATED (DQH_2-METHYL VIOLOGEN) AND QSAR-PARAMETERS FOR VARIOUS 1,4-BENZOQUINONES

The log *P* values for the substituents were taken from Ref. 12. The 'Dummy' parameter was set 0 if the substituent pattern at the quinone moiety was symmetrical, otherwise 1. For determination of pI_{50} values in the two systems, see Materials and Methods. The control rates were 95 μmol DCIP reduced per mg Chl per h and 262 μmol O_2 consumed per mg Chl per h. Calculated pI_{50} values were obtained by calculation with Eqn. 2. MV, methyl viologen.

Nr.	...-1,4-benzoquinone	log *P*	'Dummy'	pI_{50} H_2O-DCIP found	pI_{50} DQH_2-MV found	pI_{50} DQH_2-MV calculated	Δ
1	2,5-dichloro-3,6-dimethoxy	1.38	0	< 3.5	3.45	3.72	0.27
2	2,5-dibromo-3,6-diphenyl	5.64	0	4.52	4.51	4.43	0.08
3	2,5-dinitro-3-methyl-6-isopropyl	1.53	1	4.87	4.79	4.56	−0.23
4	2,5-dichloro-3,6-di-*tert*-butyl	5.40	1	5.55	4.95	5.35	0.40
5	2,6-diiodo	2.24	0	< 3	5.08	5.02	−0.06
6	2,5-dibromo-3,6-di-isopropyloxy	2.44	0	5.21	5.19	5.24	0.05
7	2-iodo-3-amino-5-*tert*-butyl	1.87	1	5.04	5.21	5.10	−0.11
8	tribromo-*n*-hexyl	5.58	1	5.23	5.31	5.07	−0.24
9	tetraiodo	4.48	0	7.06	5.55	5.67	0.12
10	tetrachloro	2.84	0	4.79	5.69	5.58	−0.11
11	2-iodo-3-methyl-6-isopropyl	3.23	1	4.12	5.76	6.38	0.62
12	tetrabromo	3.44	0	5.89	6.07	5.88	−0.19
13	2-bromo-5-*tert*-butyl	2.72	1	4.30	6.07	6.07	±0
14	2,3-dibromo-5-*tert*-butyl	3.70	1	5.26	6.27	6.46	0.19
15	tribromo-methyl	3.14	1	4.40	6.29	6.33	0.04
16	2,3-dichloro-5-*tert*-butyl	3.40	1	4.39	6.53	6.43	−0.10
17	2,3-diiodo-5-*tert*-butyl	4.22	1	6.29	6.54	6.38	−0.16
18	2,5-dibromo-3-methyl-6-isopropyl	3.83	1	4.61	6.86	6.46	−0.40

nitrosubstituted 1,4-benzoquinone for inhibition of electron transport in isolated thylakoid membranes through Photosystem II and the Cyt b_6/f-complex, respectively. Electron transport through Photosystem II was assayed using water as the native electron donor and DCIP as an artificial acceptor in the presence of DNP-INT [14] in order to prevent Photosystem I dependent DCIP-reduction. Electron transport through the Cyt b_6/f-complex was determined as methyl viologen mediated oxygen consumption with durohydroquinone as the donor [15,16]. Table I demonstrates that the various 1,4-benzoquinones differ widely in their inhibitory activity in both systems. Three different groups can be discerned. For one group, pI_{50}-values in DCIP-reduction and methyl viologen reduction are about equal (eight compounds: nrs. 1–3, 6–8, 12 and 17). The second group is characterized by a higher inhibitory activity in Photosystem II electron transport as compared to electron transport through the Cyt b_6/f-complex (two compounds: nrs. 4 and 9). In particular, tetraiodo-benzoquinone (nr. 9) is an excellent Photosystem II inhibitor (pI_{50} value, 7.06). In the third group a higher inhibition in electron transport through the Cyt b_6/f-complex rather than Photosystem II is observed (eight compounds: nrs. 5, 10, 11, 13–16 and 18). As already known, DBMIB (nr. 18) is a much better inhibitor of plastohydroquinone oxidation rather than plastoquinone reduction [2].

We have reported recently a quantitative structure-activity relationship (QSAR) for inhibition of Photosystem II electron transport by 1,4-benzoquinones [5,17]. As molecular parameters served the redox potential of the quinone, the sum of the Taft steric parameters for the positions 2 and 3, and 5 and 6, respectively, and in addition a dummy parameter accounting for an asymmetric substituent distribution in the quinone moiety [5,17]. A similar QSAR calculation was now performed for the inhibition of electron transport through the Cyt b_6/f-complex. However, no dependency of the inhibitory activity on either the redox potential,

nor the Taft steric parameter, nor the electronic σ-Hammett parameter could be established ($r < 0.3$; $F < 1$). Contrary, inhibitory activity could be well correlated to the lipophilicity of the quinone as expressed by the sum of log P for each substituent. The multiple linear regression yielded Eqn. 1 as the result:

$$pI_{50} = 0.409 + 3.139 \log P - 0.419 \log^2 P$$

$$n = 18;\ s = 0.40;\ r = 0.90;\ F = 30.73;\ (F_{15,0.01} = 6.36) \qquad (1)$$

where n represents the number of compounds, s the standard deviation, r the correlation coefficient and F the F-test. As judged from the correlation coefficient and the F-test, the correlation is statistically of high significance.

Introduction of a 'dummy' parameter D which was set 1 if the substitution pattern of the quinone is asymmetric and otherwise 0, improved the correlation significally (Eqn. 2):

$$pI_{50} = 0.390 + 2.957 \log P - 0.397 \log^2 P + 0.572\ D$$

$$n = 18:\ s = 0.27;\ r = 0.96;\ F = 49.70\,(F_{14,0.01} = 5.56) \qquad (2)$$

The pI_{50} values calculated according to Eqn. 2 are listed in Table I. In Eqn. 2 log P occurs in quadratic terms. Therefore, an optimal log P of 3.72 for maximal inhibitory activity can be evaluated.

Reaction of halogenated 1,4-benzoquinones with nucleophiles

For further characterization of the biochemical properties of halogen-substituted 1,4-benzoquinones bromanil was chosen for two reasons. Bromanil is a good inhibitor of photosynthetic electron transport through Photosystem II and the Cyt b_6/f-complex as well with pI_{50}-values of 5.89 and 6.07, respectively. Furthermore, for binding studies a ^{14}C-labeled bromanil was easily obtainable in a one-step synthesis. Halogen-substituted 1,4-quinones can be considered as vinylogous acid halides. In a Michael-type addition a nucleophile like a sulfhydryl compound can add onto the carbon atom in the quinone ring which bears the halogen substituent. In the subsequent elimination reaction the halogen anion is split off. Formally, halogen has been replaced by the nucleophile. It should be noted that this addition/elimination reaction for chemical reasons can only take place with the quinone, but not with the hydroquinone. As already stressed, the inhibitory activity of the halogen-substituted 1,4-benzoquinones is governed by their lipophilicity as expressed by log P. If a halogen is replaced by a less lipophilic substituent this will lead to a drop in inhibitory activity. An example is demonstrated in Table I. 2-Iodo-3-amino-5-*tert*-butyl-1,4-benzoquinone (nr. 7) has been synthesized from 2,3-diiodo-5-*tert*-butyl-1,4-benzoquinone (nr. 17) by reaction with aqueous ammonia. The amino group is less lipophilic than the iodo group, and consequently log P drops from 4.22 (2,3-diiodo-) to 1.87 (2-iodo-3-amino). This drop in lipophilicity is accompanied by a decrease in inhibitory activity from a pI_{50} of 6.54 (2,3-diiodo) to 5.21 (2-iodo-3-amino-) in the duroquinol-methyl viologen system. If a nucleophile is present in a test system for inhibitory activity of a halogenated benzoquinone, the nucleophile will react with the quinone. The reaction product in general will exhibit a lower pI_{50}-value and partial reversal of the electron-transport inhibition will be observed. This is only valid if a soluble nucleophilic compound is used but not if the quinone binds to its target site within the photosynthetic membrane.

The experiment as described above has been performed using electron transport through the Cyt b_6/f-complex, bromanil as the inhibitor and 0.1 mM β-mercaptoethanol as the nucleophile. The result is demonstrated in Fig. 1. With increasing incubation time the pI_{50}-value of bromanil decreases from an original value of 6.07 to 5.33 after 2 min (but to only 5.58 if bromanil is added first and then β-mercaptoethanol). In a similar experiment the incubation time was left constant, but the concentration of β-mercaptoethanol varied (Fig. 2). 1 mM β-mercaptoethanol brings the pI_{50}-value of bromanil down to 4.64.

It should be noted that the inhibition of photosynthetic electron transport by bromanil and the chemical reaction between bromanil and β-mercaptoethanol follow entirely different time-courses. Inhibition of photosynthetic electron transport is achieved within seconds and under the experimental conditions no time lag is observed. Conversely, the chemical reaction between

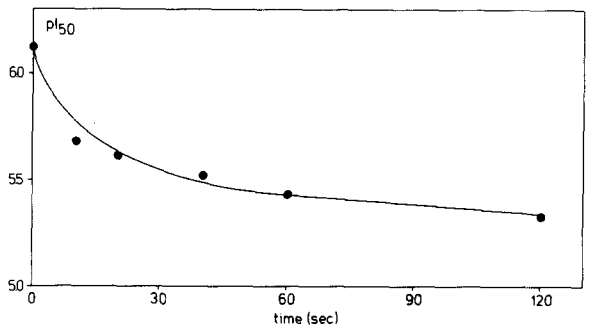

Fig. 1. Dependency of the pI_{50} value of bromanil from different preincubation times in the presence of 0.1 mM β-mercaptoethanol.

the nucleophile and bromanil is a time- and concentration-dependent step (see Figs. 1 and 2).

A similar reversal of bromanil Photosystem II inhibition by β-mercaptoethanol cannot be performed directly, because β-mercaptoethanol chemically reduces DCIP. Instead, NADP reduction was measured under conditions where the Cyt b_6/f-complex was blocked by 10^{-6} M DNP-INT [14] and by-passed by 10^{-4} M TMPD [18]. In this system the pI_{50} value of bromanil dropped from 5.43 to 3.74 in the presence of 1 mM β-mercaptoethanol after 2 min.

A covalent attachment of bromanil to the thylakoid membrane which causes an irreversible inhibition of photosynthetic electron transport can be demonstrated directly. Thylakoids are incubated with bromanil for 15 min, washed twice to remove unbound bromanil and assayed for activity in photosynthetic NADP-reduction. As can be seen in Table II, even after washing NADP-reduction remains inhibited. The degree of inhibition is dependent on the bromanil concentration and amounts to 65% inhibition at 0.1 mM.

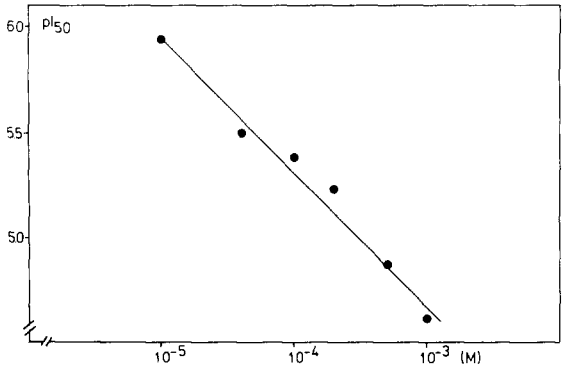

fig. 2. Dependency of the pI_{50} value of bromanil from preincubation (2 min) at different concentrations of β-mercaptoethanol.

Binding properties of bromanil

Photosystem II inhibitors displace themselves mutually in a competitive fashion from their binding site in the Photosystem II reaction-center complex. This can be easily studied by determining the binding characteristics of a radioactively labeled inhibitor in the presence of varying concentrations of another unlabeled inhibitor [19]. In a competitive displacement the binding constant of the radioactively labeled inhibitor increases at increasing concentrations of the unlabeled inhibitor, whereas the number of binding sites remains unchanged [19]. We have studied the binding of [^{3}H]DCMU in the presence of increasing concentrations of bromanil. It should be noted that [^{3}H]DCMU and bromanil were added simultaneously. The double reciprocal Lineweaver–Burk plot of the binding data is shown in Fig. 3. As can be clearly seen, the regression lines intercept at a common ordinate (number of binding sites), but at different abscissas (binding constant). Thus, bromanil behaves identical like all other Photosystem II inhibitors. In a similar experiment the binding of [^{14}C]atrazine at a constant concentration of bromanil but after varying incubation times (up to 40 min) was studied. The half-reciprocal Eadie–Scatchard plot of the binding data is shown

TABLE II

IRREVERSIBLE INHIBITION OF PHOTOSYNTHETIC NADP-REDUCTION BY BROMANIL

Thylakoids were incubated for 15 min and then wased twice and centrifuged to remove unbound bromanil. The relatively low rates in NADP-reduction are due to the washing and centrifugation procedures.

	μmol NADPH per mg Chl per h	% Inhibition
Control	58.0	0
10^{-5} M	40.7	29.8
$2.5 \cdot 10^{-5}$ M	35.6	38.6
10^{-4} M	20.4	64.8

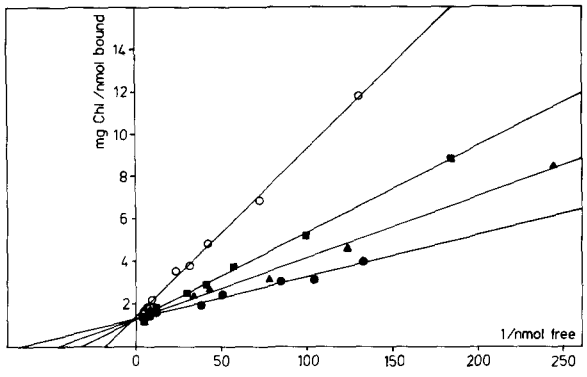

Fig. 3. Lineweaver–Burk plot of [^{3}H]DCMU binding to thylakoids in the presence of bromanil. ●——●, control; ▲——▲, +3.1; ■——■, +6.2; ○——○, +9.3 nmol.

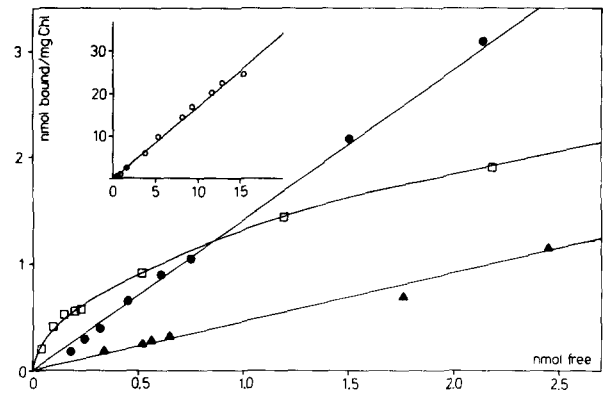

Fig. 5. Binding of [^{14}C]bromanil to thylakoids in the absence (●——●) and presence (▲——▲) of 1 mM β-mercaptoethanol and comparison to [^{14}C]atrazine binding (□——□). Inset: Binding of [^{14}C]bromanil in the concentration range up to 16 nmol.

in Fig. 4. Again there was no change in the number of binding sites (identical abscissa intercepts), but an increase in the binding constant (different ordinate intercepts). Note that abscissa and ordinata have a different meaning in the Lineweaver–Burk and the Eadie–Scatchard plot. This experiment indicates that the amount of bromanil bound to the thylakoid membrane increases upon prolonged incubation time.

To study directly the binding properties of bromanil, a radioactively labeled bromanil was synthesized by excessive bromination and simultaneous oxidation of [^{14}C]phenol. The binding curve of bromanil is shown in Fig. 5. Contrary to all other Photosystem II inhibitors studied so far,

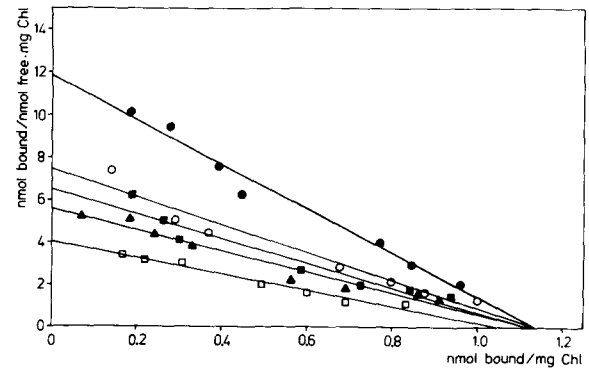

Fig. 4. Eadie–Scatchard plot of [^{14}C]atrazine binding after incubation with 10 nmol bromanil at different time intervals. ●——●, control; ○——○, 10; ■——■, 20; ▲——▲, 30; □——□, 40 min.

bromanil shows no Michaelis-Menten type binding behaviour (compare the binding curves of radioactively labeled bromanil and atrazine in Fig. 5). The amount of bromanil bound is directly proportional to the concentration of free bromanil. This is also true for relatively high concentrations of bromanil up to 16 nmol free (see inset in Fig. 5). This result is quite surprising because bromanil with a pI_{50} value of almost 6 is a good Photosystem II inhibitor. No binding parameters for bromanil could be determined. As expected from the previous experiments, 1 mM β-mercaptoethanol after an incubation time of 10 min decrease the total bromanil binding by about two-thirds (Fig. 5).

As already stressed, the Michael addition/elimination reaction can only take place with the quinone, but not with the reduced hydroquinone. The binding of [^{14}C]bromanil has been studied in continuous light for 10 min at low concentrations which will not lead to a substantial inhibition of photosynthetic electron transport. Under these conditions bromanil is reduced to its hydroquinone by the photosynthetic electron-transport chain. Its binding is then diminished by 47% (average value of eight independent experiments) as compared to the dark controls.

As is evident from the experiments with β-mercaptoethanol, bromanil can react with nucleophilic compounds. We have checked if

bromanil can also react with nucleophilic groups in proteins. If bovine serum albumin is treated with [^{14}C]bromanil and subjected to polyacrylamide gel electrophoresis, the radioactivity comigrates with the Coomassie blue stainable peak. 1 μg bovine serum albumin incorporated a total of 2302 dpm. This corresponds to 1.26 molecules bromanil per molecule bovine serum albumin. This indicates that about one molecule of bromanil binds to the free sulfhydryl group present in bovine serum albumin [20].

Isolated Cyt b_6/f-complex and thylakoids were incubated with [^{14}C]bromanil for 10 min and after detergent treatment subjected to polyacrylamide gel electrophoresis for identification of possible bromanil binding proteins. Since bromanil due to its reactivity toward nucleophiles can react with a variety of proteins, a clear pattern like with the photoaffinity labeling technique cannot be expected. However, there should be preferential binding to some proteins due to the property of bromanil as an efficient inhibitor of electron flow through the Cyt b_6/f-complex and Photosystem II as well. Photographs of the gels and radioactivity distribution therein are shown in Fig. 6 for the Cyt b_6/f-complex and in Fig. 7 for thylakoids. In isolated Cyt b_6/f-complex from the four proteins present, radioactivity is found in the 33 and 34 kDa Cyt f and the 20 kDa Rieske Fe-S protein. No radioactivity is found in the 23.5 kDa Cyt b_6

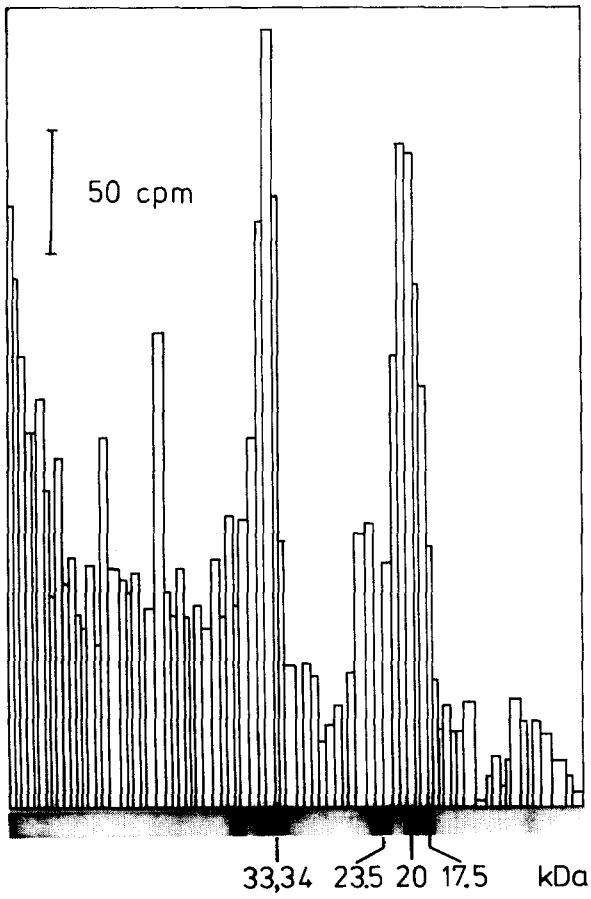

Fig. 6. Photograph of a SDS-polyacrylamide gel electrophoresis gel (10–15%) and radioactivity distribution therein of spinach Cyt b_6/f-complex labeled with [^{14}C]bromanil (2 nmol/nmol Cyt f).

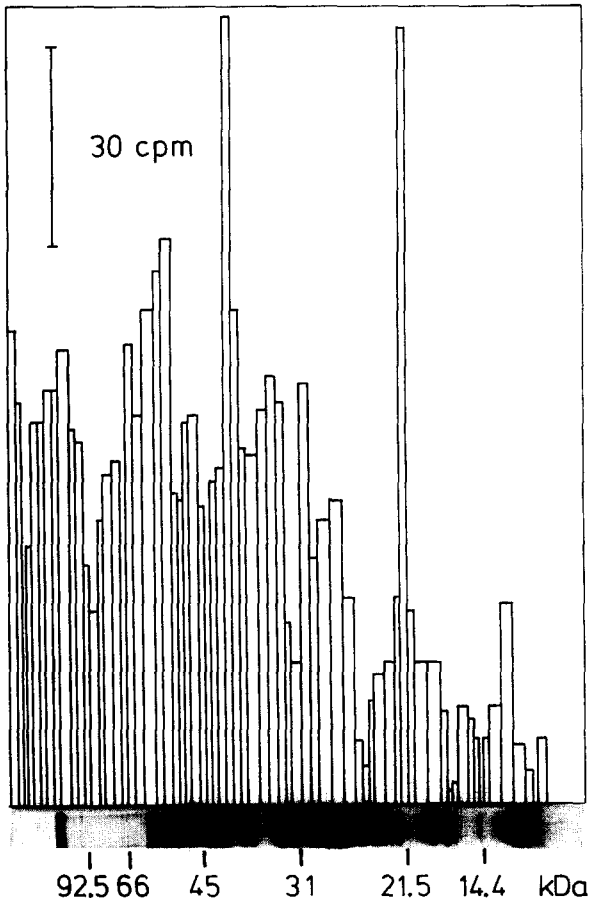

Fig. 7. Photograph of a SDS-polyacrylamide gel electrophoresis gel (10–15%) and radioactivity distribution therein of spinach thylakoids labeled with [^{14}C]bromanil (10 nmol/mg Chl). The positions of the marker proteins are indicated.

nor in the 17.5 kDa small subunit IV (Fig. 6). In thylakoids, two major labeled proteins can be identified: a 41 kDa protein and a 20 kDa protein with an R_f value identical to that of the Rieske Fe-S protein.

Discussion

The halogen-substituted 1,4-benzoquinone DBMIB has been introduced as an efficient inhibitor almost two decades ago [1]. Due to its specific block at the Cyt b_6/f-complex it facilitated investigations on the Photosystem II electron-transport pathway. DBMIB is not unique in a sense because other halogen-substituted 1,4-benzoquinones are also potent inhibitors of the Cyt b_6/f-complex (Table I). However, depending on the substitution pattern, 1,4-benzoquinones may even be more effective in Photosystem II than in the Cyt b_6/f-complex (Table I). Though on both inhibition sites plastoquinone redox reactions take place the participating electron carriers are different and hence the mode of quinone binding. This becomes also evident from the QSAR studies. Inhibition at the Cyt b_6/f-complex is governed by the lipophilicity of the quinone alone as expressed by Eqn. 2. Contrary, Photosystem II inhibition by 1,4-benzoquinones follows an entire different parameter set. Here, the redox potential (a parameter correlated to the σ-Hammett electronic constant) and steric parameters play the major role [5,17]. Similarily, a QSAR of Photosystem II inhibition by 1,4-naphthoquinones is described by the σ-Hammett constant and steric parameters [21]. In this respect, 1,4-quinones in their QSAR are different from the DCMU-type and phenolic Photosystem II inhibitors (for discussion, see Ref. 21).

The drop in bromanil's pI_{50}-value upon incubation with β-mercaptoethanol clearly demonstrates that at least one and probably more bromine substituents can be replaced by a nucleophilic group. A similar inactivation of DBMIB by thiol compounds has been reported [22]. Furthermore, Reimer et al. [23] and Robinson et al. [24] could also reverse DBMIB-inhibition by addition of bovine serum albumin. Bovine serum albumin is known to contain one reactive sulfhydryl group [20]. In both cases an identical Michael type addition/elimination reaction takes place; however, the inactivation mechanism is different. As discussed in detail in the subsection Reaction of halogenated 1,4-benzoquinones with nucleophiles, according to the QSAR a replacement of bromine by a nucleophilic group of less log P than bromine leads to a new compound with a lower pI_{50}-value as compared to bromanil or DBMIB. By reacting with bovine serum albumin, the halogenated quinone is removed from the aqueous phase and the equilibrium between free quinone and quinone bound to the thylakoid membrane is disturbed. Consequently, additional quinone dissociates from its binding site to be bound to bovine serum albumin. Indeed, we could demonstrate that approx. one molecule of [^{14}C]bromanil attaches to bovine serum albumin. Some years ago we have synthesized a radioactively labeled 2,3-diido-5-*tert*-butyl-1,4-benzoquinone (compound 17, Table I). The radioactivity was situated as [^{125}I]iodine in the two iodine substituents [25]. Due to the addition/elimination reaction as described above the release of [^{125}I]iodide into the supernatant could be demonstrated [26]. In [^{14}C]bromanil the radioactivity is located in the carbon atoms of the quinone moiety and, hence, radioactivity will not be released upon binding. [^{14}C]Bromanil does not exhibit a Michaelis–Menten type binding behaviour, i.e., a 'specific binding' [19]. This behaviour is quite exceptional as compared to other radiolabeled Photosystem II inhibitors [19]. At present we are unable to explain this unique feature. It may be correlated to the covalent binding of the quinone. It should be stressed, however, that inhibition of electron transport and covalent binding are two completely different processes. Covalent binding is not a prerequesite for inhibition of photosynthetic electron transport. Inhibition of photosynthetic electron transport in the testing system is achieved immediately after addition of the quinone. Covalent binding is a time-dependent reaction. This is judged from the fact that less reversal of inhibition is achieved the longer the incubation time is.

When the binding of [^{14}C]atrazine to thylakoids has been studied which have been incubated with bromanil for prolonged time periods, the number of binding sites for atrazine remained unchanged, whereas its binding affinity decreased (Fig. 4).

This indicates that the binding sites for bromanil and atrazine are different. If the atrazine binding site in isolated thylakoids was occupied by covalently linked azidoatrazine and subsequently [^{3}H]DCMU binding was studied, no change in the affinity of DCMU to its binding site, but a decrease in its number of binding sites was observed [27]. This suggests indeed identical binding sites among DCMU-type inhibitors. The notion of different binding sites for quinones and DCMU-type and phenolic inhibitors as well is further corroborated by a recent report by Vermaas et al. [28]. Upon covalent linkage of an azido-quinone to thylakoids they also observed no change in the number of binding sites for either atrazine nor ioxynil, but a decrease in their binding affinities instead.

[^{14}C]Bromanil preferentially labels proteins of the isolated Cyt b_6/f-complex or thylakoids, respectively. In isolated Cyt b_6/f-complex only the 33–34 kDa Cyt f and the 20 kDa Rieske Fe-S protein get tagged. A direct interaction between certain halogen-substituted 1,4-benzoquinones and the Rieske Fe-S protein has been demonstrated by means of the EPR-technique. In the $g = 1.9$ region the Rieske Fe-S protein exhibits a prominent signal at $g = 1.89$. Upon addition of DBMIB this signal vanishes and a new signal at $g = 1.94$ appears [29,30]. An identical shift was observed upon addition of quinones nrs. 13, 14 and 17 (Table I) [31]. This shift is characteristic of halogenated 1,4-benzoquinones and cannot be elicited by other inhibitors of the Cyt b_6/f-complex like UHDBT, DNP-INT or antimycin [30,31]. However, UHDBT and DNP-INT are capable of reversing the DBMIB-induced shift; i.e., they displace DBMIB from its binding site at the Rieske Fe-S protein. This notion seems to contradict our concept of covalent binding of halogen-substituted 1,4-benzoquinones. However, as already stressed, the covalent binding is a time-dependent process and it is not exactly known from Refs. 30 and 31 how much time elapses between the addition of DBMIB and the subsequent addition of UHDBT. According to Refs. 30 and 31 samples are stored and spectra recorded at cryogenic temperatures where chemical reactions cannot take place. Thus, the observation of DBMIB replacement by UHDBT is not necessarily in contradiction to our covalent binding proposal. There is, so far, no report in the literature for an interaction between quinones and Cyt f. A close neighbourhood between Cyt f and the Rieske Fe-S protein may be the reason for the labeling of Cyt f by [^{14}C]bromanil. Furthermore, in the isolated Cyt b_6/f-complex the Cyt f may have exposed a relatively high number of nucleophilic groups which are prone to an attack by bromanil. In isolated thylakoids [^{14}C]bromanil besides a 20 kDa protein, which may be the Rieske Fe-S protein, also tags a 41 kDa protein (the Cyt f labeling may be obscured in the background or does not take place at all in intact thylakoids). A labeling of a 41 kDa protein by an azido derivative of the phenolic herbicide dinoseb has been reported by us [12,32]. Azido-monuron in isolated thylakoids from *Chlamydomonas* labels the 32–34 kDa herbicide binding protein ('Q_B protein') and, in addition, a 41 kDa protein [33]. Its exact nature and function is not known so far.

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