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Reversal of quinone-induced chlorophyll fluorescence quenching

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We have used two methods to investigate the reversibility of the interaction of substituted quinones with the thylakoid membrane of plant chloroplasts. Treatment of chloroplasts with added quinones lowers the room-temperature Photosystem II chlorophyll fluorescence intensity by variable amounts depending on the identity and concentration of the quinone. The extent of restoration of the chlorophyll fluorescence level is used as a measure of the effectiveness of the reversal technique. One reversal method involves the addition of thiols to quinone-treated chloroplasts to alter the quinone in a chemical way via a nucleophilic 1,4-Michael addition. In general, the modified quinones exhibit a lower affinity for the thylakoid membrane, as evidenced by an accompanying increase in chlorophyll fluorescence. The thiol concentrations necessary for quenching reversal are found to be in the order [dithiothreitol] < [2-mercaptoethanol] < [glutathione]. The second reversal method examines the extent to which added quinones can be removed from thylakoid membranes using a concentration gradient established by resuspension of quinone-treated chloroplasts in quinone-free media. The results further support the reversible nature of the quinone inhibition and indicate that the extent of recovery is dependent upon the degree of fluorescence inhibition originally induced by the added quinone.

Introduction

The action of substituted quinones on the room-temperature chlorophyll fluorescence of plant chloroplasts has been extensively studied [1–3]. For series of substituted 1,4-benzoquinones, 1,4-naphthoquinones and 9,10-anthraquinones, variable fluorescence quenching abilities have been measured. Comparisons between quinone structural properties and experimentally measured chlorophyll fluorescence parameters have established a relation between physicochemical properties and inhibitory action for each class of quinones. In particular, both substituent hydrophobicity and electronic characteristics govern the fluorescence quenching activity of quinones.

Two mechanisms have been proposed to describe the mode of action for quinone-induced fluorescence quenching. Some studies suggest that the added quinones function to dissipate excitation energy by interaction with either the Photosystem II light-harvesting chlorophyll-protein complexes or the Photosystem II reaction center chlorophyll [4–7]. These results support a mechanism for quenching involving a through-space intermolecular electron transfer from an excited state chlorophyll molecule to the quinone, followed by a rapid decay of the charge-transfer complex to the ground state [8–10]. Differential fluorescence quenching activity would be interpreted to reflect variations in both quinone-membrane solubility and the electron-accepting ability of the quinone.

An alternative quenching mechanism [11–15] suggests that the added quinones compete with the intrinsic plastoquinone electron acceptor, Q_B, for the same or overlapping binding sites in a common binding domain [16–18] on the D-1 protein [19]. Two hydrogen bridges to the carbonyl groups are possible to orient the quinones in the binding niche, one to histidine$_{215}$ and

Abbreviations: AQ, 9,10-anthraquinone; BQ, 1,4-benzoquinone; Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DMOE, 1,2-dimethoxyethane; DMSO, dimethylsulfoxide; DTT, dithiothreitol; F_{max}, maximum chlorophyll fluorescence level with Photosystem II electron acceptor Q_A reduced; GLU, glutathione; Hepes, 4-(2-hydroxyethyl)-1-piperazine-sulfonic acid; I_0, I_Q, I_R, chlorophyll fluorescence levels in untreated chloroplast samples, in quinone-treated chloroplast samples, and in samples after resuspension of quinone-treated chloroplasts in quinone-free buffer, respectively; MER, 2-mercaptoethanol; NQ, 1,4-naphthoquinone; Q_A and Q_B, the membrane-bound and membrane-exchangeable plastoquinone electron acceptors in Photosystem II, respectively.

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the other to a peptide close to serine$_{264}$ [20,21]. A reduction in chlorophyll fluorescence emission presumably results when the added quinone inhibits Photosystem II electron transport activity by displacing Q_B and preventing the normal occurrence of a small degree of charge recombination to re-populate an excited state of chlorophyll. Variations in the extent of fluorescence quenching observed with different quinones would be interpreted to reflect varying degrees of competitive displacement of Q_B.

We have examined two methods that reverse the fluorescence quenching action of quinones to demonstrate further the dynamic nature of the quenching process and to determine whether the effectiveness of quenching reversal exhibits variability as observed for the inhibition process. One reversal method involves the addition of thiols to quinone-treated chloroplasts to alter in a chemical way the quinone and modify its inhibitory activity. Thiols are known to react with quinones via a nucleophilic 1,4-Michael addition [13,22–24] to produce a quinol with lower affinity for the membrane [25,26]. Observation of a restoration of chlorophyll fluorescence would indicate removal of the quinone inhibitor from its inhibition site. The degree of restoration of fluorescence levels upon thiol addition should reflect such factors as the accessibility of the thiol for the quinone site of action and the extent of reaction of thiol with quinone.

We have also studied the extent to which added quinones can be subsequently removed from thylakoid membranes using a concentration gradient established by resuspension of quinone-treated chloroplasts in quinone-free media [13]. In addition to examining the reversibility of the fluorescence quenching process, we have also explored whether the removability of a quinone from its site of action is related to the extent of quinone-membrane permeability as governed by hydrophobic factors.

Materials and Methods

As in previous studies [1–3], chloroplasts were isolated from freshly harvested growth-chamber barley (*Hordeum vulgare*) in a medium containing 0.4 M sucrose/50 mM Hepes-NaOH (pH 7.5)/10 mM NaCl. Centrifugation at $6000 \times g$ for 10 min was followed by resuspension of the chloroplasts in a medium of 0.1 M sucrose/10 mM Hepes-NaOH (pH 7.5)/10 mM NaCl. Following centrifugation at $6000 \times g$ for 10 min, the pellet was resuspended in a medium of 0.1 M sucrose/50 mM Hepes-NaOH (pH 7.5)/5 mM NaCl to give 16.7 μg Chl per ml. For fluorescence measurements the chloroplast suspension was diluted as described below to a concentration of 10 μg Chl per ml.

Substituted quinones were purchased from Aldrich Chemical Company unless otherwise noted and included: *1,4-benzoquinones (BQ)* – 2,3,5,6-tetramethyl; 2,5-dibromo-3-methyl-6-isopropyl (DBMIB); *1,4-naphthoquinones (NQ)* – 2-bromo; 2,3-dichloro (Alfa); 5-hydroxy; 5,8-dihydroxy; 1,4-anthraquinone (Alfa); 9,10-anthraquinone (Alfa); *9,10-anthraquinones (AQ)* – 1-chloro; 1-hydroxy (ChemService, Westchester, PA); 1,8-dihydroxy (chrysazin); 1,4-dihydroxy (quinizarin); 1,2-dihydroxy; 1,2,4-trihydroxy (Pfaltz and Bauer); 1,2,5,8-tetrahydroxy-9,10-anthraquinone (quinalizarin); 1-amino-4-hydroxy; 1-amino; 2-amino; 1,2-diamino; 1,4-diamino; 1,5-diamino (Pfaltz and Bauer); 2,6-diamino (Pfaltz and Bauer); 1,4,5,8-tetraamino; 1,5-diamino-4,8-dihydroxy (Alfa); 3,4-dihydroxy-2-sulfonic acid sodium salt; 1-amino-2-methyl-4-bromo; 2-ethyl; 2-carboxylic acid; 2-chloro; and 3-chloro-2-carboxylic acid (Pfaltz and Bauer). As necessary, quinones were further purified by recrystallization or sublimation. Stock solutions of the quinones (10 or 20 mM) were prepared in dimethylsulfoxide (DMSO), ethanol, or 1,2-dimethoxyethane (DMOE). As noted in previous studies [1–3], these solvents showed no quenching effects on chlorophyll fluorescence at the concentrations employed in diluted chloroplast samples.

Thiols used for quenching reversal studies included dithiothreitol and glutathione, purchased from Aldrich Chemical Company, and 2-mercaptoethanol, purchased from Sigma Chemical Company. Thiol stock solutions were prepared using the final chloroplast buffer as solvent. The addition of thiols to untreated chloroplasts induced small changes in the chlorophyll fluorescence level, I_0, at 684 nm; at a thiol concentration of 800 μM, an increase was observed in I_0 of $3 \pm 2\%$ for 2-mercaptoethanol, $4 \pm 3\%$ for glutathione, and $9 \pm 3\%$ for dithiothreitol.

Quinone- and thiol-enriched chloroplast samples were prepared adding appropriate volumes of quinone stock solutions, buffer and thiol solutions to concentrated chloroplast samples to form final samples with [Chl] = 10 μg/ml, [quinone] = 100 μM, and [thiol] = 0–800 μM. Incubation of chloroplast and quinone solutions for 5 min in the dark at 4°C was sufficient to achieve a time-independent level of chlorophyll fluorescence [1–3]. Thiol addition to the quinone-enriched chloroplast samples was followed by incubation on ice in the dark for 5 min to allow for complete reaction [13], again monitored by a time-independent chlorophyll fluorescence level.

Quinone-depleted samples were prepared by incubating quinone-treated chloroplasts in quinone-free measuring buffer according to the following protocol. Chloroplast samples were incubated with quinone solutions for 5 min in the dark at 4°C such that [Chl] = 10 μg/ml and [Quinone] = 100 μM. The samples were then centrifuged at $6000 \times g$ for 10 min, and the pellet was resuspended in quinone-free measuring buffer. Fluorescence measurements were conducted as described be-

low. As a control, the chlorophyll fluorescence level of untreated chloroplasts was compared with that obtained for re-centrifuged and resuspended chloroplast samples. We found no difference in emission levels at 684 nm: $I_0 = 100.0 \pm 0.8$ before centrifugation and $I_0 = 100.1 \pm 1.0$ after centrifugation and resuspension at the same chlorophyll concentration.

Room-temperature fluorescence emission spectra were recorded with a Perkin-Elmer LS-5 fluorescence spectrophotometer interfaced to a Perkin-Elmer Model 3600 Data Station. Chlorophyll fluorescence was induced by excitation at 620 nm with a pulsed xenon lamp and detected over the range 650–760 nm with a Hamamatsu R928 photomultiplier tube. Measurements of chlorophyll fluorescence were made for chloroplasts in the F_{max} state by saturating with high light intensity for 2 min to close Photosystem II reaction centers. All fluorescence samples contained [Chl] = 10 μg/ml. The fluorescence intensity values reported in Tables I–V are for measurements made at the wavelength of maximum emission, 684 nm, although we found no sizable differences either in the extent of fluorescence quenching [2,3] or in the extent of fluorescence recovery when measured at other emission wavelengths (e.g., 730 nm).

Results

Table I presents the results of thiol reversal studies using dithiothreitol (DTT), 2-mercaptoethanol (MER), and glutathione (GLU) with chloroplasts enriched with 100 μM of five various quinones. The quenching reversal is characterized by four parameters: minimum thiol concentration required to achieve a constant and maximum level of fluorescence quenching reversal; thiol concentration at which half-maximum reversal occurred; maximum chlorophyll intensity (I_R) at 684 nm obtained upon thiol addition as a percentage of the chlorophyll fluorescence level (I_0) in a quinone- and thiol-free sample; and chlorophyll fluorescence intensity (I_Q) measured in a quinone-treated sample at 684 nm as a percentage of the chlorophyll fluorescence level (I_0) in untreated samples. Fig. 1 illustrates a representative fluorescence recovery curve with data from the addition of 2-mercaptoethanol to DBMIB-treated chloroplasts. Limited reversal of fluorescence quenching by 1,8-dihydroxy-9,10-anthraquinone was observed with all thiols and could be attributed to thiol-induced effects on chlorophyll fluorescence. 2-Mercaptoethanol slightly quenched chlorophyll fluorescence further when added to chloroplasts treated with 1,4-anthraquinone. In general, when thiols were effective in restoring chlorophyll fluorescence levels, the concentrations necessary for reversal were in the order [DTT] < [MER] < [GLU]. For both benzoquinones examined, thiols increased the chlorophyll fluorescence level above that observed in the absence of quinone.

As DTT effected fluorescence restoration most effec-

TABLE I

Fluorescence quenching reversal by addition of various thiols

This table presents the results of the addition of dithiothreitol (DTT), 2-mercaptoethanol (MER), and glutathione (GLU) to barley chloroplasts treated with 100 μM quinone. Tabulated parameters for each quinone-thiol combination include: minimum thiol concentration required to achieve maximum fluorescence quenching reversal, $[thiol]_{max}$; thiol concentration for half-maximum reversal ($[thiol]_{1/2}$); maximum chlorophyll fluorescence intensity (I_R) at 684 nm obtained upon thiol addition as a percentage of the chlorophyll fluorescence level (I_0) in quinone- and thiol-free samples; and the chlorophyll fluorescence intensity (I_Q) measured in quinone-treated samples at 684 nm as a percentage of the chlorophyll fluorescence level (I_0) in untreated samples. The + symbol in the $[thiol]_{max}$ column indicates that quenching reversal had not reached a well-defined maximum level at [thiol] = 800 μM. Uncertainties are expressed as average deviations of mean values calculated using 4–6 measurements.

Quinone	DTT reversal				MER reversal			
	$[DTT]_{max}$ (μM)	$[DTT]_{1/2}$ (μM)	$\frac{I_R}{I_0} \times 100\%$	$\frac{I_Q}{I_0} \times 100\%$	$[MER]_{max}$ (μM)	$[MER]_{1/2}$ (μM)	$\frac{I_R}{I_0} \times 100\%$	$\frac{I_Q}{I_0} \times 100\%$
2,3,5,6-Tetramethyl-BQ	800+	320±20	111 ±8	73.7±1.3	800±40	340±20	81.4±1.0	74.1±1.4
DBMIB	800±40	140±10	151 ±3	14.6±0.6	400±20	210±20	112 ±1	12.8±0.9
2,3-Dichloro-NQ	140±10	100±10	66.8±0.6	18.2±0.4	480±20	350±20	72.3±1.5	17.1±0.6
1,4-Anthraquinone	140±10	20±10	56.9±0.7	32.0±0.9	180±10	60±10	25.8±0.2	30.0±0.4
1,8-Dihydroxy-AQ	480±20	100±10	22.9±0.1	20.7±0.3	560±20	180±10	21.8±0.2	20.3±0.6

Quinone	GLU reversal			
	$[GLU]_{max}$ (μM)	$[GLU]_{1/2}$ (μM)	$\frac{I_R}{I_0} \times 100\%$	$\frac{I_Q}{I_0} \times 100\%$
2,3,5,6-Tetramethyl-BQ	800±40	400±20	82.4±1.1	78.4±2.0
DBMIB	800±40	480±20	61.5±1.0	14.4±0.7
2,3-Dichloro-NQ	800±40	750±40	89.4±0.8	19.8±0.6
1,4-Anthraquinone	800+	400±20	30.2±0.1	27.8±0.5
1,8-Dihydroxy-AQ	320±20	240±20	20.3±0.1	19.7±0.9

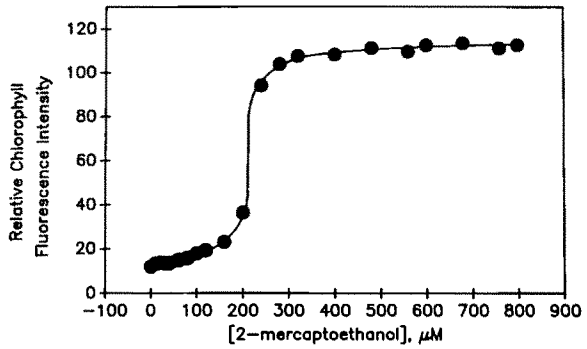

Fig. 1. The relative chlorophyll fluorescence intensity of barley chloroplasts incubated with 100 μM DBMIB and subsequently treated with various concentrations of 2-mercaptoethanol. The relative chlorophyll fluorescence intensity in the absence of quinone and thiol is equal to 100. The protocol for quinone incubation and thiol treatment is as described in the text. Chlorophyll concentrations were equal to 10 μg/ml and fluorescence intensities are reported for measurements made at 684 nm.

tively at the lowest thiol concentrations, additional reversal measurements with DTT were made for a more extensive selection of quinones. Table II presents the results of 12 thiol reversal studies using DTT with chloroplasts enriched with 100 μM quinone. Limited quenching reversal was possible with all 9,10-anthraquinones examined. This result is consistent with the inability of carbonyl groups on the central ring to react with thiols via a 1,4-Michael addition [13,22–24]. Instead, the small changes in chlorophyll fluorescence levels upon thiol addition to 9,10-anthraquinones are most likely a consequence of the previously noted thiol effects on untreated chloroplasts. Two distinct reversal regions were observed for 5-hydroxy- and 5,8-dihydroxy-1,4-naphthoquinone. For all other quinones examined, thiol concentrations required for half-maximum reversal ranged from 20 to 360 μM. The fluorescence levels of benzoquinone-enriched and 5-hydroxy-1,4-naphthoquinone-enriched chloroplasts were restored above the level measured in the absence of quinone.

Table III illustrates the effect of the sequence of addition of thiol and quinone aliquots to chloroplasts. Two addition protocols were tested: (1) the chloroplast sample was treated with a particular quinone for 5 min before adding the appropriate thiol; (2) a quinone-thiol combination was preincubated for 5 min before the mixture was added to a chloroplast sample. Generally, a smaller concentration of thiol was required to restore chlorophyll fluorescence when the quinone and thiol were mixed prior to adding to chloroplasts than when the thiol was added to quinone-treated chloroplasts. No effect of the order of addition of reagents was observed for 1,4-anthraquinone and DTT.

Table IV summarizes the results of fluorescence measurements of quinone-treated chloroplasts resuspended in quinone-free media. No simple dependence of fluorescence recovery on the single parameter of quinone hydrophobicity (as measured by Hansch π-values [27,28] for quinone substituents) was observed. However, for the 9,10-anthraquinones examined, the extent of fluorescence recovery showed a dependence on the degree

TABLE II

Fluorescence quenching reversal by addition of dithiothreitol

This table presents the results of the addition of dithiothreitol (DTT) to barley chloroplasts treated with 100 μM quinone. The parameters tabulated for each quinone tested include: minimum thiol concentration required to achieve maximum fluorescence quenching reversal, $[\text{thiol}]_{max}$; thiol concentration for half-maximum reversal ($[\text{thiol}]_{1/2}$); maximum chlorophyll fluorescence intensity (I_R) at 684 nm obtained upon thiol addition as a percentage of the chlorophyll fluorescence level (I_0) in quinone- and thiol-free samples; and the chlorophyll fluorescence intensity (I_Q) measured in quinone-treated samples at 684 nm as a percentage of the chlorophyll fluorescence level (I_0) in untreated samples. The + symbol in the $[\text{thiol}]_{max}$ column range indicates that quenching reversal had not reached a well-defined maximum level at [thiol] = 800 μM. Uncertainties are expressed as average deviations of mean values calculated using 4–6 measurements.

Quinone	$[\text{DTT}]_{max}$ (μM)	$[\text{DTT}]_{1/2}$ (μM)	$\frac{I_R}{I_0} \times 100\%$	$\frac{I_Q}{I_0} \times 100\%$
2,3,5,6-Tetramethyl-BQ	800+	360±20	111 ±8	73.7±1.3
DBMIB	800+	140±10	151 ±3	14.6±0.6
2-bromo-NQ	800+	50±10	91.9±1.6	19.3±0.6
2,3-Dichloro-NQ	140±10	100±10	66.8±0.6	18.2±0.4
5,8-Dihydroxy-NQ	60±10	40±10	33.8±0.7	26.9±1.4
	800+	500±20	58.9±1.1	
5-Hydroxy-NQ	140±10	60±10	60.8±0.7	40.7±0.7
	800+	750±40	108 ±1	
1,4-Anthraquinone	140±10	20±10	56.9±0.7	32.0±0.9
1,8-Dihydroxy-AQ	480±20	100±10	22.9±0.1	20.7±0.3
1-Amino-4-hydroxy-AQ	800+	300±20	47.6±0.7	43.8±1.3
1,2,5,8-Tetrahydroxy-AQ	800+	80±10	45.3±0.2	40.7±0.4
1,4-Dihydroxy-AQ	800+	140±10	17.0±0.3	15.9±0.8
2-Chloro-AQ	800+	300±20	56.9±0.2	51.7±1.4

of quenching originally induced by the quinone, as presented in Table V. This dependence is graphically illustrated in Fig. 2, where the maximum chlorophyll fluorescence intensity after resuspension of such chloroplasts is plotted as a function of the fluorescence intensity measured after the original incubation of chloroplasts with quinone. For those quinones which lowered the fluorescence level to below 70% of the intensity measured for untreated chloroplasts, there appears to be a linear dependence of recovered fluorescence intensity on quenched fluorescence intensity. As previously described [3], the fluorescence quenching activity of 9,10-anthraquinones is governed by numerous factors, including the hydrophobicity, position, and electronic characteristics of substituents on the terminal rings.

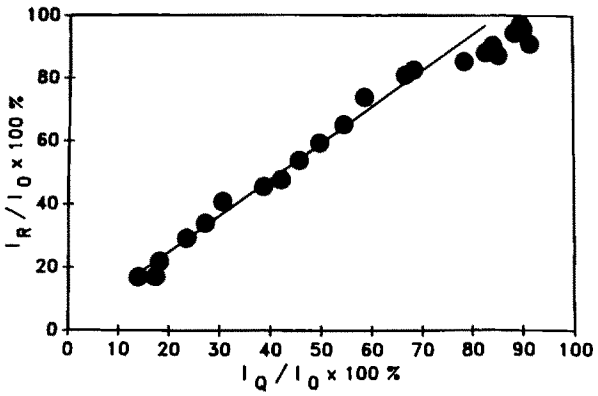

Fig. 2. The maximum chlorophyll fluorescence intensity of quinone-treated barley chloroplasts after resuspension of such chloroplasts in quinone-free media plotted as a function of the fluorescence intensity measured after the original incubation of chloroplasts with 100 μM quinone. A linear relation between recovered fluorescence intensity and quenched fluorescence intensity is indicated for quinones which lowered the chlorophyll fluorescence level to below 70% of the intensity measured for untreated chloroplasts.

Discussion

Thiol studies

The data presented support the concept of a dynamic and generally reversible interaction of added quinones with the photosynthetic membrane. The extent of reversibility, as measured by the restoration of chlorophyll fluorescence to pre-quinone incubation levels, is variable and dependent upon both the quinone and the thiol used to modify the quinone's effectiveness as an inhibitor of the photosynthetic process. For example, the quenching action of substituted benzoquinones was most likely to be affected by added thiol, while the effect of the 9,10-anthraquinones was not altered by thiol, presumably because a 1,4-Michael addition to the central ring was not possible. Furthermore, dithiothreitol, with two thiol moieties, required the lowest concentrations to induce fluorescence quenching reversal, while glutathione, the most sterically bulky of the thiols used, generally restored the quenched fluorescence by the lowest amounts.

The incomplete reversibility of fluorescence quenching suggests that the quinone inhibition site is not freely accessible to added thiol. A saturation of fluorescence restoration at thiol concentrations below the upper limit of 800 μM suggests that at least some of the quinone inhibition sites are shielded from the external medium, limiting the facility with which a thiol approaches a quinone. Furthermore, the lower chlorophyll fluorescence level when thiols are added to quinone-treated chloroplasts than when pre-incubated quinone and thiol solutions are added to chloroplasts further indicates the inaccessibility of some membrane-incorporated quinone to added thiol.

Resuspension studies

A closer examination of the results of Table V reveals a dependence for the extent of fluorescence re-

TABLE III

Effect of the order of addition of various thiols and quinones on fluorescence quenching recovery

This table presents a comparison of thiol-induced reversals of chlorophyll fluorescence quenching as the order of addition of reagents is varied. A restoration of fluorescence was achieved by either (1) preincubating quinone and thiol for 5 min before adding to a chloroplast sample or (2) incubating a chloroplast sample with a particular quinone for 5 min before adding a thiol sample. Final chloroplast samples contained [Chl] = 10 μg/ml, [quinone] = 100 μM, and [thiol] = 0–800 μM. Fluorescence measurements are reported for 684-nm emission and are expressed as the maximum chlorophyll fluorescence intensity (I_R) obtained after treatment (1) or (2) as a percentage of the chlorophyll fluorescence level (I_0) in chloroplast samples with no added quinone or thiol. Also listed are the minimum thiol concentrations needed for maximum fluorescence reversal, $[thiol]_{max}$, and for half-maximum reversal, $[thiol]_{1/2}$. Uncertainties are expressed as average deviations of mean values obtained using 4–6 measurements.

Quinone/thiol	Preincubation of quinone + thiol			Preincubation of chloroplasts + quinone		
	$\frac{I_R}{I_0} \times 100\%$	$[Thiol]_{max}$ (μM)	$[Thiol]_{1/2}$ (μM)	$\frac{I_R}{I_0} \times 100\%$	$[Thiol]_{max}$ (μM)	$[Thiol]_{1/2}$ (μM)
DBMIB/MER	122±3	220±10	180±10	110±3	480±20	210±10
2,3-Di-Cl-NQ/DTT	79±1	60±10	20±10	66±1	140±10	100±10
2,3-Di-Cl-NQ/MER	85±2	360±20	140±10	72±2	480±20	350±20
1,4-Anthraquinone/DTT	57±1	400±20	20±10	57±1	400±20	20±10

covery expressed as the parameter $I_R/I_0 \times 100\%$ on both quinone hydrophobicity and substituent position and identity. Generally, low values of this recovery parameter, i.e., less than 65%, were obtained for all quinones with a hydrophobic substituent, e.g., chloro or ethyl groups. Higher values (greater than approx. 75%) of this recovery parameter were measured for quinones with hydrophilic substituents (e.g., hydroxy, amino, and sulfonic acid groups), except for 1-hydroxy-anthraquinone and for any anthraquinone with hydroxy and/or amino substitution at two or more of the 1,4 and 8 positions with only hydrogen present at the 2-position. This latter group of quinones displayed low values of $I_R/I_0 \times 100\%$ below 55%, with particularly low degrees of fluorescence recovery for chloroplasts treated with the strongest 9,10-anthraquinone quenchers at 100 μM 1-hydroxy; 1,4-dihydroxy; and 1,8-dihydroxy-AQ ($I_R/I_0 \times 100\% = 17$–$21\%$).

Whenever an incomplete (or more than 100%) recovery of fluorescence is observed in either reversal study, consideration must also be given to the hypothesis that addition and/or removal of a quinone from its interaction site may alter the conformation of that region. Conformationally induced fluorescence variations are consistent with both proposed mechanisms of chlorophyll fluorescence quenching by added quinone. For example, it is well known that chlorophyll fluorescence emission can be affected by changes in the organization of the chlorophyll-protein complexes of the thylakoid membrane [29,30]. Furthermore, there is evidence that some Q_B inhibitors distort the spatial

TABLE IV

Fluorescence quenching recovery by resuspension of quinone-treated chloroplasts in quinone-free media

This table presents the results of experiments which attempted to remove added quinones and restore the chlorophyll fluorescence level in quinone-treated chloroplasts through a concentration gradient established by resuspension of the chloroplasts in quinone-free media. The maximum amount of the quenched fluorescence that was recovered by this treatment is tabulated as a percentage of the total amount of fluorescence quenched by added quinone at 100 μM concentration. Fluorescence recovery is reported for 684-nm emission. Also tabulated is $\Sigma\pi$, the sum of the Hansch π-values [27,28] for the substituents present on the quinone ring as a measure of the substituent hydrophobicity, assuming π-value additivity for multiple substitution [28] and assuming no intramolecular interaction of substituents [3]. Uncertainties are expressed as average deviations of mean values calculated using 5–9 measurements.

Quinone	Maximum % recovery	$\Sigma\pi$
2,3,5,6-Tetrachloro-BQ	100 ±11	1.64
2,3,5,6-Tetramethyl-BQ	80 ± 6	2.24
2-Amino-3-chloro-NQ	86 ± 3	−0.82
2-Methyl-NQ	44 ± 4	0.56
2,3-Dichloro-NQ	7.4 ± 0.7	0.84
2-Methoxy-NQ	6.2 ± 1.5	−0.02
1,2-Dihydroxy-AQ	96.7 ± 0.6	−1.34
2-Sulfonic acid-3,4-dihydroxy-AQ	85 ±12	−6.10
2,6-Diamino-AQ	53 ± 9	−2.46
1,2-Diamino-AQ	43 ± 6	−2.46
2-Carboxylic acid-AQ	43 ± 2	−0.32
Unsubstituted-AQ	39 ± 6	0.00
1,2,4-Trihydroxy-AQ	37 ± 5	−2.01
1,4,5,8-Tetraamino-AQ	34.1 ± 0.8	−4.92
1-Chloro-AQ	32 ± 1	0.41
1,5-Diamino-AQ	31.1 ± 0.8	−2.46
1-Amino-4-hydroxy-AQ	28.9 ±0.9	−1.90
2-Ethyl-AQ	24 ± 4	1.02
2-Chloro-AQ	20 ± 1	0.41
1,4-Diamino-AQ	15.3 ± 0.7	−2.46
1,5-Diamino-4,8-dihydroxy-AQ	11.3 ± 0.7	−3.80
2-Carboxylic acid-3-chloro-AQ	9.3 ± 0.3	0.39
2-Amino-AQ	5.8 ± 4.8	−1.23
1,2,5,8-Tetrahydroxy-AQ	4.5 ± 0.6	−2.68
1,4-Dihydroxy-AQ	3.6 ± 0.4	−1.34
1,8-Dihydroxy-AQ	3.2 ± 0.4	−1.34
1-Hydroxy-AQ	0.59± 0.06	−0.67
1-Amino-AQ	0 ± 2	−1.23
1-Amino-2-methyl-4-bromo-AQ	0 ± 1	0.19

TABLE V

Dependence of fluorescence quenching reversal by resuspension of quinone-treated chloroplasts on quenching activity of quinone

This table presents the following data for a series of 9,10-anthraquinones: the maximum chlorophyll fluorescence intensities (I_R) obtained after resuspension of quinone-treated chloroplasts in quinone-free media as percentages of the chlorophyll fluorescence level (I_0) in untreated samples, and the chlorophyll fluorescence intensities (I_Q) measured in quinone-treated samples as a percentage of the chlorophyll fluorescence level (I_0) in untreated samples. All uncertainties are average deviations of mean values obtained using 5–9 measurements and are less than or equal to ± 1% unless otherwise noted. Fluorescence levels refer to measurements made at 684 nm.

9,10-Anthraquinone	$\frac{I_Q}{I_0} \times 100\%$	$\frac{I_R}{I_0} \times 100\%$
1,4-Dihydroxy	14	17
1-Hydroxy	17	18
1,8-Dihydroxy	18	21
1-Chloro	24	29
1,4,5,8-Tetraamino	27	34
1-Amino-4-hydroxy	30	41
1,5-Diamino-4,8-dihydroxy	39	45
3-Chloro-2-carboxylic acid	42	48
1,4-Diamino	46	54
2-Chloro	50	60
2-Ethyl	54 ± 2	65 ± 2
1,2,4-Trihydroxy	59	74 ± 2
1,2-Diamino	67	81 ± 2
2-Carboxylic acid	68	82
2-Amino	78	86
1-Amino	85	85
1,5-Diamino	83	88
Unsubstituted	84	90
1-Amino-2-methyl-4-bromo	92 ± 2	92
2,6-Diamino	89	95
1,2-Dihydroxy	90 ± 2	97
3,4-Dihydroxy-2-sulfonic acid	90	98

arrangement of amino acid residues near the binding niche as the inhibitors approach the binding site on the D-1 protein [21]. Thus, the inability to restore fluorescence to its original intensity may partially reflect subtle quinone-induced changes in D-1 protein conformation. As a consequence, rebinding of the Q_B plastoquinone may be impaired to alter electron flow and prevent the recovery of fluorescence to normal levels. Similar alterations of Q_B binding efficiency have been observed to arise from conformational changes induced by tris treatment [21,31].

In summary, we have shown that the effect exerted by added substituted quinones on the chlorophyll fluorescence of plant chloroplasts can generally be diminished by techniques which serve to remove the quinone from its site of action. We can conclude that added quinones are not irreversibly bound inhibitors of chlorophyll fluorescence emission, but that noncovalent forces (e.g., electrostatic interactions or hydrogen bonding) are likely to be responsible for the quinone-membrane interactions. Restoration of chlorophyll fluorescence levels in quinone-incubated chloroplasts is dependent on both the quinone and the removal technique employed.

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