A Chloroplast Photosystem 2 Reaction Resistant to Salicylaldoxime

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Photoreduction of the artificial electron acceptor, cytochrome c, by isolated spinach chloroplasts in the absence of added catalyst has been found to be resistant to inhibition by 10 mM salicylaldoxime. This contrasts with the pronounced inhibition of the photoreduction of both 2,6-dichlorophenolindophenol and ferricyanide over a range of electron flow rates, and indicates an inhibition on the acceptor side rather than on the donor side of photosystem 2 (PS2). All photoreductions were susceptible to inhibition by 3-(3′,4′-dichlorophenyl)-1, 1-dimethyl urea (DCMU). Salicylaldoxime (1–10 mM) inhibited electron flow from PS2 to P700, and also altered the chlorophyll fluorescence induction of dark-adapted chloroplasts. Salicylaldoxime concentrations up to 5 mM did not change the initial fluorescence level, F_0, or the initial slope, $(dF/dt)_0$, but lowered the final steady state fluorescence level, F_m, and the value of p_0 [$p_0 = (F_m - F_0)/F_m$]; an effect similar to that seen on 1 mM ferricyanide addition, thus indicating an induced oxidation of the fluorescence quencher, Q. Both DCMU and orthophenanthroline, which block electron transport directly after Q, caused an increase in $(dF/dt)_0$, F_0 and F_m but left p_0 unchanged. This contrasted with the effect of salicylaldoxime.

It is proposed that salicylaldoxime inhibits electron transport from PS2 to photosystem 1, at a site which can probably be identified with the plastoquinone pool, by inducing a cyclic flow of electrons around PS2. Cytochrome c, under the conditions used, appears to be photoreduced at a site close to the secondary electron acceptor, R, of PS2.

Introduction

Salicylaldoxime was introduced by Green *et al.* as an inhibitor of photosynthesis with a mode of action which probably depended upon its ability to chelate copper [1]. Later work showed that it had no effect upon isolated plastocyanin, a blue copper-protein required for electron transport from photosystem 2 (PS2) to photosystem 1 (PS1), and that its site of inhibition must be sought elsewhere [2]. A recent thorough study of Berg and Izawa has shown that whereas high concentrations of salicylaldoxime were found to inhibit PS1 and PS2 irreversibly, and to alter the thylakoid membrane structure, concentrations less than 10 mM only inhibited PS2 electron transport and photophosphorylation in a reversible manner [3]. They suggested from experiments with artificial electron donors and acceptors that a site of salicylaldoxime inhibition existed on the donor side of PS2, in agreement with the conclusions of Katoh [4].

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; FeCN, potassium ferricyanide; SAL, salicylaldoxime; OP, orthophenanthroline; PS2, photosystem 2.

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Cytochrome c has been used as an artificial electron acceptor from PS1 on the addition of catalysts such as ferredoxin [5] and various quinones [6]. When no catalyst is added a comparatively slow photoreduction can still be observed, and this endogenous photoreduction has been shown by Keister and San Pietro to take place at a site distinct from that seen with added catalyst, and probably close to PS2 [5]. The endogenous cytochrome c photoreduction has been used in this report to localise more precisely the site of inhibition of 10 mM salicylaldoxime.

Materials and Methods

Chloroplasts were prepared from spinach 3–4 weeks old, grown in a greenhouse at 24 °C in a 16 h light/8 h dark regime. About 10 g leaf was homogenised in 30 ml of a medium containing 0.4 M sucrose, 10 mM NaCl and 50 mM potassium phosphate, pH 6.5, filtered through nylon net, and the filtrate centrifuged at $200 \times g$ for 1 min to remove large debris. The chloroplasts were sedimented at $5000 \times g$ for 8 min and resuspended in a hypotonic medium, containing 50 mM potassium phosphate pH 6.5 and 10 mM NaCl for all experiments. All steps were carried out at 0–4 °C. Chloroplasts prepared in this way were found to have low rates of ferricyanide photoreduction unless the uncoupler

NH_4Cl was added. The chlorophyll concentrations were measured according to Bruinsma [7]. 1 mM 2,6-dichlorophenolindophenol, sodium salt (DCPIP) was freshly prepared and filtered before use. 10 mM potassium ferricyanide (FeCN) solution was freshly prepared and stored in the dark. Horse heart cytochrome c type III was obtained from Sigma and was stated to be salt-free. Salicylaldoxime was first dissolved in methanol and then diluted with water to give a 100 mM solution in 10% methanol.

Photoreduction of Hill oxidants was followed on a Cary 14 recording spectrophotometer modified as described by Massini and Voorn [8]. The sample solution was maintained at 21 °C and stirred magnetically, it was illuminated by light passing through a Schott RG2 red cut-off filter/Balzer Calflex filter combination. The voltage to the lamp was adjustable and allowed a variation in illumination intensity. DCPIP reduction was observed by the decrease in absorption at 620 nm, employing a molar absorption coefficient at pH 6.5 of 18000 [9]. Photoreduction of ferricyanide was followed in the presence of 2 mM ammonium chloride by the decrease in absorbance at 420 nm using a molar absorption coefficient at this wavelength of 1070. Cytochrome c photoreduction was observed as an increase in absorbance at 550 nm, assuming the difference in molar absorption coefficient of oxidised and reduced forms at this wavelength to be 19000 [5]. Addition of 1 mM potassium cyanide could prevent cytochrome c reoxidation.

Chlorophyll fluorescence was measured at room temperature in an apparatus constructed by Dr. Elgersma of Philips Research Laboratories, and described in a previous publication [10]. Fluorescence was excited by 520 nm light (intensity 1 $W \cdot m^{-2}$) and monitored at 90° to the excitation beam by an EMI 9558 photomultiplier protected by a Schott RG 665 cut-off filter. The sample, in a 10×10 mm cuvette had a concentration of 2.7 μg chlorophyll $\cdot ml^{-1}$, and was dark-adapted for 5 min before the experiment. The fluorescence induction of chloroplasts kept as a concentrated suspension was found to be reproducible even after 5 h at 0 °C.

P700 photo-oxidation was measured in an Aminco-Chance dual-wavelength spectrophotometer, using broad-band blue side illumination (intensity 90 $W \cdot m^{-2}$) and monitoring the absorbance change A700 − A740. The photomultiplier was protected by a Schott RG 665 red cut-off filter. The sample had a pathlength of 10 mm and a concentration of 45 μg chlorophyll $\cdot ml^{-1}$. 0.3 M sucrose was present in the sample suspension to minimise settling of the chloroplasts. In all experiments 25 μM methylviologen (MV) was added to increase electron flow through P700.

Results

The photoreduction of cytochrome c by chloroplasts in the absence of added cofactors (endogenous photoreduction) was not inhibited by concentrations of salicylaldoxime up to 10 mM, but was completely inhibited by 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea (DCMU), (see Fig. 1 A and B). In control experiments, the addition of 1% methanol

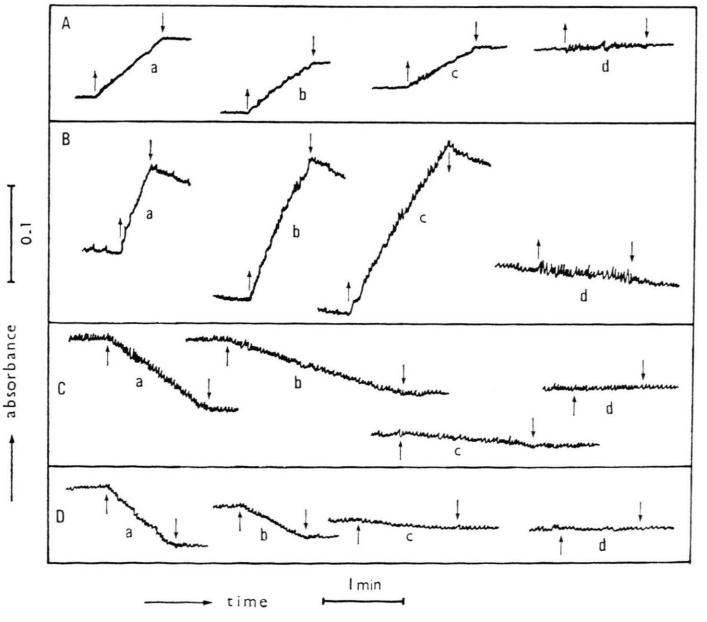

Fig. 1. The effect of salicylaldoxime and DCMU on the photoreduction of cytochrome c (A and B), ferricyanide (C) and DCPIP (D) by isolated spinach chloroplasts. The sample volume of 3 ml of 50 mM potassium phosphate buffer pH 6.5 with 10 mM NaCl, contained in (A) 1.2 mg cytochrome c, 3 μmol KCN and 16 μg chlorophyll; in (B) 2.7 mg cytochrome c, 44 μg chlorophyll and no KCN; in (C) 3 μmol potassium ferricyanide, 6 μmol NH_4Cl and 44 μg chlorophyll; and in (D) 0.3 μmol DCPIP and 16 μg chlorophyll.
The side illumination had an intensity of 20 $W \cdot m^{-2}$, an upward arrow indicates light on and a downward arrow light off. The sample temperature was 21 °C. Cytochrome c reduction was observed at 550 nm, ferricyanide reduction at 420 nm and DCPIP reduction at 620 nm. The following additions were made: (a) none, (b) 5 mM salicylaldoxime, (c) 10 mM salicylaldoxime and (d) 13 μM DCMU. Other details are given in the text.

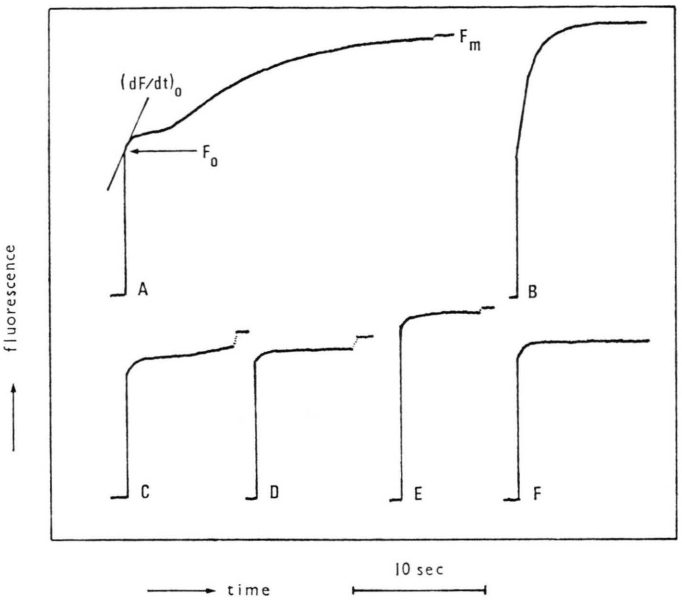

Fig. 2. Chlorophyll fluorescence induction in dark-adapted isolated spinach chloroplasts. Additions were made as follows: (A) none; (B) 50 μM orthophenanthroline; (C) 1 mM, (D) 5 mM and (E) 10 mM salicylaldoxime; and (F) 1 mM potassium ferricyanide.
The chloroplasts, at a concentration of 2.7 μg chlorophyll·ml^{-1} in 50 mM potassium phosphate buffer with 10 mM NaCl, were placed in a 10× 10 mm cuvette. Fluorescence was excited by 520 nm light, intensity 1 W·m^{-2}, and the emission monitored at 90°. In (A), (C), (D) and (E) the final steady state level (F_m) is shown. F_0 is the initial fluorescence level, and $(dF/dt)_0$ is the initial slope. Fluorescence is expressed on a linear scale in arbitrary units, which is the same for all traces.

was found to give a slight inhibition of cytochrome c photoreduction, which increased progressively with the length of incubation. This gave rise to an apparent slight inhibition (up to 20%) of cytochrome c photoreduction by 10 mM salicylaldoxime in some cases. The photoreduction of both 2,6-dichlorophenolindophenol and potassium ferricyanide was already inhibited about 50% by 5 mM salicylaldoxime, and completely inhibited by 10 mM salicylaldoxime (Fig. 1, C and D). In agreement with Berg and Izawa [3] the photoreduction of DCPIP, restored to inactivated chloroplasts by the addition of 10 mM hydroxylamine, was also inhibited by 10 mM salicylaldoxime. Katoh [4] also reported the inhibition by salicylaldoxime of electron flow from the artificial PS2 donor-couple ascorbate/hydroquinone. It is clear that the site of inhibition by 10 mM salicylaldoxime of DCPIP and FeCN photoreduction cannot be located on the donor side of PS2. When the electron flow rates with DCPIP and FeCN as Hill oxidants were reduced to the lower levels observed with cytochrome c the inhibition by salicylaldoxime was still apparent (Table I). Therefore the lack of inhibition cannot be ascribed to the slower electron flow rate with cytochrome c. The range of cytochrome c reduction rates observed (35 – 90 μmol·mg chlorophyll^{-1}·h^{-1}) agrees well with values reported by Keister and San Pietro [5] under optimal conditions of illumination, pH and cytochrome c concentration (50 – 90 μmol·mg chlorophyll^{-1}·h^{-1}).

When 10 mM salicylaldoxime was present the P700 content of chloroplasts became more oxidised upon illumination compared to untreated chloroplasts, and in addition the re-reduction of P700 in the dark was much slower. The results (not illustrated) indicate an inhibition of electron flow from PS2 to P700 by salicylaldoxime.

The effect of salicylaldoxime on room temperature chlorophyll fluorescence from dark-adapted chloroplasts is complicated and the results of Katoh [4] are in conflict with the recent report of Berg and

Table I. Comparison of the effectiveness of salicylaldoxime inhibition of 2,6-dichlorophenolindophenol, ferricyanide and cytochrome c photoreduction by spinach chloroplasts at comparable electron flow rates.

Acceptor	Uninhibited rate [μmol·mg chl^{-1}·h^{-1}]	% inhibition by 5 mM SAL
DCPIP	42 (84)	40
DCPIP	20 (40)	50
FeCN	87 (87)	59
FeCN	29 (29)	54
Cytochrome c	39 (39)	0

The uninhibited rate with DCPIP and FeCN as acceptor was adjusted approximately to that seen with cytochrome c by lowering the intensity of the side illumination. The figure given in brackets is the corresponding rate expressed in electron equivalents. Chl = chlorophyll.

Table II. Characteristics of the chlorophyll fluorescence induction in dark-adapted spinach chloroplasts on various additions.

	Addition	$(dF/dt)_0$	F_0	F_m	p_0
Experiment 1	none	7.0	41	68	0.40
	1 mM FeCN	6.8	42	47	0.11
	1 mM SAL	6.0	37	50	0.26
	5 mM SAL	3.0	41	49	0.16
	10 mM SAL	6.2	52	60	0.13
	10 mM SAL	6.0	51	57	0.11
Experiment 2	none	9.8	39	75	0.48
	1.0% MeOH	8.8	39	67	0.41
	10 mM SAL	10.0	62	70	0.11
	8 μM DCMU	63.6	55	119	0.54
	8 μM DCMU	71.5	64	125	0.48
	50 μM OP	24.6	42	82	0.49
	100 μM OP	23.6	41	77	0.48
	500 μM OP	46.0	50	102	0.51

F_0 is the initial fluorescence level, F_m is the final steady state fluorescence level, and p_0 is given by: $p_0 = (F_m - F_0)/F_m$. The initial slope, $(dF/dt)_0$, is the rate of increase of fluorescence at time zero (see Fig. 2A).

Izawa [3]. Fig. 2 (C and D) shows that the addition of salicylaldoxime up to 5 mM caused a decrease in the variable part of the fluorescence but that the constant part (F_0) was unaffected. When the salicylaldoxime concentration was increased to 10 mM (Fig. 2 E), the variable fluorescence was practically zero and F_0 increased markedly, in agreement with Berg and Izawa [3], but in contrast to the decrease in F_0 observed by Katoh with 10 mM salicylaldoxime [4]. In Table II the characteristics of these fluorescence induction curves are compared with those found on addition of DCMU, orthophenanthroline (Fig. 2 B) and ferricyanide (Fig. 2 F). The effect of increasing salicylaldoxime concentrations was to leave the initial slope, $(dF/dt)_0$, unchanged whilst p_0 decreased steadily. A very similar effect was seen on addition of 1 mM ferricyanide. In contrast, both DCMU and orthophenanthroline caused a marked increase in the initial slope compared to untreated chloroplasts but p_0 remained unchanged. It is clear that the site of action of salicylaldoxime is not identical to that of DCMU, and rather than cause a more rapid reduction of the fluorescence quencher, Q, it appears to bring about its oxidation.

Discussion

The use of artificial electron donors and acceptors which can interact with the photosynthetic electron transport chain at defined sites has been a productive approach in the investigation of the sequence of the electron transport components (see for example ref. 11). Combined with the use of inhibitors it is possible to study isolated sections of the electron transport chain, if the precise site of action of the inhibitor is known. The usefulness of salicylaldoxime as an electron transport inhibitor is restricted by our lack of knowledge of its mode of action, although the recent study of Berg and Izawa has demonstrated that for concentrations less than 10 mM, salicylaldoxime acts reversibly to block only PS2 electron transport and inhibit photophosphorylation [3]. Within the concentration range 0 – 10 mM it appears to have a relatively simple inhibitory effect which could perhaps be utilised to study the electron transport chain close to PS2. The necessity to use such high concentrations compared to other inhibitors could indicate that it acts on a component present in large amounts, or that it has a low affinity for the inhibition site. The results reported above on the effect of salicylaldoxime on P700 oxidation-reduction and chlorophyll fluorescence indicate a site of action within the thylakoid membrane rather than, for example, an interference by the inhibitor of access of Hill oxidants to the photoreduction site.

Berg and Izawa came to the conclusion that salicylaldoxime could act on the donor side as well as on the acceptor side of PS2 [3], which would introduce a complicating factor into its use as an inhibitor. However, the evidence for inhibition on the donor side rested mainly upon the ability of salicylaldoxime to inhibit DCMU-resistant oxygen evolution associated with the photoreduction of the silicomolybdate/ferricyanide couple. The same authors have stated in a later report on silicomolybdate reduction pathways, that great care must be taken in designing and interpreting experiments with silicomolybdate as a Hill oxidant [12]. It is clear from the results presented in this communication that salicylaldoxime at less than 10 mM does not inhibit the donor side of PS2 because cytochrome c photoreduction is completely unaffected by salicylaldoxime at this level (Fig. 1 and Table I). A possible explanation for the findings of Berg and Izawa [3], would be that the action of silicomolybdate is not simply that of an electron acceptor, and that in addition, it allows a release of the inhibition of electron transport by DCMU. The silicomolybdate/ferricyanide couple is then reduced by a component

of the electron transport chain located after the salicylaldoxime inhibition site, so that electron transport is susceptible to salicylaldoxime inhibition.

Katoh has also suggested that salicylaldoxime could act on the donor side of PS2 [4]. He observed that the extent of the variable fluorescence yield from chloroplasts had a similar dependence on the concentration of salicylaldoxime as was shown by the Hill activity. This evidence is however not conclusive, because a lowering in the apparent efficiency of PS2 to reduce the fluorescence quencher Q could also be brought about by a salicylaldoxime-induced oxidation of Q, as suggested by the data of Fig. 2 and Table II. It is possible that the mode of action of salicylaldoxime, acting on the acceptor side of PS2, is to disrupt the organisation of the electron transport chain and allow a reoxidation of Q by the donor side of PS2 perhaps via the plastoquinone pool.

From studies with the inhibitor dibromothymoquinone the sites of DCPIP and ferricyanide photoreduction have been shown to be situated at or after the plastoquinone pool [11]. The site of action of salicylaldoxime would therefore appear to be at or before the plastoquinone pool because both DCPIP and ferricyanide reduction are susceptible to salicylaldoxime inhibition (Fig. 1 and Table I). Cytochrome c photoreduction, although not inhibited by salicylaldoxime, is found to be inhibited by DCMU (Fig. 1), which is known to act directly after Q and probably at the level of the secondary electron acceptor, R [13]. The conclusion must therefore be that salicylaldoxime inhibits at the level of the plastoquinone pool. As plastoquinone is involved in the transport of protons across the thylakoid membrane, which is coupled to energy conservation [14, 15], the inhibition of photophosphorylation by salicylaldoxime [3], could also be explained by its action on plastoquinone function.

Finally, the site of "endogenous" photoreduction of cytochrome c would appear to lie very close to the reductive terminus of PS2, so that this Hill oxidant might be expected to be useful in an investigation of electron transport through the secondary electron acceptor, R, of PS2. The difference in the site of reduction of cytochrome c compared to that of DCPIP and ferricyanide can probably be related to the overall charge carried by these artificia electron acceptor molecules, although redox potential and molecular size will also probably play a role. There is good evidence that the thylakoid membrane surface is negatively charged (see for example recent work on 9-aminoacridine association with chloroplasts [16]). Cytochrome c may be able to interact with membrane surface sites not accessible to negatively-charged molecules because it carries a net positive charge at neutral pH (it is isoelectric at about pH 10, see for example ref. 17). This idea is supported by the inhibitory action of high cation concentrations on the "endogenous" cytochrome c photoreduction first reported by Keister and San Pietro [5]. The interaction of cytochrome c with membranes has been reviewed recently by Nicholls [18].

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