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Inhibitors affecting the oxidizing side of Photosystem II at the Ca^{2+}- and Cl^--sensitive sites

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In addition to compounds which inhibit the function of calmodulin (a ubiquitous calcium regularity protein found in plants and mammalian tissues), Ca^{2+} or Cl^- channel blockers in mammalian tissues were also found to inhibit electron transport in Photosystem II submembrane preparations. Their inhibition was overcome by electron donation to P-680 by diphenylcarbazide (for all of the compounds used) and by H_2O_2 (except with trifluoperazine). Addition of Ca^{2+} and/or Cl^- also partially prevented the inhibitory action. We postulate that the inhibitory action occurs at the level of the water-splitting system at the site of Ca^{2+} modulation of the Cl^- cofactor requirement for O_2 evolution (as hypothesized in Nakatani, H.Y. (1984) Biochem. Biophys. Res. Comm. 120, 299–304).

Introduction

The photosynthetic oxygen-evolving system is believed to involve the participation of three chloroplast thylakoid polypeptides (extrinsic to the lumenal membrane surface) having molecular weights of 16, 24 and 33 kDa [1–3]. The removal of these polypeptides from PS II submembrane preparations and inside-out chloroplast thylakoid vesicles results in a large inhibition of oxygen evolution whereas the re-addition of these polypeptides to depleted preparations has been shown to reconstitute this activity [4–7]. However, recent studies indicate that these proteins may not be directly implicated in the mechanistic photolysis of water [8,9]. It was suggested that the 16 and 24 kDa polypeptides may regulate the effect of Ca^{2+} and/or Cl^- ions [9]. Furthermore, the 33 kDa polypeptide which was initially thought to be the Mn-bearing protein, has recently been shown to be removable from Photosystem II membranes with partial loss of oxygen-evolving activity and no loss of Mn^{2+} [10].

The inhibition of water splitting in PS II preparations by calmodulin-type inhibitors [11] suggests the presence of a Ca^{2+}-binding protein in the oxygen-evolving complex. In this report, we describe the effects of a number of inhibitors, known to influence Ca^{2+} and/or Cl^- channels in mammalian tissues [12,13], which were found to affect the oxidizing side of PS II at a site sensitive to Ca^{2+} and/or Cl^-.

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Abbreviations: PS II, Photosystem II; DCIP, 2,6-dichlorophenolindophenol; Mes, 4-morpholineethanesulphonic acid; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic-stilbene; DIDS, diisothiocyano-stilbene-2,2′-disulfonic acid; DPC, diphenylcarbazide; DCMU, 3-(3,4′-dichlorophenyl)-1,1-dimethylurea.

Material and Methods

Photosystem II submembrane fractions were prepared from stroma-free thylakoids of spinach as described [9]. DCIP photoreduction was monitored as previously described [9]. Fluorescence induction kinetics were recorded at room temperature by an oscilloscope (Nicolet Instrument Corporation, Model 2090-III). The sample contained 10 μg Chl · ml^{-1} in 20 mM Mes-NaOH at pH 6.5. All of the tested chemicals were handled in dim light or complete darkness. Calmidazolium (R24571) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, trifluoperazine was a kind gift from Smith, Kline and French Laboratories, Philadelphia, PA and nifedipine was graciously supplied by Dr. J.R. Hume (Michigan State University). SITS, DIDS and antibiotics were graciously provided by Dr. E. McGroarty (Michigan State University).

Results

Several compounds which affect Ca^{2+}- or Cl^--channels of mammalian tissues (see Fig. 1) were found to inhibit DCIP photoreduction by PS II preparations. These were studied with respect to their site of action in photosynthetic electron transport. Most of the inhibitors used were more effective after a short period of incubation (up to 10 min for maximal effect). The I_{50} of these inhibitors (concentration giving 50% inhibition) for DCIP photoreduction with water as the electron source are listed in Table I. Since an incubation period was required to observe maximal inhibition, the I_{50} values may be regarded as tentative. All inhibitors affected the oxidizing side of PS II, since their inhibitory action could be overcome by DPC and $MnCl_2$, which act as electron donors to the oxidizing side of PS II.

Ca^{2+} antagonists

Calmidazolium and trifluoperazine, two inhibitors of calmodulin function [12,14], were previously reported to inhibit water photolysis at a site near H_2O_2 electron donation to PS II [11]. The inhibition of DCIP photoreduction by calmidazolium, but not trifluoperazine, could be overcome by hydrogen peroxide (Table I). That calmidazolium affects the oxidizing side of PS II is further confirmed by its effect on the chlorophyll fluorescence induction curve shown in Fig. 2. The addition of $4 \cdot 10^{-5}$ M calmidazolium resulted in a remarkable quenching of the variable fluorescence (Fig. 2). Such a quenching was previously shown to indicate effects on the oxidizing side of PS II [15]. Moreover, the inhibitory effect of both calmidazolium and trifluoperazine was overcome by DPC and $MnCl_2$ (Table I).

Nifedipine has been used as a calcium channel antagonist in mammalian skeletal and smooth

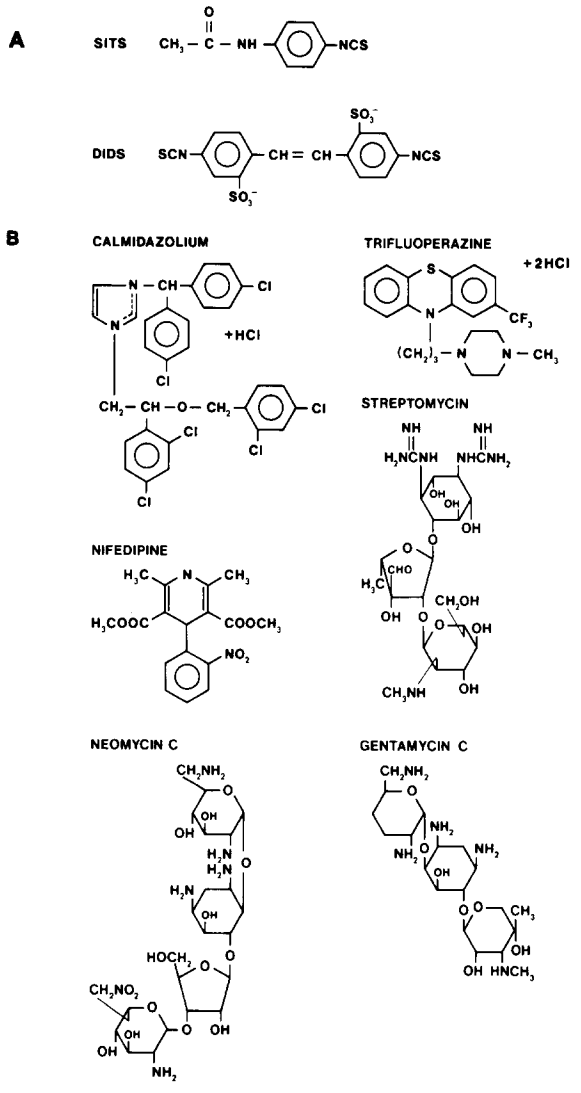

Fig. 1. Chemical structures of inhibitors of DCIP photoreduction (See Refs. 12 and 13). (A) Cl^--channel blockers; (B) Ca^{2+}-antagonists.

TABLE I

INHIBITION OF DCIP PHOTOREDUCTION BY Ca^{2+} OR Cl^- ANTAGONISTS IN PS II PREPARATIONS (SEE TEXT FOR MORE DETAILS)

Inhibitors	I_{50}	Restoration		
		H_2O_2 (0.03%)	DPC (0.5 mM)	$MnCl_2$ (0.1 mM)
Calmodulin inhibitors				
Calmidazolium	0.015 mM	++	++	+
Trifluoperazine	0.08 mM	-	++	+
Ca^{2+}-channel blocker				
Nifedipine	0.30 mM	++	++	+
Antibiotics				
Streptomycin	0.3 mg/ml	-	++	+
Neomycin	0.2 mg/ml	-	++	+
Gentamycin	0.3 mg/ml	-	++	+
Cl^--channel blockers				
DIDS	0.85 mM	++	++	+
SITS	0.85 mM	++	++	+

muscle and cardiac tissues [12]. We tested whether this compound could interact at the oxygen-evolving complex by assuming the presence of a polypeptide having similar characteristics, i.e., possible Ca^{2+}-binding characteristics rather than as a true Ca^{2+}-channel. Nifedipine was found to induce a strong inhibition of DCIP photoreduction which was, moreover, alleviated by the addition of H_2O_2 (Table I).

We also investigated the effects on PS II of some organic (antibiotic) cations (streptomycin, neomycin and gentamycin). We assumed that they might interact with the charged groups of the oxygen evolving complex at a Ca^{2+}-sensitive site, especially since neomycin was previously shown to target Ca^{2+}-channels in mammalian tissues [12]. The results are shown in Table I. All of the compounds examined were inhibitory in the DCIP photoreduction assay. The inhibition produced by these antibiotics could not be overcome by H_2O_2 as observed for trifluoperazine. Similar results were previously presented with trifluoperazine and metal cation antagonists of Ca^{2+} [9].

Chloride antagonists

The Cl^--cofactor requirement for oxygen evolution implicates a site for Cl^- binding in the oxygen-evolving complex. We, therefore, investigated the effect of SITS and DIDS (known Cl^--channel blockers in red blood cell membranes [13]). We found that they caused an inhibition of DCIP photoreduction at a site prior to the electron donation site of H_2O_2 (Table I). A site of action on the oxidizing side of PS II is also supported by the chlorophyll fluorescence data of Fig. 2. Upon addition of 1.2 mM SITS, we observed a decrease of fluorescence from the steady-state level attributable to a loss of variable fluorescence. The restoration of electron flow to P-680 by 10 mM NH_2OH reestablished a higher steady-state yield indicating action on the oxidizing side of PS II.

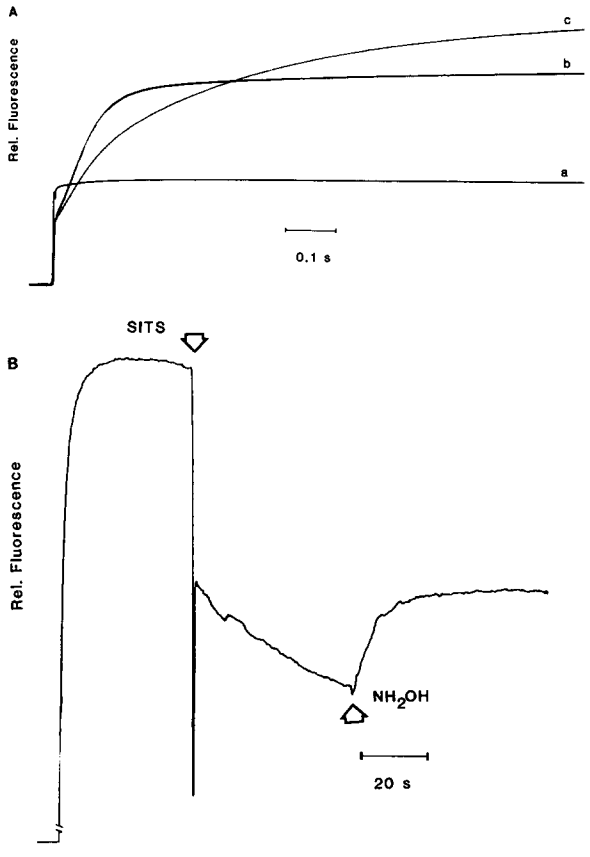

Fig. 2. (A) Effect of calmidazolium on the fluorescence induction transient: (a) $4 \cdot 10^{-5}$ M calmidazolium; (b) control; (c) $3 \cdot 10^{-6}$ M DCMU. The control sample has a higher F_{max} than that in the presence of DCMU very likely due to the presence of oxidized endogenous PQ. (B) Effect of 1.2 mM SITS on the steady-state yield of fluorescence. NH_2OH was added to a concentration of 10 mM. The initial rapid fluorescence decrease upon addition of the inhibitor is due to chlorophyll dilution and a mixing artifact.

Cl^- and Ca^{2+}-cofactor requirement

A number of salts were added to the PS II preparation prior to the introduction of the inhibitors used to determine any association of the inhibition with Ca^{2+} and/or Cl^- action. The results in Table II show that the salts generally affected the extent of inhibition in a specific fashion depending upon the presence of either Ca^{2+} or Cl^- and the type of inhibitor studied. The inhibitory effect of the calmodulin-type inhibitors was reduced by the presence of Cl^- ions and, to a lesser extent, by Ca^{2+} ions as shown in Table II. This effect was also observed for streptomycin and neomycin (Table II). In general, Ca^{2+} as the Cl^- salt relieved the extent of inhibition of all of the compounds tested with the exception of DIDS with which there was an increased inhibitory effect (see Table II). Calcium nitrate, however, had negligible effect, except with the Cl^- channel blockers where it enhanced their effectiveness. NaCl was partially effective in relieving the inhibitory effects of the calmodulin-type inhibitors. streptomycin and neomycin as well as the Cl^- channel blockers. NaCl was particularly effective in almost totally relieving the effect of SITS (see Table II). $CaCl_2$ increased the inhibitory effect of DIDS in contrast to suppressing the effect of SITS. $NaNO_3$ enhanced the inhibitory effect of nifedipine and partially relieved the effect of SITS (see Table II).

Discussion

We have identified a series of inhibitors which act on the oxidizing side of PS II in detergent-derived submembrane oxygen evolving PS II preparations from spinach (see also Ref. 20). These compounds were previously known as chloride and calcium channel blockers in other biological systems. Fig. 3 summarizes where these inhibitors are proposed to act in the electron-transport pathway involved in O_2 evolution. This model is based upon our studies of various partial reactions as summarized below.

Calmidazolium, nifedipine, DIDS and SITS cause inhibition of O_2 evolution when water acts as the electron donor, but not when H_2O_2 is the donor to PS II; gentamycin, trifluoperazine, streptomycin and neomycin block O_2 evolution when either H_2O or H_2O_2 are electron donors, although reaction center activity can be restored with electron donors thought to act at Z (DPC and Mn^{2+}). The differential activity of these inhibitors indicates the existence of an intermediate stage in

TABLE II

PREVENTION OF THE INHIBITION OF DCIP PHOTOREDUCTION BY Ca^{2+} OR Cl^--ANTAGONISTS BY THE PREADDITION OF SALTS (SEE TEXT FOR MORE DETAILS)

Values are the means of three experiments (maximal variation 12%). 100% corresponded to 564 μequiv. (per mg Chl per h). Measurements obtained directly after mixing.

Inhibitors	Concn.	Percent inhibition				
		No addition	NaCl (10 mM)	$CaCl_2$ (5 mM)	$NaNO_3$ (10 mM)	$Ca(NO_3)_2$ (5 mM)
Calmodulin inhibitors						
Calmidazolium	0.02 mM	58	42	49	49	52
Trifluoperazine	1.0 mM	64	47	53	61	75
Ca^{2+}-channel blocker						
Nifedipine	0.2 mM	39	42	25	50	36
Antibiotics						
Streptomycin	0.2 mg/ml	40	24	22	39	40
Neomycin	0.2 mg/nl	51	33	29	58	45
Gentamycin	0.2 mg/ml	37	36	32	40	35
Cl^--channel blockers						
DIDS	0.75 mM	47	34	80	46	83
SITS	0.80 mM	45	7	21	29	63

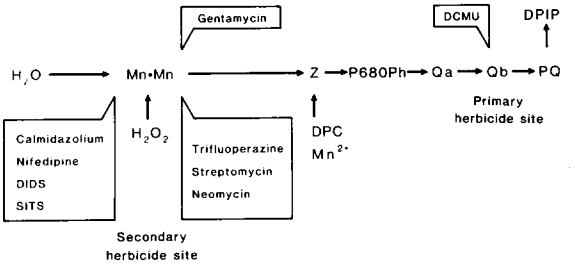

Fig. 3. Diagrammatic representation of the site of action of the Cl^-- and Ca^{2+}-antagonists on photosynthetic electron transport ($H_2O \rightarrow PQ$). P-680 Ph is the PS II reaction center chlorophyll *a* complex containing P-680 and pheophytin (Ph), see Refs. 16–18.

H_2O oxidation whose further oxidation is blocked by gentamycin, trifluoperazine, streptomycin and neomycin. The fact that all of these inhibitors are Ca^{2+} or Cl^- ion antagonists in other biological systems makes it likely that Ca^{2+} and Cl^- act to stabilize the 'conformation' of the partially oxidized ('cryptoperoxy') intermediate, presumably at the site at which Mn^{2+} is involved in the O_2 evolution reactions. This idea is born out by the observations that salt additions (Ca^{2+} and/or Cl^-), i.e., before the addition of inhibitor, can affect inhibitor activity. We conclude that the organic cations compete with Ca^{2+} for binding sites; these sites can either be at the Mn-Mn complex or at other Ca^{2+}-specific binding sites in the complex (16, 23 and 33 kDa proteins) that are known to participate in the physiological regulation of O_2 evolution [1–3,8]. The fact that high levels of Cl^- salts, in the absence of Ca^{2+}, can also affect the inhibitory action of most of these inhibitors suggests that the organization of the proteins in the oxygen-evolving complex is important where anion mediated conformational changes can be used to explain inhibitor exclusion. This is consistent with previous ideas suggesting that the 16, 23 and 33 kDa proteins are involved in Ca^{2+} and Cl^- mediated function at the oxygen evolving site [Refs. 8 and 9; see also Ref. 19].

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