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Salicylhydroxamic acid inhibits electron transfer through PSII

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Introduction

A variety of data support the existence of parts of a chlororespiratory electron transfer chain in the thylakoid membranes of higher plants and green algae which was proposed by Bennoun (1982). These data include genetic, biochemical and kinetic evidence for the existence of an NAD(P)H dehydrogenase analogous to the mitochondrial enzyme (Matsubayashi *et al.*, 1987; Groom *et al.*, 1993; Quiles *et al.*, 1996), and a little evidence supporting the presence of a succinate dehydrogenase in *Chlamydomonas reinhardtii* (Willeford *et al.*, 1989). However, Bennoun's original data showed that a variety of inhibitors and substrates of haem-containing terminal oxidases (CN^-, CO, N_3^-, NO and O_2) influence the yield of chlorophyll fluorescence from *Chlorella* or *Chlamydomonas* (Bennoun, 1982). Bennoun also showed that salicylhydroxamic acid (SHAM), an inhibitor of the mitochondrial alternative oxidase (AOX), affected the yield of chlorophyll fluorescence, an observation confirmed by Garab *et al.* (1989) in tobacco. These data might be consistent with the presence of a SHAM-sensitive AOX in the thylakoid membrane which is also consistent with the effects of O_2 observed by Damdinsuren *et al.* (1995).

We show that SHAM inhibits electron transfer through photosystem II (PSII) and that it decreases the size of the pool of electron acceptors as measured from chlorophyll fluorescence transients (Malkin and Kok, 1966; Kurreck *et al.*, 1999). While this does not eliminate the possibility of an AOX in the thylakoid membrane, the effect of SHAM on chlorophyll fluorescence can be explained by the inhibition of electron transfer through PSII.

Materials and methods

Thylakoid membranes were prepared from field-grown silver beet (*Beta vulgaris* L. cv. Fordhook Giant) according to a modification of the method of Robinson and Yocum (1980). About 50 g of leaves were ground in 100 ml of buffer (400 mM NaCl, 2 mM $MgCl_2$, 0.2% (*w*/*v*) BSA, 20 mM Tricine-AMPD pH 7.3) and filtered through two layers of miracloth. The filtrate was centrifuged at 3000 × *g* for 10 minutes and the pellet was resuspended in 40 ml of wash buffer (150 mM NaCl, 5 mM $MgCl_2$, 0.2% (*w*/*v*) BSA, 20 mM Tricine-AMPD pH 7.3) and centrifuged at 3000 × *g* for ten minutes. The pellet was resuspended in wash buffer to yield 1-2 mg chl ml^{-1}.

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Measurements of photosynthetic electron transfer were made polarographically at 25°C using a Clark-type O_2 electrode (Rank Brothers, Bottisham, UK) and data were acquired digitally (Brown and Dykstra, 1999). Actinic light was provided using a slide projector fitted with a red longpass filter (Schott RG645) to give 1000 µmoles photons m^{-2} s^{-1} at the cuvette.

Chlorophyll fluorescence transients were collected using a digital storage oscilloscope (Tektronix Instruments) and a fast-response silicon photodiode with an on-board amplifier oriented at 90° to the actinic light from which it was protected by a series of filters. The actinic light was provided by a custom-built bank of high intensity LEDs under computer control. The data were processed using software developed in-house.

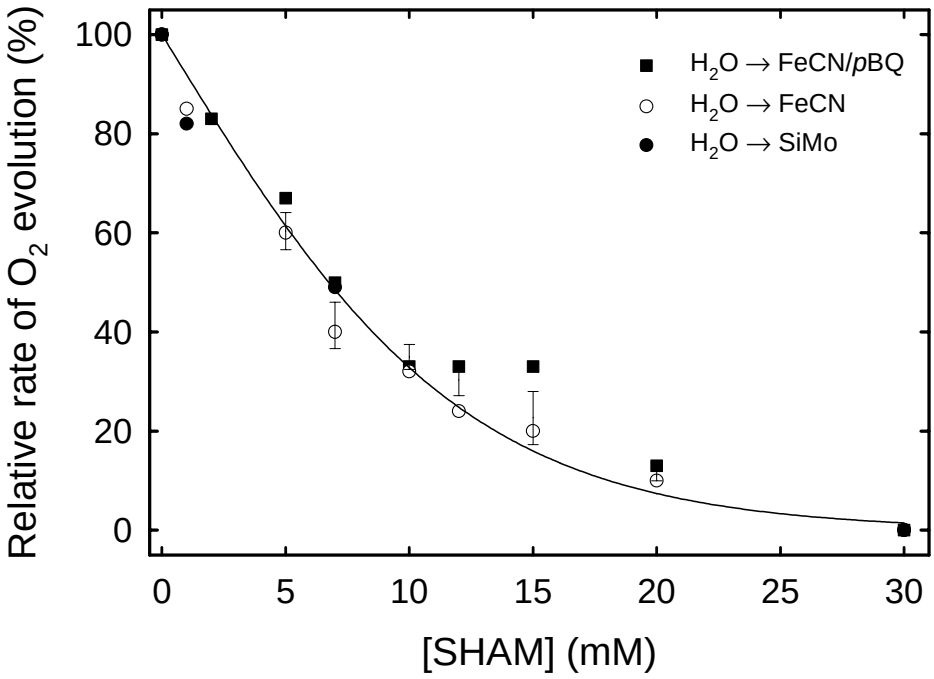

Figure 1. Effect of salicylhydroxamic acid (SHAM) on the rate of O_2 evolution by isolated thylakoid membranes. Oxygen evolution was measured polarographically in 25 mM HEPES-AMPD pH 7.0, in the presence of 2.5 mM NH_4Cl and 15 µg chl ml^{-1}. The electron acceptor was 0.5mM FeCN, with or without 130µM *p*BQ, or 50 µg ml^{-1} SiMo in the presence of 1 µM DCMU. The stock solution of SHAM was made up in 2ME. The rate of O_2 evolution in the absence of SHAM was 190-230 µmoles O_2 mg^{-1} chl h^{-1} using FeCN (± *p*BQ) or 30-50 µmoles O_2 mg^{-1} chl h^{-1} using SiMo. The titrations shown are representative of at least five experiments and the curve is a fit to the data intended to assist the eye.

Chlorophyll was determined using 80% acetone according to the method of Arnon (1949) and photon flux density was determined using an LI-189 quantum radiometer (Li-cor, Lincoln, Nebraska, USA). Salicylhydroxamic acid (SHAM) was made up in either 2-methoxyethanol (2ME) or dimethylsulphoxide (DMSO).

Results

Potassium ferricyanide (FeCN) accepts electrons from PSII or PSI, but in the presence of 1,4-benzoquinone (*p*BQ) it accepts from the PQ pool, whereas silicomolybdate (SiMo) accepts electrons from the vicinity of Q_A (Izawa, 1980). Electron transfer from H_2O to FeCN, to FeCN/*p*BQ or to SiMo, as measured by O_2 evolution, was inhibited by SHAM (figure 1), with an apparent K_I of 6.1 ± 0.2 mM. Electron transfer from H_2O to methyl viologen (MV) and

from 5,5'-diphenylcarbamate (DPC) to a variety of electron acceptors were similarly inhibited (data not shown). In contrast, electron transfer from ascorbate/2,6-dichlorophenolindophenol to MV, which represents electron transfer through the cytochrome b_6f complex and photosystem I, was not affected by up to 20 mM SHAM (data not shown).

The fluorescence induction curve in the presence of either FeCN, *p*BQ or MV as an electron acceptor was relatively slow, and the addition of 10 μM DCMU substantially accelerated the rise (figure 2) and completely eliminated O_2 evolution. However, the addition of both SHAM and DCMU accelerated the fluorescence rise even more (figure 2). The relative area over the induction curve, was decreased 22% by the addition of 20 mM SHAM in the presence of 10 μM DCMU.

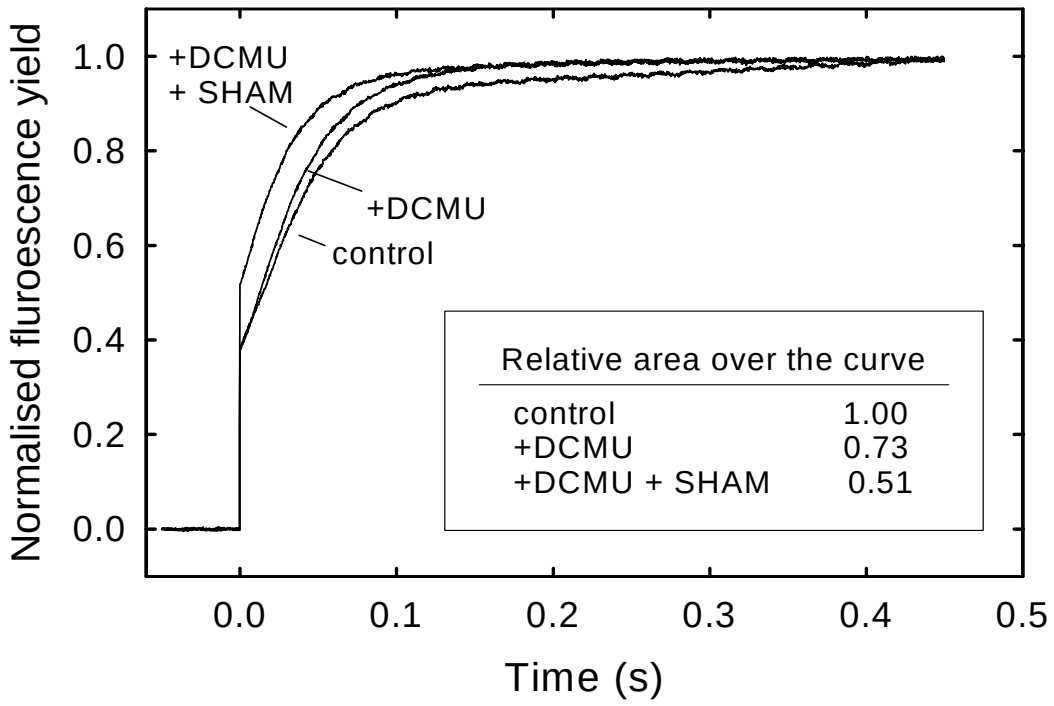

Figure 2. The effect of DCMU and SHAM on the fluorescence induction curve. Thylakoid membranes (10 μg chl ml^{-1}) were suspended in 25 mM MES-AMPD pH 6.5 (control), treated with 10 μM DCMU (+DCMU) or treated with both 10 μM DCMU and 20 mM SHAM (+DCMU +SHAM).

Discussion

We have shown that SHAM inhibits electron transfer through PSII before the site of SiMo reduction (figure 1) and that it can reduce the size of the pool of PSII electron acceptors more than DCMU (figure 2). From this we hypothesise that SHAM inhibits electron transfer from Q_A to Q_B. Since SHAM acts as an Fe(III) chelator (Špringer *et al.*, 1987), it is possible that SHAM disturbs the structure of the non-haem iron between Q_A and Q_B, thereby disrupting electron transfer. While Debus *et al.* (1986) showed that the removal of the non-haem iron from *Rhodobacter sphaeroides* reaction centres did not affect electron transfer, it is conceivable that the presence of SHAM in the vicinity of the quinones causes much more disruption than the simple removal of the iron. For example, Koulougliotis *et al.* (1993) showed that cyanide binds to the non-haem iron and affects Q_A-Q_B electron transfer. This is consistent with the effects of other chelators (such as *o*-phenanthroline) which also inhibit PSII electron transfer (Trebst, 1980). Inhibition by SHAM of electron transfer through PSII is consistent with the inhibition by SHAM of CO_2 assimilation reported by Diethelm *et al.* (1990).

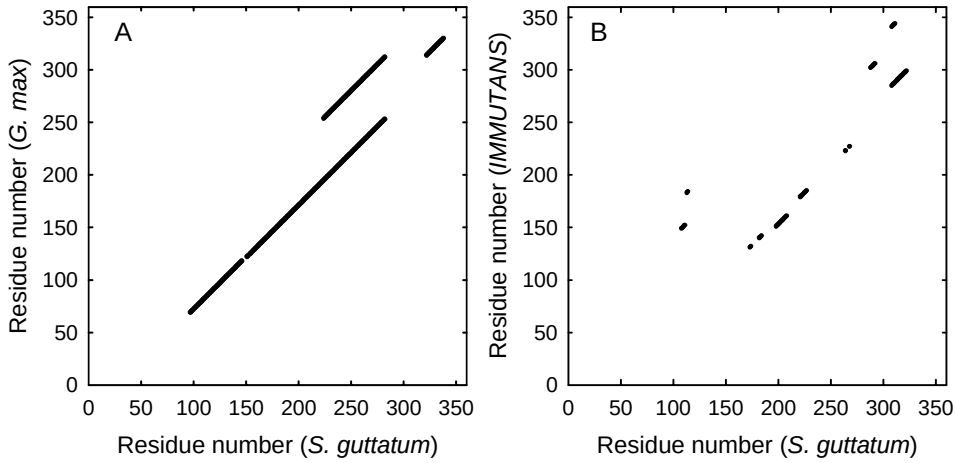

Figure 3. Intersequence comparisons between (A) alternative oxidases from soybean (*Glycine max*) and the voodoo lily (*Sauromatum guttatum*) and (B) the *IMMUTANS* protein from *A. thaliana* and the AOX from *S. guttatum*. The points represent local window scores averaging more than 1.5 using the PAM250 matrix and a 23 residue window. The average value of the scores plotted (± SEM) were (A) 3.56 ± 0.06 (n = 289) and (B) 1.64 ± 0.02 (n = 55).

In spite of the inhibition of electron transfer through PSII that we have identified, it is nevertheless possible that an oxidase does exist in the thylakoid membrane. Some support for this possibility is provided by the recent report that the 40.5 kD *IMMUTANS* protein identified in *Arabidopsis thaliana* has homology with the AOX (Wu *et al.*, 1999; Carol *et al.*, 1999). However, the *IMMUTANS* protein is at best only distantly related to the higher plant mitochondrial AOX (figure 3), as has been pointed out by Berthold *et al.* (2000).

Two models of the di-iron centre of the AOX have been proposed, but neither provides strong support for the hypothesis that the *IMMUTANS* protein has an homologous structure. Siedow *et al.* (1995) suggested that Glu270, His283, Glu319 and His322 (*S. guttatum* numbering) were among the ligands to the iron atoms. Of these four ligands, which are highly conserved among AOXs, only Glu319 and His322 are conserved in the *IMMUTANS* protein. Recently, Berthold *et al.* (2000) presented a revision of a later model in which Glu181, Glu217, His220, Glu268, Glu319 and His322 are involved in iron binding. All of these residues are conserved among the AOXs and the *IMMUTANS* protein, but these residues represent isolated regions of sequence identity that are sometimes only a single residue long (figure 4).

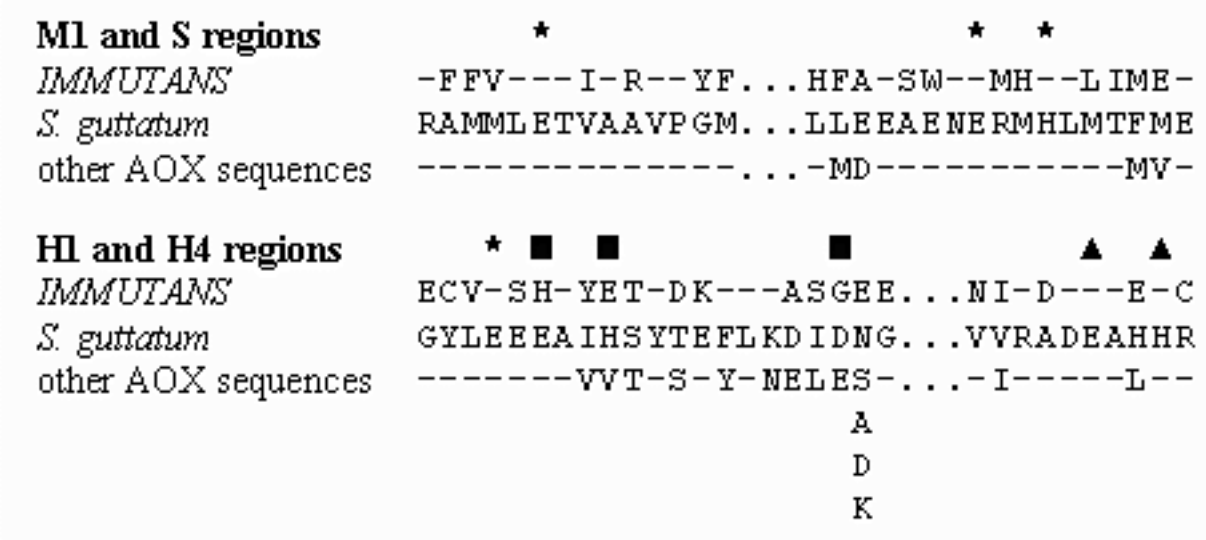

Figure 4. The M1, S, H1 and H4 regions (Vanlerberghe and McIntosh 1997) of the AOX sequence from *S. guttatum*. This was aligned with ten other AOX sequences and the *IMMUTANS* protein sequence. Variations from the *S. guttatum* sequence are shown, - indicates an identity with that sequence and . . . indicates a gap in all the sequences. Also indicated are the proposed ligands for the two iron atoms (* and ▲ are those of Berthold *et al.* (2000); ■ and ▲ are those of Siedow *et al.* (1995)).

Nevertheless, there are data which imply that the *IMMUTANS* protein does function as an oxidase. Josse *et al.* (2000) expressed the *IMMUTANS* gene in *Escherichia coli* and showed that 2 mM cyanide inhibited O_2 consumption by about 80%, compared with about 95% in membranes from the parent strain. However, the inhibition took a surprisingly long time to take effect given the cyanide concentration. They also showed that the residual O_2 consumption was inhibited by 300 µM *n*-propyl gallate, a concentration that was substantially greater than that required for inhibition in a suspension of *Arum maculatum* mitochondria (Hoefnagel *et al.*, 1995) or inhibition of the *A. thaliana* enzyme expressed in *E. coli* (Berthold, 1998). Josse *et al.* (2000) did not report on the effect of SHAM on the putative O_2 consumption by the *IMMUTANS* protein.

Our own data do not preclude the presence of a SHAM-sensitive oxidase, but they do support the suggestion that SHAM inhibits electron transfer through PSII (figures 1 and 2). However, if there is an oxidase in the higher plant thylakoid membrane, then the *IMMUTANS* protein is a possible candidate. That it has only weak sequence homology with AOXs (figures 3 and 4) and that it appears to require high concentrations of *n*-propyl gallate for inhibition (Josse *et al.*, 2000) provide only weak support for its proposed relationship with AOXs. Nevertheless, we are currently both pursuing this possibility and continuing our work on the mechanism of inhibition of PSII electron transfer by SHAM.

Acknowledgements

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