Site of action of a halogenated 4-hydroxypyridine on ferredoxin-catalysed cyclic photophosphorylation

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Tetrabromo-4-hydroxypyridine (J820) inhibited ferredoxin-catalysed cyclic photophosphorylation at micromolar concentrations but did not inhibit or uncouple the AQS-catalysed system. At 2 μM it did not abolish the slow phase of the electrochromic shift or affect the turnover of cytochromes *b*-563 and *f*. At higher concentrations (10 μM) it decreased the rate of re-reduction of cytochrome *f*, whilst inhibiting the reduction of cytochrome *b*-563. It is concluded that tetrabromo-4-hydroxpyridine does not bind to the quinone reduction site of the cytochrome *bf* complex, but inhibits the putative ferredoxin-plastoquinone reductase.

Cyclic photophosphorylation *Hydroxypyridine* *Antimycin* *Q-cycle* *Ferredoxin* *Anthraquinonesulfonate*

1. INTRODUCTION

Cyclic photophosphorylation catalysed by the endogenous cofactor ferredoxin is of physiological importance in chloroplasts [1]. A number of artificial cofactors can also catalyse cyclic phosphorylation and therefore cyclic electron transport [2,3]; however, many of these, unlike ferredoxin, bypass the cytochrome *bf* complex. Antimycin, at low concentrations, is an inhibitor of ferredoxin-catalysed cyclic phosphorylation [1], but has no effect with other cyclic cofactors [4] or with any non-cyclic system [5]. In mitochondria and purple photosynthetic bacteria, it inhibits the re-oxidation of the *b*-type cytochrome of the bc_1 complex [6,7]. By analogy, its effect in chloroplasts has been assumed to be within the Q-cycle scheme of Mitchell [8], at the site at which plastoquinone is reduced by cytochrome *b*-563 (QR site).

Certain halogenated 4-hydroxypyridines have been shown recently to inhibit both ferredoxin-catalysed cyclic phosphorylation and photosystem II, but to have no effect on other non-cyclic systems [9,10]. Hartung and Trebst have suggested that, like antimycin, they act on the QR (Q_c in [9]) site of the cytochrome *bf* complex. The most potent of the compounds studied was tetrabromo-4-hydroxypyridine (J820).

Other lines of evidence have suggested that antimycin may act on a distinct ferredoxin-plastoquinone reductase, rather than on the QR site [11]. Ferredoxin and anthraquinone-2-sulphonate (AQS) both catalyse DBMIB-sensitive phosphorylation, but only the former is sensitive to antimycin. Measurements at low light intensities have suggested that with both cofactors a Q-cyclie is involved. In support of this, a slow phase of the electrochromic effect at 518 nm ($P518_s$) is ob-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; AQS, 9,10-anthraquinone-2-sulphonate; J820, tetrabromo-4-hydroxypyridine; $P518_s$, slow phase of the electrochromic shift; QR site, quinone reduction site; QO site, quinol oxidation site; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; NQNO, 2-nonyl-4-hydroxyquinoline *N*-oxide

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served, but in neither case is it sensitive to antimycin. It has been argued that antimycin would not be expected to inhibit $P518_s$ if the electrogenic reaction is the reduction rather than the re-oxidation of cytochrome *b*-563 [9]. Nevertheless, 2-alkyl-4-hydroxyquinoline *N*-oxides (HQNO and NQNO) have been shown both to inhibit the re-oxidation of the cytochrome and to have an effect on $P518_s$, observations consistent with an effect on the QR site [12,13].

The aim of the present work was to examine the effects of J820 on a wider range of systems, in order to provide evidence as to whether it acts at the QR site of the cytochrome *bf* complex or on a distinct ferredoxin-plastoquinone reductase.

2. MATERIALS AND METHODS

Chloroplasts were isolated from 14–18-day-old pea seedlings and 28–35-day-old spinach leaves by the method of Cerović et al. [14], with the inclusion of an osmotic shock (chloroplasts exposed to 10 mM $MgCl_2$ for 20 s followed by a return to isotonic medium) between the two centrifugation steps. The broken chloroplasts were either stored at 77 K (after addition of 5% (v/v) dimethyl sulphoxide) and used for cyclic phosphorylation measurements, or used fresh for light-induced absorbance changes of cytochromes and the slow phase of the electrochromic effect ($P518_s$).

Cyclic photophosphorylation catalysed by AQS or ferredoxin was measured as described [11]; the reaction volume was 250 μl. Inhibitors were added as anaerobic solutions in ethanol; an equal volume of anaerobic ethanol was added to the controls.

The spectroscopic measurements of $P518_s$ and the redox kinetics of cytochromes *b*-563 and *f* were analysed with a computerised single-beam spectrophotometer (Applied Photophysics, London). Chloroplasts were suspended in a reaction medium containing 30 mM Tricine-KOH (pH 8.0), 0.1 M sorbitol, 20 mM KCl, 3 mM $MgCl_2$, 10 μM DCMU, 0.5 mM duroquinol and 0.1 mM methyl viologen; the chlorophyll concentration was 25 μg/ml. Saturating flashes were passed through a Schott glass filter (RG 630) and were provided by an Applied Photophysics flashlamp giving flashes with half-peak width of 2 μs. The data capture system employed a Datalab transient recorder (DL902) set to take 2048 points at 20 μs intervals. Groups of eight adjacent points were averaged in the computer to give traces with 256 points spaced at 160 μs. The traces shown are averages of 50 traces in the case of $P518_s$, or 150 traces in the cases of cytochrome measurements, taken at a frequency of 0.3 Hz.

Oxygen uptake was monitored on a Hansatech oxygen electrode at 20°C; illumination through a Schott glass filter (RG610) was provided by a 150 W tungsten/halogen projector lamp. A 1 ml reaction volume was used consisting of 330 mM sorbitol, 50 mM Hepes-KOH (pH 8.0), 10 mM KCl, 1 ml EDTA, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 10 μM DCMU, 0.5 mM duroquinol, 0.1 mM methyl viologen, and 5 mM NH_4Cl.

5-Deazaflavin was a gift from Professor V. Massey and was kept as a 1 mM stock solution in dimethylformamide at 4°C. Ferredoxin was prepared from 14–18-day-old pea seedlings following the method of Plesničar and Bendall [5] as far as the first ion-exchange column. The crude ferredoxin was then passed down a Sephadex G-75 column and a further ion-exchange column (Whatman DE-52). Fractions with A_{422}/A_{277} ratios greater than 0.6 were stored as a 1 mM stock solution at 77 K.

3. RESULTS

Fig.1 shows that the inhibition profile of J820 on ferredoxin-catalysed cyclic photophosphorylation was in good agreement with that of Trebst et al. [10]. At 2 μM J820 the ferredoxin-catalysed reaction was inhibited by 75%, but, in contrast, there was little effect on the AQS-catalysed process. Thus J820, like antimycin [1,5,11], acted as a specific inhibitor of ferredoxin cyclic phosphorylation.

If J820 were to act on the QR site of the *bf* complex it would be expected to inhibit the re-oxidation of cytochrome *b*-563, and also, provided the reoxidation is electrogenic, to inhibit $P518_s$. HQNO and NQNO have been shown to have both these effects [12,13]. Fig.2a,b shows that under flash illumination J820 had no effect on either cytochrome *b*-536 turnover or $P518_s$ at a concentration of 2 μM, which is enough to inhibit steady-state cyclic phosphorylation substantially.

At higher concentrations (10 μM) some loss of the $P518_s$ was found (fig.2c) and the rates of reduc-

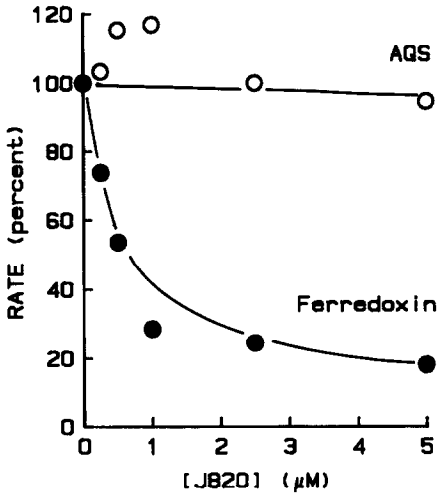

Fig.1. Effect of J820 on cyclic photophosphorylation catalysed by ferredoxin and AQS. Control rates were between 82.0 and 57.4 μmol ATP/h per mg chlorophyll with AQS as cofactor, and between 74.2 and 9.5 μmol ATP/h per mg chlorophyll with ferredoxin as cofactor.

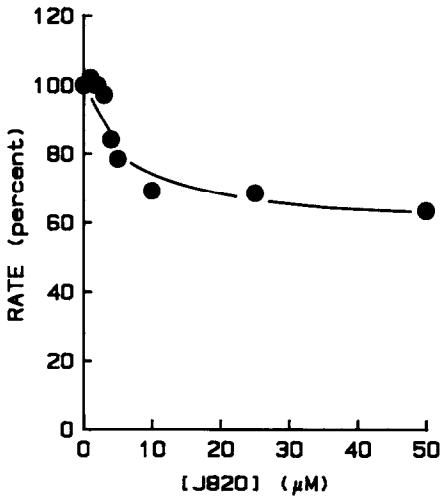

Fig.3. Effect of J820 on duroquinol catalysed non-cyclic electron flow. The control rate was 330 μmol O_2/h per mg Chl.

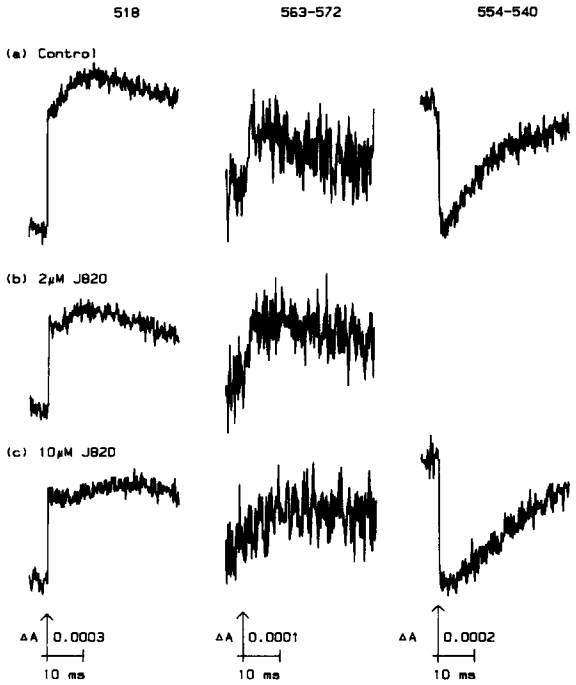

Fig.2. Effect of J820 on the kinetics of the flash-induced absorbance changes at (i) 518 nm, (ii) 563–572 nm due to cytochrome *b*-563 and (iii) 554–540 nm due to cytochrome *f*. The flash was triggered as indicated by the arrows. In addition to the reaction medium in section 2, the reaction mixture contained 5 μM gramicidin for cytochrome absorbance measurements.

tion of cytochrome *b*-563 and re-reduction of cytochrome *f* were decreased. These effects were similar to those of DBMIB [16]. Thus at high concentrations, J820 appeared to act not at the QR site but probably at the QO site of the cytochrome *bf* complex.

This proposal may be tested by investigating the effect of J820 on the photosystem I-dependent reduction of oxygen in the presence of methyl viologen as an autoxidizable acceptor and duroquinol as an artificial electron donor. This system involves the cytochrome *bf* complex and is inhibited by both DBMIB and DNP-INT [17], but was found to be insensitive to low concentrations of antimycin (not shown). Fig.3 shows that J820 had little effect at 2 μM but at higher concentrations (10 μM) inhibited uncoupled electron flow; however, the inhibition was incomplete, unlike that with DBMIB. Such inhibition is not inconsistent with the suggestion that the QO site is a secondary site of action, but the significance of the J820-insensitive electron flow is not yet clear.

4. DISCUSSION

The present data confirm that 2 μM J820 inhibits ferredoxin-catalysed cyclic phosphorylation [9] and stress the specificity of the effect. The

failure to inhibit AQS-catalysed phosphorylation and to affect significantly either $P518_s$ or the flash-induced turnover of cytochrome *b*-563 suggests that its primary site of action is not the QR site of the cytochrome *bf* complex but an independent ferredoxin-plastoquinone reductase. Its effects are like those of antimycin but unlike those of HQNO and NQNO. The latter are similar to those of antimycin on chromatophores of *Rhodopseudomonas sphaeroides* [18], and this provides further support for the view that antimycin acts at a different site in chloroplasts.

The simplest interpretation of the similar effects of antimycin and J820 is that they combine with the same site on ferredoxin-plastoquinone reductase. Comparison of the structures of the two inhibitors suggests that the dilactone portion of antimycin is not essential for inhibition, but provides an additional lipophilicity which may account for its higher affinity for the enzyme. The specificity of the binding must be provided by the aromatic nucleus and its immediate substituents.

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