A Nitroxide Diuron Analog as a Probe for the Mode of Action of Herbicides

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A new spin label nitroxide which is also a herbicide of the urea family has been synthesized. The nitroxide spin label derivative of diuron (ditempuron, DTPU) inhibits electron flow at the site of diuron inhibition of photosystem II (PSII) activity, and it is also reduced by electron flow from one of the first acceptors of PSII. The reduction of the nitroxide group of DTPU is inhibited by diuron or by trypsin treatment of the thylakoids but not by DNP-INT or DBMIB. It is suggested that DTPU binds specifically to and accepts electrons at the Q site.

Introduction

During recent years it has been established that a 32–34 kDa thylakoid polypeptide of chloroplast origin is involved in the formation of the Q site of photosystem II, and binds urea and triazine derivatives, acting as electron flow inhibitors between QA and PQ [1]. The mechanism of action of these inhibitors which are widely used as herbicides is not yet completely understood. It has been proposed that formation of the high affinity binding site of these herbicides requires not only the presence of the 32–34 kDa polypeptide but also its integration within an active PSII complex and its interaction with the 44–47 kDa polypeptides of reaction center II [2]. The light dependent, fast turnover of the 32–34 kDa polypeptide appears to be related to its function as the protein moiety of the Q site. Furthermore, it has been demonstrated that in certain thylakoids such as those of Chlamydomonas, a free pool of 32–34 kDa polypeptide might exist in an inactive form, becoming subject to turnover only upon its integration in PSII unit [3, 4]. Information on the interaction of the Q protein with its surroundings could be obtained, if a spin-labeled probe attached to herbicide were available. In the present work we present data obtained with a newly synthesized, spin-label urea derivative which acts as a specific inhibitor of electron flow between PSII and PSI.

Materials and Methods

Lettuce or spinach chloroplasts were prepared as previously reported [1]. Chlamydomonas thylakoids were prepared according to Shochat et al. [2]. The isolated thylakoids were resuspended in 0.05 M Hepes or Tris buffer, pH 7.5, containing 0.4 M sucrose and 0.01 M NaCl.

Ferricyanide or DCIP reduction measurements were carried out using an Amino-Chance dual wavelength spectrophotometer, as reported before [2, 5]. Measurements of fluorescence kinetics at room temperature were performed in an apparatus as described by Cahen et al. [5]. ESR measurements were done with a Varian E-12 spectrometer equipped with a slide projector to illuminate the sample within the ESR cavity.

Competition experiments to assess binding of DTPU to isolated thylakoids were carried out using [1H]diuron [2]. Treatment of isolated thylakoids with trypsin was performed at 25 °C, using bovine crystalline trypsin, type III, as previously described [2]. DTPU was synthesized and purified by the reaction of dichloroaniline with phosgene. The resulting isocyanate derivative was reacted with the spin label tempanime, to give the final product N-(3,4-dichlorophenyl)-N’-(3,3,5,5-tetramethyl-piper-

Abbreviations: DBMIB, dibromothymoquinone; DCIP, dichlorophenol indophenol; DCMU (diuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2,4-dinitrophenylether of 2-iodo-4-nitrothymol; DTPU (ditempuron), N-(3,4-dichlorophenyl)-N’-(3,3,5,5-tetramethylpyperidine-4-oxyl)urea; ESR, electron spin resonance; PQ, plastoquinone; PS, photosystem; QA, primary quinone electron acceptor of photosystem II; Q site, secondary quinone electron acceptor.

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Results and Discussion

The ability of DTPU to act as an electron flow inhibitor of PSII was assessed by comparing its action to that of diuron on ferricyanide and DCIP reduction. The concentration dependence of this inhibition is shown in Fig. 1. It is evident that DTPU causes a 50% inhibition of these reactions at \(6 \times 8 \times 10^{-5}\) M, a concentration considerably higher than that found for diuron \((2 \times 10^{-7}\) M\). The slopes of the curves are sigmoidal, indicating a cooperative effect (Fig. 1). Since these measurements were carried out at optimal conditions for electron flow, one would expect that the only limiting factor in electron flow from \(H_2O\) to the acceptors used would be at the Q\(_B\) site blocked by the herbicide. In such a case one would expect a linear relationship between the residual activity (or % of inhibition) and the inhibitor concentration. The sigmoidality of the inhibition curves suggests the presence of two binding sites with different affinities. Results of the experiments in which DTPU was used as a competitor for \([^3H]duron\) binding, also indicate the presence of a non-competitive and a competitive binding site of slightly lower affinity (Fig. 2). The inhibitory effect of DTPU on PSII is also demonstrated by its effect on the kinetics of variable fluorescence in presence of the inhibitor, which is very similar to that of diuron (Fig. 3).
In Fig. 4 the ESR spectra of DTPU in solution and in the presence of thylakoids are shown. The spectrum in the buffer is typical of the fast motion limit and exhibits an isotropic coupling constant of $A_0 = 17.25$ Gauss. The spectrum of the nitroxide in the chloroplasts is more complex and appears to consist of two superimposed spectra: a narrow signal similar to that in the buffer solution, $A_0 = 17.37$ Gauss, and a broader spectrum which may be identified as that of a spin labeled molecule in the membrane. This signal is too broad to determine its hyperfine splitting constant. Estimation of the relative concentration of DTPU molecules in the "membranal" and "aqueous" phases shows the presence of 90–95% of the probe in the broad membrane spectrum and only about 10% or less contributed to the narrow signal.

Fig. 4c shows an ESR signal of the spin label tempamine in chloroplasts, which exhibits only the narrow peak spectrum. As an amine, tempamine was shown to penetrate into the inner space of the thylakoid membrane without residing in the membrane [7], and thus it does not exhibit the broad membranal signal.

When the chloroplast membranes in the ESR cavity are illuminated by continuous light, the DTPU signal disappears and slowly reappears in the dark. The spectrum of the probe scanned in the dark (a), the spectrum taken during 4 min of illumination (b), and the spectrum taken 30 min after cessation of illumination (c) are shown in Fig. 5. It can be seen that most of the signal disappears after 1 min of illumination, namely, the lower field hyperfine, whereas the recovery after 30 min is not complete. It is clear that both the narrow and the broad signals disappear together within the time resolution of the spectrometer; if one of the signals disappears first, then the repartitioning between phases is faster than the scanning time. These results indicate that light dependent electron flow in the chloroplasts reaches the nitroxide group and reduces it, thereby diminishing the size of the paramagnetic signal. No decrease in signal intensity was detected when DTPU was illuminated in buffer solution without chloroplasts.

Following addition of DCMU to the chloroplasts, there is an increase of the narrow signal and a slight

<table>
<thead>
<tr>
<th>Table I. Effect of light on DTPU signal.</th>
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<tr>
<td>No addition</td>
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<tr>
<td>+ 2.0 x 10^{-4} M DCMU</td>
</tr>
<tr>
<td>+ 1.2 x 10^{-4} M DNP-INT</td>
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<tr>
<td>+ 5.0 x 10^{-4} M DBMIB</td>
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<td>After trypsin treatment</td>
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The assay system consisted of a suspension of spinach thylakoids (2 mg chlorophyll/ml) without or with addition of the inhibitors, as indicated. Trypsin treatment was as in Table II. The suspension was illuminated in the flat cell within the ESR cavity. The DTPU concentration was 2 x 10^{-4} M.
Table II. Effect of trypsin treatment of lettuce chloroplasts on the rate of photosynthetic ferricyanide reduction and its inhibition by DCU or DPTU.

<table>
<thead>
<tr>
<th>Trypsin (µg/mg chlorophyll)</th>
<th>No addition</th>
<th>Activity (µmol ferricyanide reduced/mg chl x h) after trypsinization</th>
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<tbody>
<tr>
<td></td>
<td>+ DCU (2 x 10^{-6} M)</td>
<td>+ DPTU (2 x 10^{-4} M)</td>
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<tr>
<td>0</td>
<td>134</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>98</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>75</td>
<td>13</td>
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<td>200</td>
<td>44</td>
<td>24</td>
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Lettuce thylakoids (1 mg chlorophyll/ml) were incubated with trypsin (bovine pancreas, type III) in a buffer containing 0.05 M Hepes, pH 7.5, 0.4 M sucrose and 0.01 M NaCl, at 25 °C for 10 min. The reaction was terminated by the addition of a trypsin inhibitor (1 mg/ml) and the thylakoids were washed twice by centrifugation in the same buffer before measurements of ferricyanide reduction were performed.

decrease of the broad one. Illumination of the chloroplasts in the presence of DCU brings about an increase in the narrow signal of free DPTU. None of its signals disappear during the illumination in presence of DCU. This can be explained by the fact that in the presence of DCU, electrons cannot reach the site where DPTU is reduced.

The effect of light on DPTU ESR signal was also assayed in the presence of either DNP-INT or DBMIB. These inhibitors are known to inhibit electron transport beyond the plastoquinone pool [8]. Their effect is summarized in Table I. Addition of these inhibitors to the sample containing DPTU could not prevent the light induced reduction of the DPTU ESR signal, contrary to the inhibitory effect of DCU. Thus, we conclude that the site of reduction of DPTU is at or beyond the DCU blocking site but not beyond the plastoquinone pool.

It has been reported before that following trypsinization of isolated thylakoids, a surface exposed segment of the Q_b protein is cleaved, resulting in loss of herbicide binding and inhibition of electron transport [9]. Similar results were obtained when using thylakoids of spinach, lettuce and Chlamydomonas, and when the effects of diuron or DPTU were assessed on intact or trypsin-treated thylakoids. Electron flow from H_2O to ferricyanide was drastically reduced after prolonged trypsinization. The residual activity was only partially inhibited by DCU or DPTU (Table II). It should be noted, however, that these effects were obtained at high trypsin concentrations which caused extensive degradation of thylakoid polypeptides, as detected by changes in the electrophoretic pattern of treated thylakoids (not shown). No drastic change was observed in the hyperfine structure of the ESR spectrum of DPTU added to trypsinized membranes. Nevertheless, a change did occur in the rotational correlation time τ_c of the narrow signal. The value of τ_c for non-treated thylakoids was 2 x 10^{-9}s as compared to 4.5 x 10^{-10}s for trypsin treated thylakoids and 9.4 x 10^{-11}s for the buffer control. Hence, in the trypsinized membranes the spin label molecule can tumble more than four times faster than in the untreated membranes. These results suggest that the narrow signal is not a simple representative of the DPTU in the aqueous phase but rather manifests environmental changes in the membrane itself.

The results presented so far show that DPTU inhibits electron flow at or close to the site of DCU inhibition, is reduced by electron flow from either Q_A, A_b or PQ pool, and also senses changes in the binding site environment of the thylakoid membrane. The inhibition by trypsinization of its light dependent reduction shows that the damage caused by trypsinization, possibly to the Q_b protein, does not permit electrons to reach this probe, although still in the membrane, and this suggests specificity of the site of its reduction.

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