Properties of the Photoactive Chlorophyll-aII in Photosynthesis

G. DöRING, G. Renger, J. Vater, and H. T. Witt

Max-Volmer-Institut, I. Institut für Physikalische Chemie, Technische Universität Berlin


1. The complete difference spectrum of the reaction of the photoactive chlorophyll-aII is presented.
2. The reaction of excited chlorophyll-aII is of the type of a sensitizer. It is not engaged directly in the electron transfers. This is in contrast to the photoactive chlorophyll-a which is an electron donor in its excited state.
3. The chlorophyll-aII-reaction can be separated from the overall reaction by heating chloroplasts 5 min at 50 °C.
4. Chlorophyll-aII is the reaction center of the well-known poison DCMU.
5. Properties of chlorophyll-aII are depicted in Tab. 1. They are compared with those of chlorophyll-a and the O₂-evolution system.

The electron transport chain of photosynthesis is driven by the light reactions of chlorophyll-a and chlorophyll-aII. Chlorophyll-aII has been detected by absorption changes with a half-life time of 2·10⁻⁴ sec at 22 °C. We found two characteristic absorption changes at 435 nm and 682 nm. Therefore the pigment was called chlorophyll-aII-435-682. It is located within the electron transport chain between plastoquinone and water (see fig. 1).

In fig. 1 the substance X-320 is that molecule out of the pool of ~8 PQ-molecules which is complexed with Chl-aII and which is converted into a semiquinone.

In the following we have extended the measurements on the properties of Chl-aII.

Material and Methods

Whole chloroplasts of spinach were prepared as described in l.c.¹. Suspensions in which the reaction system II is enriched are prepared according to Boardman². The spectroscopic measurements were performed by the repetitive flash technique described in l.c.

The oxygen yield per flash was measured with the Clark-electrode⁴. The double-flash experiments were performed by combining the techniques in l.c.⁷ and l.c.⁸. Further details are described in the legend of the figures.

Results

a) Fig. 2 (left) shows the typical time course of the absorption changes at 690 nm with a superposition of chlorophyll-a and chlorophyll-aII changes. In fig. 2 (right) the relative changes are plotted in a log scale. The fast component (τ₁ = 0.24 ms) belongs to Chl-aII, the slow component (τ₁ = 16 ms) to Chl-aI.

The absorption changes of chlorophyll-aII have been measured in the whole visible region between 420 and 725 nm in suspensions in which the reaction system II is enriched. The results are depicted in fig. 3. They represent the complete diff-

Fig. 1. Simplified electron transfer system in photosynthesis. NADP\(^+\) = nicotinamide adenine dinucleotide phosphate, Chl-a\(_I\) = chlorophyll-a\(_I\), PQ = plastoquinone pool, X = plastosemiquinone, Chl-a\(_II\) = chlorophyll-a\(_II\), PMS\(^-\) = reduced N-methylphenazonium-methylsulfate.

Fig. 2. Left: Absorption changes at 690 nm in whole chloroplasts as function of time. At t=0 a flash was fired. Right: Relative absorption changes as function of time plotted in a log scale. The fast phase (0.24 ms) is caused by Chl-a\(_II\), the slow phase (16 ms) by Chl-a\(_I\). Chlorophyll content 5\(\times\)10\(^{-5}\) M, activity of O\(_2\)-production 88 moles O\(_2\)/mole Chl.h. Tris-buffer pH 7.2 0.05 M. Phosphorylation uncoupler: NH\(_4\)Cl 2\(\times\)10\(^{-3}\) M, electron acceptor: benzylviologene 10\(^{-4}\) M. Optical path length through the cuvette 1.2 mm. Excitation: 610—710 nm (1 mm RG 610 + T8), 380—500 nm (2 mm BG 28 + T8), duration 2\(\times\)10\(^{-5}\) sec. Repetitive flash technique, frequency 10 cps, saturating intensity. For each measuring point 4.096 flashes were fired. Measuring beam: grating monochromator, optical band width 7 nm. Temperature 22 °C. Electrical bandwidth 0.1 cps–13 kcps.

Fig. 3. Absorption changes with a life time of 2\(\times\)10\(^{-4}\) sec as a function of the wavelength in spinach chloroplasts. It was used the 10,000 g precipitate of digitonin-treated chloroplasts, preparation see l. c. 5. Chlorophyll content 5\(\times\)10\(^{-5}\) M, activity of O\(_2\)-production 103 moles O\(_2\)/mole Chl.h. Tris-buffer pH 7.2 0.05 M. Phosphorylation uncoupler: NH\(_4\)Cl 2\(\times\)10\(^{-3}\) M, electron acceptor: K\(_2\)Fe(CN)\(_6\) 5\(\times\)10\(^{-4}\) M. Optical path length through the cuvette 1.2 mm. Excitation: 610—710 nm (1 mm RG 610 + T8), 380—500 nm (2 mm BG 28 + T8), duration 2\(\times\)10\(^{-5}\) sec. Repetitive flash technique, frequency 10 cps, saturating intensity. For each measuring point 4,096 flashes were fired. Measuring beam: grating monochromator, optical band width 5 nm. Temperature 22 °C. Electrical band width 0.1 cps–13 kcps.

For such information we heated the chloroplasts 5 min at higher temperatures and measured the absorption changes always at 22 °C. The magnitude of the chlorophyll-a\(_II\)-absorption change at 690 nm in dependence of the heating temperature is depicted in fig. 4. The characteristic temperature of desactivation is observed for Chlorophyll-a\(_II\) at 55 °C. (50% of maximal value)

Fig. 4. Relative changes of absorption of Chl-a\(_II\) at 690 nm and relative oxygen yield per flash as a function of the heating temperature in spinach chloroplasts. The chloroplasts had been exposed to the marked temperatures for 5 min before the measurement. Chlorophyll content: 5\(\times\)10\(^{-5}\) M, activity of O\(_2\)-production (before heating): 152 moles O\(_2\)/mole Chl.h. Tris-buffer pH 7.2 0.05 M. Phosphorylation uncoupler: NH\(_4\)Cl 2\(\times\)10\(^{-3}\) M, electron acceptor: benzylviologene 10\(^{-4}\) M (resp. K\(_2\)Fe(CN)\(_6\) for the O\(_2\)-measurements). All other details see fig. 3, except: frequency of the flashes 10 cps for Chl-a\(_II\) and 8 cps for O\(_2\).
Corresponding measurements have been made simultaneously for the \( \text{O}_2 \)-production per flash during the cleavage of \( \text{H}_2\text{O} \) (see fig. 1). According to fig. 4 the characteristic temperature of desactivation is observed for \( \text{O}_2 \)-production at 44 \( ^\circ \text{C} \). (50\% of maximal value)

The oxygen yield per flash is practically zero at 50 \( ^\circ \text{C} \), whereas the absorption change of chlorophyll-\( \text{a}_{\text{II}} \) at 50 \( ^\circ \text{C} \) is influenced nearly not at all (see fig. 4 and also fig. 5).

\[ \text{Fig. 5. Relative change of absorption of Chl-\( \text{a}_{\text{I}} \), X and Chl-\( \text{a}_{\text{II}} \), and relative oxygen yield per flash in spinach chloroplasts. Left: normal conditions, center: after 5 min heating to 50 \( ^\circ \text{C} \), right: after 5 min heating to 50 \( ^\circ \text{C} \) and addition of 2 \( \times 10^{-5} \) M DCMU. Chl-\( \text{a}_{\text{I}} \) was measured by absorption changes at 703 nm, X at 335 nm and Chl-\( \text{a}_{\text{II}} \) at 690 nm. All other details see fig. 4.} \]

The difference of the results in l.c.\(^9\) in which a temperature of desactivation for the \( \text{O}_2 \)-production was reported at 35 \( ^\circ \text{C} \) is caused by different preparations of the chloroplasts. In l.c.\(^9\) we prepared the suspensions without dimethylsulfoxide, which has a stabilizing effect against temperature desactivation.

c) It is of interest to know which other members of the electron transport chain are blocked when the reaction of \( \text{O}_2 \)-production is totally desactivated at 50 \( ^\circ \text{C} \). We checked this question by measuring the absorption changes of chlorophyll-\( \text{a}_{\text{II}} \) at 703 nm and X-320 at 335 nm. The results are depicted in fig. 5. With the desactivation of the \( \text{O}_2 \)-production the reaction of chlorophyll-\( \text{a}_{\text{I}} \) and X-320 are also strongly reduced.

To avoid misconceptions it should be mentioned that for the chlorophyll-\( \text{a}_{\text{I}} \)-reaction itself [separated from the overall reaction by addition of the artificial electron donor PMS\(^6\)] (see fig. 1) the temperature of desactivation is 65 \( ^\circ \text{C} \)\(^{10,11}\) (see also table 1).

\[ \text{Fig. 6. Top. Relative oxygen yield per flash, bottom, relative change of absorption of Chl-\( \text{a}_{\text{II}} \) at 690 nm as a function of the DCMU-concentration in spinach chloroplasts before and after heat treatment. The heated chloroplasts had been exposed to 50 \( ^\circ \text{C} \) for 5 min before the measurements. Frequency of the flashes 10 cps. For further details see Fig. 3 and Fig. 4.} \]

d) It is long known that DCMU poisons the \( \text{O}_2 \)-production\(^12\). Firstly, we repeated such measurements for the oxygen production in dependence of the DCMU concentration (see fig. 6 top). The characteristic concentration of the desactivation is for \( \text{O}_2 \)-production at \( \sim 10^{-7} \) M. (50\% of maximal value)

Secondly, corresponding measurements were done after heating the chloroplasts for 5 min at 50 \( ^\circ \text{C} \). At these conditions the \( \text{O}_2 \)-production is nearly zero at all DCMU-concentrations (see fig. 6 top).

Thirdly, corresponding measurements were carried out for the reaction of chlorophyll-\( \text{a}_{\text{II}} \). According to fig. 6 bottom the characteristic concentra-

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Properties Chlorophyll-ai Chlorophyll-an H2O/O2

<table>
<thead>
<tr>
<th>Type of Reaction</th>
<th>Electron Donor</th>
<th>Sensitizer</th>
<th>Cleavage of Water</th>
</tr>
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<tbody>
<tr>
<td>Characteristic Change</td>
<td>430, 655, 680–703 nm</td>
<td>435, 640, 682 nm</td>
<td>2–10–2 sec</td>
</tr>
<tr>
<td>of Absorption</td>
<td>Band Splitting</td>
<td>No band splitting</td>
<td>6–10–4 sec</td>
</tr>
<tr>
<td>Half-Life Time (20°C)</td>
<td>2–10–2 sec *</td>
<td>6–10–4 sec</td>
<td>6–10–4 sec</td>
</tr>
<tr>
<td>Redox Potential</td>
<td>+0.46 V (Chl-ai/Chl-ai)</td>
<td>+0.81 V (pH 7)</td>
<td>5–9</td>
</tr>
<tr>
<td>Range of Excitation</td>
<td>λ &lt; 730 nm</td>
<td>λ &lt; 700 nm</td>
<td>44°C</td>
</tr>
<tr>
<td>Range of pH</td>
<td>3–11</td>
<td>3–11</td>
<td>4°C</td>
</tr>
<tr>
<td>Temperature of Inactivation</td>
<td>65°C</td>
<td>55°C</td>
<td>6°C</td>
</tr>
<tr>
<td>Sensitive to Aging</td>
<td>no</td>
<td>t1/2 ≈ 95 h (~0°C)</td>
<td>t1/2 ≈ 36 h (~0°C)</td>
</tr>
<tr>
<td>Sensitive to DCMU</td>
<td>no</td>
<td>ci/2 » 10–7 M/1</td>
<td>no</td>
</tr>
</tbody>
</table>

* When the electron donor is in the oxidized state.


Propagion of desactivation is the same as for the O2-production, namely

Chlorophyll-an at ~ 10–7 M.

(50% for maximal value)

Fourthly, the measurements were performed for chlorophyll-an after heating the chloroplasts for 5 min at 50°C after which all electron transfers are blocked (see h and c). According to fig. 6 bottom the chlorophyll-an-reaction has nearly not changed in comparison with the behavior of chlorophyll-ai before heating. It shows the same dependence on the DCMU concentration with a characteristic concentration of desactivation

Chlorophyll-an at ~ 10–7 M.

(50% of maximal value)

This indicates that DCMU acts directly on chlorophyll-ai (see discussion).

The DCMU dependency depends on the chlorophyll content. In the experiments reported above this content was 5–10–5 M (s. also fig. 6).

e) From measurements of the O2-production and of the absorption change of X-320 in double flashes it follows that the rate limiting time of the electron transport from water to X-320 is 6·10–4 sec (characteristic time distance of the second flash for 50% of maximal value) 13. In case that the chlorophyll-an-reaction is an electron carrier between H2O and X-320, this reaction should also decrease to 50% in its absorption change in a second flash when this flash has a time distance of t4 = 6·10–4 sec from the first one. Fig. 7 shows, however, that even with t4 = 2.5·10–4 sec the absorption change in the second flash is decreased not at all. This means that chlorophyll-an is not engaged directly in the electron transfer between H2O and X-320.

It is to be mentioned that the double-flash experiment was possible only by using the high frequency flash technique and by using the high frequency modulated measuring light technique be-

![Fig. 7. The kinetics of the absorption changes of Chl-an at 690 nm in a double-flash experiment in spinach chloroplasts.](image)

cause of the extremely high fluorescence in the red region.

Joliot\(^{15}\) found after addition of DCMU in the dark that only the first flash is able to cause an electron transfer from which it was concluded that DCMU acts possible to an excited state in vivo.

Bertsch et al.\(^{16}\) concluded from delayed light emission that DCMU blocks the electron flow in light reaction II. Zweig et al.\(^{17}\) had assumed from oxygen evolution and fluorescence that DCMU may inhibit the energy migration to light reaction II. Wessels et al.\(^{18}\) had supposed that the phenyl ureas might associate the active cyclopentanone ring of chlorophyll through hydrogen bonds. Such association would prevent the transfer of excitation energy from chlorophyll to an acceptor.

In respect to the results with DCMU in d) the following should be mentioned.

We have checked if DCMU has any influence on the photochemistry of chlorophyll reactions in vitro. Chlorophyll solved in "oxygen free" (\(<10^{-7}\) mole/l) butanol is not influenced in the kinetics of its singulet as well as its triplet state by the addition of DCMU up to \(10^{-2}\) M\(^{14}\).

**Discussion**

1. In l. c.\(^{10}\) evidence was given how it is possible to separate the reaction of chlorophyll-\(\text{a}\) from the overall reaction. From the results above it is now known how to separate also the reaction of chlorophyll-\(\text{a}\) from the overall reaction. It can be simply done by heat-treating the chloroplasts for 5 min at 50 °C.

2. The fact that DCMU has the same influence on the chlorophyll-\(\text{a}\)-reaction before and after heat-treating (see fig. 6) and the fact that heat-treating blocks all electron transfer events (see fig. 5) indicates clearly that in vivo the action center of DCMU is chlorophyll-\(\text{a}\).

3. DCMU has without heat-treating the same influence on the chlorophyll-\(\text{a}\)-reaction as on the \(O_2\)-production (see fig. 6). This indicates together with the conclusion in 2 that chlorophyll-\(\text{a}\) is the active pigment in the cleavage of \(H_2O\).

This has already been shown in other ways in l. c.\(^{1}\).

4. The fact that heat-treating stops \(O_2\)-production indicates that no linear electron transfer from \(H_2O\) to \(NADP^+\) takes place. The fact that under this condition also the reactions of the members of the electron chain, as chlorophyll-\(\text{a}\) and X-320 are blocked indicates that also no cyclic electron transfer is in action (see fig. 1).

When without any electron movements in linear and cyclic ways nevertheless chlorophyll-\(\text{a}\) is in action, it can be concluded that the reaction of chlorophyll-\(\text{a}\) is probably not of the type of a light induced redox reaction, but of the type of a sensitizer \(*\). The same conclusion follows from the double flash experiment in fig. 7 during intact electron transfers. If chlorophyll-\(\text{a}\) donates or accepts electrons in its excited state, chlorophyll-\(\text{a}\) should be able to act in a following second flash not before the electron transfer time from \(H_2O\) to X-320 \((6 \times 10^{-4}\) sec\). This is, however, not the case.

In contrast to this behaviour of chlorophyll-\(\text{a}\) it has clearly been shown that the reaction of chlorophyll-\(\text{a}\) is a light induced redox reaction\(^{19, 10, 11}\). In its excited state chlorophyll-\(\text{a}\) donates an electron to an acceptor.

5. The properties of chlorophyll-\(\text{a}\) have been investigated in detail in l. c.\(^{10, 11, 19, 20}\). The properties of chlorophyll-\(\text{a}\) have been investigated as yet only in combination with the \(H_2O/O_2\)-system and this only indirectly. This was done by the analysis of the positive absorption change of chlorophyll-\(\text{a}\)\(^{21}\). These positive changes indicate the reduction of chlorophyll-\(\text{a}\)\(^{21}\) or the production of electrons in the chlorophyll-\(\text{a}\)-\(H_2O/O_2\)-system. By the difference spectrum of chlorophyll-\(\text{a}\) its properties can now be measured directly and (by heating to 50 °C) separately from the properties of the \(H_2O/O_2\)-system. The results so far obtained here and in l. c.\(^{1, 2}\) are summarized in Tab. 1. Additionally, the properties of chlorophyll-\(\text{a}\)\(^{19, 10, 11, 20}\) and those of the oxygen evolution system\(^9, 13\) are depicted in Tab. 1.

The most remarkable fact is that the two photoactive chlorophylls are obviously engaged in two completely different types of reactions. It is unknown in which way the excited chlorophyll-\(\text{a}\) sensitizes the electron transfer from \(H_2O\) to X-320.

\(^{15}\) P. Joliot, private information.


\(^{17}\) J. S. C. Wessels and R. Van der Veen, Biochim. biophysica Acta [Amsterdam] 66, 196 [1963].

\(^{18}\) B. Kok, Biochim. biophysica Acta [Amsterdam] 48, 527 [1961].

\(^{19}\) G. Döring, J. L. Bailey, W. Kreutz, J. Weikard, and H. T. Witt, Naturwissenschaften 55, 219 [1968].

\(^{*}\) To assume aster heat-treating an electron back reaction between Chl-\(\text{a}\) and an unknown intermediate is also unlikely because the kinetics of Chl-\(\text{a}\) does not change.