The Effect on Ammonium Chloride on the Kinetics of the Back Reaction of Photosystem II in *Chlorella fusca* and in Chloroplasts in the Presence of 3-(3,4-Dichlorophenyl)-1,1-dimethylurea

Gerhard Vierke

Institut für Physikalische Biochemie und Biochemie, Universität Frankfurt, Sandhofstr. 2-4, D-6000 Frankfurt 71

Z. Naturforsch. 35 c, 451–460 (1980); received February 29, 1980

Kinetics of the Back Reaction, Photosystem II, Ammonium Chloride

The effect of NH₄Cl on the kinetics of the back reaction of photosystem II as derived from luminescence measurements was investigated in dark adapted *Chlorella* in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at different temperatures. The kinetics of the back reaction, under these conditions, leads to the reduction of the S₂ state by the primary electron acceptor Q⁻ of photosystem II was observed to be considerably slowed down in the presence of NH₄Cl.

Analysis of the kinetic results in the light of the theory of the back reaction developed by Mar and Roy (J. Theor. Biol. 48, 257–281 (1974)) revealed two opposite effects of NH₄Cl to be present simultaneously:

1) The enthalpy of activation of the back reaction was lowered (catalyzing effect of NH₄Cl)
2) The frequency factor which indicates the number of collisions of the reacting molecules in the membrane per second is largely decreased (inhibitory effect of NH₄Cl).

This reduction of the mobility of the recombining species of the back reaction is the predominant effect of NH₄Cl. It is suggested that this effect is due to a change of the conformational state of the membrane induced by dissolution of relative large amounts of NH₃ within the lipid phase of the thylakoid membrane. This hypothesis is supported by the observation that the value of the exciton yield of the back reaction changes upon addition of NH₄Cl.

Introduction

It has long been known that oxygen evolution in chloroplasts is inhibited in the presence of high concentration of NH₄Cl [1, 2]. The active species is the uncharged base NH₃ [1—3]. The inhibition site of NH₃ was observed to be located before the donor site of NH₂OH within photosystem II [2] thus indicating that NH₃ inhibits electron transport near the water splitting reaction. More recently, the effect of NH₄Cl on the S states of the oxygen evolving system has been investigated in detail in chloroplasts [4, 5] and in *Chlorella* [5]. It has been shown by luminescence measurements [4] and by measuring the turnover times of the S states [5] that NH₃ directly inhibits the oxygen evolving reaction S₂ → S₂⁺. Furthermore, NH₄Cl strongly affects the kinetics of deactivation of S₂ and S₃. Additional experiments suggested that these effects may possibly be attributed to binding of NH₃ to the states S₂, S₂⁺, and S₃.

In this article the effect of NH₄Cl on the kinetics of deactivation of S₂ in *Chlorella* in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was investigated in more detail. The S₂ state was created by continuous illumination of dark adapted *Chlorella* cells in the presence of DCMU. Under these conditions the deactivation of S₂ cannot be followed by oxygen evolution measurements. Therefore, the kinetics of deactivation was monitored by the kinetics of the back reaction as derived from luminescence measurements. Experimental evidence accumulated thus far suggests that there is a close correspondence between both reactions [4, 5].

This method is based on the observation that in *Chlorella* the deactivation reaction of the S₂ state is initiated by electron back transfer from the primary electron acceptor Q⁻ of photosystem II to its oxidizing side [6, 7]. According to the recombination hypothesis the back reaction is accompanied by luminescence [8, 9]. This offers the possi-
bility to evaluate the kinetics of the back reaction from luminescence measurements [10–12].

Using this method, investigation of the effect of NH₄Cl on the kinetics of the back reaction of S₂ in Chlorella in the light of the theory of the back reaction developed by Mar and Roy [10] revealed that stabilization of the S₂ state in the presence of NH₄Cl cannot be attributed to binding of NH₃ to S₂ but rather is due to the restriction of the mobility of the recombining species because of changes of the conformational state of the membrane induced by dissolution of high amounts of NH₃ within the lipid phase of the thylakoid membrane.

Materials and Methods

Preparation of the Chlorella samples

Chlorella fusca was cultivated as described by Soeder et al. [13]. Chlorella cells were taken from a synchronous culture always at the same time shortly after the release of the autospires (in the 24th hour of the synchronous cycle). They were kept in dark until use within one hour later. The cells were then harvested by centrifugation at 25 °C, washed and resuspended in 67 mM potassium phosphate at pH values and Chl concentrations as indicated in the legends to figures. After addition of 20 μM DCMU the suspension was continuously stirred for 10 min. The incubation time for NH₄Cl was 30 min. DCMU was obtained from K & K Lab. and was recrystallized twice from benzene. Chlorophyll was determined as described previously [12].

Preparation of spinach chloroplasts

Chloroplasts were isolated from market spinach according to the following procedure. Approximately 20 g of fresh spinach leaves (without ribs) were suspended in a buffer solution containing 50 mM TES-buffer pH = 7.9, 0.4 M sucrose, 10 Mm NaCl, 20 mM ascorbate, and 5 mM MgCl₂. They were homogenized for 10 sec in a blender, filtered through two layers of nylon cloth (mesh width 70 x 70 μm), and centrifuged for 5 min at 200 x g. The supernatant was then centrifuged at 1000 x g for 15 min. The sediment was incubated in the isolation buffer and was stored at 0 °C until use one hour later. Chlorophyll was determined by the method of Arnon [14]. The chloroplasts were incubated with DCMU for 10 min in the dark.

Luminescence measurements

Luminescence was excited by monochromatic light (478 nm ± 10 nm) obtained from a 900 W Xenon lamp (XBO 900 W, Osram, placed in the LH 151 NZ lamp house, Schoeffel Instr.). The exciting light beam was passed through a water filter (10 cm), a IR reflection filter and through a monochromator (Bausch & Lomb 33-86-02 with grating 33-86-25-02, blaze 500 nm). The monochromatic light was focussed on the cuvette (made of quartz glass suprasil, Hellma) containing the Chlorella suspension. The intensity of the exciting light measured at the surface of the cuvette was 22 mW/cm². The optical pathlength of the suspension was 5 mm. Chl concentration was usually kept well below 50 μg/ml Chl tot in order to minimize reabsorption of delayed light.

The temperature of the cuvette was regulated by a thermostat and was measured by a calibrated copper-constantan thermocouple. Luminescence was measured in the direction of the excitation beam. The sample was placed in the center of a cylindrical shutter with two openings arranged at an angle of 85° so that the sample was either illuminated with exciting light and the emission window was closed or after rotating the shutter by an electrical pulse within 10 ms luminescence was measured with the excitation window closed. The time resolution of the spectrometer, therefore, is 10 ms.

The emitted light was measured by an EMI photomultiplier 9658 A which was kept at -30 °C by use of a thermoelectrically refrigerated photomultiplier tube housing (TE 104, Products for Res., Inc., USA) in order to improve the signal-to-noise ratio. The photomultiplier was protected from stray light by a cut-off filter (WG 655, 10 mm, Schott) thus permitting the measurement of the whole spectrum of luminescence. The photomultiplier signal was fed to a rapid DC amplifier (GV 9031, EGB) and then was recorded by a light beam galvanometer recorder (Lumiscript-150-13, Hartmann & Braun). Millisecond flash-induced luminescence was measured by placing an electronic shutter (Compur electronic 5 FS) in the excitation beam. The shortest flash duration available was 16 ms. The electromagnetically driven shutter of the luminescence spectrometer was triggered by an electric pulse from a phototransistor placed at the opening of the electronic beam shutter with the help of a...
special electronic device. This device also allowed for the selection of various delay times between the incoming pulse of the phototransistor and the trigger pulse for the electromagnet of the spectrometer shutter. Delay times were variable between 0 and 140 ms. So, by using delay times shorter than the flash duration of the electronic beam shutter, even a one millisecond flash could be generated.

Before each measurement the sample was kept in the dark for 15 min.

Determination of the kinetics of the back reaction

The kinetics of the back reaction in *Chlorella* in the presence of DCMU was determined from the luminescence decay curve according to the method described earlier [11, 12]. This method correlates the partial and total light sums of luminescence with the time course of the oxidation of the reduced primary electron acceptor Q⁻ of photosystem II in the seconds region. The theory [11, 12] leads to the following expression

\[
\frac{[Q^{-}]}{[Q^{-}]_{0}} = \frac{[1 + 2 A B N(t)]^{1/2} - 1}{A}
\]

which is valid in *Chlorella* for times \( t \approx 0.3 \) sec. \( A \) and \( B \) are constants depending on the values of \( p \), the mean probability for excitation transfer between different photosystem II centers, and of the ratio \( \varphi_{\infty}/\varphi_{0} \) of the fluorescence yields when \( [Q^{-}] = [Q^{-}]_{0} \) or \( [Q^{-}] = 0 \), respectively. For *Chlorella* \( p \) is equal to 0.45 and \( \varphi_{\infty}/\varphi_{0} \) to 5 [11, 12]. For chloroplasts the same values were used.

\[
N(t) = 1 - \frac{\mathcal{L}(t)}{\mathcal{L}_{tot}}
\]

\( \mathcal{L}(t) \) and \( \mathcal{L}_{tot} \) are the partial or total light sums, respectively. The light sums were calculated by integrating numerically the luminescence decay curve. Integration was done by making use of the integration program of the Hewlett-Packard calculator 9815 A.

Theoretical concepts

Interpretation of the effect of \( \text{NH}_4\text{Cl} \) on the kinetics of deactivation of \( S_{2} \) will severely depend on the underlying theory of the kinetics of the back reaction. Two theoretical concepts have been put forward as yet: first order theory [8] and the theory of Mar and Roy [10] which is used in this paper in order to interpret the experimental results.

Lavorel [8] has emphasized that the high photochemical rate of the reaction center of photosystem II can hardly be explained by assuming that the components of the reaction center are freely diffusable. Within a fixed reaction center complex the kinetics of the back reaction is then expected to be strictly monomolecular.

Contrary to this view, Mar and Roy [10] hold that, in spite of the relatively rigid structure of the reaction center, the rate of the back reaction is nevertheless controlled by the rate of diffusion of the primary electron acceptor \( Q^{-} \) and of the oxidized electron donor \( Z^{+} \) within a limited solid state-like lipoprotein region of the membrane. Elaboration of this idea [10] leads to the following expression for the kinetics of the back reaction.

\[
\frac{[Q^{-}](t)}{[Q^{-}]_{0}} = \frac{e^{-ct}}{1 + D[1 - e^{-ct}]}
\]

The rate constant \( D \) solely depends of the entropy of activation \( AS^{*} = k \ln \Omega \) [11] **.

\[
D = n \frac{\Omega_{i}}{\Omega_{0}}
\]

\( \Omega_{0} \) and \( \Omega_{i} \) denote the first two terms of the development in the series of the partition function \( \Omega \). \( n \) is the number of nearest neighbour reaction centers. Eqn (2) states that the value of \( D \) is low when the entropy of activation is high and vice versa.

The second rate constant \( C \) is given by [10, 11]

\[
C = \nu \Omega_{0} W^{\alpha} \exp \left( - \frac{\Delta H^{*}}{kT} \right)
\]

Here \( \nu \) denotes the vibration frequency of \( Q^{-} \) in its initial site. \( \Delta H^{*} \) is the enthalpy of activation of the back reaction. \( W \) is an expression that originates from the Goldman equation \( V = kT \ln W \) which determines the potential of the permeable ions across the membrane. \( \alpha \) is the polarization constant.

* In the presence of DCMU the ultimate oxidized electron donor of PS II is the \( S_{2} \) state.

** Eqns. (3) and (4) are simplified versions of the expressions given by Mar and Roy [10]. The simplification can be made because the value of the exciton yield of the back reaction \( S \) is of the order of \( 10^{-4} \) [11, 12].
Effect of NH₄Cl on the Back Reaction of Photosystem II

of the membrane. W is given by *

\[ W = \sum_{A} P_{A}[A^{-}]_o + \sum_{c} P_{c}[c^{+}]_i \]

Here \( P_{A} \) is the permeability of the anion \( A^{-} \), \( P_{c} \) is the permeability of the cation \( c^{+} \), \( i \) denotes the inside, and \( o \) the outside of the membrane. Once \( [Q_{1}^-] \) has been determined from Eqn (1) the kinetic parameters \( D \) and \( C \) may be evaluated from Eqn (2) by plotting the expression

\[ \ln \frac{[Q_{0}]/[Q_{1}^-] + D}{D + 1} \]

against time. The plot yields a straight line for the correct value of \( D \). \( C \) is given by the slope of the line.

It should be emphasized that the theory of Mar and Roy allows for the determination of the enthalpy of activation \( \Delta H^{*} \) from the temperature dependence of \( C \) but allows only for qualitative statements on the change of the values of \( \Delta S^{*} \) and \( W \).

Results and Discussion

Contrary to the effect of other uncouplers [15] the kinetics of the integrated luminescence intensity and consequently that of the back reaction also (see Eqn (1)) is markedly delayed in the presence of NH₄Cl**. The effect strongly depends on the concentration of NH₄Cl present. It is observed that the kinetics is increasingly slowed down with increasing NH₄Cl concentration both in Chlorella (Fig. 1) and in isolated chloroplasts (Fig. 2). Addition of Valinomycin enhances this effect of NH₄Cl in chloroplasts (Fig. 2).

This delay effect of NH₄Cl on the kinetics of the back reaction cannot be attributed to the uncoupling action of this compound. This conclusion may be supported by several experimental findings. First, uncoupling of chloroplasts by a high concentration of Valinomycin or by Gramicidin D does not lead to delayed kinetics of the back reaction [15]. Second, the kinetics is not slowed down after flash excitation either [15] which does not create a pH gradient across the thylakoid membrane. Third, the delay effect of NH₄Cl is also observed in this flash-induced state of the membrane (\( \Delta pH = 0 \)) though not as much pronounced as in the light-adapted state (Fig. 3).

Further information on the mechanism of action of NH₄Cl was obtained by investigating the kinetics of the back reaction in Chlorella in more detail. This was done using the theory of Mar and Roy. It generally predicts non-first order kinetics (Eqn (2)). First order kinetics may only be derived from theory as an approximate solution at very small values of the kinetic constant \( D \) (high value of the entropy of activation \( \Delta S^{*} \)).

The theory of Mar and Roy was preferred because our results do not lend support to first order theory as a generally applicable concept. In most chloroplast preparations the kinetics of the back reaction was observed to be first order but deviations from first order kinetics were sometimes found to occur. In Chlorella, the kinetics is generally not first order in the stationary light-adapted state but after flash excitation it was found to be first order or not first order depending on the culture conditions. These observations indicate that, in spite of the fact that non-first order kinetics may always be formally decomposed into two first order components, two different chemical reactions are not involved but only one. If in principle two back reactions occurred, they would be expected to occur in any case regardless whether the back reaction is initiated by flash or continuous illumination. Therefore it was assumed in the following that the back reaction involves only one electron donor \( (Q_{-}, in the presence of DCMU) \) and one electron acceptor on the oxidizing side of photosystem II (the \( S_{2} \) state) and that the differences in kinetic order are due to changes in membrane ultrastructure.

In fact, it has been demonstrated earlier [11, 12] that, in Chlorella, the kinetic results obtained in the light-adapted state fit well into the theory of Mar and Roy. It is justified, therefore, to study the
Fig. 1. Kinetics of the back reaction in Chlorella in the presence of DCMU and various amounts of NH₄Cl. ○ Control; ● 50 mM NH₄Cl; △ 100 mM NH₄Cl; ▲ 200 mM NH₄Cl; □ 400 mM NH₄Cl; ■ 700 mM NH₄Cl. Phosphate buffer, pH = 8.0; T = 25 °C; DCMU, 20 μM. Chl, 33.5 μg/ml; preillumination time, 30 sec. C₀ denotes the maximal concentration of Q⁻ and C the concentration of Q⁻ at time t.

Fig. 2. Flash-induced kinetics of the back reaction in isolated chloroplasts of spinach in the presence of DCMU and various amounts of NH₄Cl. □ Control; ■ 50 mM NH₄Cl; ○ 100 mM NH₄Cl; ● 100 mM NH₄Cl + 2 μM Valinomycin. TES buffer, pH = 7.9, containing 0.4 m sucrose, 10 mM NaCl, 20 mM ascorbate, and 5 mM MgCl₂. T = 25 °C; Chl, 50 μg/ml; DCMU, 20 μM; preillumination time, 66 ms. C₀ and C denote the maximal concentration of Q⁻ or its concentration at time t, respectively.
Fig. 3. Effect of NH₄Cl on the flash-induced and continuously excited kinetics of the back reaction in Chlorella in the presence of DCMU. ■ Flash-induced kinetics in the absence of NH₄Cl; □ kinetics in the light-adapted state in the absence of NH₄Cl; ● flash-induced kinetics in the presence of 250 mM NH₄Cl; ○ kinetics in the light-adapted state in the presence of 250 mM NH₄Cl. Phosphate buffer, pH = 8.0; T = 25 °C; DCMU, 20 μM; Chl, 33.5 μg/ml; preillumination time, 15 ms (flash-induced kinetics) or 30 sec (kinetics in the light-adapted state), respectively. C₀ and C denote the maximal concentration of Q⁺ or its concentration at time t, respectively.

The effects of NH₄Cl on the kinetics of the back reaction were studied in more detail by making use of the theory of enzyme kinetics.

Evaluation of the kinetics according to Eqn (2) in terms of the two rate constants D and C (Fig. 4) shows that the entropy of activation ΔS⁺ is not changed in the presence of NH₄Cl because the value of D remains constant. But the rate constant C is greatly diminished. According to Eqn (4) this could be due to a change of the value of the enthalpy of activation ΔH⁺, to generation of a diffusion potential across the membrane (V = kT ln W) because of an electrogenic influx of NH₄Cl+, or to a change of the value of the frequency factor v.

ΔH⁺ may be determined by measuring the temperature dependence of the rate constant C. This has been done in the presence of 100 mM NH₄Cl in Chlorella (Fig. 5). Evaluation of the kinetic data (Fig. 6) shows that the value of ΔH⁺ is 0.50 eV. Determination of ΔH⁺ in the absence of NH₄Cl from luminescence and fluorescence induction measurements [10, * Electrogenic influx of NH₄Cl has been discussed as a possible alternative to the widely accepted neutral influx of NH₄Cl [16].
yields $\Delta H^* = 0.60 \text{ eV}$. Repeating this measurement we obtained the same result. It is seen that the enthalpy of activation $\Delta H^*$ is diminished by 0.1 eV in the presence of NH$_4$Cl.

This should give rise to an enhanced rate of the back reaction. The opposite is true, however. Hence either a diffusion potential induced by electrogenic influx of NH$_4$Cl which is of opposite polarity as the light-induced membrane potential is created or the vibration frequency of Q~ is reduced because of high amounts of NH$_3$ being solved in the lipid phase of the membrane. Both hypothesis are supported by the finding that the delay effect of NH$_4$Cl increases with increasing NH$_4$Cl concentration (Fig. 1). But it was observed that the kinetics of the back reaction in the presence of 750 mM NH$_4$Cl – an amount which is sufficient to abolish O$_2$ evolution – progressively is slowed down with increasing pH (Fig. 7). This indicates that the delay effect depends on NH$_3$ concentration and not on that of NH$_7^-$ – a result clearly not compatible with the first hypothesis.

Therefore, it has to be concluded that dissolution of NH$_3$ within the thylakoid membrane inhibits the diffusion controlled recombination reaction of the electron donor Q~ and electron acceptor S$_2$ of the back reaction. Since NH$_3$ is thought to bind to the S$_2$ state [4, 5], an attractive explanation of the effect of NH$_3$ on the back reaction would be to assume that binding of NH$_3$ to S$_2$ restricts the mobility of the charge carrying prosthetic group of the water splitting enzyme. However, this explanation can be ruled out because after complete inhibition of O$_2$ evolution by NH$_3$ the kinetics of the back reaction should remain unaffected when the concentration of NH$_4$Cl is further increased. This was not observed. Though O$_2$ evolution is abolished in the presence of 250 mM NH$_4$Cl (Fig. 8), the kinetics is further slowed
down considerably at higher NH₄Cl concentrations (Fig. 1).

It is suggested, therefore, that dissolution of high amounts of NH₃ within the lipid phase of the thylakoid membrane affects its structure in such a way that the diffusion controlled reduction reaction of S₂ is inhibited. This means that the conforma-

<table>
<thead>
<tr>
<th>[NH₄Cl] mM/L</th>
<th>( L₀ )</th>
<th>( \mathcal{S} _\text{tot} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>112</td>
<td>38.7</td>
</tr>
<tr>
<td>50</td>
<td>59</td>
<td>45.7</td>
</tr>
<tr>
<td>100</td>
<td>63</td>
<td>58.4</td>
</tr>
<tr>
<td>200</td>
<td>62</td>
<td>61.5</td>
</tr>
<tr>
<td>400</td>
<td>21</td>
<td>29.3</td>
</tr>
<tr>
<td>700</td>
<td>6.5</td>
<td>18.1</td>
</tr>
</tbody>
</table>

The values of \( L₀ \) and \( \mathcal{S} \_\text{tot} \) are given in arbitrary units.

This conclusion is supported by the finding that the value of the exciton yield of the back reaction is changed upon addition of NH₄Cl. The exciton yield is the probability that a Chl molecule of the reaction center will be excited during the back reaction. This quantity is expected to be quite sensitive to changes in membrane ultrastructure. The change of the value of the exciton yield can be derived from the observation that the total light sum \( \mathcal{S} \_\text{tot} \) of luminescence strongly depends on the concentration of NH₄Cl added (Table I). The total light sum may be determined by the expression [11, 12]

\[
\mathcal{S} \_\text{tot} = \varphi_0 SC_0 \left\{ 1 - \left( \frac{\varphi_{\infty}}{\varphi_0} - 1 \right) \frac{1 - p}{p} \left( 1 + \ln \left( \frac{1 - p}{p} \right) \right) \right\},
\]

\( p \) denotes the mean probability of excitation transfer between reaction centers of photosystem II. \( \varphi_0 \) and \( \varphi_{\infty} \) are the fluorescence quantum yields when the concentration of \( Q^- \) is zero or maximal, respectively. \( S \) is the exciton yield of the back reaction and \( C_0 \) is the concentration of photosystem II reaction centers.
Fig. 7. Kinetics of the back reaction in *Chlorella* in the presence of DCMU and 750 mM NH$_4$Cl at various pH values. ○ pH = 6.5; ● pH = 7.0; □ pH = 7.5. Phosphate buffer, $T = 25^\circ$C; DCMU, 20 µM, Chl, 42 µg/ml. Preillumination time, 30 sec. Kinetic data are represented as indicated in the section "Theoretical concepts". $C_0$ and $C$ denote the maximal concentration of $Q^-$ or its concentration at time $t$, respectively.

The values of $\varphi_0$ and $\varphi_{\infty}$ were found to be only slightly decreased (<10%) in the presence of 100 mM NH$_4$Cl [18]. Measurement of the fluorescence induction curves in the presence of NH$_4$Cl at various concentrations up to 400 mM as indicated in Table I revealed that addition of NH$_4$Cl (incubation time 30 min) did not affect the value of $p$ within experimental accuracy*. Since the effect of NH$_4$Cl on the values of $\varphi_0$, $\varphi_{\infty}$ and $p$ is negligible, the change of $I_{\text{tot}}$ monitors the change of the exciton yield $S$.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. The technical assistance of Mrs. Ute Bergmann is gratefully acknowledged. The *Chlorella* samples were kindly provided by the Institute of Botany (Prof. A. Ried), University of Frankfurt.

* The variable fluorescence was nearly entirely suppressed, however, upon addition of 700 mM NH$_4$Cl but not at lower NH$_4$Cl concentration.