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# STUDIES ON THE PROTON TRANSPORT AT SYSTEM II IN TRYPSIN-TREATED SPINACH CHLOROPLASTS

G. RENGER and R. TIEMANN

*Max Volmer Institut für Physikalische Chemie und Molekularbiologie, der Technischen Universität Berlin, Strasse des 17. Juni 135, D-1000 Berlin 12 (Germany)*

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## Summary

The proton transport coupled with the DCMU-insensitive oxygen evolution mediated by $K_3[Fe(CN)_6]$ in trypsin-treated chloroplasts (Renger, G. (1976) FEBS Lett. 69, 225—230) has been investigated with the aid of the pH indicator bromcresol purple. It was found that (1) the proton uptake from the outer aqueous phase observed in normal chloroplasts is completely suppressed by mild trypsin treatment; (2) a rather slow proton release into the external phase is detected which is insensitive to DCMU; (3) in the presence of DCMU, the extent of the proton release depends on the incubation time with trypsin in a similar manner as the average oxygen yield per flash.

The results are interpreted by the assumption, that: (i) the reduced primary electron acceptor of System II, X $320^-$, does not become protonated, and (ii) the external acidification is caused by a passive efflux of protons, which are released by the watersplitting enzyme system Y into the inner phase of the thylakoids. The p$K$ value of X $320^-$ in trypsinated chloroplasts is estimated to be below 4.5. A possible p$K$ shift caused by a modification of the proteinaceous barrier, which earlier (Renger, G. (1976) Biochim. Biophys. Acta 440, 287—300) was postulated to cover up the primary electron acceptor X 320, is discussed.

Furthermore, the watersplitting enzyme system Y is inferred to be sensitive to deletereous attack from the outer aqueous phase mainly by secondary structural effects. Trypsination does not change the direction of the proton release in system Y.

## Introduction

Within the framework of the widely accepted zig-zag-scheme of electron transport from water to $NADP^+$ the primary reactions of Systems I and II lead

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 2-(*N*-morpholino)sulfonate.

to a vectorial electron shift from inside to outside across the thylakoid membrane (for reviews see refs. 1 and 2). Subsequently, protonations at the acceptor side cause proton uptake from the external aqueous phase, whereas deprotonations giving rise to internal proton release accompany the secondary reactions at the donor side of Systems I and II. The external proton uptake at System I is finally due to $NADP^+$-reduction. Analogously, proton uptake by System II was found to be coupled by a 1 : 1-stoichiometry to the reduction of the plastoquinone pool [3,4]. However, as three different types of functional plastoquinones, acting as primary acceptor (X 320, refs. 5 and 6), as pool [6] and as connector molecule B [7,8], respectively, can be distinguished, the site of proton uptake has to be clarified.

Very recently, in dark-adapted chloroplasts the proton uptake from the outer aqueous phase was shown to oscillate with a periodicity of two [9], which was ascribed to be due to different p$K$ values of the redox states $B^-$ and $B^{\cdot}$. Likewise, on the basis of spectral evidence, the protonation of the primary acceptor in its reduced semiquinone form, $X\ 320^-$, was inferred to be prevented under normal conditions [18]. This might be caused either by thermodynamical reasons (low p$K$ value of X 320 · H) or by a kinetic effect, if the protonation of $X\ 320^-$ becomes markedly slower than its reoxidation [5,6] by B or $B^-$ due to the retardation of proton transport by a diffusion barrier. Based on kinetic studies of proton uptake from the outer aqueous phase the reducing sides of both Photosystems were inferred to be covered up by a shield acting as barrier to proton diffusion [10]. Furthermore, investigations of the effect of mild trypsination on the activity of System II and its blockage by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) led to the conclusion that X 320 is embedded into a proteinaceous component, which prevents the direct reoxidation of $X\ 320^-$ by external electron acceptors [11]. This proteinaceous component could also be responsible for the limitation of the proton transport rate. Hence, it seems to be worthwhile to investigate the influence of trypsin on the external proton uptake at System II. The presented data show that trypsination completely suppresses the proton uptake from the outer phase under conditions where more than 50% of all watersplitting enzyme systems Y remain fully intact. Instead of a transient proton uptake, a rather slow proton efflux is observed. On the basis of the present results the p$K$ value of the $X\ 320^-$-plastosemiquinone is estimated to be below 4.5. A possible p$K$ shift caused by trypsination or some other treatments making X 320 accessible to the outer aqueous phase is discussed.

## Materials and Methods

Class II subchloroplasts lacking the envelope have been prepared according to the method of Winget et al. [13], as described in ref. 14. Trypsination was performed at room temperature by injection of commercially available trypsin (Boehringer) into the chloroplast suspension, at a concentration of 20 $\mu$g/ml suspension and 50 $\mu$g/ml chloroplast suspension for the measurements of absorption changes and oxygen, respectively. The measurements were made after an incubation time indicated in the abscissae or the legends of the figures. The dependency on the time of trypsination was measured in the same sample.

As the time required for the detection of a signal was short enough in comparison to the trypsination rate, in most of the experiments no trypsin inhibitor was applied to stop the digestion process, so that a complete trypsination pattern was obtainable from the same sample (experiments in the presence of trypsin inhibitor confirm this procedure to be applicable).

The reaction mixture contained: chloroplasts (10 $\mu$M and 50 $\mu$M chlorophyll for the measurements of absorption changes and oxygen, respectively), 300 $\mu$M $K_3Fe(CN)_6$, 10 mM KCl, 2 mM $MgCl_2$. For the detection of the flash-induced pH changes in the outer aqueous phase 25 $\mu$M bromocresol purple was added and the pH was adjusted to 6.5 by injection of a small amount of NaOH. Instead of bromcresol purple the assay for the oxygen measurements contained additionally 20 mM (2-(*N*-morpholino)ethanesulfonate (MES)-NaOH, pH 6.5.

The absorption changes at 571 nm due to the pH indicator bromocresol purple and due to the turnover of X 320 are recorded by a repetitive flash spectrophotometer, as is outlined in ref. 4 (for the details of the X 320-measurements via the absorption change at 334 nm see ref. 6). The device for the oxygen detection is described in ref. 15.

## Results

In normal chloroplasts the kinetics of the turnover of X 320 indicated by the absorbance change at 334 nm and of the proton uptake from the outer aqueous phase reflected by an absorbancy increase of bromcresol purple at 571 nm are quite different, as is shown in Fig. 1 (top). The rather slow proton uptake was shown to be caused by a barrier to proton diffusion [10]. The kinetical arguments, however, cannot exclude a protonation of the X $320^-$-semiquinone form, because internal proton pools could donate protons very rapidly ($\mu$s range) to X $320^-$, but would equilibrate only slowly (a few tens of milliseconds) with the outer aqueous phase. Likewise, an oscillatory pattern with a periodicity of 2 [9] could not only be caused by different p*K* values, but also by a barrier whose permeabilities switch between "closed" and "open" states, depending on the conformation which might be regulated allosterically by the redox state of B. Therefore, a simpler situation would arise if this barrier could be removed.

If one tentatively assumes that the proteinaceous shield, which covers X 320 [11], acts simultaneously as a barrier to proton transport at System II, trypsination would be a useful tool to study the possibility of the protonation of X $320^-$. Furthermore, in trypsinated chloroplasts DCMU does not inhibit ferricyanide-mediated oxygen evolution. On the contrary, 1 $\mu$M DCMU completely suppresses System II activity in normal chloroplasts, as shown in Fig. 1 (middle). Mild treatment with trypsin was shown to interrupt the internal connection between X 320 and the pool [11,12] (probably at the stage of B), concomitantly with a drastic increase of the accessibility of X $320^-$ to external ferricyanide. The reoxidation of photoreduced X $320^-$ by ferricyanide is slower than the in vivo electron transfer to B or $B^-$ and becomes dependent on the ferricyanide concentration [16]. In the presence of 0.3 mM ferricyanide the reoxidation of X $320^-$, refelected by the decay of the 334 nm absorbance change, has a half-life time of a few ms, as is shown in Fig. 1 (bot-

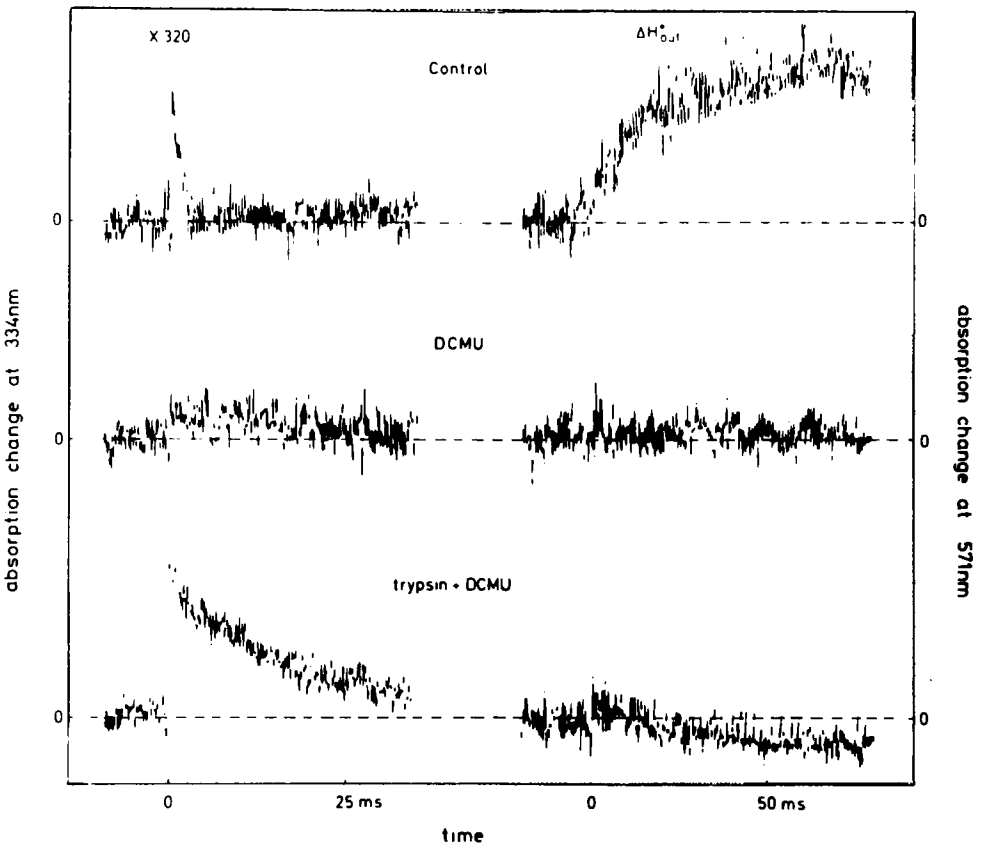


Fig. 1. Absorption changes at 334 nm, reflecting the turnover of X 320, and at 571 nm, indicating via the response of the indicator dye bromcresol purple the change of the external proton concentration (absorbance increase is caused by external alkalization), as a function of time. Excitation, short flashes (20 μs) at a repetition rate of 4 Hz; 512 signals were averaged. Top, normal chloroplasts; middle, normal chloroplasts in the presence of 1 μM DCMU; bottom, chloroplasts trypsinated for 5 min, measurements in the presence of 1 μM DCMU.

tom, left side). The accessibility of X 320$^-$ to $K_3Fe(CN)_6$ suggests a rather hydrophilic surrounding of X 320 in trypsinated chloroplasts. Accordingly, as the photoreduction of X 320 is accomplished in less than 1 μs [17] a very fast protonation is expected, provided that the p$K$ value of X 320 · H is sufficiently high. In a subsequent reaction the semiquinone becomes reoxidized in a few ms, so that a deprotonation back into the hydrophilic environment would occur, whose kinetics should nearly coincide with the electron abstraction from X 320 · H by $K_3Fe(CN)_6$. If indicator dye molecules are located very close to the primary electron acceptor of System II, a transient proton uptake coupled with the turnover of X 320 would be directly reflected by the indicator. A transient alkalization of the outer aqueous phase, characterized by the above mentioned kinetics, has not been detected, as is shown in Fig. 1 (bottom right), but rather a slow acidification due to protons liberated by water oxidation (vide infra) takes place. The failure to observe a transient proton uptake could therefore simply be explained by the p$K$ of X 320 · H being significantly

smaller than the pH of the outer aqueous phase. However, the result can be explained also by a kinetic effect, if the indicator dye molecules are separated from X 320$^-$ even in trypsinated chloroplasts by a specific barrier slowing down the proton transport rate below the turnover rate of X 320. The latter effect can be excluded, because in the presence of the potent protonophore *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone no transient proton uptake was found, but the acidification becomes drastically accelerated (data not shown). Therefore, the present results lead to the conclusion that at pH 6.5 the semiquinone anion X 320$^-$ is not protonizable for thermodynamical reasons, i.e. the p$K$ value should be markedly below 6.0. In order to obtain a better estimation of the p$K$ value, chloroplasts were trypsinated at pH 6.5 for 10 min and after the subsequent addition of trypsin inhibitor adjusted to pH 5.4 by injection of HCl. Even under these conditions no transient alkalization could be observed. Therefore, it appears reasonable to suggest that the p$K$ value of X 320$^-$ is less than 4.5. This conclusion is in agreement with results of van Gorkom [18] who has shown, on the basis of spectral evidence, that in digitonon-fractionated subchloroplasts the reduced primary electron acceptor of Photosystem II, X 320$^-$, does not become protonated even at pH 4.

On the other hand, it was found that under mild trypsination a significant

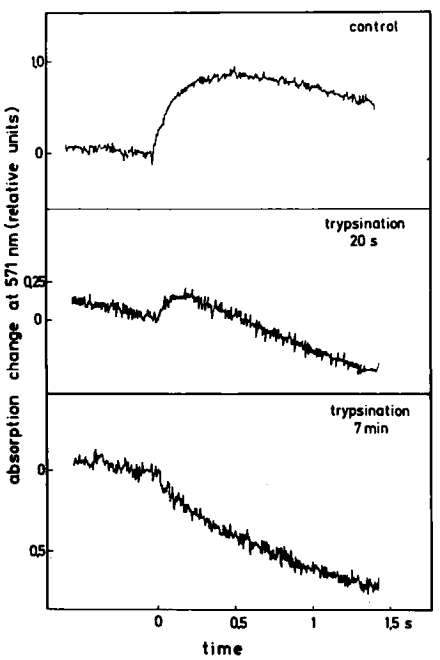


**Fig. 2. Absorption change at 571 nm as a function of time. Excitation, short flashes (20 μs) at a repetition rate of 0.1 Hz; 16 signals were averaged.**

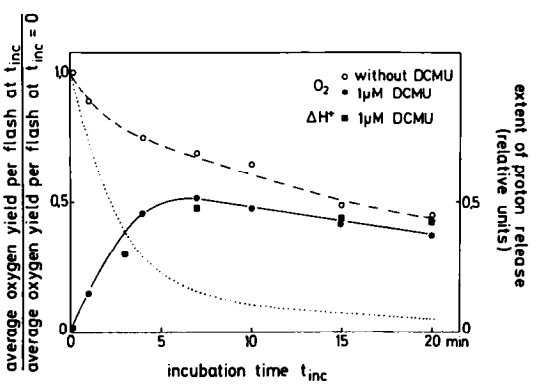


**Fig. 3. Relative average oxygen yield per flash and relative extent of proton release into the outer aqueous phase as a function of the incubation time with trypsin, $t_{inc}$, in normal chloroplasts in the presence of 1 μM DCMU. For comparison the dependence on $t_{inc}$ of the relative average oxygen yield per flash in the absence of DCMU is depicted. The dotted line is the difference of the oxygen evolution in trypsinated chloroplasts in the absence and presence of DCMU, respectively.**

fraction of the watersplitting enzyme system Y remains intact for oxygen evolution, which has to be accompanied by proton release. In Fig. 2 (top) the proton uptake in normal chloroplasts is shown. After a short incubation time with trypsin the proton uptake is drastically reduced (Fig. 2, middle). With a prolonged trypsin treatment, however, a slow proton release is observed, which appears to be insensitive to DCMU (Fig. 2, bottom and Fig. 3). As this slow acidification is assumed to be caused by the protons liberated due to water oxidation, the extent (under our excitation conditions the proton release pattern of system Y (see refs. 19—21) does not influence the average number of protons released per flash) should depend on the time of incubation with trypsin in the same manner as the average oxygen yield per flash. The data are shown in Fig. 3. In the absence of DCMU the oxygen yield gradually declines, indicating a slow degradation of the watersplitting enzyme system Y. On the contrary, in the presence of DCMU, the oxygen yield first increases and afterwards decreases. The difference between both curves (represented by the dotted curve) can be interpreted as the digestion of the proteinaceous component, which covers up X 320 and is simultaneously responsible for the DCMU blockage [22]. In the presence of DCMU, the extent of proton release as a function of the incubation time has to correspond with the dependence of the average oxygen yield per flash. In the absence of DCMU, however, the scheme becomes complicated at short times because of the overlapping of faster proton uptake in systems still surviving the tryptic attack and of the slow subsequent proton efflux of the systems already modified (Fig. 2, middle and bottom, respectively). Therefore, only the data obtained in the presence of DCMU are presented in Fig. 3. They correspond fairly well with the oxygen measurements.

## Discussion

The proton transport pattern in mildly trypsinated chloroplasts coupled with the $K_3Fe(CN)_6$ supported DCMU-insensitive System II electron transport can be explained by the assumptions, that (a) the p*K* value of X $320^-$ is low enough to prevent its protonation, and (b) the protons released due to the irreversible water oxidation lead to a comparitively slow external acidification. The scheme in Fig. 4A describes the above discussed proton transport pattern. Theoretically, the same overall proton release could also be obtained by the overlapping of a transient proton uptake coupled with the X 320 turnover and a fast proton release into the outer aqueous phase by the water splitting enzyme system Y, as is shown in Fig. 4B. However, the latter proton transport pattern can readily be discarded, because the rate of the external acidification is much slower than the rate of X $320^-$ reoxidation and is strongly dependent on the permeability of the thylakoid membrane (vide infra).

According to the present results X $320^-$ is inferred to remain unprotonated in trypsinated chloroplasts for thermodynamic reasons. The p*K* is estimated to be below 4.5. This estimation is in close correspondence with conclusions drawn by van Gorkom [18] on the basis of spectroscopic measurements made at pH 4.0 in digitonin-fractioned chloroplasts.

Similarly, in normal chloroplasts the X 320 turnover was also claimed not to

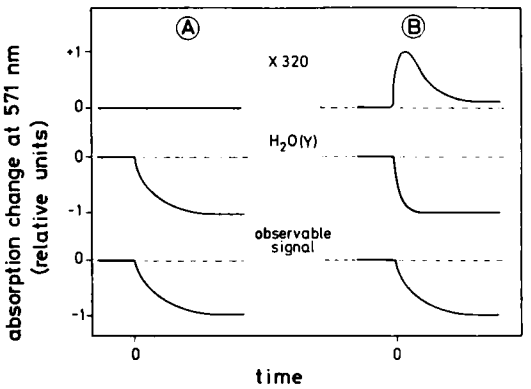


**Fig. 4. Scheme of two different proton transport pattern at System II in trypsinated chloroplasts giving rise to an acidification of the outer aqueous phase (for explanation see text). X 320 and $H_2O(Y)$ symbolize the proton transport due to a possible transient protonation of X 320 and to the irreversible water oxidation, respectively. Negative absorption changes at 571 nm caused by bromcresol purple reflect acidification.**

be coupled with a transient protonation [18,23]. In the simplest way this effect could analogously be explained by thermodynamics, i.e. by X 320 · H having a low p$K$. However, at least two experimental findings cast serious doubts on this interpretation:

(a) A low p$K$ in normal chloroplasts appears to be in conflict with titration experiments, indicating the midpoint potential of the primary acceptor of System II (X 320) to be strongly pH dependent in the pH range of 6—9 [24, 25]. This effect was interpreted to be due to the fact that X 320 can chemically be reduced only via the connector molecule B, so that the observed pH dependence would really be caused by the properties of B [18]. However, if one admits the existence of a true equilibrium between the redox states of X 320 and B [31], this explanation is not easily understandable without serious restrictions of the principle of microreversibility.

(b) As X 320 is proposed to be a special plastoquinone molecule, p$K$ values below 4.5 are not easily reconcilable with the dissociation constants of closely related compounds, such as ubisemiquinone and durosemiquinone, which were found to be of the order of $10^{-6}$ M in polar solvents like methanol or water/ethanol mixtures [26,27]. However, as different lines of evidence suggest X 320 to be situated in a special hydrophobic site [11,32,33], the p$K$ value of X 320 is expected to be even higher [28] *, in correspondence with the pH dependence reported for the redox potential of X 320. It should be mentioned that a high polarity microenvironment closed up to the outer aqueous phase by a strong diffusion barrier would be reconcilable with a low p$K$ value [31], but there is no any experimental hint to favour this assumption.

Accordingly, on the basis of the above mentioned arguments the p$K$ value in normal chloroplasts is thought to be too high ($\geqslant$5.5) to provide a thermody-

* Of course, a comparison with in vitro systems cannot provide unambigious evidence for a rather high p$K$ value because of the special state of the X 320-plastoquinone in vivo, which is reflected by the inability to accept two electrons in chloroplasts blocked by DCMU-type inhibitors.

namic explanation for X 320$^-$ remaining unprotonated down to pH 6.4 [23].

Therefore, the interpretation of the failure of the X 320 turnover to be coupled with a transient proton uptake in normal chloroplasts by a kinetic rather than by a thermodynamic effect seems to be more attractive.

The protonation of X 320$^-$ is assumed to be prevented by a restricted diffusion due to the proteinaceous shield, which covers up X 320 [11], so that the proton transfer is slow in comparison with the reoxidation of X 320$^-$ by B or B$^-$. In this way the proteinaceous shield would regulate not only the electron transport of the acceptor side of System II and its inhibition by DCMU-type inhibitors [11,22], but also influence the protonation pattern. After the modification of this component by trypsin treatment the functional connection to the plastoquinone pool becomes interrupted together with a significant polarity change of the environment of X 320. This effect shifts the p$K$ value towards the acidic range, so that under these conditions X 320$^-$ cannot become protonated for thermodynamic reasons. Similar effects could be responsible for the results obtained on detergent or low pH-treated chloroplasts [18,29]. The present results indicate that in mildly trypsinated chloroplasts the $K_3[Fe(CN)_6]$-mediated oxygen evolution involves only one protolytic reaction: the proton release due to water oxidation. In normal chloroplasts the proton release at the water splitting enzyme system Y was shown to be directed into the inner space of the thylakoids [30]. It could be possible that trypsination would lead to a direct release into the outer aqueous phase of the protons expelled from system Y. However, preliminary experiments with neutral red (Ausländer, W. and Renger, G., see ref. 22) indicate that trypsination does not seriously modify the rapid proton release at system Y into the inner thylakoid space. Accordingly, the external acidification (see Fig. 2) is caused by the passive efflux of the internally released protons and its kinetics is limited by the permeability of the thylakoid membrane (in the presence of uncouplers the kinetics becomes markedly faster, data not shown). Thus, mild trypsination does not influence the water splitting enzyme system Y with respect to its polarity of proton transport. This provides further evidence for the assumption that a deleterious attack of trypsin on system Y might be caused by secondary structural effects [22].

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