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STUDIES ON THE STRUCTURAL AND FUNCTIONAL ORGANIZATION OF SYSTEM II OF PHOTOSYNTHESIS

THE USE OF TRYPSIN AS A STRUCTURALLY SELECTIVE INHIBITOR AT THE OUTER SURFACE OF THE THYLAKOID MEMBRANE

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SUMMARY

The effect of trypsin on the photosynthetic electron transport has been investigated in the presence of various electron acceptors (benzyl viologen, *p*-benzoquinone, $K_3[Fe(CN)_6]$) by measurements of flash-induced oxygen evolution and of the absorption changes at 334 nm, indicating the primary electron acceptor of System II, X 320, and at 515 nm, indicating via electrochromism the electrical potential gradient across the thylakoid membrane. It was found that the effect of trypsin is strongly dependent on the nature of the electron acceptor:

(1) Oxygen evolution is completely inhibited in the presence of *p*-benzoquinone, but remains nearly unaffected by $K_3[Fe(CN)]_6$.

(2) The initial amplitude ΔA_0 of the 334 nm absorption change is insensitive to trypsin in the presence of $K_3[Fe(CN)_6]$, but the absorption change is abolished if benzyl viologen is used as acceptor.

(3) The initial amplitude ΔA_0 of the 515 nm absorption change decreases by trypsin down to 50 % with $K_3[Fe(CN)_6]$ and is completely suppressed with benzyl viologen.

(4) In trypsinated chloroplasts, the above-mentioned activities appear to be rather insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea, in contrast to normal chloroplasts.

On the basis of these results it is inferred that the primary electron acceptor of System II, X 320, is covered up by a proteinaceous component susceptible to tryptic digestion. In addition, it is postulated that this component acts as well as an allosteric protein responsible for the regulation of the electronic interaction between X 320 and the plastoquinone pool, as for the inhibitory effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Various other possible effects caused by the proteinaceous shield and its modification by trypsin are discussed.

The present results are in complete agreement with asymmetric membrane

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, tris(hydroxymethyl)methylglycine.

In memoriam to my parents (deceased on August 28th, 1975 and September 20th, 1975).

models postulating a zig-zag arrangement of the electron transport chain with the reducing side located towards the outer phase and the oxidizing side near the inner phase of the thylakoids.

INTRODUCTION

In a previous paper [1], indirect experimental evidence has been presented in favor of the assumption that the site of action of the well known inhibitory effect on electron transport caused by trypsin treatment in spinach chloroplasts [2–4] is probably located near the primary acceptor of Photosystem II. This interpretation is plausible on the basis of recent models of the structural organization of the electron transport chain within the thylakoid membrane. Within the basic framework of the membrane model postulated by Singer and Nicholson [5] the proteinaceous reaction centers of System I and II are assumed to be integral membrane proteins (or lipoproteins) anisotropically arranged perpendicular to the plane of the thylakoid membrane. The reaction centers are oriented in such a way that the primary electron acceptors of System I and II, an Fe-S protein [6] and X 320 [7, 8], respectively, are located near the outer phase, whereas the primary donor molecules, chlorophyll a_I [9, 10] and chlorophyll a_{II} [11, 12] are directed towards the inner phase of the thylakoid membrane [12–16]. This specific orientation of the reaction centers causes the light-induced charge separation to be a vectorial electron flux from the inside of the thylakoid membrane to its outside, followed by a net proton flux via protonation/deprotonation reactions (for review, see ref. 16). If the basic ideas of the above-mentioned models about the structural organization of photosynthetic electron transport are correct, one would then anticipate that large water-soluble impermeable molecules such as the proteolytic enzyme trypsin, can attack only those proteinaceous components of the electron transport chain which are located near the outer phase, i.e. mainly the reducing side of the reaction centers and/or structurally correlated secondary electron acceptors. Recently, it has been shown that trypsin digestion not only blocks System II electron transport but also inhibits $NADP^+$ reduction mediated by System I electron donors, such as DCIP plus ascorbate, whereas the Mehler reaction supported by viologens [17] remains uneffected [18]. This effect was shown to be caused by an interference of trypsin with the enzyme ferredoxin-NADP reductase [18]. Immunological investigations proved ferredoxin-NADP reductase to be located at the outer phase of the thylakoid membrane [19]. Thus, this result and our indirect conclusion, i.e. that the location of the tryptic attack on System II is also on the reducing side [1], strongly support the above-mentioned consideration. However, in addition, it has been inferred by some groups [2–4, 18] that trypsin digestion also interrupts the electron transport between the water-splitting enzyme system Y and the reaction centers of System II. A direct attack of trypsin should be possible only if either some electron carriers of the oxidizing side of System II are accessible from the outer aqueous phase by comparatively large impermeable water-soluble molecules like trypsin, or if the membrane structure is so strongly modified that trypsin can penetrate into the inner phase of the thylakoid. However, the second alternative is not easily reconcilable with the experimental finding that trypsin penetration requires ultrasonic treatment of the chloroplasts [18]. On the other hand, an indirect effect

of trypsin, leading to blockage of the donor side of System II by a modification of membrane structure, cannot be excluded.

Hence, it seems to be worthwhile to clarify the effect of trypsin treatment on System II in order to get further information about the structural organization of System II within the thylakoid membrane.

In the present paper, the effect of trypsin on the oxygen evolution and on the absorption changes at 334 nm indicating the function of the primary electron acceptor X 320 [7, 8] and at 515 nm indicating via electrochromism the electrical field across the thylakoid membrane [20, 21] has been investigated under different conditions. The obtained results clearly indicate that, under the experimental conditions applied here, trypsin does not seriously interfere with the oxidizing side of System II. This corroborates earlier assumptions about the localization of the water-splitting enzyme system Y near the inner phase of the thylakoid membrane [22, 23].

MATERIALS AND METHODS

Stripped spinach chloroplasts have been prepared according to the method of Winget et al. [24], as described in ref. 25.

The standard reaction mixture for the measurements of oxygen evolution contained: chloroplasts (50 μM chlorophyll), 100 μM *p*-benzoquinone or 1 mM $K_3[Fe(CN)_6]$+1 mM $K_4[Fe(CN)_6]$ as electron acceptor, 10 mM KCl, 2 mM $MgCl_2$, 20 mM Tricine/NaOH, pH 7.0.

The standard reaction mixture for the measurements of the absorption changes contained chloroplasts (100 μM chlorophyll) and 100 μM benzyl viologen instead of *p*-benzoquinone as electron acceptor, and other additions as for the oxygen measurements.

Trypsin treatment of the chloroplasts occurred in the same way as in ref. 1. Details about the incubation time are given in the legends of the figures.

The oxygen measurements were performed with a Clark type electrode by a repetitive technique as was described in ref. 26.

The method for the measurement of the absorption changes using a repetitive flash photometer similar to that of ref. 27 is described in ref. 1. The measurements were carried out under excitation conditions where a contribution due to a slow back reaction around System II can be neglected (for details see ref. 25).

RESULTS

Fig. 1 shows the effect of trypsin treatment on the average oxygen yield per flash. Whereas in the presence of *p*-benzoquinone as electron acceptor, trypsin progressively inhibits the oxygen production in a way similar to that which has been already reported for the initial amplitudes of photoinduced absorption changes of the electrochromic effect and of chlorophyll a_1, in the presence of benzyl viologen [1], practically no influence of trypsin on oxygen evolution is observed in the presence of $K_3[Fe(CN)_6]$ as electron acceptor (see Fig. 1A). The small effect in the presence of $K_3[Fe(CN)_6]$ is caused mainly by slow thermal degradation of the water-splitting enzyme system Y, occurring independently of the presence of trypsin. Correspondingly, at a constant incubation time, the inhibitory degree of oxygen evolution increases

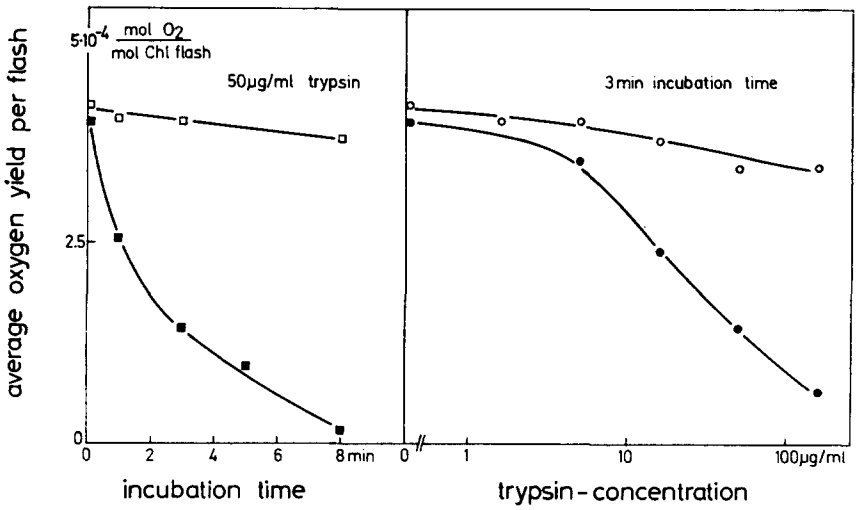

Fig. 1. Average oxygen yield per flash as a function of incubation time of trypsin or of trypsin concentration in the presence of *p*-benzoquinone (closed symbols) or K_3 [$Fe(CN)_6$] (open symbols) as electron acceptor in spinach chloroplasts. Chloroplast suspension was as described in Materials and Methods. Trypsin concentration and incubation time are given in the figure. After the indicated dark incubation time the measurements began. Excitation: 120 white flashes (20 µs, saturating intensity), time t_d between the flashes 250 ms.

with increasing trypsin concentration if *p*-benzoquinone acts as electron acceptor, whereas in the presence of $K_3[Fe(CN_6)]$ only a slight effect occurs (Fig. 1B).

The results strongly support the conclusion that generally the electron transport of the oxidizing side of System II and especially the water-splitting enzyme system Y are rather resistant to tryptic digestion. The obvious importance of the nature of the electron acceptors for the sustenance of oxygen evolution in the presence of trypsin, indicates a possible modification of the reducing side of System II. This corroborates our earlier conclusion about the mode of action of tryptic digestion [1]. For a further clarification of this point, it seems interesting to investigate the effect of inhibitors specifically interfering with the reducing side of System II, on the oxygen evolution in trypsin-treated chloroplasts. In Fig. 2, the effect of DCMU, known to act as a very effective inhibitor of electron transport from reaction centers of system II into the plastoquinone pool [28, 29], has been studied on oxygen production. The results show that in the presence of *p*-benzoquinone at all trypsin concentrations and at different incubation times (not explicitly shown) the oxygen evolution remains blocked by DCMU. On the contrary, in the presence of $K_3[Fe(CN)_6]$ as electron acceptor, the oxygen-evolving capacity reappears in the presence of DCMU after a suitable treatment at high enough concentrations and incubation times of trypsin. The degree of regeneration amounts to up to 85 % of the control, as is seen by comparison with the corresponding control values (in the absence of trypsin and DCMU) given by horizontal bars on the ordinate. For a maximal recovery, a sufficient incubation time of 5–6 min and a trypsin concentration of approx. 50 µg/ml is required. The injection of $K_3[Fe(CN)_6]$ into chloroplast suspensions incubated in the presence of *p*-benzoquinone with 50 µg/ml trypsin for a few minutes which are not able to produce oxygen (see Fig. 2) leads to a recovery of oxygen-evolving capacity (data and shown).

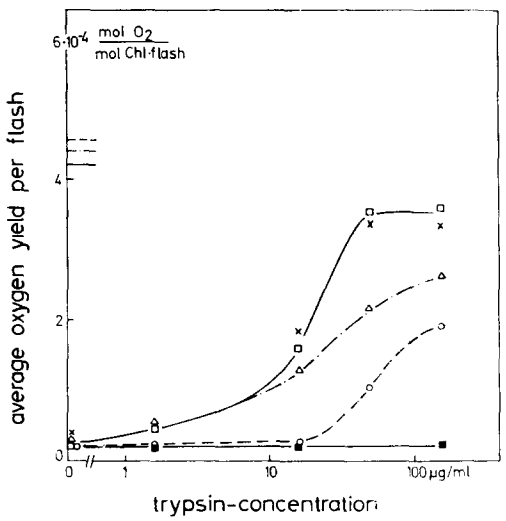

Fig. 2. Average oxygen yield per flash as a function of trypsin concentration at different incubation times in the presence of *p*-benzoquinone (■) or $K_3[Fe(CN)_6]$ (○, △, □, ×) as electron acceptor and 0.2 µM DCMU in spinach chloroplasts. Incubation time of trypsin: 1 min (○ – – ○), 3 min (△ – · – △), 6 min (□–□) and 10 min (×–×). Other conditions as in Fig. 1. Bars on the ordinate represent the average oxygen yield per flash with $K_3[Fe(CN)_6]$ as electron acceptor in the absence of DCMU and trypsin after 1 min (– – –), 3 min (– · –) and 6 min (–), respectively.

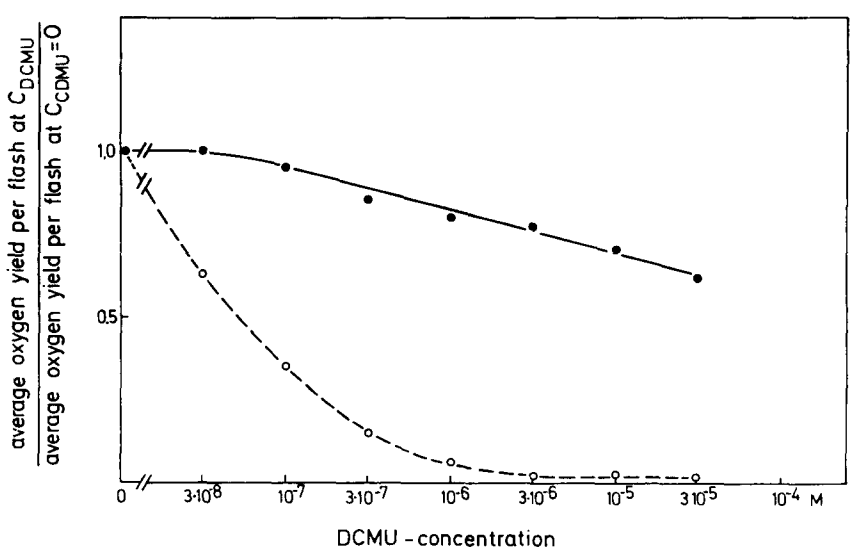

Fig. 3. Average oxygen yield per flash as a function of DCMU concentration with $K_3[Fe(CN)_6]$ as electron acceptor in normal (○ – – –○) and in trypsinated (●–●) chloroplasts. Trypsinated chloroplasts were obtained by incubation with 50 µg/ml trypsin for 8 min.

In Fig. 3 the dependence of the average oxygen yield per flash of normal chloroplasts on DCMU concentration [30] is compared with that of trypsinated chloroplasts. The results show, that in the presence of $K_3[Fe(CN)_6]$ as electron acceptor in trypsin-treated chloroplasts, oxygen evolution persists at comparatively high DCMU concentrations. This indicates that the reaction center of System II and its oxidizing side are rather stable to DCMU inhibition.

These results provide direct evidence for the conclusion that trypsin attacks near the primary electron acceptor X 320 of Photosystem II. In order to clarify this point, measurements of the absorption changes at 334 nm* indicating the reactions of X 320 [7, 8] were made under different conditions.

Figs. 4A and 4E show the normal absorption changes in the presence of the electron acceptors benzyl viologen and $K_3[Fe(CN)_6]$, respectively, characterized by a half-decay time of approx. 500 μs, indicating the kinetics of the electron transfer from reduced X 320$^-$ into the plastoquinone pool [7] probably via a secondary acceptor B [31]. The 334 nm absorption changes are suppressed by DCMU, irrespective of the nature of the external artificial electron acceptor (Figs. 4B and 4F). On the contrary, the effect of trypsin treatment is strongly dependent on the nature of the electron

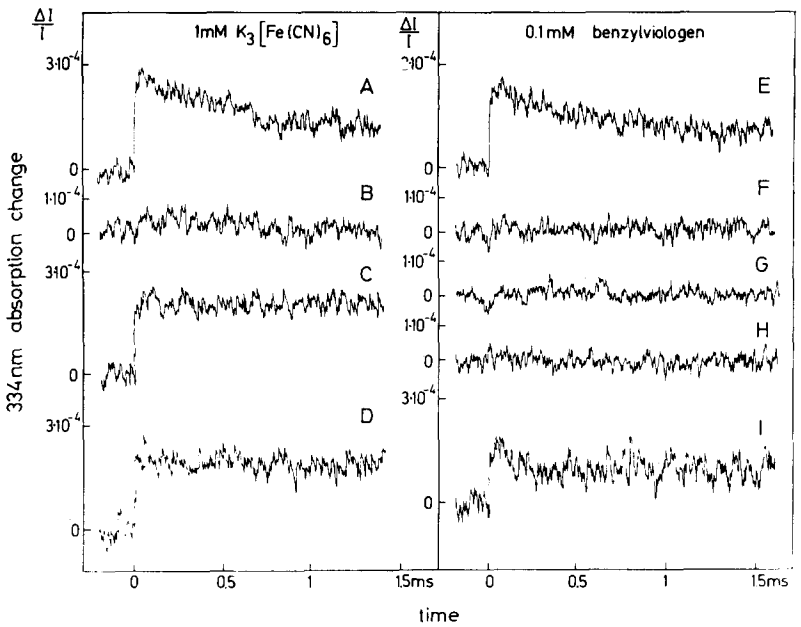

Fig. 4. Absorption changes at 334 nm as a function of time in spinach chloroplasts. Chloroplast suspension as described in Materials and Methods, electron acceptor as indicated in the figure. A and E without further additions; B and F, with 2 μM DCMU; C and G, dark incubation with 50 μg/ml, trypsin for 8 min before the measurements; D and H, dark incubation with 50 μg/ml trypsin for 8 min, then addition of DCMU (final concentration 2 μM) and beginning of the measurements; I, injection of $K_3[Fe(CN)_6]$ (final concentration 1 mM) into the suspension used for experiment H and illumination by a second flash train. Excitation: saturating red flashes (see ref. 1) ,time t_d between the flashes 250 ms; 1024 signals were averaged.

* See Note Added in Proof, 1, p. 298.

acceptor. In the presence of $K_3[Fe(CN)_6]$, trypsin does not seriously influence the initial amplitude of the 334 nm absorption change, but slows its decay kinetics (Fig. 4C)*. On the other hand, in the presence of *p*-benzoquinone, trypsin completely inhibits the 334 nm absorption change (Fig. 4G). DCMU does not significantly modify the 334 nm absorption changes in trypsin-treated chloroplasts (Figs. 4D and 4H), in close agreement with the results of the oxygen evolution experiments. Furthermore, as has been already noted for oxygen production, injection of $K_3[Fe(CN)_6]$ into the inactive chloroplast suspension preincubated with trypsin in the presence of *p*-benzoquinone (Fig. 4H) regenerates the 334 nm absorption change (Fig. 4I). The effect of trypsin is independent of the presence of DCMU during dark incubation.

The results reported so far can be explained by the assumption that trypsin digestion leads to an interruption of the functional connection between X 320 and the plastoquinone pool, so that only external electron acceptors of sufficient oxidizing power, such as $K_3[Fe(CN)_6]$, are able to mediate System II electron transport. In this interpretation is correct, one would expect that in the absence of System I electron donors the absorption changes at 703 nm due to chlorophyll a_I reaction should be suppressed by trypsin treatment, irrespective of the nature of external electron acceptors. This has been found to occur (see refs. 1 and 68). On the other hand, the flash-induced absorption changes at 690 nm caused by chlorophyll a_{II} should be dependent on the nature of the external electron acceptor in trypsinated chloroplasts in a way similar to oxygen evolution and the absorption change of X 320.

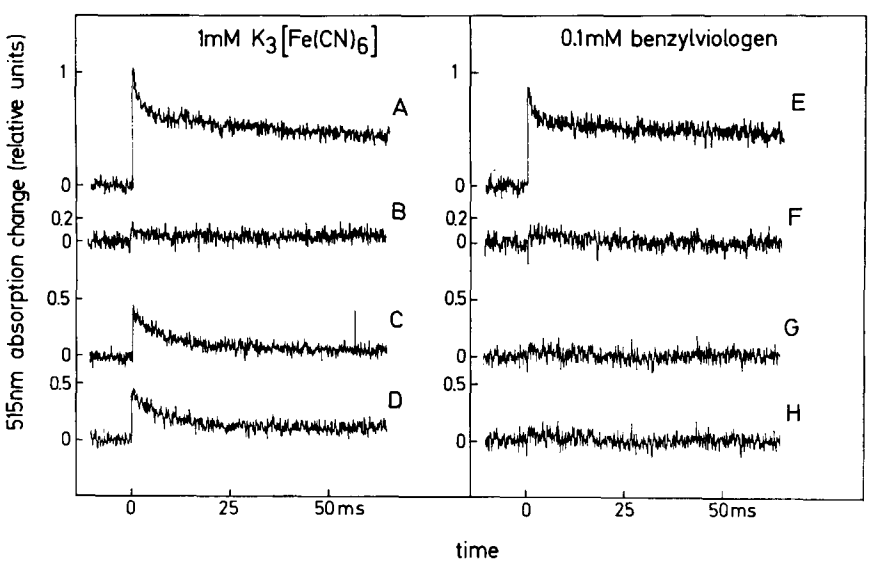

Fig. 5. Absorption changes at 515 nm as a function of time in spinach chloroplasts. Experimental conditions as described in Fig. 4. A and E, without further additions; B and F, with 2 μM DCMU; C and G, dark incubation with 50 μg/ml trypsin for 8 min before the measurements; D and H, dark incubation with 50 μg/ml trypsin for 8 min, then addition of DCMU (final concentration 2 μM) and beginning of the measurements.

* See Note Added in Proof, 2, p. 298.

This has been confirmed by our measurements [1] and recent experiments of Döring [68].

Furthermore, the 515 nm absorption change indicating the generation of an electrical field across the thylakoid membrane by Photosystem I and II, respectively [13, 16], should decrease down to 50 % by trypsin treatment in the presence of $K_3[Fe(CN)_6]$, but should be completely suppressed in the presence of benzylviologen. The results in Fig. 5 show that this really occurs. In addition it is seen that trypsination leads to a modification of the membrane permeability. In chloroplasts frozen for storage in liquid nitrogen and rethawed for the measurements the decay of the 515 nm absorption change appears to be biphasic. The fast component has been ascribed to thylakoids with impaired membranes. It is interesting to note that by trypsin treatment this fast decay becomes decelerated, whereas the decay kinetics of the slow phase of the 515 nm absorption change indicating thylakoids with intact membranes are accelerated.

If one presupposes that the changes in the membrane permeability may be caused mainly by interference of trypsin with coupling factor proteins [32, 33] in a way similar to what has been reported for other treatments of the energy coupling system in chloroplasts [34, 35], one could tentatively speculate that the fast phase of aged chloroplasts reflects a membrane leakage at the coupling factor sites. However, further experiments are required to clarify this point which will not be discussed here.

DISCUSSION

In the previous paper [1], indirect evidence has been given for the localization of trypsin inhibition on the reducing side of System II. As a confirmation of this suggestion, the present data directly prove the inhibition of System II electron transport to be caused by an interruption of the functional connection between the reaction center and the plastoquinone pool, whereas the oxidizing side appears to be rather resistant to tryptic attack under our experimental conditions. Since trypsin is a large water-soluble impermeable molecule, the results are in complete agreement with asymmetric membrane models postulating that the reducing side of the reaction centers is located near the outer phase and the oxidizing side is arranged near the inner phase of the thylakoid membrane [12–16]. However, the present data do not only confirm the general concept of the zig-zag arrangement of the electron transport chain within the thylakoid membrane, but provide further insight into the details of the structural and functional organization of System II.

On the basis of these results *a* structural model of System II depicted in Fig. 6 has been derived. It is postulated: (1) In normal chloroplasts the primary electron acceptor X 320 acting as the negative pole of the photochemically active reaction center C_{II} (see ref. 36) is located near the outer phase of the thylakoid membrane and is covered up by a proteinaceous component. (2) The proteinaceous component acts as an effective shield, leaving X 320 highly inaccessible to external redox agents. (3) The proteinaceous component is an essential structural element probably by its orientation effects (e.g. orbital steering, see ref. 37) for the functional connection between X 320 and the plastoquinone pool via a special plastoquinone molecule B [31, 38]. Thus, the proteinaceous component functions as an allosteric regulator of the

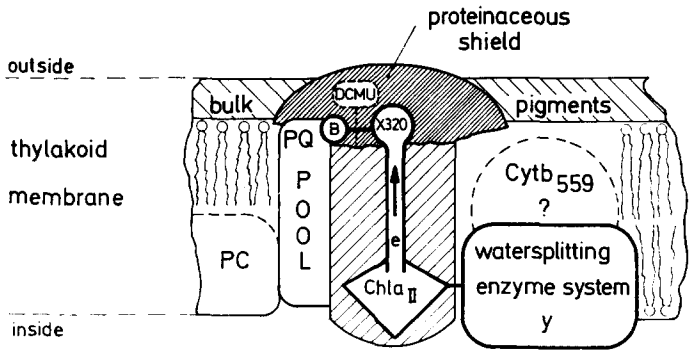

Fig. 6. Simplified scheme of the functional and structural organization of System II. For details and explanation see text.

electron flux from X 320$^-$ into the plastoquinone pool. (4) The proteinaceous component probably contributes to a geometric separation of the plastoquinone pool from the bulk pigment system in order to prevent dissipative non-photochemical exciton quenching. (5) The water-splitting enzyme system Y and intermediate electron carriers building up the functional connection between Y and chlorophyll a_{II} are located near the inner phase of the thylakoid membrane.

Within the framework of this model, the present results are simply understandable by the assumption that trypsin is very effective in the digestion of the proteinaceous shield covering up the primary electron acceptor X 320. On one hand, this effect causes the breakdown of the functional connection between X 320 and the plastoquinone pool, leading to an interruption of the linear electron transport; but on the other hand, the destruction of the proteinaceous component concomitantly enhances the accessibility of X 320 to exogeneous redox agents.

Thus, System II electron transport in trypsinated chloroplasts can be mediated only by redox components which are able to oxidize the reduced primary electron acceptor X 320$^-$. According to its function, this type of electron acceptors will be referred to as X-type electron acceptors (see ref. 39).

The reducing power of X 320$^-$ is comparatively weak ($E_{m,7} \approx -50$ mV, see refs. 40 and 41). Very recently, the midpoint potential of the primary electron acceptor of System II, X 320, has been found to depend on pH, with $E_{m,7} \approx 0$ mV and $E_{m,9} = -130$ mV. Furthermore,it was inferred that due to the existence of a proton barrier in normal chloroplasts X 320$^-$ equilibrates in the unprotonated form, i.e. the real midpoint potential under physiological conditions should be about -130 mV (see ref. 42).

However, trypsin could modify the above-mentioned barrier, so that the midpoint potential under our experimental conditions in trypsinated chloroplasts is expected to be nearly 0 mV. Hence, only redox reagents with a positive midpoint potential, $E_{m,7}$, should be able to act as artificial electron acceptors in chloroplasts treated with trypsin. This readily explains the inability of viologens to function as electron acceptors of X 320$^-$. However, a high enough midpoint potential alone is not sufficient for a redox substance to mediate System II electron transport in trypsinated chloroplasts, as is shown by failure of *p*-benzoquinone, characterized by a compara-

tively high midpoint potential of $E_{m,7} = +280$ mV, to function as an X-type acceptor. Similar results have been obtained for other substances [43]. Obviously, the electron transfer from the one-electron carrier X 320$^-$ to a two-electron acceptor like *p*-benzoquinone requires a special mechanism (see ref. 43). In this respect it is interesting to note that also in vivo a special electron carrier B mediates the electron transfer between X 320 and the plastoquinone pool [31, 38, 44, 45].

X-type electron acceptors are very important. Because DCMU is known to interrupt the electron transfer between X 320 and the plastoquinone pool [28, 29], X-type electron acceptors should be able to support a DCMU-insensitive System II electron flow, thereby allowing a functional separation of this part of photosynthetic electron transport from the overall reaction (see Fig. 1 of ref. 39). However, according to the present model either a suitable modification or the removal of the proteinaceous X 320 shield are indispensible prerequisites for the possibility of exogeneous redox components to act as X-type electron acceptors. This assumption readily explains the resistance of the $K_3[Fe(CN)_6]$-mediated System II electron transport to DCMU in trypsinated chloroplasts reported here (see Figs. 2–5). Furthermore, the lack of X-type electron acceptors in normal chloroplasts is understandable. On the other hand, it has been found that in subchloroplast fractions or in special chloroplast preparations, a DCMU-insensitive System II electron transport can be mediated by certain redox components which cannot function in the same manner in normal chloroplasts [46, 47]. It is easy to understand that this effect is caused by modifications of the proteinaceous X 320 shield due to the specific preparation conditions. Recently, silicomolybdate and silicotungstate have been reported to act also in normal chloroplasts as X-type electron acceptors, giving rise to a DCMU-insensitive oxygen evolution [48, 49]. This result could cast some doubts about the above-mentioned shielding function of the proteinaceous component. However, because the activity in the presence of silicomolybdate and silicotungstate persists only for a short while (about 1 min, see ref. 49), the acceptor function of these substances is obviously accompanied by secondary effects. This has been confirmed by very recent experiments supporting clear evidence for structural changes in System II caused by silicomolybdate and silicotungstate treatment of chloroplasts [50]. Thus, all data so far known for DCMU-insensitive System II electron transport are compatible with the above-stated assumption that modification of the proteinaceous X 320 shield is indispensible for exogeneous redox components to function as X-type electron acceptors.

According to the third postulate of the present model, the proteinaceous X 320 shield is assumed to be an essential structural element for the functional connection between X 320 and the plastoquinone pool via B. Hence, structural modifications should influence the reoxidation of X 320$^-$ by the plastoquinone pool. In this way the proteinaceous component might function as an allosteric regulator. If one assumes that light-induced conformational changes occur in the proteinaceous shield, the large differences of the apparent equilibrium constant for the reaction between X 320 and the plastoquinone pool obtained for dark-adapted and for illuminated chloroplasts [51, 52], can be explained by a structurally determined regulatory role of the proteinaceous component. Recently, it has been speculated that this electron flow might be controlled by a regulatory molecule in redox equilibrium with the pool [53]. This could occur by the above-mentioned mechanism.

In this respect, it must be noted that the basic idea of the regulation of the

electronic interaction between the primary electron acceptor of Photosystem II (X 320) and the plastoquinone pool by structural changes has been already discussed by Malkin and Michaeli [54], but the details of their mechanism clearly differ from the model presented here.

For allosteric regulation of electron flux on the reducing side of System II, structural changes in the proteinaceous component play a key role. Now the existence of such structural changes appears to be experimentally confirmed. On the basis of diazonium benzene sulfonic acid binding measurements during the last years, ample evidence has been presented for the occurrence of conformational changes at the outer surface of the thylakoid membrane coupled to the light-induced electron transport from the reduced primary electron acceptor of System II, X 320, into the plastoquinone pool [55, 56].

The introduction of the proteinaceous component as the regulatory structural element for electron transfer on the reducing side of System II could also lead to a reinterpretation of the well known inhibitory effect of DCMU [28, 29]. It can be assumed that DCMU binds reversibly to a special place of the proteinaceous X 320 shield, thus leading to an allosteric inhibition of the electron transfer from $X\,320^-$ to the connector molecule B (see Fig. 6). A reversible protein-DCMU complexation at specific inhibitor sites [57] is plausible, as the molecular architecture of this compound contains a peptide-like –CO–NH group, as has been already discussed [58]. On the other hand, van Rensen [59] assumes the direct complexation of plastoquinone in its oxidized state by DCMU to be responsible for the inhibitory effect. If the proteinaceous component containing the binding site for allosteric inhibition by DCMU acts simultaneously as an X 320 shield and as an allosteric regulator of the electron transfer from $X\,320^-$ into the plastoquinone pool, the term "DCMU-insensitive electron transport of System II" would become ambiguous with respect to the details of the mechanism responsible for DCMU insensitivity. Theoretically the effect could be caused: (a) by a pure accessibility effect to external redox compounds leading to an unshielding of X 320 without serious influence on the inhibitory site of DCMU and on the functional connection between X 320 and plastoquinone, or (b) by a pure inaccessibility effect to DCMU by destroying the binding site for DCMU without remarkable modification of the other two activities, or (c) by a combined effect leading to the loss or modification of more than one of the above-mentioned properties of the proteinaceous component. Further experiments are required to clarify this point.

On the basis of DCMU-type inhibition experiments it has been inferred that two reaction centers of System II cooperate functionally [60, 61]. It would be interesting to speculate about a possible role of the proteinaceous shield of X 320 for this twin cooperativity.

The fourth postulate assumes that the proteinaceous component additionally provides a spatial separation between plastoquinone and the bulk pigment system*. Because quinones are known to quench the lowest excited singlet state of chlorophyll *a*

* It must be emphasized that the representation in Fig. 6 does not reflect the real structural organization of the bulk pigment system and its functional connection with chlorophyll a_{II} within the thylakoid membrane. It shall only symbolize a geometrical separation between plastoquinone and the bulk pigments.

[62, 63], the spatial separation of the plastoquinone molecules from the bulk pigment system is important in order to avoid dissipative energy loss. Hence, a destruction of the proteinaceous component could lead to an increased interaction between plastoquinone and the bulk pigment system, giving rise to enhanced fluorescence quenching. Thus, the decrease of the extent of variable fluorescence observed in trypsinated chloroplasts [1] could be explained by interaction between plastoquinone and the bulk pigments. The invariance of the initial fluorescence might be caused by a significant contribution of System I (for details see refs. 64 and 65).

The present results show the oxidizing side of System II to be rather resistant to tryptic attack. This favours the assumption that especially the water-splitting enzyme system Y, known to be very sensitive to treatments which modify the structure of proteins (pH, temperature, chaotropic agents, see ref. 66) is located at the inner side of the thylakoid membrane (postulate 5 of the present model). This is in accord with similar conclusions based on other experimental data (see refs. 22, 23, 36 and 67). The chemical nature of the proteinaceous X 320 shield is unknown. Because trypsin primarily attacks arginine and lysine, these amino acids can be assumed to play a key role for the structure of this component. In contrast to the very sensitive water-splitting enzyme system Y (for review see ref. 66), the proteinaceous X 320 shield appears to be rather stable. Furthermore, it would be reasonable to assume that the proteinaceous X 320 shield also acts as a barrier for proton transport. Therefore, a high degree of hydrophobicity should be expected. The existence of a barrier limiting the proton transfer rate has been already postulated (for review see ref. 67).

Recently, the conformational changes connected with the electron transfer from X 320 into the plastoquinone pool have been speculated to be related also to energy coupling at so-called phosphorylation site II [48]. However, it seems to be premature to discuss here a possible role of the proteinaceous component.

In the present paper, the basic concept of the proteinaceous X 320 shield has been introduced. The details of the structural and functional role of this component remain to be confirmed and clarified by further experiments.

NOTE ADDED IN PROOF (Received June 3rd, 1976)

1. Though the maximum of the absorbtion changes due to X 320 turnover is approx. 325 nm, the measurements were made at around 334 nm because at this wavelength there is an emission maximum of the ultraviolet lamp (Hanovia) which gives rise to a higher signal/noise ratio.

2. The decay kinetics of the 334 nm absorption change in trypsinated chloroplasts is dependent on the $K_3[Fe(CN)_6^-]$ concentration (see ref. 43).

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