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STUDIES ON THE FUNCTIONAL ORGANIZATION OF PHOTOSYSTEM II

THE EFFECT OF PROTEIN-MODIFYING PROCEDURES AND OF ADRY AGENTS ON THE REACTION PATTERN OF PHOTOSYSTEM II IN TRIS-WASHED CHLOROPLASTS

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The role of the protein matrix embedding the functionally active redox components of Photosystem II reaction centers has been studied by investigating the effects of procedures which modify the structure of proteins. In order to reduce the influence of the electron transport involving secondary donor and acceptor components, Tris-washed chloroplasts were used which are completely deprived of their oxygen-evolving capacity. The functional activity was detected via absorption changes, reflecting at 334 and 690 or 834 nm the turnover of the primary plastoquinone acceptor, X320, and of the photochemically active chlorophyll *a* complex, Chl a_{II}, respectively, and at 520 nm the transient formation of a transmembrane electric potential gradient. Under repetitive flash excitation of Tris-washed chloroplasts it was found that: (a) The relaxation kinetics at 690 nm become significantly accelerated in the presence of external electron donors. (b) Trypsin treatment blocks to a high degree the turnover of Chl a_{II} and X320 unless exogenous acceptors are present, which directly oxidize $X320^-$, such as $K_3Fe(CN)_6$. (c) In the presence of $K_3Fe(CN)_6$ the recovery kinetics of Chl a_{II} and X320 are retarded markedly by trypsin, followed by a progressive decline in the extent thereof. (d) 2-(3-Chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT 2p), known to reduce the lifetime of S_2 and S_3 in normal chloroplasts, significantly accelerates the recovery of Chl a_{II}. 10 μs kinetics are observed which correspond with the electron-transfer rate from D_1 to Chl a_{II}^+. ANT 2p simultaneously retards the decay kinetics of $X320^-$ and of the electrochromic absorption changes. (e) The kinetic pattern of the electrochromic absorption changes is also affected by the salt content of the suspension. Under dark-adapted conditions, the 10 μs relaxation kinetics of the 834 nm absorption change due to the first flash are hardly affected by mild trypsinization of 5–10 min duration, whereas the amplitude decreases by approx. 30%. The data obtained in Tris-washed chloroplasts could consistently be interpreted as a modification of the back reaction between $X320^-$ and Chl a_{II}^+ which is caused solely by a change in the reactivity of X320 due to trypsin-induced degradation of the native X320-B apoprotein. Furthermore, ADRY agents are inferred to stimulate cyclic electron flow, which leads to reduction of D_1^+ between the flashes. A simplified scheme is discussed which describes the functional organization of the reaction center complex.

Introduction

Photosynthetic water cleavage by visible light requires the generation as well as the cooperation of four redox equivalents of sufficient oxidizing power. The former goal is achieved by exciton dissociation

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; ANT 2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; Tricine, *N*-tris(hydroxymethyl)methylglycine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; PS, Photosystem.

within the PS II reaction center, the latter by a mangano protein catalyst, referred to as water-splitting enzyme system Y (for reviews see Refs. 1 and 2). The PS II reaction center contains a special Chl *a* complex, referred to as Chl a_{II} [3] and a special plastoquinone molecule (designated as X320, see Ref. 4) which is required for 'stable' charge separation and the concomitant formation of a transmembrane electric potential gradient [5].

Another redox component, D_1, on the donor side is assumed to connect Chl a_{II} with system Y. However, it still remains an open question as to whether D_1 is an integral part of system Y. Tris washing of chloroplasts suppresses the function of system Y accompanied by manganese release [6,7] and the occurrence of cyclic pathways at the expense of linear electron flow from water to Chl a_{II}^+. After exhaustion of the capacity of the donor component D_1, which was inferred to be a one-electron redox component directly connected with Chl a_{II} [8], under repetitive flash excitation conditions the light-induced charge separation at the reaction center is dissipated mainly via an electrogenic back reaction between $X320^-$ and Chl a_{II}^+. This back reaction is characterized by a half-life of 100–200 μs [9–12]. Accordingly, Tris-washed chloroplasts appear to be an appropriate system to study the functional mechanism of the PS II reaction center.

The reaction center redox active groups participating in the light-induced charge separation are embedded in proteins, which are postulated to play an essential functional role as apoenzymes [2]. In order to reveal a possible functional role of the protein matrix, the effects of different procedures which are known to modify the structure of proteins have been studied. Trypsin treatment was analyzed because this proteolytic enzyme has been found to attack the X320-B apoprotein which regulates the electron flow from $X320^-$ via B into the plastoquinone pool [13–15]. Structural changes in proteins can also be achieved by modification of the electrostatic interaction. Accordingly, it seems worthwhile to study the effect of salt depletion on the reactivity of reaction centers in normal and Tris-washed chloroplasts [15].

In normal chloroplasts, a number of chemicals, referred to as ADRY reagents [16], accelerate the decay of the charge accumulation states S_2 and S_3 of system Y [17]. The mechanism of this effect still remains to be clarified. Tris-washed chloroplasts seem suitable for studying the possible participation of reaction center components in the ADRY effect.

The results presented in this contribution support evidence for the functional role of the protein matrix in the reaction center complex. Furthermore, the data indicate the existence of an additional component, tentatively referred to as component C, which contributes to the reaction sequence at PS II in Tris-treated chloroplasts. On the basis of these data and previous results the functional and structural organization scheme of PS II is discussed.

Materials and Methods

Class II chloroplasts were prepared from market spinach according to a method described by Winget et al. [18], except that 10 mM ascorbate was present in the grinding medium. 5% dimethyl sulfoxide was added for storage in liquid nitrogen. Tris-washed chloroplasts were obtained by incubating isolated chloroplasts with 0.8 M Tris-HCl, pH 8.0, according to the method of Yamashita and Butler [19]. These Tris-washed chloroplasts are completely deprived of their oxygen-evolving capacity. Trypsin treatment was performed as described in Ref. 20. The standard reaction mixture contained: chloroplasts (5 μM Chl), 10 mM KCl, 2 mM $MgCl_2$, 20 mM Tricine-NaOH and 100 μM benzyl viologen or 100 μM $K_3Fe(CN)_6$ as electron acceptor. Other additions were as indicated in the figure legends.

The absorption changes at 334 and 520 nm were recorded by a repetitive flash double-beam photometer resembling that described in Ref. 21. Absorption changes in the red (690 nm) were recorded by a modified repetitive flash photometer developed by Dr. Buchwald using a 14 MHz modulated detecting light beam in order to eliminate fluorescence artifacts due to the actinic flashes [22].

Measurements of absorption changes in the far red were performed using a method similar to that described in Ref. 10. For measurements under dark-adapted conditions, a flow system was used in order to change the sample after each flash illumination. The absorption changes are not corrected for the flattening effect which significantly affects only the 334 nm absorption changes of the present study [23].

Signal averaging (32–1024 samples) was performed in an Nic 1127 apparatus. Excitation of photosynthesis was achieved either with xenon flashes of 10–20 μs duration passing Schott filters BG 23/3 or RG 1 for measurements in the red or ultraviolet and green spectral range, respectively, or with a Q-switched Nd/YAG laser (λ 530 nm and pulse width 20 ns) for measurements at 834 nm. The repetition rate of the flashes was 0.2–4 Hz.

Results

The effect of artificial PS II electron donors

Under repetitive flash excitation at a repetition rate of a few hertz the turnover of the PS II reaction center in Tris-washed chloroplasts is limited by the electrogenic back reaction between Chl a_{II}^+ and $X320^-$ which is characterized by a half-life of 100–200 μs [11]. In dark-adapted Tris-washed chloroplasts excited with a single turnover flash, Chl a_{II}^+ was found to become reduced via 5–10 μs kinetics in the pH range 7–8 [8]. This reaction is assumed to be caused by the electron transfer from D_1 to Chl a_{II}^+, with D_1 being modified in its reactivity due to Tris washing. Artificial electron donors are known to restore the linear electron flow in Tris-washed chloroplasts. In order to decide if this electron transport occurs via D_1, the recovery kinetics of the 690 nm absorption change have been measured. The results obtained in the absence and presence of the donor couple hydroquinone/ascorbate are depicted in Fig. 1. The analysis of the data obtained in the presence of the donor indicates multiphasic kinetics with half-lives of 10–15 and 40–50 μs and slower kinetics in the millisecond range. The fast component is probably limited by the time resolution of our equipment and therefore might be underestimated in amplitude. Irrespective of these shortcomings, the data indicate that the main pathway of the artificially induced electron flow involves D_1 as internal donor component. This conclusion is in agreement with the latest findings of Yerkes and Babcock [24]. However, the possibility of participation of a further internal component, D_2, as a mediator for electrons from external donors cannot be totally excluded. This transfer could account for the 40–50 μs kinetics, which amount to approx. 25–40% of the total amplitude of the 690 nm absorption changes observed in

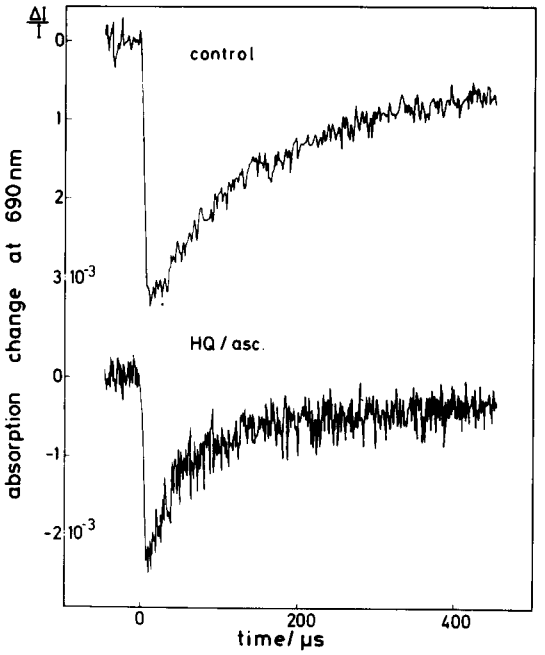

Fig. 1. Absorption changes at 690 nm as a function of time in Tris-washed chloroplasts in the absence and presence of exogeneous PS II electron donors (Hydroquinone/ascorbate, HQ/asc.) Excitation: xenon flashes. Other conditions as described in Materials and Methods.

the absence of hydroquinone/ascorbate. The existence of a donor component D_2 was proposed by Diner [25] and by Den Haan et al. [26]. Results similar to those depicted in Fig. 1 have been obtained with other PS II electron donors, such as Mn^{2+} and diphenylcarbazide. In the latter case, however, complications often arise which might be due to secondary reactions caused by oxidation products of diphenylcarbazide.

The effect of trypsin on the reaction pattern of Chl a_{II} and X320 in Tris-washed chloroplasts

Trypsin treatment of normal chloroplasts under carefully selected experimental conditions was found to attach primarily the X320-B apoprotein located at the outer side of the thylakoid membrane [13,20]. Accordingly, this method seems to provide a promising stereospecific tool also for the investigation of the functional role of the protein matrix embedding the redox components of the reaction center.

Fig. 2 depicts the initial amplitudes of the 690 nm absorption changes obtained in Tris-washed chloro-

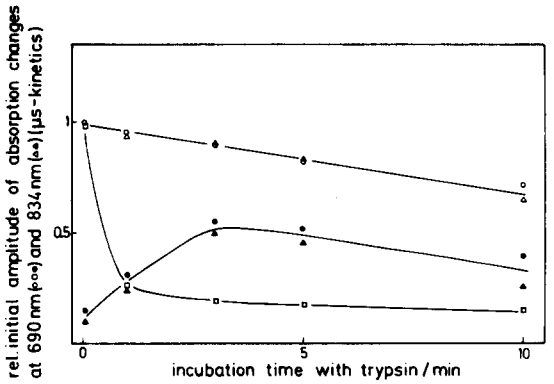

Fig. 2. Initial amplitudes of absorption changes at 690 nm and of the microsecond components of absorption changes at 834 nm in Tris-washed chloroplasts as a function of incubation time with trypsin. Full symbols: 3 μM DCMU. (○, ●, △, ▲) 100 μM $K_3Fe(CN_6$, (□) 100 μM benzyl viologen, Excitation: xenon flashes (half-width 10–15 μs). Other conditions as in Materials and Methods. The time resolution of the measurements was the same as in Fig. 1 (ΔA_{690}) and Fig. 3 (ΔA_{834}).

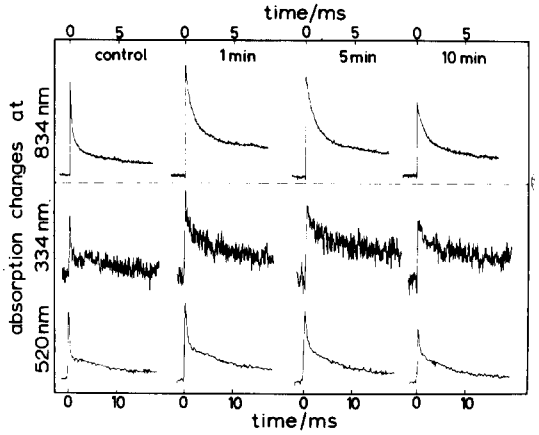

Fig. 3. Absorption changes at 834, 334 and 520 nm as a function of time in Tris-washed chloroplasts treated with trypsin for different incubation periods. The incubation time (0, 1, 5 and 10 min) is given at the top of the figure.

plasts under repetitive flash excitation in the presence of benzyl viologen or $K_3Fe(CN)_6$ as a function of the incubation time with trypsin. In the presence of benzyl viologen a rapid decline in the initial amplitude, ΔA_{690}, is observed. On the other hand, only a slight decrease in ΔA_{690} arises in the presence of $K_3Fe(CN)_6$. These results confirm that the back reaction between Chl a_{II}^+ and $X320^-$ can be established only if the donor capacity of D_1 is exhausted via a prior photochemical turnover of the PS II reaction center and $X320^-$ is oxidizable either via B or directly with the aid of an appropriate external acceptor (photoreactions of Chl a_{II} under conditions of $X320^-$ staying reduced, which involve a further internal acceptor component, X_a, will not be discussed here; see Ref. 5).

Fig. 3 depicts, as a function of incubation time with trypsin, absorption changes at 834, 334 and 520 nm reflecting the turnover of Chl a_{II}, X320 and the transient formation of a transmembrane electric gradient, respectively. The results reveal a striking retardation of the relaxation kinetics, which is already significant after short incubation with trypsin. As a general pattern, the amplitude of the faster kinetics which are of the order of a few hundred microseconds decreases, while slower kinetics with half-lives in the millisecond time range increase in extent. The faster components, characterized by 400–800 μs kinetics, probably reflect the electrogenic back reaction between $X320^-$ and Chl a_{II}^+, because the relaxation kinetics of the corresponding absorption changes at 334 and 834 nm and of the electrochromic effect practically coincide in this time range. The electron transfer appears to be retarded due to the modification of the protein matrix by trypsin. These kinetics are not monophasic. The slower relaxation kinetics in the millisecond range of the absorption changes at 334 and 834 nm markedly increase after mild trypsinization. They probably reflect to a high degree the turnover of processes other than the reoxidation of $X320^-$ and the rereduction of Chl a_{II}^+. The increase at 834 nm could not be ascribed to a Chl a_I reaction, because this process was shown to be suppressed in trypsinized chloroplasts in the absence of a PS I electron donor [27], nor to a Chl a_{II} reaction because a corresponding increase at 690 nm has not been observed. Furthermore, the difference spectrum in the range 750–900 nm of these millisecond kinetics significantly differs from the typical spectrum of the Chl a^+ radical formation [10] (data not shown). Therefore, only the microsecond relaxation kinetics of the 834 nm absorption changes are assumed to reflect the turnover of Chl a_{II}. This idea is corroborated by the dependency on the incubation time with trypsin corresponding with

that of the 690 nm absorption changes either in the absence or in the presence of DCMU (see (Fig. 2).

The analysis of the absorption changes at 690 nm observed in trypsinized Tris-washed chloroplasts reveals at least two phases, one with a half-life of 70–100 μs which contributes to a minor extent (appox. 1/3) to the overall kinetics, and a slower one with a half-life of 600–800 μs being the predominant component (approx. 2/3). These data could reflect heterogeneity of the reaction centers towards the attack of trypsin. However, similar multiphasic kinetics are also observed in trypsin-treated Tris-washed chloroplasts in the presence of DCMU. If one accepts that in the presence of DCMU a modification of the X320 apoprotein is the prerequisite for restoration of the back reaction under repetitive flash excitation, the latter data would favor heterogeneity of the reaction centers with respect to their back reaction pattern. Unfortunately, unambiguous conclusions cannot be drawn because even in the absence of trypsin complete suppression of the 690 nm reaction could not be achieved in the presence of 3 μM DCMU. The nature of this resistant reaction which amount to a few percent of the activity observed in the absence of DCMU still remains to be clarified.

The data reported in Figs. 2 and 3 could consistently be interpreted as a modification of the back reaction between $X320^-$ and Chl a_{II}^+ in Tris-washed chloroplasts, which is caused solely by a change in the reactivity of $X320^-$ due to trypsin-induced degradation of the native X320-B apoprotein. In order to explain the decrease in amplitudes within this framework, one has additionally to assume that in some centers trypsin treatment disconnects X320 completely from the reaction center complex, as has been reported, e.g., for lipase treatment [28].

However, the data could also be explained by a modification of the donor side. In order to test a possible effect of trypsin on the donor side, absorption changes at 834 nm were measured in dark-adapted Tris-washed chloroplasts excited by a 20 ns laser flash. The recovery kinetics of the 834 nm absorption change, which is assumed to reflect the electron transfer from D_1 to Chl a_{II}^+, were found to be sensitive to pH and strongly dependent on temperature [29]. Accordingly, a possible modification within the Chl a_{II}-D_1 segment due to trypsin treatment should lead to significant changes in the 834 nm relaxation kinetics. The data depicted in Fig. 4 (upper) show the expected 5–10 μs kinetics. This reaction is resistant to DCMU. Mild trypsinization does not modify the 5–10 μs kinetics (see Fig. 4, lower), but the extent is reduced by approx. 30%. Therefore, the data of Fig. 4 indicate that mild trypsinization does not affect the functional connection between Chl a_{II} and D_1. This result provides further evidence for the conclusion that the Chl a_{II}-D_1 segment is located close to the inner side of the thylakoid membrane [8,40]. Furthermore, it proves trypsin to be acting as a structurally selective proteolytic enzyme which modifies PS II primarily at the X320-B apoprotein. Accordingly, the blockage of PS II centers is inferred to be caused by a trypsin-induced functional disconnection of X320 from Chl a_{II}. This assumption would also explain the decline in oxygen-evolving capacity in trypsinized normal chloroplasts [15,20] to be due to inhibition of the PS II reaction center rather than a specific attack on the water-splitting enzyme system Y. This conclusion is in line with previous findings that trypsin treatment is not accompanied by manganese release [30], in contrast to procedures which are well known to destroy system

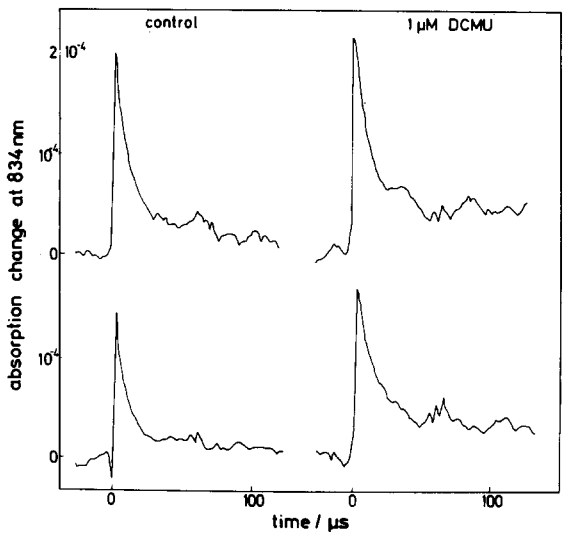

Fig. 4. Absorption changes at 834 nm as a function of time in Tris-washed dark-adapted chloroplasts. Lower traces: trypsinized chloroplasts; upper traces: normal chloroplasts. Chlorophyll concentration: 10 μM. Other additions as indicated in the figure and as described in Materials and Methods Excitation: 20 ns laser flashes. Optical pathlength: 13 mm.

Y selectively, such as Tris washing or hydroxylamine treatment [6,7]. On the other hand, experiments performed in the absence and presence of diphenylcarbazide suggest an additional effect of trypsin on system Y [31].

Modifications of system Y were shown to affect the complex rereduction pattern of Chl a_{II} [32]. Therefore, absorption changes at 690 nm were measured in trypsinized normal chloroplasts in order to detect a possible effect on system Y. The results obtained are depicted in Fig. 5.

In the presence of 100 μM $K_3Fe(CN)_6$ the amplitude remains nearly invariant, while the relaxation kinetics are markedly retarded. On the other hand, in the presence of 100 μM benzyl viologen the amplitude is greatly diminished because of the blockage of the intrinsic $X320^-$ reoxidation by trypsin treatments. In the presence of $K_3Fe(CN)_6$ the relaxation kinetics progressively slow down as a function of time

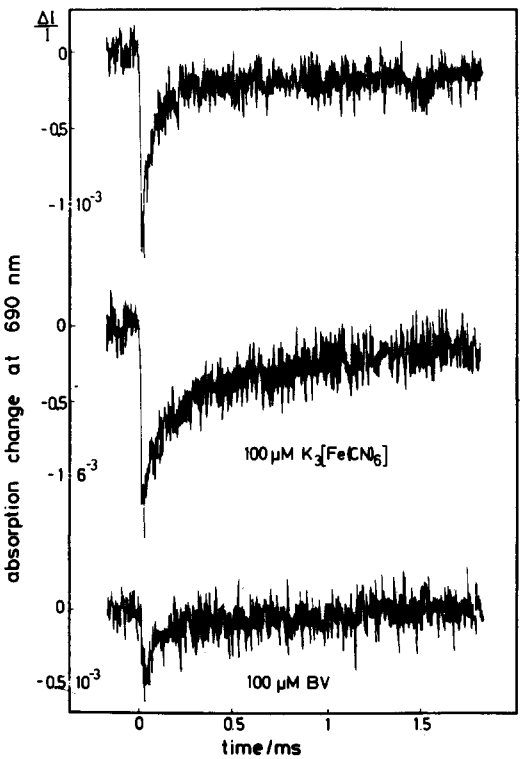

Fig. 5. Absorption changes at 690 nm as a function of time in normal control (top trace) and trypsinized chloroplasts (center and lower traces). Electron acceptors are given in the figure, trypsin incubation for 5 min. BV, benzyl viologen.

of trypsin treatment, but the amplitude of the 690 nm absorption changes is practically unchanged.

The variation of the recovery kinetics of the 690 nm bleaching might suggest an effect of trypsin on the functional connection between Chl a_{II} and system Y, eventually giving rise also to a back reaction. However, due to the limited time resolution the reduction kinetics of Chl a_{II}^+ are not completely detecable, so that unambiqious conclusions cannot be drawn. Further experiments are required to clarify this point.

The effect of salt depletion on the reaction pattern of Tris-washed chloroplasts

Recent experiments on normal chloroplasts led to the conclusion that salt depletion markedly changes the structural interrelationship of functionally essential protein moieties within the thylakoid membrane such as the X320-B apoprotein [15]. Accordingly, salt depletion could also affect the reaction pattern in Tris-washed chloroplasts. Absorption changes at 520 nm measured under repetitive flash excitation in Tris-washed chloroplasts suspended in 'low salt' or 'high salt' solution are depicted in Fig. 6. In order to avoid significant excitation due to the detecting light, the measuring light beam has been pulsed, as described in Ref. 33. A comparison of the data reveals that salt depletion enhances the relative extent of slower decay kinetics at the expense of the 100–200 μs kinetics, accompanied by a decrease in the total amplitude. These findings, which are in contrast to previous data obtained in normal chloroplasts [34], indicate that changes in the electrostatic interaction due to salt depletion really cause a modification of the PS II reaction pattern. Comparative measurements at 334 and 690 nm (data not shown, see Ref. 35) favor the assumption of accelerated D_1 reduction during the dark time between flashes. Single flash experiments are in line with this idea [8].

The effect of ADRY agents on the reaction patterns of Tris-washed chloroplasts

In normal chloroplasts with intact water-splitting enzyme system Y, the amplitude of the 690 nm absorption change was found to be modified by a class of substances referred to as ADRY reagents [36]. As these compounds are known to destabilize in isolated chloroplasts the holes stored in the water-splitting enzyme system Y [17], the results were interpreted

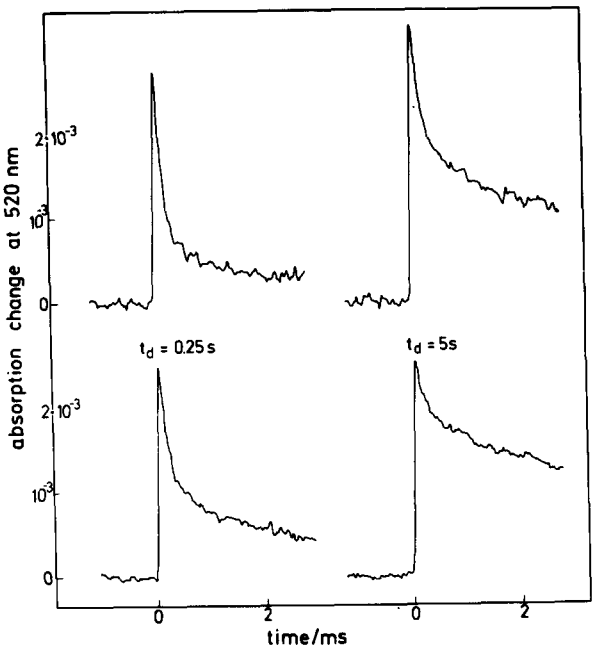

Fig. 6. Absorption changes at 520 nm as a function of time in Tris-washed chloroplasts suspended in 'low' or 'high' salt (upper traces) medium. Chlorophyll concentration: 15 μM. Detecting beam: pulsed (pulse duration approx. 20 ms). In the low salt medium, KCl and $MgCl_2$ were omitted, other experimental conditions as described in Materials and Methods.

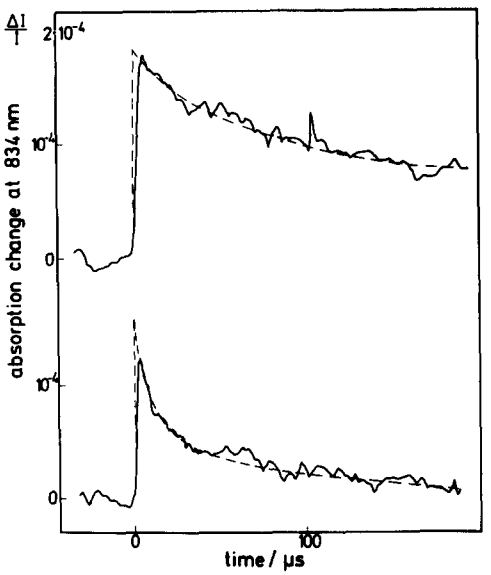

Fig. 7. Absorption changes at 834 nm as a function of time in Tris-washed chloroplasts in the absence (upper trace) and presence (lower trace) of 30 nM ANT 2p. Chlorophyll concentration: 10 μM. Excitation: 20 ns laser flashes, repetition rate 5 Hz. Optical pathlength: 10 mm. The dashed line indicates extrapolation to the time of the laser flash excitation.

to indicate a dependency of the rereduction kinetics of Chl a_{II}^+ on the charge accumulation state of system Y [36]. However, ADRY agents could also interfere with intermediary redox components which interconnect Chl a_{II}^+ and the water-splitting enzyme system Y. In order to clarify this question, the effect of ADRY reagents on the back reaction has been investigated in Tris-washed chloroplasts, which are completely deprived of their oxygen-evolving capacity.

The most powerful ADRY reagent was found to be ANT 2p. The effect of 30 nM ANT 2p on the 834 nm absorption changes obtained under repetitive flash (20 ns laser pulses) excitation of Tris-washed chloroplasts is shown in Fig. 7. A remarkable modification of the relaxation kinetics is shown even at the low ratio of one ANT 2p molecule per 350 chlorophylls. In the absence of ANT 2p these kinetics are determined predominantly by the 100–200 μs back reaction, whereas ANT 2p causes the apparance of a much faster recovery of Chl a_{II}, which is characterized by a half-time of 5–10 μs. These kinetics correspond with the rate constant reported for the electron transfer from the donor component D_1 to Chl a_{II}^+ [8]. Therefore, the effect of ANT 2p could easily be explained by the assumption that the ADRY reagent ANT 2p catalyzes a cyclic electron flow, thus giving rise to a partial reduction of D_1^+ during the dark time (t_d = 200 ms) between the flashes.

A donor function of ANT 2p analogous to that of typical PS II donor couples, such as hydroquinone/ascorbate, leading to D_1 reduction (see Fig. 1) can be excluded for stoichiometry reasons.

If D_1 is partially reduced between the flashes the electrogenic back reaction cannot become 'switched on' in those reaction centers with reduced D_1. Accordingly, the reoxidation kinetics of $X320^-$ and the decay of the light-induced transmembrane electric field have necessarily to be changed by ANT 2p. The data depicted in Fig. 8 indicate a retardation of these kinetics. The decay kinetics of the 520 nm absorption change reflect the collapse of the transmembrane electric potential gradient due to the ANT 2p-induced passive proton flux (uncoupling effect of ANT 2p).

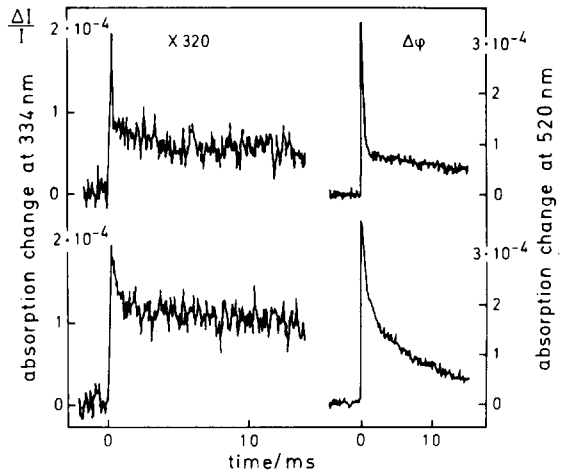

Fig. 8. Absorption changes at 334 and 520 nm as a function of time in Tris-washed chloroplasts in the absence (upper traces) and presence (lower traces) of 30 nM ANT 2p. Optical pathlength: 20 mm.

At a concentration ratio of 1 ANT 2p molecule per 170 chlorophylls in normal chloroplasts the stability of oxidizing redox equivalents stored in the water-splitting enzyme Y is significantly reduced without affecting the oxygen-evolving capacity per system Y (see Ref. 15). As system Y is irreversibly destroyed in the Tris-washed chloroplasts, the data of Figs. 7 and 8 indicate that at PS II, ANT 2p does not specifically react with the intact water-splitting enzyme Y exclusively. On the other hand, the ADRY effect in normal chloroplasts cannot be explained by a competitive D_1 reduction (see Discussion). In any case, ANT 2p obviously destabilizes oxidizing redox equivalents on the donor side of PS II, localized either at the connector molecule D_1 (Tris-washed chloroplasts) or in the water-splitting enzyme system Y (normal chloroplasts). Now the question arises as to the mechanism of this catalytic degradation of the oxidizing equivalents. Preliminary experiments favor the assumption of a participation of cytochrome *b*-559 (data not shown), which is well known to be affected in its reactivity by ADRY reagents [37,38]. However, further experiments are required to clarify this mechanism.

Discussion

The results obtained in Tris-washed chloroplasts treated with trypsin show that the proteolytic enzyme primarily attacks the reaction center matrix at the level of the X320-B apoprotein because after a rather short trypsinization period a repetitive turnover can be achieved only in the presence of $K_3Fe(CN)_6$, which is able to react directly with $X320^-$. The back reaction between $X320^-$ and Chl a_{II}^+ was claimed to occur via a tunnel mechanism [39]. Likewise, the electron transport from $X320^-$ to B is inferred to proceed via tunneling through a specific channel established by the X320-B apoprotein [15]. Trypsin treatment completely blocks the electron transport between $X320^-$ and B in normal as well as in Tris-washed chloroplasts (Weiss and Renger, unpublished results). On the other hand, the reactivity of the reaction center involving Chl a_{II} and X320 is affected in a heterogeneous manner in Tris-washed chloroplasts. Some (about 30% after 5–10 min trypsinization, see Figs. 2 and 3) of the reaction centers are completely blocked, while in the fraction remaining still active the back reaction becomes retarded only by less than one order of magnitude. A kinetic analysis reveals that the recovery kinetics probably reflecting the back reaction between $X320^-$ and Chl a_{II}^+ are not continuously slowed down with increasing time of trypsin incubation, but rather the number of active centers declines progressively. If one admits that the kinetics of an electron tunneling between redox groups embedded in a protein matrix are highly sensitive to structural modifications of the protein, the present result can be interpreted by the assumption that the functional connection between Chl a_{II} and X320 (probably involving an intermediary redox carrier, see Refs. 5 and 41) requires a specific structure of the protein matrix. This conclusion is based on the analysis of the data of Fig. 3, which indicate that trypsin-induced modifications leading to only slight variations of the tunnel barrier for the back reaction (even less retardation can be achieved than by thermal variations, see Ref. 29) are tolerable, beyond this threshold the functional activity completely breaks down.

Recently, two different types of PS II reaction centers have been discovered [42] which are distinguished by their X320 redox potential as well as by their antenna composition [43]. As the difference in the redox potential is very likely due to specific X320 environments, it appears reasonable to assume that the protein matrices also vary in their sensitivity to

trypsin. Accordingly, a 30% blockage of the reaction centers could be explained by a trypsin-induced functional disconnection of X320 from Chl a_{II} predominantly occurring within the β-centers. In the PS II reaction centers remaining still active (eventually mainly the α-centers) the functional connection between X320 and Chl a_{II} is only slightly affected by trypsin, despite the complete blockage of the electron transport from $X320^-$ to B. Experiments are in progress to study possible differences in the protein matrices of the α- and β-centers.

The trypsin-induced changes reported here are inferred to be due to the modification of the reaction center protein matrix around X320 exclusively, because the kinetics of the electron transfer from D_1 to Chl a_{II}^+ are practically insensitive to trypsin. Accordingly, trypsin under appropriate experimental conditions can be used as a structurally selective modifier of the PS II reaction center protein matrix at the level of the primary plastoquinone acceptor X320. This conclusion suggests that the decline of the average oxygen yield per flash induced by trypsin in normal chloroplasts [15,20] is caused by a blockage of the reaction center complex rather than by the destruction of the water-splitting enzyme system Y. The latter assumption is corroborated by the finding that trypsinization does not lead to manganese release [30], in marked contrast to procedures which are known to attack system Y exclusively, such as Tris washing or hydroxylamine treatment. The effect of diphenylcarbazide in trypsinized chloroplasts, however, which cannot be explained by the above-mentioned conclusion, still remains to be clarified.

Another interesting modification of the PS II reaction pattern is induced in Tris-washed chloroplasts (completely deprived of their oxygen-evolving capacity) by ANT 2p known to be one of the most powerful ADRY agents [16]. The data of Figs. 7 and 8 suggest an ADRY agent-accelerated D_1 reduction probably caused by stimulation of a cyclic electron flow. Likewise, in normal chloroplasts, ADRY agents are known to accelerate the reduction of oxidizing redox equivalents stored in states S_2 and S_3 within the water-splitting enzyme system Y [17]. Therefore, the question arises as to whether the same component is involved as the target of ADRY agents to induce the destabilitzation of oxidizing equivalents on the donor side of PS II in normal and Tris-washed chloroplasts. One could simply assume that ADRY agents catalyze the reduction of D_1. However, if one accepts that D_1 functionally connects Chl a_{II} and system Y, competitive D_1^+ reduction can be readily excluded, because D_1^+ becomes reduced by system Y in less than 1 ms which is not seriously changed by the ADRY agent carbonyl cyanide *m*-chlorophenylhydrazone [44]. These kinetics are much faster than the ADRY agent-induced discharge of S_2 and S_3 [17]. Therefore, a further redox component, tentatively referred to as C, is assumed to be the target of ADRY agents. As cytochrome *b*-559, which is located on the donor side of PS II, is well known to become affected in its reactivity by ADRY agents [37,38], component C might be substantiated to be cytochrome *b*-559. In this respect, it is interesting to note a previous hypothesis, which also claims cytochrome *b*-559 to be responsible for the ADRY effect [45]. However, if one takes into consideration appropriate redox equilibria between the D component and the S_i states, a possible participation of D_1^+ in the ADRY effect cannot be totally excluded.

Within this framework, also the salt-induced changes of the reaction pattern in Tris-washed chloroplasts could be explained by a structural rearrangement of PS II redox proteins, which change the kinetics of cyclic electron flow around PS II. Electrostatic interactions are known to play an essential functional role for the electron transfer between redox enzymes, such as cytochrome *c* and cytochrome oxidase or cytochrome peroxidase [46].

On the basis of the results presented in this contribution and of previous results, the electron-transport scheme depicted in Fig. 9 appears to be the simplest model for the functional organization of the electron transport in PS II. The existence and the functional role of the components X_a and X_b have been discussed previously [5,47] and will be outlined in a forthcoming paper. The role and the nature of X_b remain completely unresolved problems, especially its relation to C (X_b could be identified as C).

As an unambigious proof for the participation of cytochrome *b*-559 in the ADRY effect is still lacking, the description of the function of ADRY agents in Fig. 9 has to be considered as a tentative mechanism only (a direct effect on Y cannot be excluded).

In the scheme of Fig. 9, component D_1 is assumed to be the donor component, which is directly con-

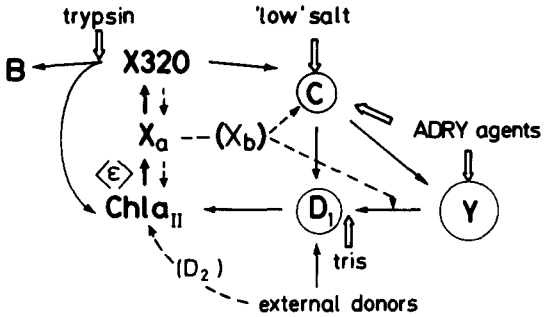

Fig. 9. Simplified scheme of the PS II electron transport in Tris-washed chloroplasts. ⟨ε⟩ symbolizes an exciton localized at Chl a_{II}, which leads to the primary electron-transfer step. For other details see text.

nected with Chl a_{II} irrespective of the state of system Y. Based on different lines of evidence, the reduction kinetics of Chl a_{II}^{+} in normal chloroplasts with intact system Y were inferred to vary significantly under different physiological conditions, such as inner thylakoid pH or the S_i states [36,40,48]. Therefore, it is reasonable to assume that destruction of system Y by Tris washing markedly modifies the functional contact between Chl a_{II} and D_1, thus giving rise to a slower electron-transfer rate. A further donor component is not required to explain the different recovery kinetics of Chl a_{II} in normal and Tris-washed chloroplasts. The scheme of Fig. 9 only gives a minimum functional organization scheme. However, it has to be emphasized that the functional redox groups are embedded in protein or lipoprotein matrices, so that the mode of mutual orientation of these proteins could seriously affect the reaction pattern. In normal chloroplasts the donor groups of PS II are not accessible to exogenous redox couples, such as hydroquinone/ascorbate or diphenylcarbazide [49]. Tetraphenylboron as a highly lipophilic compound is the only donor known thus far which effectively competes with water oxidation in normal chloroplasts [50]. Accordingly, the endogenous donor groups of PS II are covered up by the water-splitting enzyme system Y. This is of physiological relevance in order to prevent dissipative sidepath reactions which could additionally cause oxidative destruction. It remains to be clarified if D_1 is an integral part of system Y, which survives Tris treatment (a tentative static structural model of PS II is described in Ref. 2).

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References

1 Renger, G. (1978) in Photosynthetic Oxygen Evolution Metzner, H., ed.), pp. 229–248, Academic Press, London
2 Renger, G. and Eckert, H.J. (1980) Bioelectrochem. Bioenerg. 7, 104–124
3 Döring, G., Renger, G., Vater, J. and Witt, H.T. (1969) Z. Naturforsch. 24c, 1139–1143
4 Stiehl, H.H. and Witt, H.T. (1969) Z. Naturforsch. 24c, 1588–1598
5 Eckert, H.J. and Renger, G. (1980) Photochem. Photobiol. 31, 501–511
6 Cheniae, G.M. and Martin, I.F. (1980) Biochim. Biophys. Acta 197, 219–239
7 Cheniae, G.M. and Martin, I.F. (1978) Biochim. Biophys. Acta 502, 321–344
8 Conjeaud, H. and Mathis, P. (1980) Biochim. Biophys. Acta 590, 353–359
9 Renger, G. and Wolff, C. (1976) Biochim. Biophys. Acta 423, 610–614
10 Haveman, J. and Mathis, P. (1976) Biochim. Biophys. Acta 440, 346–355
11 Renger, G. (1979) Biochim. Biophys. Acta 547, 103–116
12 Conjeaud, H., Mathis, P. and Paillotin, G. (1979) Biochim. Biophys. Acta 546, 280–291
13 Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300
14 Renger, G. (1976) FEBS Lett. 69, 225–230
15 Renger, G., Hagemann, R. and Dohnt, G. (1981) Biochim. Biophys. Acta 636, 17–26
16 Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439
17 Renger, G., Bouges-Bocquet, B. and Delosme, R. (1973) Biochim. Biophys. Acta 292, 796–807
18 Winget, G.D., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Commun. 21, 438–443
19 Yamashita, T. and Butler, W.L. (1968) Plant Physiol. 43, 1978–1984
20 Renger, G. (1977) in Membrane Bioenergetics (Packer, L., Papageorgiou, G.C. and Trebst, A., eds.), pp. 339–350, Elsevier, Amsterdam
21 Döring, G., Stiehl, H.H. and Witt, H.T. (1967) Z. Naturforsch. 22b, 639–644
22 Buchwald, H.E. (1968) Thesis, Technical University, Berlin
23 Pulles, M.P.J. (1978) Thesis, Rijksuniversiteit, Leiden

24 Yerkes, C.T. and Babcock, G.T. (1981) Biochim. Biophys. Acta 634, 19–29
25 Diner, B. (1977) in Proceedings of the 4th International Congress on Photosynthesis (Hall, D.O., Coombs, J. and Godwin, T.W., eds.), pp. 359–372, Biochemical Society, London
26 Den Haan, G.A., Gorter de Vries, H. and Duysens, L.M.N. (1976) Biochim. Biophys. Acta 430, 265–281
27 Renger, G., Erixon, K., Döring, G. and Wolff, C. (1976) Biochim. Biophys. Acta 440, 278–286
28 Okayama, S., Epel, B.L., Erixon, K. and Butler, W.L. (1971) Biochim. Biophys. Acta 253, 476–482
29 Reinman, S. and Mathis, P. (1981) Biochim. Biophys. Acta 635, 249–258
30 Selman, B.R., Bannister, T.T. and Dilley, R.A. (1973) Biochim. Biophys. Acta 292, 566–581
31 Selman, B.T. and Bannister, T.T. (1971) Biochim. Biophys. Acta 253, 428–436
32 Renger, G., Eckert, H.J. and Buchwald, H.E. (1978) FEBS Lett. 90, 10–14
33 Renger, G. and Wolff, C. (1975) Z. Naturforsch. 30c, 161–171
34 Govindjee, Mathis, P., Vernotte, C., Wong, D., Saphon, S., Wydrzynski, T. and Briantais, J. (1979) Z. Naturforsch. 34c, 826–830
35 Renger, G. and Eckert, H.J. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Balaban International Science Services, Rehovot, in the press
36 Gläser, M., Wolff, C. and Renger, G. (1976) Z. Naturforsch. 32c, 712–721
37 Heber, U., Kirk, M. and Boardman, W. (1979) Biochim. Biophys. Acta 546, 291–306
38 Maroc, J. and Garnier, J. (1979) Biochim. Biophys. Acta 548, 374–382
39 Ke, B. and Dolan, E. (1980) Biochim. Biophys. Acta 590, 401–406
40 Renger, G., Gläser, M. and Buchwald, H.E. (1977) Biochim. Biophys. Acta 461, 392–402
41 Shuvalov, V.A., Klimov, V.V., Dolan, E., Parson, W.W. and Ke, B. (1980) FEBS Lett. 118, 279–282
42 Melis, A. and Homann, P.H. (1976) Photochem. Photobiol. 23, 343–350
43 Melis, A. and Duysens, L.M.N. (1979) Photochem. Photobiol. 29, 373–382
44 Renger, G. (1971) Z. Naturforsch. 26c, 149–153
45 Butler, W.L. (1978) FEBS Lett. 95, 19–25
46 Poulos, T.L. and Kraut, J. (1980) J. Biol. Chem. 255 10322–10330
47 Eckert, H.J. and Renger, G. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Balaban International Science Services, Rehovot, in the press
48 Sonneveld, A., Rademaker, H. and Duysens, L.M.N. (1979) Biochim. Biophys. Acta 548, 536–551
49 Vernon, L.P. and Shaw, E.R. (1969) Plant Physiol. 44, 1645–1649
50 Erixon, K. and Renger, G. (1974) Biochim. Biophys. Acta 333, 95–106