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PROPERTIES OF THE PROTEINACEOUS COMPONENT ACTING AS APOENZYME FOR THE FUNCTIONAL PLASTOQUINONE REDOX GROUPS ON THE ACCEPTOR SIDE OF SYSTEM II

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The electron-transfer reactions between the plastoquinone molecules of the acceptor side of photosystem II have been inferred to be regulated by a proteinaceous component (apoenzyme), which additionally contains the receptor site for DCMU-type inhibitors (Renger, G., (1976) Biochim. Biophys. Acta 440, 287–300). In order to reveal the functional properties of this apoenzyme, the effect of procedures which modify the structure of proteins on the photosystem II electron transport have been investigated in isolated spinach chloroplasts by comparative measurements of O_2 evolution and absorption changes at 334 nm induced by repetitive flash excitation and of fluorescence induction curves caused by continuous actinic light. It was found that: (1) The release of blockage of O_2 evolution by the DCMU-type inhibitor SN 58132 due to mild tryptic digestion correlates kinetically with the deterioration of the binding properties. (2) Glutaraldehyde fixation of chloroplasts does not markedly modify the reoxidation kinetics of the reduced primary plastoquinone acceptor component, $X320^-$, of photosystem II, but it greatly reduces the fluorescence yield of the antenna chlorophylls and slightly retards the ADRY effect. Furthermore, it prevents the attack of trypsin on the apoenzyme. (3) Incubation of chloroplasts in 'low' salt medium markedly diminishes the ability of trypsin to release the blockage of O_2 evolution by SN 58132 and completely presents the effect on inhibition by DCMU. Based on these results and taking into account recent findings of other groups, the functional mechanism of the electron transport on the acceptor side of photosystem II is discussed. Assuming a tunnel mechanism, the apoprotein is inferred to act as a dynamic regulator rather than changing only the relative levels of the redox potentials of the plastoquinone molecules involved in the transfer steps. It is further concluded that salt depletion does not only cause grana unstacking and a change of the excitation energy transfer probabilities, but it additionally modifies the orientation of functional membrane proteins of photosystem II and their structural interaction within the thylakoid membrane.

Introduction

Photosynthetic water cleavage by visible light into molecular oxygen and metabolically bound hydrogen is initiated by exciton dissociation at the PS II reaction centres. These reaction centres contain a special chlorophyll *a* complex (referred to as $Chla_{II}$, see Ref. 1) acting as photoreactive primary electron donor and associated electron acceptor components. A special plastoquinone molecule (designated as X320, see Ref. 2) was inferred to act as secondary

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; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; metribuzin, 4-amino-6-isopropyl-3-methylthio-1,2,4-triazine-5-one; SN 58132, 3-isopropoxycarbonylaminophenyl-*N*-ethylcarbanilate; Tricine, *N*-tris(hydroxymethyl)methylglycine; atrazine, 2-chloro-4-(2-propylamino)-6-ethylamino-*S*-triazine; Mes, 4-morpholineethanesulphonic acid; PS, photosystem.

electron acceptor which is required for 'stable' charge separation at the reaction centre whereas another redox component functions as the primary electron acceptor (see Ref. 3). Hence, the reoxidation of $X320^-$ is the essential step for the regeneration of the functional state of the reaction centre II which is indispensable for photosynthetic water oxidation. In normal chloroplasts, $X320^-$ is reoxidized by the plastoquinone pool via a special plastoquinone connector molecule (B or R, see Refs. 4 and 5). The electron-transfer processes at the acceptor side of PS II were shown to be regulated by a proteinaceous component acting as an apoenzyme for the functional plastoquinone redox groups [6]. Accordingly, this protein will be referred to as X320-B-apoprotein. The X320-B-apoprotein not only regulates the endogenous electron transport and protects X320 from the attack by exogenous redox agents, but additionally provides the binding area for the well known class of DCMU-type herbicides [7–10]. Trypsin treatment leads to the release of the DCMU-type blockage of the PS II electron transport [6,11,12]. Under appropriately selected experimental conditions, the digestion rate of the X320-B-apoprotein can become significantly faster than the deterioration of other PS II activities, so that a highly DCMU-resistant O_2 evolution is achieved [6,12]. These findings support the zig-zag scheme of the structural arrangement of the photosynthetic electron-transport components, claiming the acceptor side to be located near to the outer surface of the thylakoid membrane, whereas the donor side (system Y) is situated near the inner surface (for a review see Ref. 13). Therefore, the X320-B-apoprotein is expected to be easily susceptible to procedures which modify the structural and functional pattern of proteins. In order to reveal the properties of the X320-B-apoprotein, the effects of treatments have been investigated, which affect the structural pattern of proteins, such as digestive attack by proteolytic enzymes, glutaraldehyde fixation and modification of the electrostatic interactions by salt depletion. The results obtained lead to the conclusion that electrostatic forces are not only responsible for the regulation of the overall structural arrangement of thylakoid membranes (grana stacking) and of excitation energy transfer, but also for the modification of the structural arrangement of functionally active components within the membrane.

Materials and Methods

Class II chloroplasts were prepared from marked spinach according to the method described by Winget et al. [14], except that 10 mM ascorbate was present in the grinding medium. 5% dimethylsulfoxide was added for storage of chloroplasts in liquid N_2. Salt-depleted chloroplasts were obtained in a similar way except using solutions free from KCl and $MgCl_2$. After final centrifugation, one-half of the chloroplasts were resuspended in pure buffer solution (Tricine-NaOH, pH 7.2, Na^+ content less than 1 mM), the other in salt-containing buffer (Tricine-NaOH, pH 7.2, 10 mM KCl, 2 mM $MgCl_2$). These types of chloroplast will be referred to in this paper as 'low' and 'high' salt chloroplasts, respectively. Trypsin treatment of chloroplasts was performed as described in Ref. 15 using trypsin commercially available from Boehringer. Glutaraldehyde fixation was achieved by two different methods outlined in Refs. 16 and 17. According to the first method [16], chloroplasts were incubated with sufficient glutaraldehyde so as to give a chlorophyll: glutaraldehyde ratio of 1 : 100. After 5 min the suspension was diluted 5-fold with buffer (Tricine-NaOH, pH 7.2), centrifuged and resuspended. Applying the second method [17], the chloroplasts were centrifuged through a concentration gradient made of five layers of different sucrose concentrations (0.33, 0.3 0.25, 0.2, 0.1 M). The middle layer contained glutaraldehyde, chloroplasts were put onto the top layer. Binding studies were performed as described in Ref. 18 by using the ^{14}C-labelled DCMU-type inhibitor SN 58132 supplied by Dr. Kötter (Schering AG, Berlin). The experiments were performed in a medium containing 20 mM Mes-NaOH, pH 6.5, 10 mM KCl, 2 mM $MgCl_2$ and various concentrations of ^{14}C labelled SN 58132 (the total amount of CH_3OH as solvent for SN 58132 was kept below 1% of the suspension). O_2 evolution induced by repetitive flash excitation (flash frequency 4 Hz) was measured with a Clark-type electrode as described in Ref. 19. The reaction medium contained chloroplasts (50 μM chlorophyll), 100 μM $K_3[Fe(CN)_6]$ and 50 mM Tricine-NaOH, pH 7.2, for measurements with low salt chloroplasts; in the case of the high salt chloroplasts, 10 mM KCl and 2 mM $MgCl_2$ were added.

Chlorophyll fluorescence was induced by a broad-band blue-green beam transmitted by 6-mm Schott

BG 28 broad-band filters and an electromechanical shutter. Fluorescence emission was detected by a photomultiplier in a straight line screened by an interference filter (693 nm) and appropriate red cut-off filters to suppress the actinic light. Measurements were made with chloroplasts suspended in Tricine-NaOH, pH 7.2 (10 mM KCl and 2 mM $MgCl_2$ were added when experimenting on high salt chloroplasts) at a chlorophyll concentration of 2 μM.

Flash-induced absorption change techniques at 334 and 520 nm were applied to follow the kinetics of the electron transport away from the primary acceptor X320 and of the electric field across the membrane, respectively [20]. Conditions were the same as above except that 100 μM $K_3[Fe(CN)_6]$ was added.

Results

Fig. 1 depicts the binding properties of SN 58132 and their modifications by trypsin treatment compared with the concomitant release of the inhibition of O_2 evolution. In untreated chloroplasts, SN 58132 was found to be bound with a dissociation constant of 2 μM at a molar ratio of one binding site per 200 chlorophyll molecules. The dissociation constant indicates that the binding strength of SN 58132 is about 3-fold lower compared to that of metribuzine or DCMU [18]. Trypsin treatment slightly reduces the attractive forces towards the inhibitor as reflected by an increase of the dissociation constant. This effect is accompanied by a more drastic reduction of the number of binding sites. This pattern corresponds qualitatively with the effect of trypsin treatment on the binding of other DCMU-type inhibitors in Tris-washed chloroplasts [18]. Furthermore, Fig. 1 shows that the decrease of the inhibitor binding is correlated with the release of inhibition of O_2 evolution with $K_3[Fe(CN)_6]$ as electron acceptor. These results indicate that mild trypsinization of the X320-B-apoprotein simultaneously leads to a serious modification of the binding area of DCMU-type inhibitors and of the accessibility of $X320^-$ towards exogenous hydrophilic electron acceptors, such as $K_3[Fe(CN)_6]$ Trypsin treatment was shown to interrupt the electron transport from $X320^-$ via B to the plastoquinone pool completely [6,36]. As the X320-B-apoprotein has been claimed to play a regulatory role for the electron transport between the functional plastoquinone molecules X320 and B, a slight structural modification of the protein moiety might affect the kinetics of this reaction rather than block it completely. Trypsin primarily attacks lysine and arginine residues of proteins. Hence, these groups could be of essential importance for the function of the X320-B-apoprotein. As glutaraldehyde is known to react with lysine [21] and because this amino acid residue was inferred to play probably a functional role in the X320-B-apoprotein (see Discussion and Ref. 6), glutaraldehyde fixation appears to be a promising tool to study possible effects on the reoxidation kinetics of $X320^-$ due to structural modifications of the proteinaceous environment. These kinetics are reflected by the relaxation of absorption changes at 334 nm [2]. The time course of the 334 nm absorption changes in normal and glutaraldehyde-fixed chloroplasts is shown in Fig. 2, top. The amplitude of the 334 nm absorption changes in glutaraldehyde-fixed chloroplasts is reduced compared to the control. This decrease reflects an inhibition of 20–40% of PS II. The relaxation of the signal, however, is not significantly changed due to glutaraldehyde fixation. The reoxidation kinetics of $X320^-$ appear to be slightly slowed down by a factor of 1.5–4. Accordingly, that part of the X320-B-apoprotein which regulates the reactivity of the functional group, X320, is not seriously modified by glutaraldehyde fixation. This conclusion is confirmed by the finding of an unimpaired capacity of DCMU-type inhibitors to block the electron transport (see Fig. 2, bottom). Therefore, glutaraldehyde fixation probably modifies other types of protein predominantly. Changes of the structural arrangement of proteins as well as of grana stacking are reflected by fluorescence changes (for a review, see Ref. 22). The fluorescence induction curves of normal and glutaraldehyde-fixed chloroplasts are shown in Fig. 3. Glutaraldehyde fixation causes a decrease of the maximum fluorescence yield, whereas the induction kinetics are not changed markedly. As the fluorescence induction curve reflects the functional connection of X320 with the plastoquinone pool, shown by the drastic effect of DCMU, the fluorescence data are coherent with the measurements of 334 nm absorption changes, indicating that glutaraldehyde fixation only marginally affects the function of the X320-B-apoprotein. This

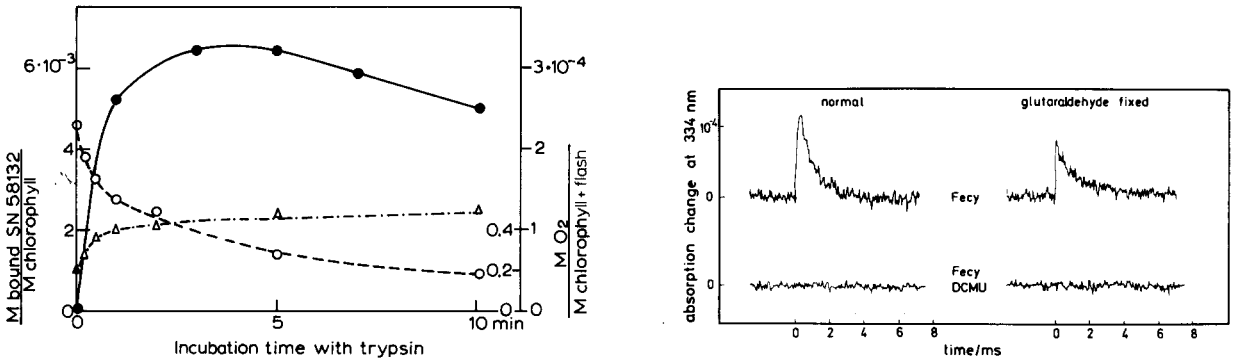


Fig. 1. Number of binding sites (○) and dissociation constant (K_D, μM) (△) of SN 58132 and average O_2 yield per flash (●) as a function of trypsin incubation time. Experimental conditions as described in Materials and Methods.

Fig. 2. Absorption changes at 334 nm as a function of time in normal and glutaraldehyde-fixed chloroplasts. Optical path length: 20 mm, 1024 signals were averaged. Other conditions as described in Materials and Methods.

comparatively small effect probably gives rise to the slight change of the induction curves in the absence of DCMU. On the other hand, the remarkable decrease of the maximum level of fluorescence observed in the absence as well as in the presence of DCMU reflects structural changes in the protein matrix of the antennae chlorophylls.

Another functional protein of PS II is the water-splitting enzyme system Y. It has been found that a number of chemicals referred to as ADRY reagents [19] accelerate the decay of oxidizing redox equivalents stored in system Y [23]. A mathematical analysis favoured the assumption of a statistical rather than of a fixed-place mechanism to be responsible for the ADRY effect [24]. Accordingly, cross-linking by glutaraldehyde even of proteins other than system Y might indirectly affect the action of ADRY agents. The data of Fig. 4 confirm this to be the case. Glutaraldehyde fixation reduces the efficiency of ADRY reagents. The mechanism which is responsible for this effect still remains to be clarified. Despite the rather weak direct effects of glutaraldehyde fixation on the

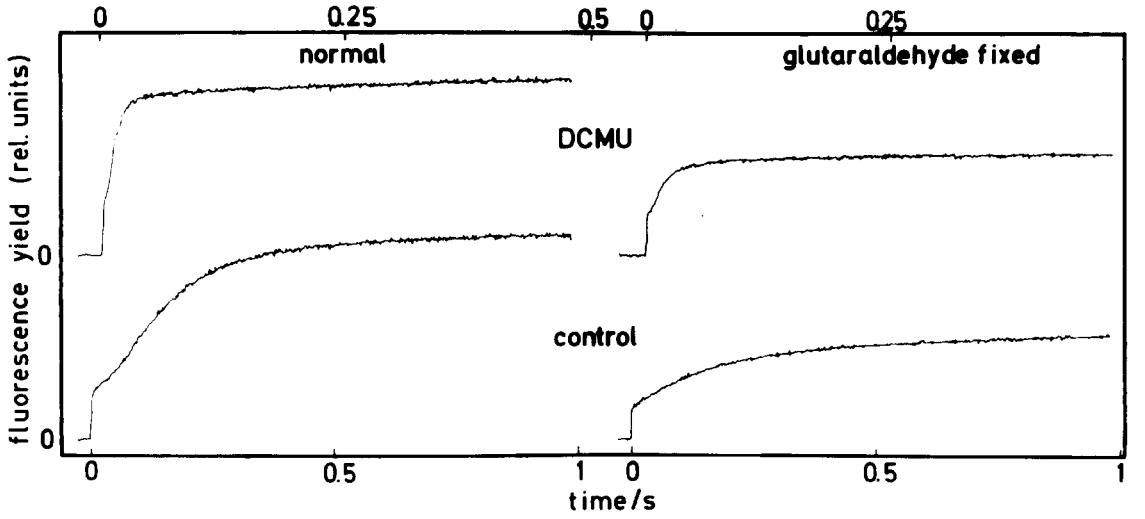
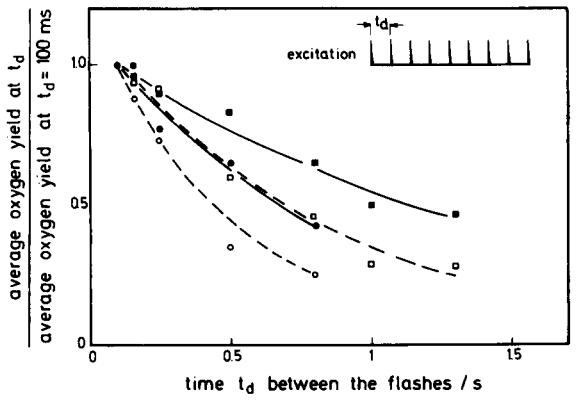


Fig. 3. Fluorescence induction in normal and glutaraldehyde-fixed chloroplasts in the absence and presence of 3 μM DCMU.

functional moiety of the X320-B-apoprotein, the accessibility to proteolytic attack by trypsin could still be modified. In order to test this possibility, the effect of trypsin treatment on the removal of blockage by DCMU of the average O_2 yield per flash has been measured in normal and glutaraldehyde-fixed chloroplasts. The data presented in Fig. 5 clearly show that the accessibility towards trypsin is greatly diminished in glutaraldehyde-fixed chloroplasts. The 30% difference in the absence of DCMU between the average O_2 yield per flash in normal and glutaraldehyde-fixed chloroplasts corresponds with the differences observed for the amplitudes of the 334 nm absorption changes (see Fig. 2). It is due to an inhibitory effect of glutaraldehyde fixation on PS II.

The failure of trypsin to attack the functional connection between X320 and B and the binding of DCMU-type inhibitors in glutaraldehyde-fixed chloroplasts is confirmed by measurements of the fluorescence induction curves and of 334 nm absorption changes [25]. Two different mechanisms could be responsible for this effect: (a) glutaraldehyde could still attack the X320-B-apoprotein at an outer segment without remarkably affecting the functional active site(s), but preventing or highly retarding further proteolytic cleavage by trypsin, (b) glutaraldehyde could induce a cross-linking of different proteins and even fix grana stacking, so that the activity of trypsin would generally be diminished. In order to study these phenomena, trypsinization experiments were performed in low and high salt chloroplasts. Incubation of chloroplasts in buffer solution was shown to cause grana unstacking and modification of excitation energy transfer (for a review, see Ref. 26). Accordingly, if one adopts the current topological scheme of the anisotropic arrangement of the electron-transport components suggesting the acceptor sides of PS I and II to be located near the outer surface of the thylakoid membrane, the X320-B-apoprotein is anticipated to be much more susceptible to trypsin in low salt than in high salt chloroplasts. The effect of trypsinization on the average O_2 yield per flash of low and high salt chloroplasts in the absence and presence of DCMU, respectively, is shown in Fig. 6. It has been found that the average O_2 yield per flash reflecting the number of functionally active PS II (including the water-splitting enzyme system Y) is generally higher by 30–50% in high salt compared to low salt chloroplasts. Resuspension of low salt chloroplasts in a high salt buffer restores the activity to nearly that of high salt chloroplasts. These salt effects, which have been observed for different preparations of spinach chloroplasts, probably reflect a heterogeneity of PS II with some of them being reversibly 'switched off' due to salt depletion. However, the much more interesting phenomenon is the failure of



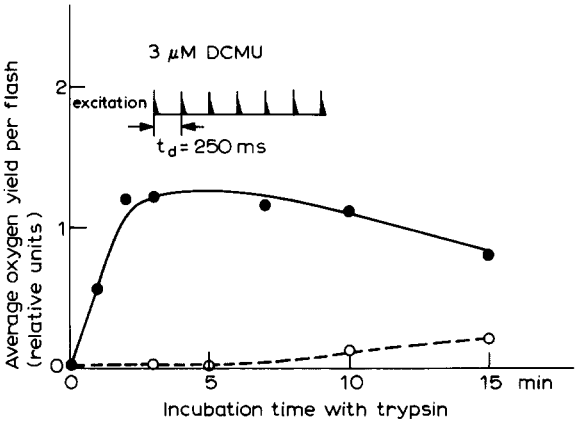


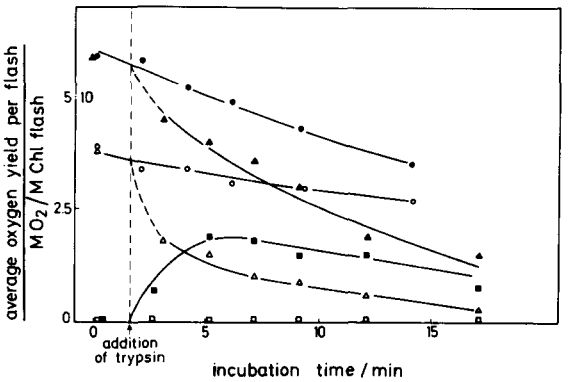
Fig. 4. Average O_2 yield per flash as a function of the time t_d between the flashes in normal and glutaraldehyde-fixed chloroplasts in the absence and presence of ANT 2p. (○,□) Normal, (●,▲) glutaraldehyde fixed. ANT 2p; 30 nM (□,■), 100 nM (○,●).

Fig. 5. Average O_2 yield per flash as a function of the incubation time with trypsin in normal (●) and glutaraldehyde-fixed (○) chloroplasts in the presence of 3 μM DCMU.

trypsin to remove the DCMU blockage of O_2 evolution under repetitive flash excitation (4 Hz) in low salt chloroplasts. On the other hand, in the absence of DCMU, the trypsin-induced degradation of the O_2-evolving capacity, takes place with at least the same rate in low and high salt chloroplasts. These data can be explained by the assumption that salt depletion induces structural changes of functional membrane proteins which make the X320-B-apoprotein less susceptible to tryptic digestion, whereas the sensitivity of the donor side of PS II (including the water-splitting enzyme system Y and the reaction centre core) remains invariant or becomes even higher. In order to test this idea, experiments have been performed using SN 58132 instead of DCMU, because the former inhibitor was found to be less tightly bound to the X320-B-apoprotein and its inhibitory effect is easier to remove by trypsin [10]. The data in Fig. 7 indicate that the inhibitory effect of SN 58132 disappears faster in high than in low salt chloroplasts. Previously, the difference between the curves of the average O_2 yield obtained in the absence and presence of an inhibitor as a function of the incubation time with trypsin had been interpreted as the degradation kinetics of the X320-B-apoprotein [15]. This interpretation, however, only remains correct if the tryptic digestion rate of the X320-B-apoprotein is markedly faster than the decline of the O_2-evolving capacity. A simple way to take into account the degradation kinetics of the latter activity can be achieved by the assumption that the effects of trypsin on both, the X320-B-apoprotein and the water-splitting enzyme system Y, are independent of each other. In the absence of a DCMU-type inhibitor, the average O_2 yield per flash as a function of the incubation time, t_{inc}, with trypsin, $M_{O_2}(t_{inc})$, reflects the number of the water-splitting enzyme systems remaining still active after t_{inc}. Therefore, $P^y(t_{inc}) = M_{O_2}(t_{inc})/M_{O_2}(t_{inc} = 0)$ is the probability of system Y surviving after an incubation time, t_{inc}, of trypsin. On the other hand, in the presence of DCMU-type inhibitors, O_2 evolution can only be obtained at PS II which attained a modified X320-B-apoprotein but simultaneously remains unaffected at the water-splitting enzyme system Y. If $M_{O_2}^{DCMU}(t_{inc})$ indicates the average O_2 yield per flash measured at t_{inc}, this amount is given by:

$$M_{O_2}^{DCMU}(t_{inc}) = P^{X320\text{-}B}(t_{inc}) \cdot P^y(t_{inc}) \cdot M_{O_2}(t_{inc} = 0) \quad (1)$$

where $P^{X320\text{-}B}(t_{inc})$ is the probability of the X320-B-



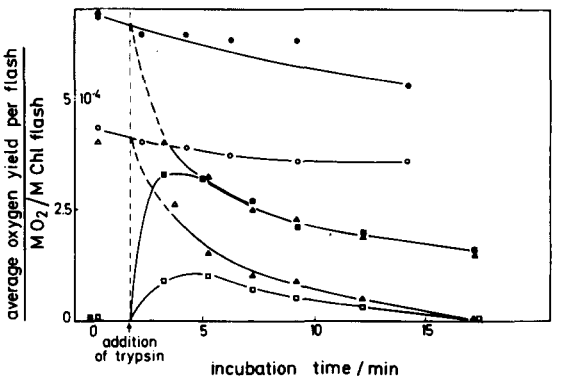


Fig. 6. Average O_2 yield per flash as a function of incubation time in low and high salt chloroplasts in the absence and presence of 50 μg trypsin/ml suspension. DCMU addition as indicated in the figure. Other experimental conditions as described in Materials and Methods. Open symbols represent measurements with low salt, full symbols those with high salt chloroplasts. (○,●) Control, (△,▲) trypsin, (□,■) 3 μM DCMU + trypsin.

Fig. 7. Average O_2 yield per flash as a function of incubation time in low and high salt chloroplasts in the absence and presence of 50 μg trypsin/ml suspension. SN 58132 addition as indicated in the figure. Other experimental conditions as described in Materials and Methods. Open symbols represent measurements with low salt, full symbols those with high salt chloroplasts. (○,●) Control, (△,▲) trypsin, (□,■) 3 μM SN 58132 + trypsin.

apoprotein being modified at its functional moiety at incubating the time t_{inc}. Therefore, $P^{X320\text{-}B}(t_{inc})$ directly reflects the digestion kinetics, which are given by the quotient $M_{O_2}^{DCMU}(t_{inc})/M_{O_2}(t_{inc})$ according to Eqn. 1 and taking into account the expression for P^y as to be $M_{O_2}(t_{inc})/M_{O_2}(t_{inc} = 0)$, i.e., the deterioration rate of system Y is assumed to be invariant to binding of DCMU-types inhibitors. The quotients obtained from the experimental data of Figs. 6 and 7 are depicted in Fig. 8. The results clearly indicate that in salt-depleted chloroplasts the X320-B-apoprotein becomes less susceptible to tryptic attack. This reduced sensitivity towards trypsin in low salt chloroplasts is not due to a diminished activity of the enzyme because in the absence of a DCMU-type inhibitor, the degradative activity on the average O_2 yield per flash in low and high salt chloroplasts is at least the same or even faster in low salt preparations (see Figs. 6 and 7).

Experiments were performed in the presence of 0.4 M sucrose to exclude osmotic effects as the main reason for the different responses of low and high salt chloroplasts to trypsin, concerning the ability to release DCMU-type inhibition. Practically the same results as in Fig. 6 were obtained so that osmotic effects seem to be of only minor importance.

Therefore, the X320-B-apoprotein and/or its localization within the thylakoid membrane is assumed to be dependent on electrostatic interactions, which become modified by salt depletion. On the other hand, the binding area for DCMU-type inhibitors is highly resistant to salt-induced modifications because in the absence of trypsin the dependency of the average O_2 yield per flash on the DCMU concentration is practically the same in low and high salt chloroplasts (data not shown).

The data of Fig. 8 also show a remarkable difference of salt depletion on the trypsin-induced release of inhibition of SN 58132 and DCMU. This effect can, at least in part, be accounted for by different modes of inhibitor binding by the X320-B-apoprotein, in agreement with former conclusions based on various lines of evidence [7,9]. However, another effect has to be taken into consideration as well, namely the ADRY effect [19]. In trypsinized high salt chloroplasts, DCMU does not exert an ADRY effect, even at rather high concentrations [27]. This situation could be changed in low salt chloroplasts. Preliminary

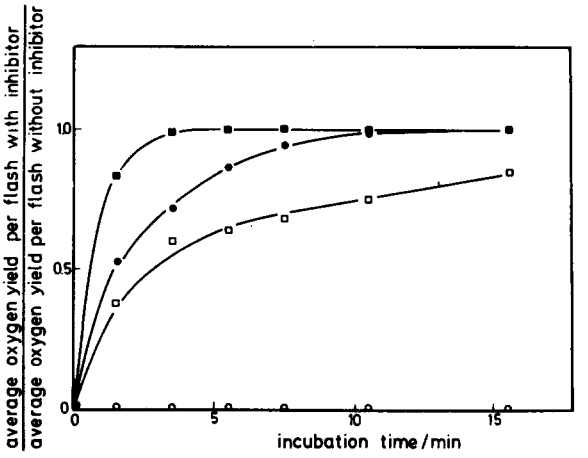


Fig. 8. Ratio of the average O_2 yield per flash measured in the absence and presence of inhibitor, respectively, as a function of incubation time with trypsin in low (○, □) and high salt (●, ■) chloroplasts. Experimental data of Figs. 6 and 7. (□, ■) 3 μM SN 58132, (○, ●) 3 μM DCMU.

experiments indicate some ADRY-type activity in trypsinized low salt chloroplasts in the presence of DCMU. Therefore, the real degradation kinetics of the release of the DCMU block are masked by the ADRY effect. Further experiments are required to clarify these quantitative aspects.

Discussion

The present study indicates that the release of the inhibition by DCMU-type inhibitors of O_2 evolution induced by mild trypsinization coincides kinetically fairly well with a decrease of the number of binding sites and an increase of the dissociation constant of the inhibitors. Furthermore, previous studies have shown that these kinetics are also correlated with the interruption of the electron transport from $X320^-$ via B into the plastoquinone pool [10]. Additionally, these phenomena were found to be coupled with the suppression of proton uptake by PS II [28]. These findings can be interpreted as reflecting a very close correlation between the structure of the binding site for DCMU-type inhibitors and the function of the B-component as electron carrier. Furthermore, the binding area seems to be situated in close proximity to X320, because a significant enhancement of the

accessibility towards $K_3[Fe(CN)_6]$ has not been achieved without modification of the binding properties for DCMU-type inhibitors (see Fig. 1).

It appears reasonable to suggest a membrane-bound protein which contains two functional groups: X320 and B. This protein complex will be referred to as X320-B-apoprotein. It could be composed of at least two distinguishable protein units, one enwrapping B, the other one X320, which is a part of the reaction center complex. This assumption is in agreement with recent findings [29], indicating that this protein on the acceptor side of PS II contains two polypeptides of molecular weights 27 000 and 32 000. Generally, two different mechanisms have to be taken into consideration for the mode of function of the X320-B-apoprotein and the way it is affected by DCMU-type inhibitors [10]: (a) thermodynamic effects; (b) kinetic effects.

The blockage of the electron transport from $X320^-$ to B by DCMU-type inhibitors is assumed to be caused by a shift of the redox potential of the B/B^- couple towards more negative values [9]. This idea is supported by the finding of DCMU-induced fluorescence changes, which are interpreted as reflecting a back electron transfer from B^- to X320 due to the above-mentioned redox shift [5,30]. Within the framework of this basic thermodynamic model, the latest theoretical studies [31] attempt to understand the effect of DCMU-type inhibitors by the assumption that the binding site contains a protein salt bridge or the terminus of an α-helix in the neighbourhood of B, which functions as binding site, predominantly via coulombic interactions with polar components of the inhibitor. An inhibitor induced shift of the redox-equilibrium, $X320^- B \rightleftharpoons X320 B^-$, to the left could be achieved either by a decrease of the electrical field generated by inhibitor binding or by causing conformational constraints which enhance the levels of the Franck-Condon states determining the energetics of an electron located at B^-.

In this model, arginine was suggested to play a key role as a constituent of the binding area. However, preliminary experiments with arginine-specific proteolytic enzymes were not successful in reducing the inhibition of PS II electron transport by DCMU. On the contrary, a lysine-specific proteolytic enzyme circumvents, at least partially, the inhibition by SN 58132 (Hagemann, R. and Renger, G., unpublished results). These results might favour lysine rather than arginine as an essential residue of the binding area.

Despite the fact that the blockage of the electron transport from $X320^-$ to B by DCMU-type inhibitors might be predominantly due to a redox shift (thermodynamic effect) of the functional B/B^- plastoquinone group, the X320-B-apoprotein is assumed to play a key role in the kinetics of the electron transfer at the acceptor side of PS II. Very recently, it was shown that the electron transport between the redox-active haem groups of cytochrome *c* and cytochrome *c* peroxidase over a rather large distance of 1.5 nm is supported by a specific structural arrangement of a few functional amino acid residues [34]. Furthermore, small structural changes in the neighbourhood of the haem group of cytochrome *c* were found to accompany a transition of the redox state [35]. An analogous mechanism is postulated for the function of the X320-B-apoprotein, which mediates the electron transfer between the two plastoquinone redox groups, X320 and B, via an 'electron channel' established by a specific arrangement of functional amino acid residues. This channel is buried in the interior of the X320-B-apoprotein, and it is not accessible without serious modification to the exogenous redox couples [12,32]. Similarly, the X320 reoxidation kinetics are only slightly dependent on the external pH in the range pH 6–8 [33]. The association of the subunits enwrapping B and X320 is probably strongly supported by the thylakoid membrane because both components are membrane bound; whereas in cytochrome *c*-cytochrome *c* peroxidase (or oxidase) systems, cytochrome *c* is often a mobile component so that the association is achieved by mirror-image charged, specific areas of the proteins [34,35]. More direct support for the postulated mechanistic role of the X320-B-apoprotein is given by the latest findings in *Rhodospirillum rubrum* chromatophores. Applying Mössbauer spectroscopy, it has been shown that during the electron transfer between the functional ubiquinone groups, UQ_1 and UQ_2, the iron centre (^{57}Fe nucleus) attached to UQ_1 and indispensable for its function is delocalized over a rather large distance of 180 pm [37]. The authors conclude that the electron transfer from UQ_1 to UQ_2 occurs via a tunnel mechanism involving conformational relaxations which are required for the stabilization of the transfer step. It was found that the reaction pattern of the

acceptor side of PS II, including the reaction centre complex, closely resembles that of photosynthesizing bacteria [38]. In green plants and in algae special plastoquinone molecules, X320 and B, provide the secondary electron transport instead of UQ_1 and UQ_2. Analogously to bacteria, the primary quinone acceptor, X320, also acts as a one-electron carrier only [39], probably complexed with a transition metal centre (Fe). According to the mechanism postulated for the function of the X320-B-apoprotein, a structural modification of the protein units in the area which forms the electron channel is expected to change the kinetics of the electron transfer. The present study shows that glutaraldehyde fixation does indeed retard the reoxidation of $X320^-$. However, the kinetic effect is rather small, while some reaction centres of PS II are completely blocked (this effect is in agreement with results reported previously [40]). Obviously, glutaraldehyde fixation is a method too crude to induce only slight and definite structural modifications of the X320-B-protein in the area of the electron channel. Similarly, the effect of DCMU-type inhibition is not modified by glutaraldehyde fixation (see Figs. 2–4). However, genetic variations are expected to be a more appropriate tool to induce well defined structural changes of proteins. It was recently found that mutants which are highly resistant to inhibition of the $X320^-$ reoxidation by atrazine are characterized by at least 10-times slower reoxidation kinetics compared to the normal atrazine-susceptible variety [41]. This result shows that structural modifications of the X320-B-apoprotein in the area of the binding sites for DCMU-type inhibitors simultaneously affect the functional plastoquinone groups, X320 and B. Therefore, the inhibition by DCMU inhibitors could also be caused by a destruction of the electron channel (kinetic effect) rather than by a redox shift of the B/B^- group (thermodynamic effect).

If one admits that the X320-B-apoprotein kinetically regulates the electron transfer from $X320^-$ to B (and vice versa from B^- to X320) via the above-mentioned electron channel, an additional type of allosteric regulation has to be taken into consideration, i.e., the allosteric modification of the electron transport by structural changes in other proteins, which are in contact with the X320-B-apoprotein or by gross structural changes of the membranes. The results obtained in glutaraldehyde-fixed and salt-depleted chloroplasts support the idea that this type of indirect regulation is of minor functional importance, because neither the sensitivity to DCMU nor the reoxidation kinetics of $X320^-$ are markedly changed. On the other hand, salt depletion was found to retard significantly the effect of trypsin to circumvent the inhibition by DCMU of the O_2 evolution under repetitive flash excitation. Trypsin is by no means a highly specific proteolytic enzyme which selectively reacts only with the X320-B-apoprotein, it also impairs the water-splitting enzyme system Y and even modifies the reaction centre complex (Renger, G. and Eckert, H.J., unpublished results). Accordingly, $P^Y(t_{inc})$ in Eqn. 1 does not reflect a specific destruction of system Y only (such as caused by Tris-washing) but symbolizes an overall deterioration of PS II activity. A salt-induced reorientation of the functional proteins within the thylakoid membrane could change the reaction pattern of a tryptic attack. The data of Figs. 6–8 indicate that this is the case. They corroborate previous findings about the strong influence of the pH in the suspending medium on the relative efficiency of trypsin as a selective modifier of the X320-B-apoprotein [15]. Accordingly, trypsin only provides an appropriate tool for the specific modification of the X320-B-apoprotein under carefully selected experimental conditions. Furthermore, the data obtained in low and high salt chloroplasts clearly show that salt depletion not only causes grana unstacking and a change of the excitation energy transfer probabilities in the antennae, but also leads to a rearrangement of the functional proteins of PS II within the thylakoid membrane. This conclusion is in line with recent electron microscopic studies [42]. At the present stage of knowledge it appears to be premature to speculate about the possible physiological role of these effects.

Note added in proof

Latest data obtained in Spirodela thylakoid membranes provide convincing evidence that the X320-B-apoprotein contains a 32 kdalton unit. The proteolytic cleavage of a small subunit of less than 1 kdalton appears to be sufficient for the induction of a DCMU-resistent electron transport from H_2O to $K_3[Fe(CN)_6]$ at PS II (Matoo, A.K., Pick, U., Hoffman-Falk, H. and

Edelman, M. (1981) Proc. Natl. Acad. Sci. U.S.A., in the press).

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