Inhibition by Tetranitromethane of Photosynthetic Electron Transport from Water to Photosystem II in Chloroplasts

P. V. Sane * and Udo Johanningmeier

Lehrstuhl Biochemie der Pflanzen, Ruhr-Universität, Postfach 10 21 48, D-4630 Bochum 1

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Low concentrations (10 μM) of tetranitromethane inhibit noncyclic electron transport in spinach chloroplasts. A study of different partial electron transport reactions shows that tetranitromethane primarily interferes with the electron flow from water to PS II. At higher concentrations the oxidation of plastohydroquinone is also inhibited. Because diphenyl carbazide but not Mn^{2+} ions can donate electrons efficiently to PS II in the presence of tetranitromethane it is suggested that it blocks the donor side of PS II prior to donation of electrons by diphenyl carbazide. The pH dependence of the inhibition by this protein modifying reagent may indicate that a functional-SH group is essential for a protein, which mediates electron transport between the water splitting complex and the reaction center of PS II.

Introduction

Protein modifying agents in the recent past have proved useful in understanding the functional amino acid residues of biologically important proteins. Tetranitromethane (TNM) – a reagent introduced first by Wormall [1] and subsequently studied in detail by Riordan and his coworkers [2, 3] modifies only –SH residues at pH 6.0 but both tyrosine and –SH groups at higher but physiological pH [2]. This makes it very useful in examining the functional tyrosine or –SH groups of proteins of biological importance. It was recently used to demonstrate the involvement of a tyrosine residue of ribulose-bisphosphate carboxylase in catalysis [4]. In following our earlier observation that proton translocation through the chloroplast membrane is essential for electron transport [5] and the result of Konishi and Packer [6] that in bacteriorhodopsin a tyrosine residue is involved in proton translocation we undertook to study the effect of TNM on the photosynthetic electron transport of isolated chloroplasts. Although the data presented in this communication do not throw light on the involvement of tyrosine in proton channels of the electron flow system they do indicate that a functional –SH group of a protein on the donor side of PS II is necessary for electron transport between water splitting complex and the reaction center of PS II.

Materials and Methods

Chloroplasts were isolated from market spinach by the procedure described earlier [7]. A 10 mM solution of tetranitromethane was made in methanol and was used in amounts such that the methanol concentration in the reaction mixture never exceeded 1%. The conditions of assay for different electron transport reactions have been stated under the tables and figures. Except for experiments involving treatment of chloroplasts at pH 6.0 the buffer used was tricine. For pH 6.0, MES buffer was used. The oxygen consumption or oxygen evolution during different reactions was monitored by an oxygen electrode (Rank Brothers). The light provided by the projector lamp was passed through a set of 2 filters (type KG 2 and RG 645 from Jenaer Glaswerk Schott). The intensity of light was 10^5 ergs $\cdot$ cm^{-2} $\cdot$ sec^{-1} and was saturating under the conditions of experiments used.

Results

The incubation of chloroplasts with 10 µM TNM results in severe inhibition of the electron transport from

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* On leave from Bhabha Atomic Research Centre, Bombay, India.

Abbreviations: DAD, diaminodurene; DBMIB, 2,5-dibromothymoquinone; DCIP, 2,6-dichloro-phenol-indophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; DMMDBQ, dimethyl-methylen-dioxy-*p*-benzoquinone; DPC, diphenylcarbazid; DQ, duroquinol; Fecy, ferricyanide; MES, 2-morpholinoäthansulfonsäure; Mv, methylviologen; PQ, plastoquinone; PS, photosystem; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylendiamin; TNM, tetranitromethane.

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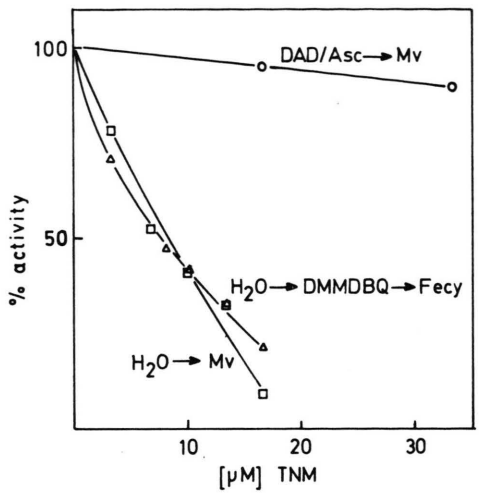

Fig. 1. Effect of TNM on the photoreduction of Mv and DMMDBQ/Fecy with water or DAD/ascorbate as electron donor. The reaction mixture in a total volume of 3 ml contained tricine-NaOH, pH 8.0, 50 mM; NaCl, 50 mM; $MgCl_2$, 5 mM and chloroplasts equivalent to 50 μg chlorophyll. In addition either Mv, 0.1 mM; NaN_3, 0.3 mM; NH_4Cl, 10 mM (for water to Mv) or Fecy, 3 mM; DMMDBQ, 0.4 mM; DBMIB, 1 μM (for water to DMMDBQ to Fecy) or DAD, 1 mM; ascorbate, 3 mM; DCMU, 1 μM; Mv, 0.1 mM; NaN_3, 0.3 mM (for DAD/ascorbate to Mv) were added. The control rates without TNM were 650, 297 and 1653 μequiv. per mg chlorophyll per hour respectively.

H_2O to methyl viologen (Fig. 1). The electron transport was studied in the presence of an uncoupler (NH_4Cl) thus avoiding any effects of TNM on the phosphorylation system. In the experiment shown in Fig. 1 the TNM at different concentrations was added to the reaction mixture. A similar inhibition by TNM was observed when a chloroplast suspension was pretreated with TNM for 10 min and the chloroplast activity was measured after one wash with tricine, pH 8.0, 10 mM; sucrose, 0.4 M; NaCl, 10 mM to remove excess of TNM. The ratio of TNM to chlorophyll used in these pretreatment experiments was the same as used in Fig. 1. The fact that TNM pretreatment shows similar results indicates that TNM reacts with the chloroplast protein and brings about permanent modification which is not reversed by mere washing with buffer. This is expected considering the known effect of TNM on proteins [8]. Since pretreatment and addition of TNM to the reaction mixture exhibit same behavior, in all the subsequent experiments the latter procedure was used as it is more convenient to study concentration effects.

In order to localize whether TNM interferes with PS II or PS I, a study of the partial reactions of photosynthetic electron transport was made. The results presented in Fig. 1 demonstrate that the electron flow from the donor couple DAD/ascorbate to methyl viologen through PS I is totally insensitive to TNM whereas the photoreduction of ferricyanide by PS II with water as electron donor is as sensitive as the electron flow from water to methyl viologen. This shows that TNM at low concentrations blocks the electron transport before the reduction of PS II acceptors such as DMMDBQ.

The electron transport from reduced plastoquinone to methylviologen was examined to find out if any component between the two photosystems is affected. This was done using duroquinol as the donor for methyl viologen reduction [10, 11]. The results show that basal as well as uncoupled rates (in the presence of NH_4Cl) are to some extent inhibited by TNM (Fig. 2). At 33 μM concentration of TNM the inhibition of uncoupled electron flow was around 30%. However, addition of catalytic amounts of TMPD which has been shown to bypass the native plastohydroquinone oxidation site [12] completely restores the electron flow. This indicates that TNM

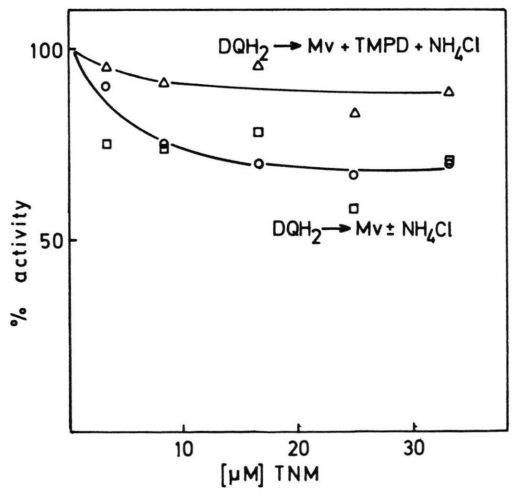

Fig. 2. Effect of TNM on the reduction of Mv with duroquinol as the donor. The reaction mixture for Mv reduction as in Fig. 1 additionally contained DCMU, 1 μM and duroquinol, 0.5 mM (freshly prepared in methanol). NH_4Cl was added after observing the basal rates. For TMPD bypass, TMPD, 30 μM and DBMIB, 1 μM were added. In the absence of TNM the basal rate was 522, the uncoupled rate 1054 and the TMPD bypass rate 1262 μequiv. per mg chlorophyll per hour.

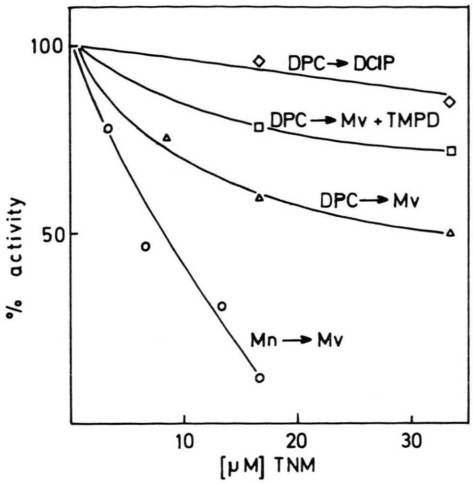

Fig. 3. Effect of TNM on donor systems for photosystem II in heat treated chloroplasts. The reaction mixture for Mv reduction as in Fig. 1 additionally contained DPC, 0.5 mM or $MnCl_2$, 5 mM. TMPD, 30 μM, was added to the reaction mixture after observing the reaction rates for a few seconds. In case of DCIP reduction the reaction mixture contained DCIP, 0.1 mM, DMMDBQ, 0.4 mM, DBMIB, 1 μM and DPC, 0.5 mM. DCIP reduction was measured spectrophotometrically at 600 nm. The chloroplasts were heat treated. The rates from water to Mv or DCIP were negligible. In the absence of TNM the rates were 226 and 141 μequiv. for Mn^{2+} and DPC to Mv respectively, and 73 μequiv. per mg chlorophyll per hour for DPC to DCIP.

also interferes with the plastohydroquinone oxidation although not as severely as the PS II reaction.

In order to find out whether the donor or the acceptor side of PS II is affected, electron flow from artificial donors to PS II like Mn^{2+} and DPC to methyl viologen as also from DPC to DCIP was studied. In these experiments the oxygen evolution system of chloroplasts was first inactivated by mild heating. The treated chloroplasts showed no electron transport with H_2O as the donor. These chloroplasts however can use Mn^{2+} or DPC as the donor (13–15) and transport electrons to methyl viologen at respectable rates that are totally sensitive to 1 μM DCMU. Fig. 3 shows that electron flow from Mn^{2+} to methyl viologen is as severely affected as the electron flow from water to methyl viologen. The reduction of methyl viologen from DPC, however, is not severely affected by TNM and shows inhibition pattern similar to electron transport from duroquinol to methyl viologen. The addition of catalytic amounts of TMPD restores the rate of electron flow from DPC to methyl viologen. Also the electron flow from DPC to DCIP (sensitive to DCMU) is not affected by TNM. These data show that TNM treatment of chloroplasts affects the donor side of PS II and specifically interferes with the ability of chloroplasts to use Mn^{2+} as the donor but not DPC as the donor.

The effect of pH on the inhibition pattern of TNM was studied. The inhibition of the rate of electron flow from H_2O to methyl viologen is almost the same at pH 7.0, 7.5, and 8.0 (Table I). At pH 8.5 the electron flow is more sensitive to TNM but this could be because at higher pH, the photosystem II may be more accessible to chemical modifiers. Since the rates of electron transport reactions are very low at pH 6.0, a separate experiment was carried out to compare the treatment of chloroplasts with TNM at

Table I. The effect of different concentrations of TNM on the uncoupled electron transport from water to Mv at different pH-values.

TNM[μM]	Percent activity at pH			
	7.0	7.5	8.0	8.5
control	100	100	100	100
3.3	74	85	71	43
6.6	65	63	58	30
9.9	52	48	42	15
13.2	35	44	23	–
16.5	23	30	21	–

The reaction mixture in a total volume of 3 ml contained tricine-NaOH with the different pH-values, 50 mM; NaCl, 50 mM; $MgCl_2$, 5 mM; Mv, 0.1 mM; NaN_3, 0.3 mM; NH_4Cl, 10 mM; and chloroplasts equivalent to 50 μg chlorophyll. The control rates without TNM were 650, 763, 678 and 424 μequiv. per mg chlorophyll per hour at pH 7.0, 7.5, 8.0 and 8.5 respectively.

Table II. Electron transport rates from water to Mv with chloroplasts pretreated at pH 8.0 and 6.0 with different TNM concentrations.

Pre-treatment with TNM[μM]	μequiv./mg Chl/h	
	pH 8.0	pH 6.0
control	395	395
50	282	312
100	127	127

0.16 ml of a chloroplast suspension (3 mg/ml) in tricine, 10 mM; sucrose, 0.4 M; NaCl, 10 mM was added to 0.84 ml of 50 mM MES-buffer, pH 6.0 or to 0.84 ml of 50 mM tricine-buffer, pH 8.0. To a sample of each chloroplast suspension 5 μl or 10 μl of a 10 mM TNM-solution was added. After 30 s incubation 0.1 ml of the suspension was pipetted into 2.9 ml of the reaction mixture as in Table I.

pH 6.0 and at pH 8.0. In this case the chloroplasts (500 μg/ml) at these pHs were treated with 50 μM and 100 μM TNM. After an incubation of 30 seconds the non-cyclic electron transport activity of an aliquot containing 50 μg chlorophyll was measured at pH 8.0. The final (carried over) TNM concentration in the reaction mixture was 3 μM. The data presented in Table II show that at pH 6.0 the extent of inhibition is as much as it is at pH 8.0.

Discussion

The results presented in this communication show that TNM interferes with the electron transport at two places: [1] it affects the ability of chloroplasts to split water, or to use Mn^{2+} as the donor in heat treated chloroplasts without causing any effect on their ability to use DPC as the donor and [2] it affects oxidation of plastohydroquinone through the native oxidation site but not in the presence of the TMPD bypass. The former reaction is very much sensitive ($pI_{50} = 8$ μM) whereas the latter is not very sensitive. The fact that addition of TMPD which bypasses native PQH_2 oxidation site [12] completely overcomes the inhibition of electron flow from PQ to methyl viologen could be interpreted to suggest that a component between PQH_2 oxidation and plastocyanin is affected by TNM. This interpretation is based on the observation that $TMPD_{ox}$ oxidizes PQH_2 at the inner surface of the chloroplast membrane and $TMPD_{red}$ is oxidized through plastocyanin [16].

Since the PQH_2 oxidation is relatively less sensitive to TNM we concentrated our efforts on localizing the step blocked by TNM in the PS II reaction. The ability of chloroplasts to use DPC as the donor but not Mn^{2+} as the donor argues for the effect of TNM on a component preceeding DPC donation but following Mn^{2+} donation.

TNM has previously been shown to modify both the tyrosine and −SH group of proteins specifically [2]. At pH 6.0 TNM does not modify tyrosine residue but specifically modifies the −SH group.

The fact that at pH 6.0 the inhibition of electron transport by TNM is the same as that at pH 8.0 indicates that a functional −SH group on the donor side of PS II is affected by TNM. However, the possibility that a special tyrosine residue which could be modified by TNM at pH 6.0 may be responsible for the observed effects cannot be ruled out. In view of the fact that electron donation by DPC to PS II is insensitive but that by Mn^{2+} is sensitive, it could be argued that the −SH group modified by TNM probably belongs to the protein which takes part in electron transport from water or Mn^{2+} to PS II. Thiol reagents have previously been shown to interfere with the photosynthetic electron transport [17]. Kobayashi *et al.* [18] have shown that treatment of chloroplasts in light with *p*-nitrothiophenol modifies the oxidation pattern of O_2 evolution indicating that a component close to or involved in H_2O splitting was blocked by this compound. Our results also show that TNM blocks the water splitting ability by modifying an −SH group/s of a protein functioning on the donor side of PS II.

The rapid inactivation of the donor side of PS II prior to donation by DPC at very low concentrations of TNM could be very useful in probing the organization of protein components on the oxidation side of PS II. Since TNM covalently binds to the protein it may facilitate identification of polypeptides functioning on the donor side of PS II. The results presented in this communication for the first time show the involvement of a functional −SH group of a protein in the electron transport between water splitting complex and the reaction center of PS II. The characterization of the second but less sensitive site is being attempted.

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