Biochimica et Biophysica Acta, 460 (1977) 1–16

BBA 47271

THE LIGHT DEPENDENT UPTAKE OF *N*-METHYLPHENAZINIUM CATIONS BY THE THYLAKOIDS OF ISOLATED CHLOROPLASTS

PETER H. HOMANN

Department of Biological Science and Institute of Molecular Biophysics, Florida State University, Tallahassee, Fla. 32306 (U.S.A.)

(Received August 30th, 1976)

SUMMARY

The absorption of *N*-methylphenazinium methylsulfate (MP^+ methylsulfate) in suspensions of envelope-free chloroplasts is reversibly lowered in the light. When the electron transport system of the chloroplasts is inhibited by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), the photobleaching reflects an uptake of MP^+ into the thylakoids. Its magnitude is a function of the composition and of the pH of the suspension medium and, most importantly, is controlled by the availability of permeant anions which apparently accompany MP^+ into the thylakoid as counterions. Consequently, the rate of the bleaching is strongly dependent on the permeability of the thylakoid to the available anion. At pH 7.5, the thylakoids of DCMU poisoned pokeweed chloroplasts are able to hold at least 6 MP^+/chlorophyll.

It is proposed that, in the presence of MP^+, the light reaction of Photosystem I in DCMU-inhibited chloroplasts causes a conformational change of the membranes which exposes nucleophilic sites inside the thylakoids. These sites appear to have a high affinity for MP^+, but may bind protons or other cations under certain experimental conditions. The uptake of MP^+ has a hypochromic effect on its absorption band in the near ultraviolet due to the resulting heterogeneous distribution of the dye cation between medium and chloroplast grana.

INTRODUCTION

N-Methylphenazinium methylsulfate (phenazine methosulfate, MP^+ methylsulfate) is a very efficient artificial cofactor in Photosystem I-mediated cyclic photophosphorylation of isolated chloroplasts. The experimental conditions necessary for an optimal action, and its mechanism, have been studied extensively but still are not fully understood. The difficulties in the assessment of the role of added MP^+ in

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; MP^+-methylsulfate, *N*-methylphenazinium methylsulfate, i.e. phenazinemethosulfate; MP^+, the *N*-methylphenazinium cation; MPH, 5-methyl-5,10-dihydrophenazine, the reduced form of MP^+; $MPH^{\cdot+}$, the cation radical of MPH.

chloroplast reactions are caused not only by the light sensitivity of MP^+ which results in the formation of photoreduced MP^+ (MPH) [1] and, under aerobic conditions, of pyocyanine [2], but also by different types of interaction with illuminated chloroplasts. For example, MP^+ can be photoreduced to MPH by system I of chloroplasts and thus serve as a Hill oxidant in non-cyclic electron transport [3]. MPH, in turn, may be reoxidized by molecular oxygen [4], or it can act as an electron donor to system I [5]. While acting as an electron carrier between the reducing and the oxidizing end of system I, the MP^+/MPH couple carries a proton from the external medium into the lumen of the thylakoid [4]. This artificially induced proton shuttle is made possible by the permeability of the thylakoid membrane to MP^+ and MPH [6], and sets up a proton-motive force capable of driving ATP synthesis [4, 7].

In addition to its participation in the reactions mentioned above, we have found [8] that MP^+ becomes reversibly bleached by illuminated intact thylakoids of envelope-free chloroplasts in vitro. The available evidence led to the suggestion that the observed reversible absorption decrease was the consequence of a binding of MP^+ to energized thylakoids. Lynn [9] and Izawa [10] had previously noted an uptake of MP^+ or MPH by illuminated chloroplasts, and the former author already interpreted the uptake as a binding of the cationic dye to the photosynthetic membranes. However, these earlier studies were performed under conditions which permitted a photoreduction of MP^+ to MPH by non-cyclic electron transport. An involvement of MPH in the observed light-dependent association of phenazine-methosulfate with the chloroplast particles was indeed indicated by its high sensitivity to an inhibition of non-cyclic electron transport [9].

Studying the interaction between MP^+ and illuminated thylakoids in the presence of the electron transport inhibitor 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), we have now been able to prove that the bleaching of MP^+ by illuminated chloroplasts is an expression of a reversible association of MP^+ with the thylakoids. The extent of this association in media with relatively low salt concentration is limited by the concentration of anions. Anions are required as counterions when MP^+ is taken up across the thylakoid membrane, and its permeability to the available anion influences the rate of MP^+ uptake during the illumination. It is proposed that MP^+ is bound to nucleophilic sites which, under different experimental conditions, may be involved in binding protons or cations like metal or NH_4^+.

MATERIALS AND METHODS

Class II chloroplasts were prepared from spinach (*Spinacia oleracea* L., var. Bloomsdale Long Standing) grown in a greenhouse, or from field-grown pokeweed (*Phytolacca americana* L.). The isolation procedure was identical to that used in a previous study. The isolation medium was either the "standard medium" at pH 7.3 (400 mM sucrose, 25 mM Tricine/NaOH, 5 mM KCl and 5 mM $MgCl_2$), or a "salt-free medium" at pH 7.5 (400 mM sucrose, 25 mM Tricine, 5 mM $Mg(OH)_2$). Chloroplasts at a concentration of about 1–1.5 mg chlorophyll/ml were stored at 0 °C for no longer than 6 h in a medium identical to that used for their isolation. When experiments at different pH were planned, the buffer concentration in the final suspension medium was lowered to 5 mM.

The "standard reaction medium" contained 400 mM sucrose, 5 mM $MgCl_2$,

5 mM KCl, 8 μM DCMU, and a buffer at 25 mM: piperazine-*N,N'*-bis(2-ethane sulfonic acid)/NaOH for pH 6.0, 6.5 and 7.0, and Tricine/NaOH for pH > 7.0. Similarly, a "salt-free reaction medium" was obtained by supplementing the "salt-free medium" with 8 μM DCMU and substituting, if necessary, a different buffer, i.e. piperazine-*N,N'*-bis(2-ethanesulfonic acid) plus $Mg(OH)_2$ for pH $\leqslant$ 7. In experiments on the pH dependence of the bleaching in salt-free reaction media the buffer concentration was adjusted to give a Mg^{2+} concentration of 8 mM.

The absorption changes were measured with a set-up described earlier and either recorded as such, or as transmission changes [8]. For direct determinations of a binding of MP^+ by illuminated chloroplasts, 5 ml of an appropriate chloroplast suspension was illuminated from above for 30–90 s and filtered by suction through a millipore filter RA 1.2 μm with the light still on. Controls were filtered in the dark with or without preillumination. All absorption values were corrected for an adsorption to the filter of about 7 % of the free MP^+.

Illumination was provided by projection lamps the light of which passed through a round bottom flask connected to running tap water, and a series of collecting lenses plus a Rohm and Haas red plastic filter and a 600 nm Optics Technology Inc. longwave pass cut-off filter. Unless otherwise noted, the intensity was saturating for the events under study, i.e. higher than $5 \cdot 10^{-2}$ J $\cdot$ s^{-1} $\cdot$ cm^{-2} (note that the light intensities given on the abscissa of Fig. 3 of ref. 8 are too high by one order of magnitude).

The number of n of available binding sites, and the dissociation constant k_d, were determined from "Scatchard plots" [11] in which the ratio of the concentration of bound dye over that of free dye is plotted against the concentration of bound dye, according to the following equation:

$$\frac{\text{dye}_{\text{bound}}}{\text{dye}_{\text{free}}} = \frac{n}{K_d} - \frac{\text{dye}_{\text{bound}}}{K_d}$$

Chlorophyll was determined according to MacKinney [12]. Among the special chemicals used in this study, calf thymus DNA was obtained from Worthington Biochemical Corp., polystyrenesulfonic acid from Aldrich Chemical Co., and MP^+ methylsulfate (PMS) from Sigma Chemical Co. The concentration of the latter in aqueous solutions was determined using an absorption coefficient of $2.6 \cdot 10^4$ cm^{-1} $\cdot$ M^{-1} [13]. Tetrabutylammonium chloride was purchased from Eastman Kodak Co., and methyltriphenyl phosphonium bromide from K and K Laboratories, Inc. *N*-Methyl-acridinium hydrochloride was prepared by methylation of commerical acridine (Aldrich Chem. Co.) with dimethylsulfate. The salt was precipitated from an aqueous solution with NaCl and recrystallized from ethanol [14].

The nigericin analog X464 was kindly provided by Dr. R. L. Harned of the Commercial Solvents Corp., Terre Haute, Ind., and A 23187 was generously donated by Dr. R. J. Hosley from Eli Lilly and Co., Indianapolis, Ind.

RESULTS

Demonstration of a light-dependent association of MP^+ with chloroplast thylakoids

In his experiments with unpoisoned chloroplasts, Lynn [9] had observed that

illuminated chloroplasts retained MP^+ when they were separated from their suspension medium by filtration. Addition of the electron transport inhibitor CMU, a monochlorinated analog of DCMU, reduced this interaction of chloroplasts and MP^+ by 80 % to approximately 1 MP^+ retained per 1/8 chlorophyll. Since the photobleaching of MP^+ observed by us with DCMU-poisoned chloroplasts suggested a binding of at least 1 MP^+/chlorophyll, it was essential to establish that the bleaching did indeed reflect a reversible association of MP^+ with the thylakoids of the kind observed by Lynn [9].

By the filtration method described in Materials and Methods it was possible to ascertain that the filtrate contained less MP^+ than was originally added, and that this deficit was dependent on the presence of light during the filtration process itself. If the lamp was turned off prior to the application of suction, or when no light was given at all, nearly all MP^+ was recovered in the filtrate.

The quantitative evaluation of the data from the filtration experiments was unsatisfactory because of experimental errors arising from a retention of some free MP^+ by the filter, and perhaps also from uncontrollable effects of the filtration process on the thylakoid – MP^+ interaction. A mutual shading of the chloroplasts packed on the filter surface was evident with small filters, but not with the larger ones

TABLE I

BINDING OF MP^+ AS DETERMINED SPECTROPHOTOMETRICALLY AND BY FILTRATION

Chloroplast suspensions were either illuminated in a cuvette (3 ml) for recording of the absorption change at 388 nm, or they were illuminated on a Millipore filter and subsequently filtered in the light as described in Materials and Methods. Standard reaction medium, pH 7.2 (spinach chloroplasts) or salt-free reaction medium, pH 7.5, supplemented with 60 mM KCl where indicated (pokeweed chloroplasts). A_{dk}(expected) is the absorbance at 388 nm of an unfiltered sample in the absence of chloroplasts. A_{dk} (found) and A_{lt} are the absorbances actually measured for the dark control and the illuminated sample, resp. The maximal absorbance decrease ΔA_{max} in the bleaching experiment with excess chloroplasts was A_{dk}(found)(1–0.51) for spinach, and A_{dk}(found)(1–0.30) for pokeweed chloroplasts. The results are expressed as μg chlorophyll/ml.

		9	17	33	11	11	20
		Standard medium			Salt-free medium		
					No KCl	Plus KCl	
	A_{dk}(expected)	0.355	0.355	0.355	0.570	0.570	0.570
Absorption	A_{dk}(found)	0.350	0.330	0.320	0.550	0.550	0.560
experiment	A_{lt}	0.265	0.200	0.165	0.480	0.205	0.180
	$\Delta A/\Delta A_{max}$	0.50	0.80	0.95	0.18	0.90	0.97
Filtration experiment	A_{dk}	0.34	0.30	0.26	0.54	0.56	0.54
	A_{lt}	0.20	0.10	0.08	0.45	0.12	0.07
	$1-\frac{A_{lt}}{A_{dk}(\text{found})}$	0.41	0.67	0.70	0.16	0.79	0.87
	$1-\frac{A_{lt}}{A_{dk}(\text{expected})}$	0.44	0.72	0.78	0.20	0.79	0.88

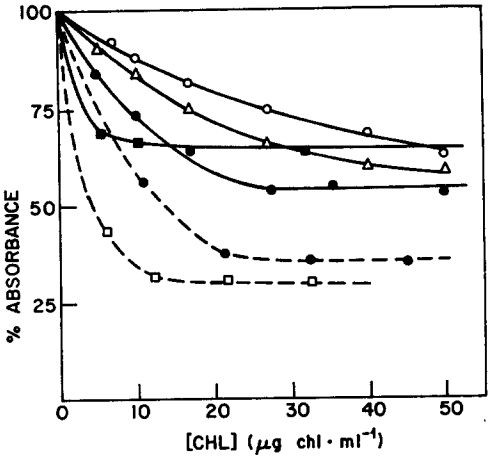

Fig. 1. Dependence of the extent of the MP^+ photobleaching on the chloroplast concentration. Total volume 3 ml. Solid lines, spinach chloroplasts; dashed lines, chloroplasts from pokeweed. Standard reaction medium, except for (■–■) which contained less than 10 mM sucrose, and (□–□) which contained 60 mM KCl. ○–○, pH 6.0, 12 μM MP^+ methylsulfate; △–△, ph 6.5, 12 μM MP^+ methylsulfate; ●–●, ■–■, □–□, pH 7.5, 20 μM MP^+ methylsulfate. CHL, chlorophyll.

finally used. With the latter, a change in the light intensity by a factor of 2–3 did not affect the result.

In spite of the encountered difficulties, the absorbance difference at 388 nm between filtered illuminated and dark control samples was consistently larger than that measured as reversible absorption change (Table I). This can be explained if it is assumed that the absorption of thylakoid-associated MP^+ at 388 nm is not zero. We checked, therefore, by how much the absorption of MP^+ would maximally decrease when chloroplasts were added in excess. A plot of the extent of photobleaching vs. chloroplast concentration is presented in Fig. 1 for suspensions of different pH, and solute concentrations. In each case a limiting value for the absorption decrease was approached which ought to represent the absorption at 388 nm when all MP^+ was taken up (cf. refs. 15 and 16). The same values were obtained when the MP^+ concentration was doubled. From the graph it can be deduced that the absorption coefficient of chloroplast-associated MP^+ depended on the composition of the suspension medium as well as on the type of chloroplast used. It also varied slightly from preparation to preparation.

The data in Table I show that significant amounts of MP^+ were detected in the filtrate even when an almost complete uptake by the thylakoids was expected. This inconsistency supported our suspicion that some MP^+ might have become unbound during the filtration procedure. The occasional retention or trapping of much MP^+ by large amounts of chloroplasts in darkness (Table I) was a further complication. Since a slightly lower absorption of MP^+ in unilluminated chloroplast suspensions could usually be accounted for by a filter effect, the measured retention of MP^+ in the dark apparently was not accompanied by any bleaching. Nevertheless, the quantitative evaluation of the filtration data, especially at high chloroplast con-

centrations, was confounded by the problem of selecting the proper dark control value.

In spite of such difficulties, the binding studies proved unequivocally that the observed bleaching of MP^+ by illuminated DCMU-poisoned chloroplasts did indeed reflect a reversible association of MP^+ with the thylakoids.

The role of salts during the light-dependent association of MP^+ with the thylakoids

It was seen at an early stage of our investigation that an omission of $MgCl_2$ from the standard reaction medium strongly inhibited the extent of the bleaching of MP^+ by illuminated chloroplasts. An explanation of this finding on the basis of the well documented influence of Mg^{2+} on the chloroplast structure [17, 18] became tenuous when a dramatic increase of the chloroplasts' ability to bleach, and bind, MP^+ was noted in a Tris-buffered reaction medium, in the presence of methylammonium chloride, or when the concentration of KCl in the reaction medium was raised (Fig. 1, Table I). Further experiments ruled out general effects of osmotic or ionic strength of the medium as the cause for the enhanced bleaching, and established a relation to the type of anion added. This was a situation distinctly different from the better known "salt effects" on the chloroplast structure which invariably are cation dependent [17–19].

In studies on the influence of added salts on the photobleaching of MP^+ by DCMU-poisoned chloroplasts we added MP^+ to a chloroplast suspension in our salt-free reaction medium and illuminated it until no further bleaching occurred. With the light still on, the salt was injected and the resumed bleaching recorded (Fig. 2). The salt-induced absorption change followed first-order kinetics and its

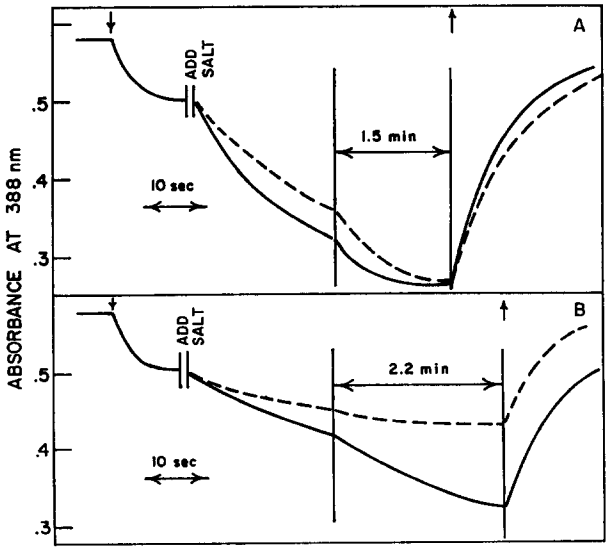

Fig. 2. Effect on MP^+ photobleaching of salts added to a salt-free reaction medium. Pokeweed chloroplasts, 25 μg chlorophyll/3 ml, 22 μM MP^+ methylsulfate. Where indicated, addition of 50 mM KCl (A, solid line), 25 mM K_2SO_4 (A, broken line), 12.5 mM $K_4[Fe(CN)_6]$(B, solid line), or 50 mM KF (B, broken line). Downward arrow, light on; upward arrow, light off.

TABLE II

RATE CONSTANTS FOR THE PHOTOBLEACHING OF MP^+ AS FUNCTION OF THE AVAILABLE ANION

Pokeweed chloroplasts (24 μg chlorophyll/3 ml) were illuminated in the salt-free reaction mixture at pH 7.5 with 22 μM MP^+ until maximal bleaching was recorded. Then the salt was introduced to give a concentration of 50 mequiv. The salt-dependent, additional absorption decrease was between 0.23 and 0.25, but 0.28 for $MgCl_2$ and Cs_2SO_4, and quite difficult to estimate because of the slow bleaching rate for $K_4[Fe(CN)_6]$ (<0.10) and, in particular, for KF and K_2HPO_4. K_2HPO_4 adjusted with KH_2PO_4 to pH 7.5.

Salt	KF	NaCl	KCl	CsCl	$MgCl_2$	$BaCl_2$
k (s^{-1})	very small	0.071	0.064	0.060	0.065	0.074

Salt	KBr	KNO_3	K_2SO_4	Cs_2SO_4	K_2HPO_4	$K_4[Fe(CN)_6]$
k (s^{-1})	0.073	0.073	0.039	0.035	very small	< 0.02

rate was relatively independent of the cation, but strongly dependent on the anion. From Table II it can be seen that anions which are known to penetrate chloroplast membranes slowly [20], also supported only a very slow bleaching, and a lower total absorption change (Fig. 2). Surprising was the almost complete absence of any effect when potassium phosphate or KF were added suggesting that the thylakoids were impermeable to HPO_4^{2-} and F^- under our experimental conditions. We ruled out any inhibitory action of these anions by ascertaining that they would not interfere with the KCl-mediated bleaching (not shown). Chaotropic anions such as I^- and SCN^-, also produced only a small "salt effect", but they were inhibitors of the bleaching.

The findings described above strongly suggested that the bleaching of MP^+ depended on the availability of anions which were capable of entering the thylakoid as charge-balancing counterions. Halogen salts of the permeable cations tetrabutylammonium and methyltriphenyl phosphonium [21] inhibited the bleaching and often led to a marked reversal of the bleaching during prolonged illumination. Initially we interpreted this observation with a successful competition of the cations with MP^+ for binding sites in the thylakoids. However, since I^- and SCN^- acted quite similarly, membrane-modifying properties of the large permeable cations could very well have been a cause for their inhibitory effect.

In the experiments described so far, and presented in Fig. 2 and Table II, the bleaching rate appeared to be limited by the permeability to the available anion. Preincubation of the chloroplasts with the salt often changed the first-order kinetics of the absorption decrease to a more complex, biphasic course, indicating some equilibration of the salt across the thylakoid and an efflux of the cation during the light-dependent MP^+ uptake. In fact, in a second illumination the bleaching with slowly permeating anions invariably was very much faster than during the first illumination, and the kinetics became complex and sometimes clearly biphasic (Fig. 3A).

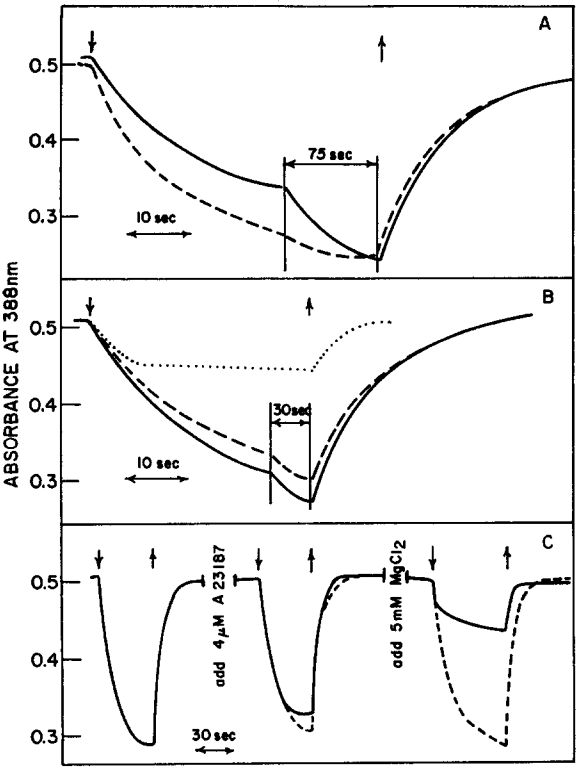

Fig. 3. Various experimentally induced changes in extent and kinetics of MP^+ photobleaching by pokeweed chloroplasts (25 μg chlorophyll/3 ml). 20 μM MP^+ methylsulfate. Downward arrows, light on; upward arrows, light off. (A) Time course of bleaching in salt-free reaction medium supplemented with 25 mM K_2 SO_4. Solid line, first illumination; broken line, second illumination. (B) Effect of valinomycin on bleaching in salt-free medium supplemented with 50 mM KCl. Solid line, no addition; broken line, plus 1 μM valinomycin; dotted line, plus 5 μM valinomycin. (C) Effect of ionophore A23187 in a medium as in B. Additions as indicated, but in experiment represented by broken line 25 μl ethanol were added instead of 25 μl ethanol containing the ionophore.

One can understand this phenomenon if one assumes that the release of MP^+ after termination of the first light period is accompanied by an influx of cations as well as an efflux of some anions. The second illumination would then lead to an uptake of MP^+ which is balanced not only by an inward movement of anions, but in addition by an efflux of previously accumulated cations. Alternately, structural changes during the first illumination may have changed the thylakoid permeability, but any such changes must have been persistent in the dark because the effect of the first light period was not reversed in subsequent darkness.

It should be noted that the response of the bleaching process to different anions varied somewhat from day to day indicating different thylakoid permeabilities due to uncontrolled properties of the chloroplast preparations. This was also true in respect to effects of ionophores like valinomycin, the nigericin analog X464, A 23187, and the anion carrier tripropyl tin [22]. As reported earlier [8] valinomycin and X 464 inhibited slightly the extent of the bleaching and, according to results from more

recent experiments, occasionally stimulated the rate of the dark reversal of the bleaching. As can be seen from Fig. 3B, valinomycin had to be present at a concentration of more than 1 μM in order to have any significant inhibitory effect at saturating light intensities. As was expected from its known specificity for divalent cations [23], any action of A 23187 was strictly dependent on the presence of Mg^{2+} in our reaction medium (Fig. 3C). Tripropyl tin at 20 μM in the standard reaction medium, finally, inhibited the bleaching by approx. 50 %, and doubled the rate of the reversal in the dark (not shown).

Binding parameters for the interaction of MP^+ with illuminated thylakoids

In attempts to determine quantitatively the number of the alleged binding sites for MP^+, and its affinity to them, we assumed that within a limited range of variation of the MP^+ concentrations in the thylakoids (2–3-fold) the ratio $\Delta A/\Delta A_{max}$ of the actual over the maximal absorption change was approximately proportional to the concentration of bound MP^+. Initially, Scatchard plots were made for conditions which we now know limited the extent of binding due to low concentrations of anions. Fig. 4A shows that at pH 7.3 the expected linear relation was obtained only for relatively high MP^+ concentrations, while for lower concentrations the assumptions required in our analysis were obviously wrong. Similar results were obtained by Dell'Antone et al. [16] for the binding of neutral red to particles from beef heart mitochondria. Our graph in Fig. 4A indicates a maximal occupation of approximately one binding site per chlorophyll, regardless of pH. Assuming that this number is the limit imposed by a roughly equal distribution of anions inside and outside the thylakoid, and taking into account a chloride concentration of 15 mM in our standard reaction medium, the internal volume of the thylakoid can be calculated to be 65 μl/mg chlorophyll, in good agreement with data of other investigators [24].

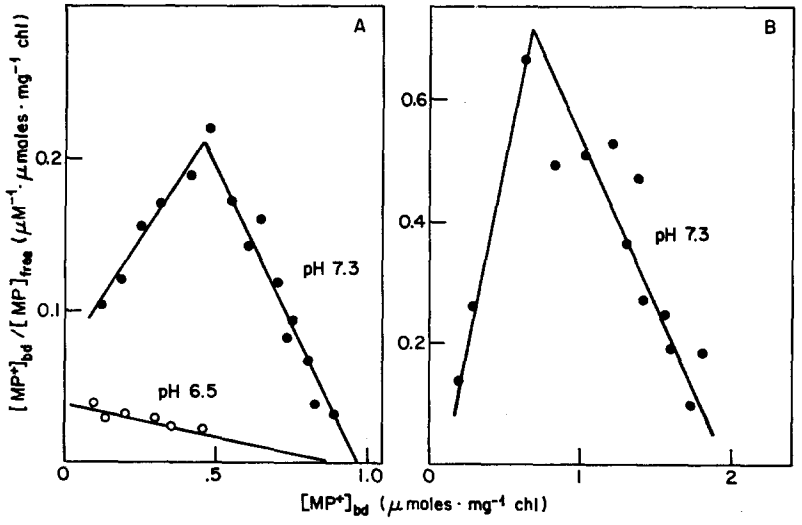

Fig. 4. Scatchard plots for the binding of MP^+ to illuminated spinach chloroplasts. 28.5 μg chlorophyll/3 ml. (A) Standard reaction medium, pH 7.3 and pH 6.5, as indicated. (B) Standard reaction medium containing only 4 mM sucrose, pH 7.3. Chl, Chlorophyll; bd, bound.

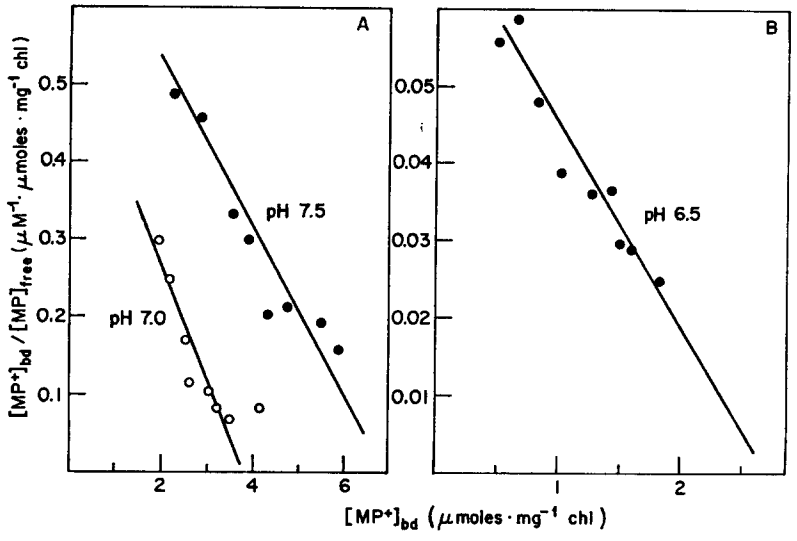

Fig. 5. Scatchard plots for the binding of MP^+ to illuminated pokeweed chloroplasts. 24 μg chlorophyll/3 ml salt-free reaction medium supplemented with 60 mM KCl at the pH indicated. chl, chlorophyll; bd, bound.

When sucrose was omitted from the standard reaction medium, the maximal binding was increased 2-fold (Fig. 4B). This observation can be explained with a swelling of the thylakoids in the hypotonic medium, allowing more anions to accompany MP^+ into the thylakoid until a final equilibrium concentration is reached.

An elevation of the anion concentration to 60 mM (Fig. 5) permitted an accumulation of 6–7 MP^+ per chlorophyll at pH 7.5, but much less at pH 7.0 and 6.5. Because of possible limitations imposed on the uptake of MP^+ by the accompanying inward movement of anions, it is not possible to interpret the slopes of the Scatchard plots (Figs. 4 and 5) in terms of true dissociation constants for the interaction of MP^+ with binding sites in the illuminated thylakoids. However, at pH 7.0 in the presence of 60 mM KCl a limitation by the Cl^- concentration can probably be ruled out, and k_d can be estimated to be 6 μM. This value is of the same order of magnitude as those calculated by others for the binding of cationic dyes to energized mitochondrial membranes [16]. At pH 6.5, the affinity of the illuminated thylakoids to MP^+ appeared to be greatly decreased (Fig. 5B).

The spectral change of MP^+ in the presence of illuminated chloroplasts

When 60 mM KCl were present, a strong photobleaching of MP^+ occurred in suspensions of relatively few chloroplasts (cf. Fig. 1). Under such conditions the spectrum of photobleached MP^+ could be determined with only little interference from light scattering. It is presented in Fig. 6. The lack of any red shift of the absorption band clearly distinguished the photobleaching from the spectral response which was observed when the synthetic polymer polystyrene sulfonate was added to solutions of MP^+ (Fig. 7). Hence, the photobleaching was not a metachromic effect of the kind seen when cationic dyes become bound to nucleophilic sites of synthetic or biological polymers and interact with each other, or with π-electron systems of the

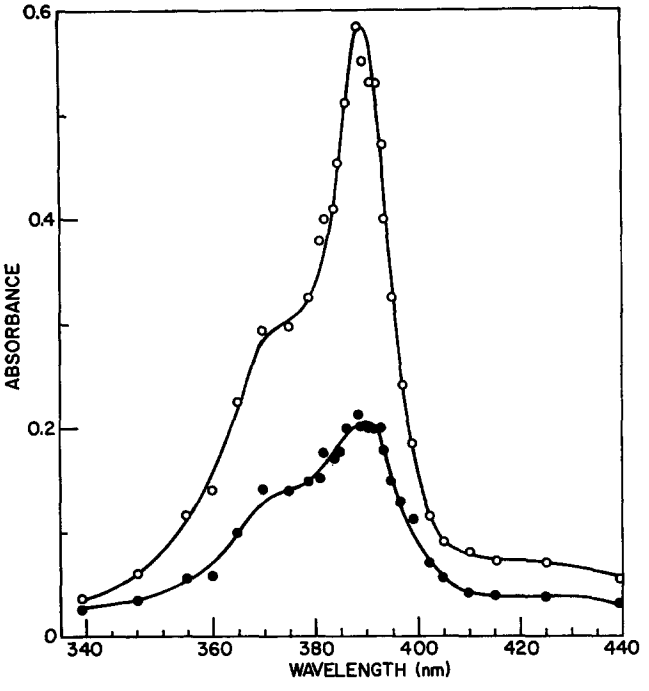

Fig. 6. Absorption spectra of MP^+ methylsulfate in the presence of pokeweed chloroplasts in the dark (○–○), and in the light (●–●). 30 μg chlorophyll/3 ml standard reaction medium supplemented with additional 55 mM KCl. 22.5 μM MP^+ methylsulfate.

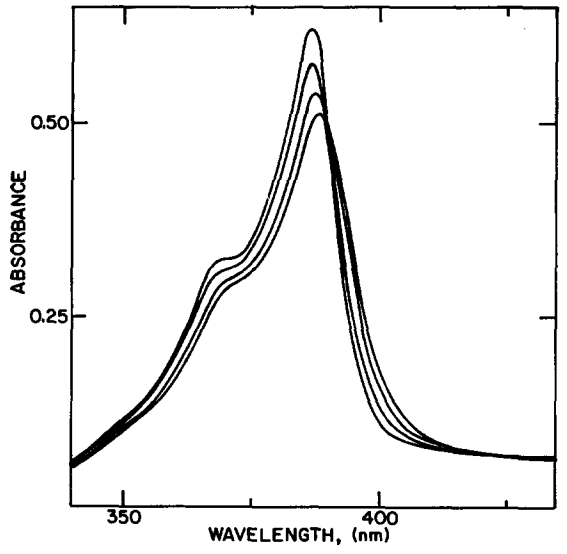

Fig. 7. Absorption spectrum of MP^+ in the presence of increasing amounts of polystyrenesulfonic acid. 10 mM Tricine/NaOH, pH 7.0; 24 μM MP^+ methylsulfate; highest curve, no polystyrenesulfonic acid; below 0.005, 0.010, and 0.030 % polystyrenesulfonic acid, respectively.

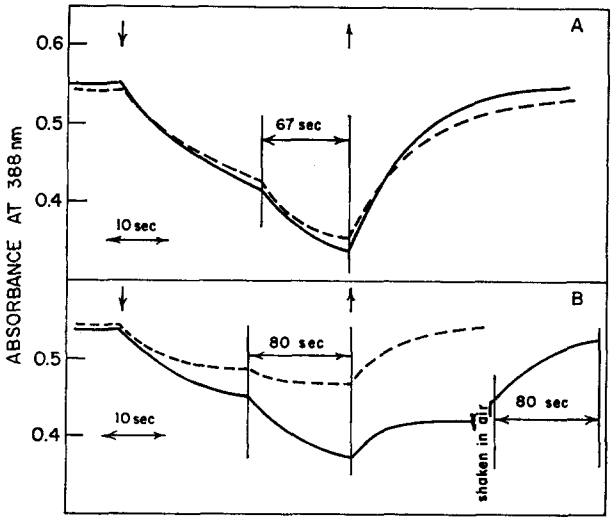

Fig. 8. Effect of anaerobiosis on the photobleaching of MP^+ methylsulfate by pokeweed chloroplasts. 24 μg chlorophyll/3 ml salt-free reaction medium supplemented with 60 mM KCl, pH 7.5. 20 μM MP^+ methylsulfate. All samples repeatedly evacuated in Thunberg cuvettes, and flushed with N_2, but those represented by broken lines subsequently bubbled with air for 20 s. (A) Normal reaction medium. (B) Reaction medium devoid of DCMU.

polymer [25–28]. Rather, the spectrum of photobleached MP^+ superficially resembled that of a mixture of MP^+ and MPH. However, no isosbestic point was evident at 350 nm to indicate the formation of MPH, and no absorption increase was detected around 440 nm which could be attributed to the semiquinoid form $MPH^{\cdot+}$ [13, 26].

In addition to this spectral evidence, we also confirmed experimentally that a photoreduction of MP^+ was not responsible for the decrease of its absorption in the presence of illuminated chloroplasts. The test was based on the assumption that the photobleaching should be sensitive to oxygen if it were due to a formation of the autoxidizable MPH. In Fig. 8 the photobleaching of MP^+ under aerobic conditions is compared with that measured under anaerobic conditions. Because of a detrimental influence of repeated evacuations on the chloroplast activity, the aerobic sample was prepared by aerating a mixture previously made anaerobic. No significant difference was detected. However, when chloroplasts were illuminated in the absence of the inhibitor DCMU, a formation of MPH was quite evident. These conditions allowed the formation of a proton gradient and, like an addition of ascorbate to DCMU-poisoned chloroplasts [8], suppressed the bleaching attributable to an uptake of MP^+.

Investigations with other dyes

Some better understanding of the interaction of MP^+ with illuminated thylakoids was hoped to come from the use of other dyes. However, while phenazine-ethosulfate responded like MP^+, we did not detect a bleaching by illuminated, DCMU-inhibited chloroplasts of pyocyanine, atebrin, proflavin, neutral red, acridine orange, phenosafranine, or *N*-methylacridinium chloride.

Because of its close structural similarity to MP^+, we studied the action of *N*-methylacridinium ions in some more detail. We found that *N*-methylacridinium in-

hibited the bleaching of MP^+, but filtration experiments gave no hint that a competition between the two dye cations might have been involved. In the absence of DCMU, and under anaerobic conditions, illuminated chloroplasts photoreduced *N*-methylacridinium almost as rapidly as MP^+. Aerobically, however, *N*-methylacridinium mediated a five times faster photoreduction of oxygen than MP^+. This was unexpected because *N*-methylacridinium could be reduced chemically under aerobic conditions, for example with NADH. The resistance to autoxidation of the reduced product, *N*-methylacridan, has been noted also by other investigators [29].

The behavior of *N*-methylacridinium can be understood if it is assumed that the aerobic photoreduction of the dye cations yielded predominantly the half-reduced radicals. In contrast to the thus produced half-reduced derivative of *N*-methylacridinium, $MPH^{\cdot+}$ may have been protected from autoxidation by binding to the thylakoids in the same manner as it is stabilized in air when bound to DNA [26]. Our preliminary data would suggest, therefore, that the inhibition of the bleaching of MP^+ by *N*-methylacridinium occurred by a mechanism similar to the inhibition by methyl viologen [8] and may involve a drain of electrons from the DCMU-poisoned electron transport system.

DISCUSSION

The data of this, and a previous [8], paper support the contention that MP^+ is bleached by illuminated, DCMU-poisoned chloroplasts as a result of a reversible, light-dependent association of MP^+ with intact thylakoids. The involved spectral change could not be attributed to a metachromic effect of the MP^+ – thylakoid interaction, and it was not caused by a photoreduction of MP^+. We postulate instead that the lowering of the absorption of MP^+ during its uptake into the thylakoids was due to the resulting heterogeneous distribution of MP^+ between the medium and the chloroplast grana suspended in it. Our explanation was supported by the dependence of the magnitude of the absorption coefficient of chloroplast-associated MP^+ on the type of chloroplasts used, and on osmotically or otherwise induced structural changes of the chloroplast membranes. If it is true, an exact quantitative analysis of the relation between bleaching and uptake could take advantage of the concepts developed by Duysens [30] and Itoh et al. [31] for the absorption "flattening" in particles.

The photobleaching of MP^+ is not linked to a proton gradient across the thylakoid membrane [8]. The same is true for a related phenomenon, the light-dependent, reversible lowering of the chloroplast fluorescence by MP^+ [8, 32]. Yet, the sensitivity of both events to a mechanical disruption of the membranes, to the phosphorylation inhibitor carbonylcyanide *m*-chlorophenylhydrazone [8], and to the ionophores valinomycin and A 23187, suggested a dependence on events at the membrane related to energy conservation.

Yamashita et al. [33] have observed that the absorption of DCMU-poisoned chloroplasts between 500 and 630 nm in the presence of MP^+ increased reversibly during an illumination due to a shrinkage of the thylakoids. This conformational change may have resulted from cyclic electron-transport in Photosystem I [8] and conceivably could make large amounts of nucleophilic sites available with a rather high affinity for MP^+. The dependence of the uptake of MP^+ on a presence of membrane

permeant anions suggests that the binding sites become exposed due to a dissociation from non-diffusible, positively charged ions or functional groups at the inner surface of the thylakoid membranes.

It is not certain at this point, but very probable, that the involved membrane sites bind protons or other cations under different experimental conditions. For example, the photobleaching of MP^+ diminished under experimental conditions which permit a deposition of protons inside the thylakoids via the MP^+/MPH proton shuttle (Fig. 8, ref. 8) or which make the membrane permeable to inorganic cations (Fig. 3). Our data furthermore corroborate the earlier suggestion by Dilley and Rothstein [34] that an increase in the H^+ concentration reduces the number of fixed negative charges in the thylakoids.

In preliminary, unpublished experiments we saw only very little MP^+ uptake with illuminated thylakoids from the granaless chloroplasts of the tobacco mutant N.C. 95 var. These thylakoids resemble other stroma thylakoids in that they lack an active Photosystem II, but are capable of cyclic photophosphorylation [35] which we have now found (Coggin and Homann, unpublished) to the relatively insensitive to uncoupling by NH_4Cl. It has been suggested [36] that such an insensitivity of ATP synthesis by stroma thylakoids is due to an impermeability of the membrane to charge balancing anions and/or to a smaller number of binding sites for protons in the energized condition of the membrane.

Although done under proton translocating conditions in the absence of DCMU, some experiments by other investigators may be relevant to our findings. For example, Gaensslen and McCarty [24] observed an uptake of up to seven molecules of ethylamine (ethylammonium?) per chlorophyll which, at high Cl^- concentrations, was accompanied by an uptake of an equal amount of Cl^-. Izawa [10] measured the ability of chloroplasts to form ATP in darkness immediately following a light period, and he noted a formation of 1 ATP/3 chlorophylls at high MP^+ and Cl^- concentrations although no proton uptake was measurable. It is tempting to relate the latter observation to the stimulation of the postillumination ATP yield by valinomycin plus K^+ [37] and postulate an increased electrochemical activity of internally liberated protons due to a diffusion potential of Cl^- according to the concept of Dilley and Giaquinta [38]. Furthermore, in the presence of low concentrations of anions a shuttle of protons via $MPH \cdot ^+$ in competition with the uptake of MP^+ is perhaps severely limited. Is it possible that this explains the discrepancy between the low steady-state rates of ATP formation by DCMU-poisoned chloroplasts measured with MP^+ under anaerobic conditions by Hauska et al. [7], and the rather high ones reported by Jagendorf and Margulies [1] who used much more Cl^- in their reaction media?

Because MP^+ methylsulfate is a strong base, its uptake by illuminated thylakoids cannot be related easily to the known uptake and binding of weakly basic dyes by electron transport membranes [16, 28]. However, Fiolet et al. [39] have observed that the binding of atebrin to illuminated chloroplasts requires its protonation, and quaternary ammonium dyes like safranine 0 have been shown by Colonna et al. [40] to bind to intact mitochondrial membranes. The large spectral change of MP^+ during its uptake should make this dye useful in studies on the conformational changes of the thylakoid membrane brought about by the photoreaction in Photosystem I of the chloroplasts. The dependence of the uptake on permeant anions

furthermore allows a quick assessment of the relative permeability of the thylakoid membrane to various anions. Finally, the MP^+-thylakoid interactions described in this paper may help to explain many puzzling observations with this compound when it was used as artificial cofactor of photophosphorylation or as electron acceptor.

ACKNOWLEDGEMENTS

This investigation was aided by grant BMS 7200076 from the National Science Foundation, and performed in the Institute of Molecular Biophysics which is supported in part by contract E-(40-1)-2690 with the U.S. Energy Research and Development Administration. The assistance of Mrs. Karen Anderson Williams and Mr. Dean Chapman during different stages at the beginning of this work is gratefully acknowledged. The author also thanks Drs. R. A. Dilley, D. Mauzerall, and W. R. Rhodes for helpful comments.

REFERENCES

1 Jagendorf, A. T. and Margulies, M. (1960) Arch. Biochem. Biophys. 90, 184–195
2 McIlwain, H. (1937) J. Chem. Soc. II, 1704–1711
3 Zweig, G., Shavit, N. and Avron, M. (1965) Biochim. Biophys. Acta 109, 322–346
4 Hauska, G. (1972) FEBS Lett. 28, 217–220
5 Schwartz, M. (1967) Biochim. Biophys. Acta 131, 548–558
6 Deamer, D. W., Prince, R. C. and Crofts, A. R. (1972) Biochim. Biophys. Acta 274, 323–335
7 Hauska, G., Trebst, A. and Draber, W. (1973) Biochim. Biophys. Acta 305, 632–641
8 Homann, P. H. (1976) Plant Physiol. 57, 387–392
9 Lynn, W. S. (1967) J. Biol. Chem. 242, 2186–2191
10 Izawa, S. (1970) Biochim. Biophys. Acta 223, 165–173
11 Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
12 MacKinney, G. (1941) J. Biol. Chem. 140, 315–322
13 Zaugg, W. S. (1964) J. Biol. Chem. 239, 3964–3970
14 Kaufmann, A. and Albertini, A. (1909) Berichte 42, 1999–2012
15 Blake, A. and Peacocke, A. R. (1968) Biopolymers 6, 1225–1253
16 Dell'Antone, P., Colonna, R. and Azzone, G. F. (1972) Eur. J. Biochem. 24, 553–565
17 Izawa, S. and Good, N. E. (1966) Plant Physiol. 41, 533–543
18 Gross, E. L. and Prasher, S. H. (1974) Arch. Biochem. Biophys. 164, 460–468
19 Homann, P. H. (1969) Plant Physiol. 44, 932–936
20 Schuldiner, S. and Avron, M. (1971) Eur. J. Biochem. 19, 227–231
21 Liberman, E. A. and Skulachev, V. P. (1970) Biochim. Biophys. Acta 216, 30–42
22 Watling-Payne, A. S. and Selwyn, M. J. (1974) Biochem. J. 142, 65–74
23 Telfer, A and Barber, J. (1974) Biochim. Biophys. Acta 333, 343–352
24 Gaensslen, R. E. and McCarty, R. E. (1971) Arch. Biochem. Biophys. 147, 55–65
25 Michaelis, L. (1947) Cold Spring Harbor Symp. Quant. Biol. 12, 131–142
26 Ishizu, K., Dearman, H. H., Huang, M. T. and White, J. R. (1969) Biochemistry 8, 1238–1246
27 Lerman, L. S. (1961) J. Mol. Biol. 3, 18–30
28 Dell'Antone, P., Colonna, R.and Azzone, G. F. (1972) Eur. J. Biochem. 24, 566–576
29 Leach, S. J., Baxendale, J. H. and Evans, M. G. (1953) Aust. J. Chem. 6, 409–420
30 Duysens, L. N. M. (1956) Biochim. Biophys. Acta 19, 1–12
31 Itoh, M., Izawa, S. and Shibata, K. (1963) Biochim. Biophys. Acta 69, 130–142
32 Slovacek, R. E. and Bannister, T. T. (1976) Biochim. Biophys. Acta 430, 165–181
33 Yamashita, K., Itoh, M. and Shibata, K. (1968) Biochim. Biophys. Acta 162, 610–613
34 Dilley, R. A. and Rothstein, A. (1967) Biochim. Biophys. Acta 135, 427–443
35 Homann, P. H. and Schmid, G. H. (1967) Plant Physiol. 42, 1619–1632
36 Arntzen, C. J., Dilley, R. A. and Neumann, J. (1971) Biochim. Biophys. Acta 245, 409–424

37 Schuldiner, S., Rottenberg, H. and Avron, M. (1973) Eur. J. Biochem. 39, 455–462
38 Dilley, R. A. and Giaquinta, R. T. (1975) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 7, pp. 49–107, Academic Press, New York
39 Fiolet, J. W. T., van der Erf-TerHaar, L., Kraayenhof, R. and van Dam, K. (1975) Biochim. Biophys. Acta 387, 320–334
40 Colonna, R., Massari, S. and Azzone, G. F. (1973) Eur. J. Biochem. 34, 577–585