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PROPERTIES OF ATP-DRIVEN REVERSE ELECTRON FLOW IN CHLOROPLASTS *

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Summary

1. The reverse reactions induced by coupled ATP hydrolysis were studied in spinach chloroplasts by measurements of the ATP-induced increase in chlorophyll fluorescence reflecting reverse electron flow, and of the ATP-induced decrease in 9-aminoacridine fluorescence, representing formation of the transthylakoidal proton gradient (ΔpH). ATP-driven reverse electron flow was kinetically analysed into three phases, of which only the second and third one were paralleled by corresponding phases in ΔpH formation. The rapid first phase and formation of a ΔpH occur also in the absence of the electron transfer mediator phenazine methosulfate.

2. The rate and extent of the reverse reactions were measured at temperatures in the range from 0 to 30°C. The rate of formation of ΔpH and of reverse electron flow were faster at high temperatures, but the maximal extent of ΔpH and chlorophyll fluorescence increase were observed at the lowest temperature. Considering rate and extent of the ATP-stimulated reactions, a temperature optimum around 15°C was found. Light activation of the ATPase occurred throughout the range studied. At 0°C and in the presence of inorganic phosphate the activated state for ATPase was maintained for more then 10 min.

3. The ATP-induced rise in chlorophyll fluorescence yield was found to be of similar magnitude as the rise induced by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), when both were measured with an extremely weak measuring

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q, primary electron acceptor of Photosystem II; R, secondary electron acceptor of Photosystem II; ΔpH, proton concentration gradient across the thylakoid membrane.

beam. It is concluded, that both effects, although derived via distinctly different pathways, are limited by the same electron donating or electron accepting pool.

Introduction

It was previously shown [1–4] that, after light activation of the latent adenosine triphosphatase (ATPase) of chloroplasts [5], addition of ATP in the dark leads to the formation of a transthylakoidal pH gradient, and to the reduction of Q (the primary electron acceptor of Photosystem II) by reverse electron flow. The reaction was most easily followed by observing the ATP-induced increase in chlorophyll fluorescence which accompanies the reduction of Q [6], and the quenching of 9-aminoacridine fluorescence which accompanies the buildup to ΔpH [7]. We have recently constructed an apparatus which allowed us to monitor simultaneously the two signals [3] and reported briefly on their interrelation in response to a variety of treatments.

In this communication we report on a kinetic analysis of the responses, their temperature dependence, and the relation of the reverse reaction to the electron transport steps adjacent to Photosystem II.

Materials and Methods

Spinach chloroplasts were prepared [8] and chlorophyll determined [9] essentially as previously described. The chloroplasts were finally suspended in a small volume of 0.4 M sucrose, 0.05 M NaCl at a concentration of about 2 mg chlorophyll/ml. The instrument used and the methodology employed were as previously described [3] with variants as indicated in the figure and table legends.

Results

Kinetics

Fig. 1 illustrates a simultaneous recording of the ATP-driven buildup of ΔpH and reduction of Q. The sample was preilluminated for 3 min in the presence of dithiothreitol with strong white-light to activate the ATPase [5], and monitoring was initiated after the activating-light was turned off. The initial changes in the figure illustrate the decay of the pH gradient ('Acridine') and the oxidation of the reduced Q ('Chlorophyll'), produced by the activating-light. After about 90 s, when these decays reached a steady state, ATP was injected, and the ATP-driven buildup of ΔpH and reduction of Q were observed.

Several features of the time course of the ATP-induced changes are worth nothing: (a) Both the quenching of acridine fluorescence and the increase of chlorophyll fluorescence show three phases. Separation between the second and third phase is not always apparent, particularly with acridine fluorescence quenching. Therefore, in the following we will mostly refer to the rapid phase and the 'main' phase. (b) The initial jump in acridine fluorescence quenching

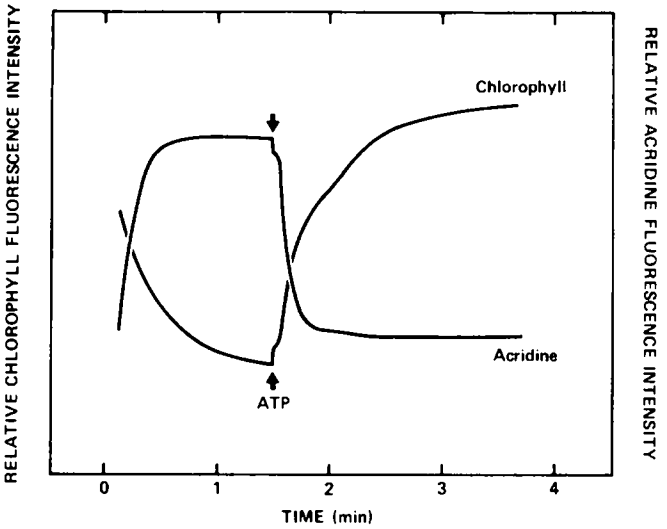

Fig. 1. Simultaneous recording of ATP-induced Q reduction (chlorophyll) and ΔpH formation (acridine). The reaction mixture contained: 15 mM tricine, pH 8.0, 20 mM NaCl, 5 mM $MgCl_2$, 1 μM phenazine methosulfate, 5 mM dithiothreitol, 2 μM 9-aminoacridine, and chloroplasts containing about 20 μg chlorophyll/ml. The system was preequilibrated and kept at 15°C by water flow from a thermostated bath [3]. To activate the ATPase, the mixture was illuminated for 3 min with intense heat-filtered white-light (about 10^5 ergs · cm^{-2} · s^{-1}). Monitoring was initiated about 5 s after the light was turned off. Where indicated, 5 μl of 10 mM ATP was injected into the stirred reaction mixture with a microsyringe [3]. The ATP-induced change in chlorophyll fluorescence is about a doubling of the dark adapted level. The ATP-induced quenching of 9-aminoacridine fluorescence is about 50%, corresponding to a ΔpH of 3—4.

was analysed to be artifactual, due to the chemical quenching by adenine nucleotides [5]. It persists in the presence of high uncoupler concentration, and does not require an active ATPase. However, the rapid increase in chlorophyll fluorescence does require an active ATPase, and is eliminated in the presence of uncouplers. (c) The main decrease in acridine fluorescence and the main increase in chlorophyll fluorescence occur after distinct lag periods, which last approx. 2 s. Thus, the rapid phase of the chlorophyll fluorescence increase precedes the detection of a substantial ΔpH. (d) The time course of ΔpH formation and of Q reduction have similar although not identical kinetics [3].

In Fig. 2 the role of phenazine methosulfate in the reverse reaction is analysed. The presence of phenazine methosulfate is not required for activation of the ATPase, as indicated by the formation of an almost normal ATP-induced ΔpH. However, only the first phase of the ATP-induced chlorophyll fluorescence increase is observed. The disappearance of the second and third phase seems to be related to the previously demonstrated requirement for an electron donor for ATP-induced Q reduction [2]. Dithiothreitol appears to be an effective electron donor to the photosynthetic electron transport chain only in the presence of the electron carrier phenazine methosulfate. This interpretation is in agreement with the observation (see Fig. 2) that later addition of phenazine methosulfate does restore the main phase of Q reduction. The first phase, then, is either unrelated to reverse electron flow or is due to reverse electron flow within internal electron carriers of the chain.

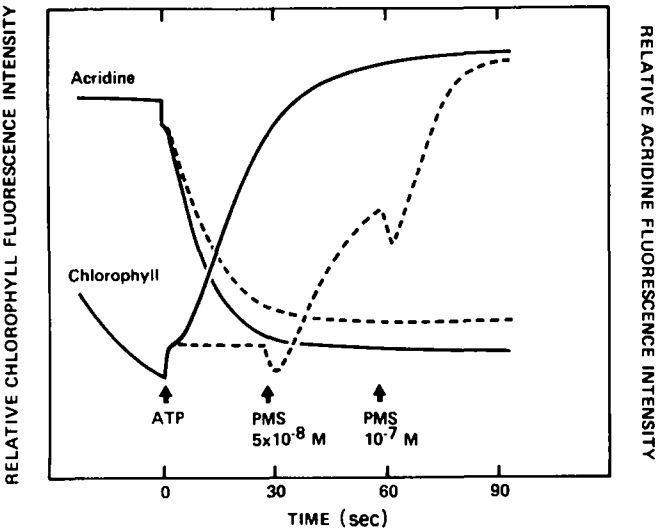

Fig. 2. Effect of phenazine methosulfate on the kinetics of ATP-induced Q reduction and ΔpH formation. Conditions as described under Fig. 1 except for the initial omission of phenazine methosulfate (PMS) in the experiment described by the dotted lines, and its presence at a concentration of $5 \cdot 10^{-8}$ M in the experiment described by the solid lines. In the former experiment phenazine methosulfate was added where indicated to the stated final concentration.

Fig. 3 illustrates the effect of the ATP-induced reverse reaction on the kinetics of the *light-induced* rise of chlorophyll fluorescence. The electron acceptor pool available for Photosystem II (represented by the area above the fluorescence rise curve) is markedly decreased in a similar way to that found in

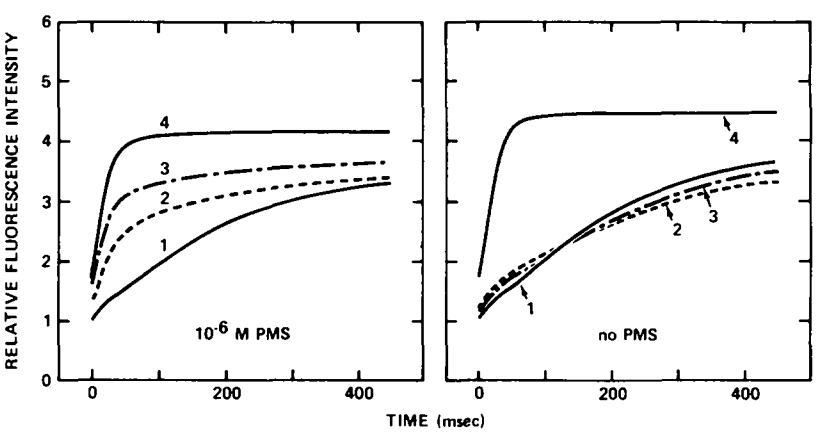

Fig. 3. The effect of ATP-induced reverse electron flow conditions on the light-induced chlorophyll fluorescence rise kinetics. Conditions as described under Fig. 1, except for omission of phenazine methosulfate in the experiments described on the right hand side of the figure, and in: (1) fluoescence exciting light turned on (time 0 in figure) 130 s after turning off the activating light. No ATP added. (2) 0.2 mM ATP added 120 s after turning off the activating light. Fluorescence exciting light turned on 10 s after ATP addition. (3) 0.2 mM ATP added 120 s after turning off activating light. Fluorescence exciting light turned on 90 s after ATP addition. (4) As 3, but 1 μM DCMU added in place of ATP. Temperature, 15°C; Intensity of blue-green fluorescence exciting light, $2 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}.

the presence of DCMU [6]. The increase of the initial fluorescence level reflects prereduction of Q. The steeper rise kinetics could be due either to a prereduction of the plastoquinone pool, by the ATP-induced reverse reaction or to an effect of the reverse coupling on the equilibrium constant between the primary acceptor Q and the secondary acceptor R, in a manner similar to that suggested for DCMU [15]. It is also apparent that the substantial ΔpH which develops even in the absence of phenazine methosulfate (see Fig. 2) does not cause any dramatic change in the light-induced fluorescence kinetics.

Temperature dependence and stability

The temperature dependence of the ATP-induced reactions is shown in Fig. 4. Note that the ATPase activation period was in all cases performed at 15°C so that the data reflect essentially the temperature dependence of only the ATP-driven reaction. The higher the temperature, the lower the extent of reaction and the faster the rate of both the ATP-driven ΔpH formation and Q reduction. The lower extent may be due to a faster non-coupled decay of the ΔpH at the higher temperatures. This is indeed corroborated by the data of Fig. 5 which shows the rate of formation and decay of *light-driven* ΔpH formation as a function of temperature. Clearly the decay rate is a strong function of temperature (see also refs. 10 and 11).

Fig. 6 illustrates the effect of temperature on the ATP-induced reduction of Q, when activation, decay and the ATP-driven reaction are all carried out at the indicated temperature. Clearly, activation can occur at 0°C, but is optimal around 10–15°C. At the lower temperature the second and third phase of the reaction are slowed down, accentuating the appearance of the first phase.

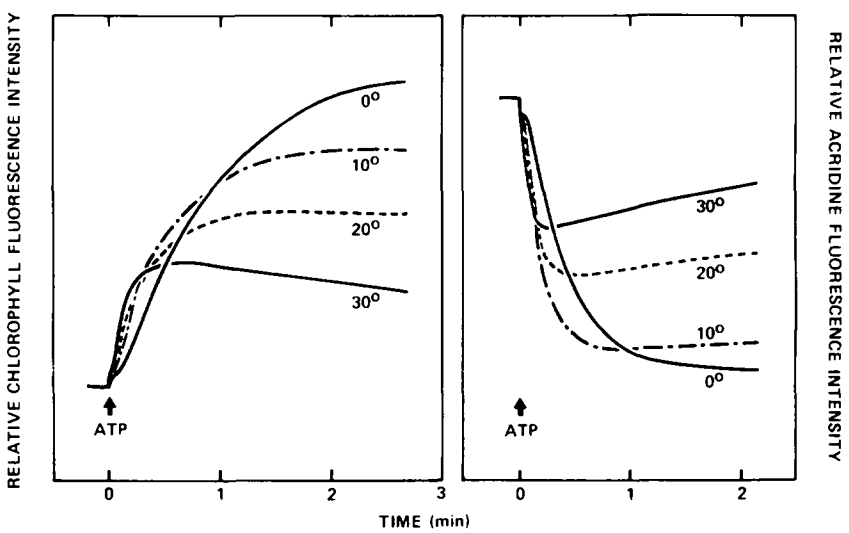

Fig. 4. Temperature dependence of ATP-driven Q reduction and ΔpH formation. Reaction conditions as described under Fig. 1, except: All samples were activated at 15°C, kept in the dark for a further 30 s at 15°C at which point the water from a second thermostated bath kept at the temperature indicated on the figure was passed through the sample [3]. After 60 s in the dark at the indicated temperature, ATP was injected to a final concentration of 50 μM.

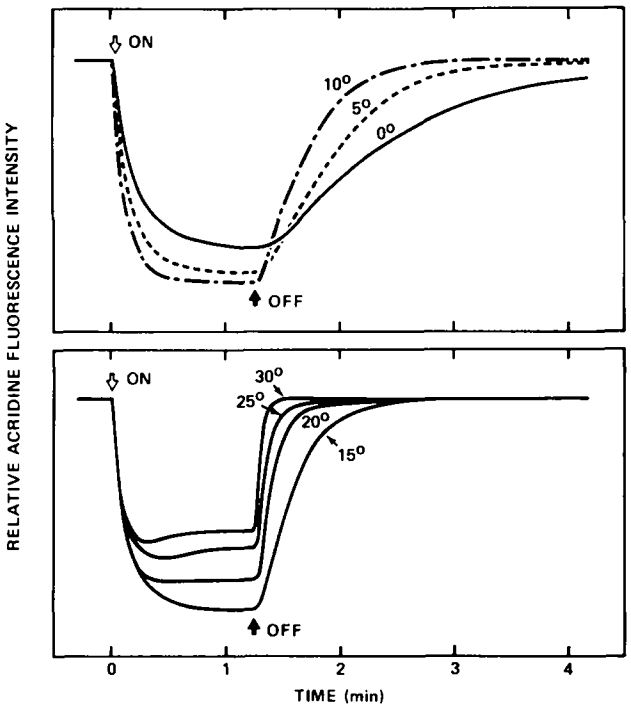

Fig. 5. The effect of temperature on light-induced ΔpH formation, and dark decay. Reaction conditions as described under Fig. 1, except for the omission of light activation and dithiothreitol from the reaction mixture, illumination was with red light (>600 nm), 10^5 ergs · cm^{-2} · s^{-1}. Each sample was preequilibrated for 2 min to the indicated temperature before illumination.

It was previously suggested that the dark decay of the capacity for ATPase activity after light-activation, is retarded by the addition of inorganic phosphate [5,12]. This is demonstrated in Fig. 7, where the light-activated state was maintained at 0°C in the dark for 9 min in the presence of inorganic phosphate. In the absence of inorganic phosphate complete decay of the capacity to

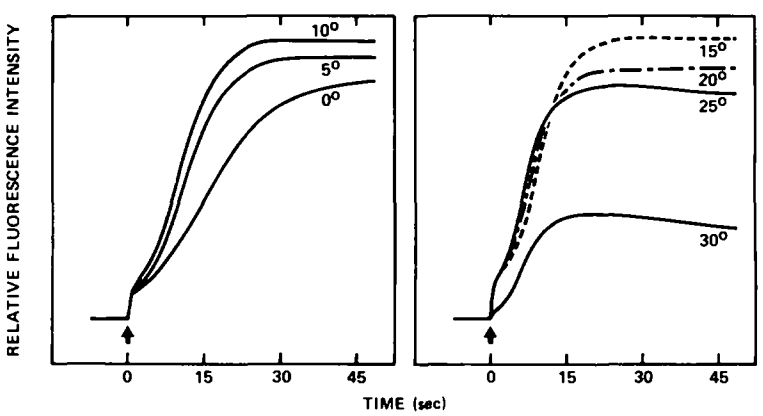

Fig. 6. The effect of temperature on the activation, decay and reaction phases of ATP-driven Q reduction. Conditions as described under Fig. 1, except for the temperature throughout which was as indicated.

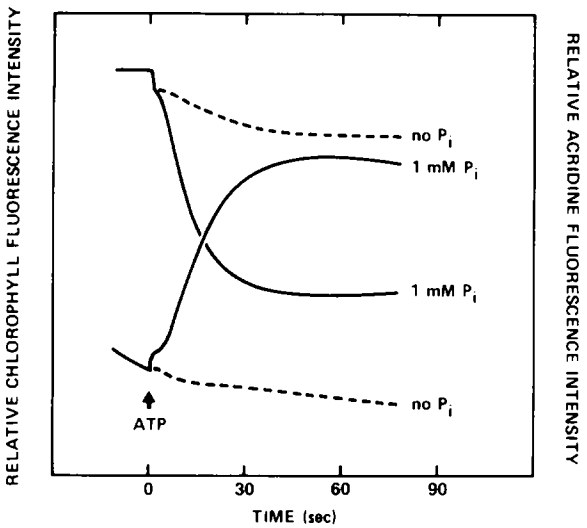

Fig. 7. The effect of inorganic phosphate in retarding the decay of the activated state of ATPase in the dark. Conditions as described under Fig. 1, except that light-activation was for 2 min at 15°C, at which point the temperature was lowered to 0°C, and light activation continued for another minute. The samples were kept for a further 8 min at 0°C in the dark, temperature was raised to 15°C and 1 min later (0 time on figure) 50 μM ATP was added. Where indicated 1 mM phosphate was added immediately after light activation.

catalyse ATP hydrolysis occurred even at 0°C. The stabilizing effect of phosphate makes it possible to perform treatments, such as removal or substitution of reaction components by centrifugation, during the lengthened period of maintainance of the activated state.

Inhibitors

It was previously shown that addition of phenylurethane or DCMU causes a rapid increase of the initial fluorescence yield [13—15]. The magnitude of this inhibitor-induced fluorescence increase (doubling of the initial fluorescence yield) is similar to that observed on ATP addition. Fig. 8 shows an experiment designed to clarify the relation between the ATP- and DCMU-induced effects. Fluorescence was measured with an extremely weak measuring beam, to avoid light-induced Q reduction. The ATP-driven rise in fluorescence yield was fully reversed by uncouplers, like SF 6847 [2,3], whereas the DCMU-induced rise was unaffected. This result is in agreement with the interpretation that the ATP-induced rise requires the production of a high energy state through the ATPase reaction, and thus is sensitive to uncouplers, while the DCMU-induced rise is due to a change in the relative redox potentials of the Q-R couple [15] which leads to a greater reduction of Q and is thus expected to be insensitive to uncouplers. The stimulation of fluorescence by DCMU is also independent of the presence of dithiothreitol or phenazine methosulfate (see Fig. 11).

The similar extent of the two effects raised the question whether, though operating via different mechanisms, both effects are limited by the same pool of electron donors or acceptors. This possibility appears to be substantiated by the experiment of Fig. 9, where it is shown that when DCMU was introduced

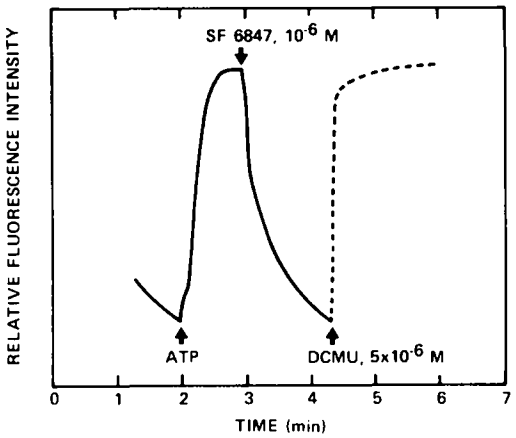

Fig. 8. Comparison of the ATP-induced and the DCMU-induced rise in fluorescence yield. Conditions as described under Fig. 1, except that the intensity of the measuring light was decreased from the usual 50 ergs · cm^{-2} · s^{-1} [3] to 0.1 ergs · cm^{-2} · s^{-1}. Where indicated the uncoupler SF 6847 and DCMU were injected to the stated final concentrations.

after ATP had already raised the fluorescence level to its maximum extent, its effectiveness was drastically reduced, and vice versa.

A more quantitative documentation is presented in Fig. 10 where ATP was added following different concentrations of DCMU, and the DCMU- and ATP-induced rises were recorded. Clearly, the higher the DCMU-induced effect the lower the ATP-induced one. Finally, it was previously shown that far-red pre-illumination can oxidize the electron carriers between the two photosystems, and thus decrease the energy-dependent reverse electron flow reaction; and that the effect is fully reversible by a subsequent preillumination with Photosystem II sensitizing light [16]. Fig. 11 illustrates that such preilluminations also markedly decrease and increase the DCMU-induced rise in fluorescence yield,

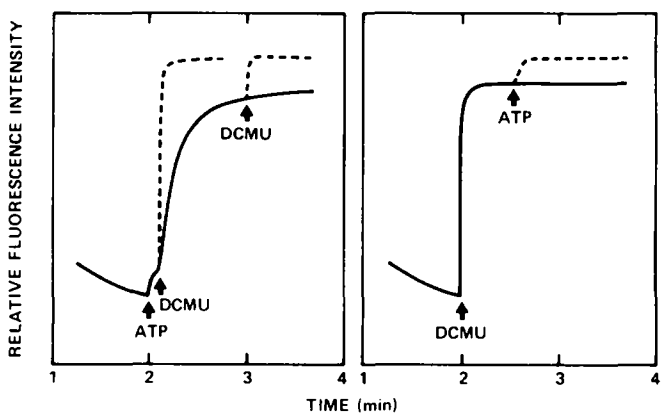

Fig. 9. The effect of consecutive additions of ATP and DCMU on the chlorophyll fluorescence yield. Conditions as described under Fig. 8. The solid curves represent the data with only the first additions; the dashes curves upon further additions of the indicated compounds.

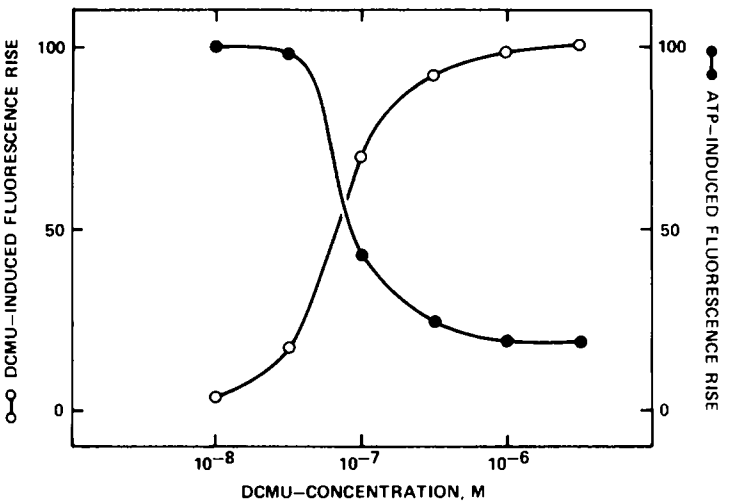

Fig. 10. The effect of increasing concentration of DCMU on the DCMU- and ATP-induced rise in fluorescence yield. Conditions as described under Fig. 8, except that DCMU, to the indicated final concentrations, was injected 90 s after light activation and ATP 30 s thereafter. The values plotted for DCMU were those measured 30 s after its addition, and for ATP, 2 min after its addition.

supporting the contention [15] that back electron transfer between components of the electron transport chain is involved.

n-Heptyl-hydroxyquinoline-*N*-oxide, which inhibits electron transport in a manner similar to DCMU [17] shows multiple effects (Fig. 12, see also ref. 2). At the lower concentration range (10^{-7}—10^{-5} M) it inhibits the ATP-induced fluorescence rise without in itself causing any significant rise in fluorescence

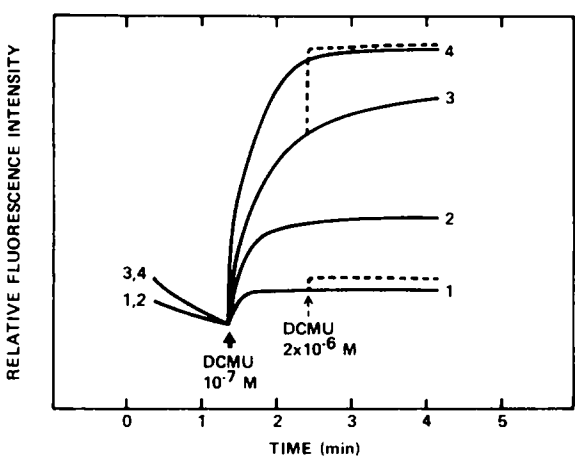

Fig. 11. The effect of preillumination with light preferentially sensitizing Photosystem I or Photosystem II on the DCMU-induced rise in fluorescence yield. Reaction mixture as described under Fig. 1 except for the omission of dithiothreitol and phenazine methosulfate, and the measuring light intensity which was 0.1 erg · cm^{-2} . s^{-1}. Preillumination was as follows: (1) 2 min with far-red light (>715 nm), 10^4 ergs ·

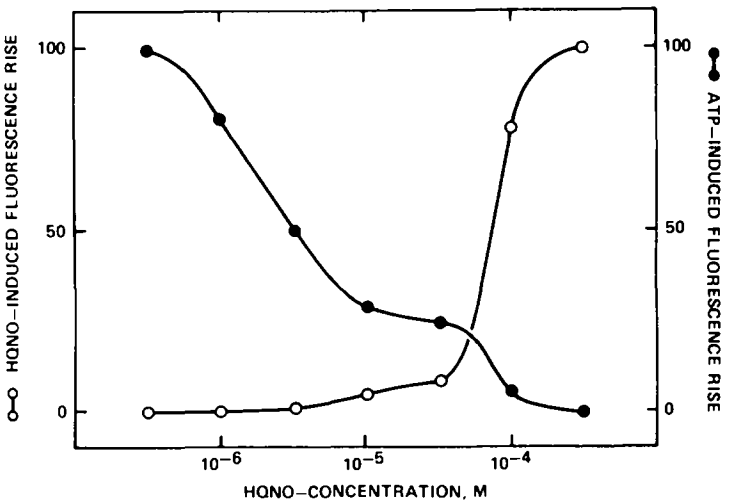

Fig. 12. The effect of increasing concentrations of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) on the HQNO- and ATP-induced rise in fluorescence yield. Conditions as described under Fig. 10, except that HQNO replaced DCMU.

$cm^{-2} \cdot s^{-1}$. (2) 2 min dark. (3) 5 s blue (390—550 nm), $5 \cdot 10^4$ ergs $\cdot$ $cm^{-2} \cdot s^{-1}$, followed by 2 min dark. (4) 10 s blue, followed by 2 min dark. Temperature, 15°C.

yield. However at higher concentration ($>5 \cdot 10^{-5}$ M) it does markedly increase fluorescence yield in a manner resembling DCMU. At the higher concentration range ($>5 \cdot 10^{-5}$ M) it acts also as an uncoupler, as indicated by its ability to dissipate the ATP-induced buildup of ΔpH (Fig. 13).

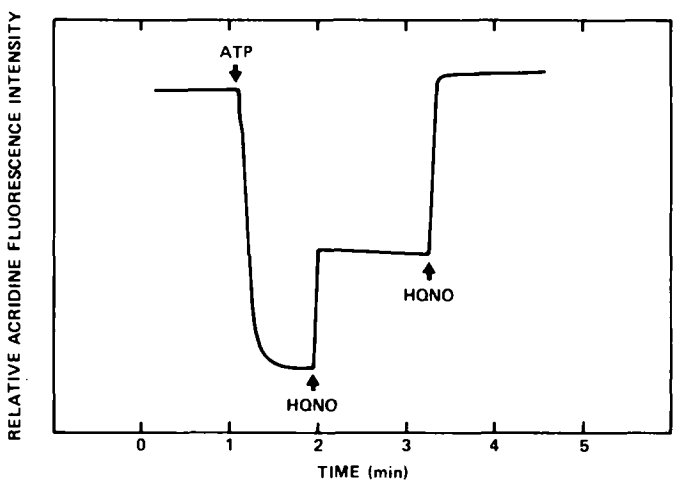

Fig. 13. Uncoupling by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). Conditions as described under Fig. 1, except that only the 9-aminoacridine fluorescence was monitored. HQNO was injected in the first addition indicated to a final concentration of $6 \cdot 10^{-5}$ M and in the second to $2 \cdot 10^{-4}$ M. Further additions were without effect.

Discussion

The data presented illustrate the rather complex nature of the ATP-induced buildup of ΔpH and of the ATP-induced increase in chlorophyll fluorescence yield. The simultaneous recordings of these two phenomena in Figs. 1, 2, 4 and 7, suggest a close kinetic correlation between ΔpH formation and the main portion of Q reduction [3]. Such behaviour is in agreement with the chemiosmotic hypothesis [18], which predicts that ATP-driven reverse electron flow operates through the intermediary creation of a transmembrane pH gradient.

A new aspect is introduced by the finding, illustrated in Fig. 2, of a rapid phase in the chlorophyll fluorescence increase. This rapid phase is not correlated with any detectable rapid step in ΔpH formation (as indicated, the initial step found in acridine fluorescence quenching was analysed to be artifactual). The rapid chlorophyll fluorescence increase can be separated from the main rise by omitting phenazine methosulfate from the reaction mixture, indicating that, contrary to the main phase, no exogenous electron donor is required. Possibly, this could mean that the rapid phase does not involve Q reduction. Whether Q reduction is involved or not, it is important to note that with the rapid phase of the chlorophyll fluorescence rise, a link between the ATPase reaction and reactions at the pigment level is given, which appears not to involve the intermediary formation of a ΔpH. In a following report [24] this conclusion is further strengthened by observations on ATP-stimulated chlorophyll luminescence [4].

Phenomenologically there is some similarity between the effect of an active ATPase and of DCMU on the Photosystem II electron acceptor pool. This similarity is evident in Fig. 3, where light-induced fluorescence kinetics are compared, and in Figs. 8—11, where the ATP and DCMU increases of the initial fluorescence yield are analysed. Clearly the mechanisms by which ATP and DCMU exert their effects are markedly different, as indicated by the insensitivity of the latter towards uncouplers (Fig. 8), as well as its lack of requirement for activation by preillumination. Nevertheless, we interpret the phenomenological similarity as indicating a similar effect of the two treatments on the state of the Q-R redox couple [15,19—22]. Velthuys and Amesz [15] suggested that DCMU causes a lowering of the midpoint potential of the secondary electron acceptor R, thus inducing reduction of Q by R^-. The state $Q \cdot R^-$ was found to decay relatively slowly, with a half-time of about 3.5 min around 16°C [15]. Therefore, under the conditions of our experiments a plentiful supply of $Q \cdot R^-$ is present at the moment of ATP or DCMU injection. However, in the absence of phenazine methosulfate, DCMU induced a full increase in fluorescence yield whereas ATP does not (see e.g. Fig. 3). For the maximum ATP-induced increase to occur an additional condition, related to the phenazine methosulfate action, has to be fulfilled. It appears likely that this reflects a requirement for a reduced plastoquinone pool. Thus, the ATP-induced reverse electron flow seems to involve an electron transfer from a reduced plastoquinone pool, while the DCMU-induced Q reduction seems to involve an electron transfer from R^- to Q. This interpretation suggests the existence of an energy coupling site between R and plastoquinone (see also ref. 23).

The relative insensitivity to low temperature of the ATP-driven reverse elec-

tron flow is surprising. The reaction proceeds well down to 0°C (Fig. 4) which makes it possible to perform long term experiments with minimal inactivation. The activation reaction has an optimum around 15°C (Fig. 6), and the activated state decays in the dark rather rapidly even at 0°C, unless inorganic phosphate is added (Fig. 7). Taking advantage of the temperature- and phosphate-stabilizing effects, it is possible to design long term experiments where the contents of the activation and reaction phases may be varied without losing activity. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide, an inhibitor whose effects on electron flow resemble those of DCMU [17], does not induce the change in the redox properties of the Q-R couple if concentrations exceeding $5 \cdot 10^{-5}$ M are avoided. Nevertheless in the low concentration range it markedly inhibits ATP-induced Q reduction (Fig. 12). Such inhibition occurs in a concentration range where its uncoupling effect is negligible. Thus, at low concentrations, it appears to be a less complex inhibitor than DCMU since it effectively inhibits forward [17] and reverse [2] electron transport, without a detectable effect on the Q-R equilibrium.

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