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AFFINITY OF FERREDOXIN FOR ELECTRONS FROM WATER AND THE REGULATION OF CYCLIC PHOTOPHOSPHORYLATION

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New evidence is presented how, in chloroplasts, an auxiliary electron flow from water, induced by photosystem II, maintains the redox balance (poising) needed for the functioning of ferredoxin-catalyzed cyclic photophosphorylation, a process driven by photosystem I. Optimal poising of cyclic photophosphorylation was attained when ferredoxin was kept in a predominantly reduced state by electrons from water but only when the electron pressure from water was greatly attenuated to prevent overreduction. An unexpected finding was the unusually high affinity of ferredoxin for electrons from water. Ferredoxin was kept in a predominantly reduced state by the severely restricted electron flow from water that was generated by 715nm illumination, or under 554nm illumination in the presence of diuron.

Recent work has established that, although the physiological type of cyclic photophosphorylation in chloroplasts (the aerobic type catalyzed by low concentrations of ferredoxin) is driven by photosystem I, it is regulated by an auxiliary electron flow from water induced by photosystem II. At a greatly reduced electron pressure from water and in the presence of oxygen, a mere trickle of electrons from water maintained the proper redox balance (poising) of electron carriers that was required for the operation of cyclic photophosphorylation at optimal rates (1-9).

What specific chloroplast electron carriers of cyclic photophosphorylation had their redox state regulated by the trickle of electrons from water was unknown. The new evidence presented here indicates that the carrier affected was ferredoxin. Optimal poising of cyclic photophosphorylation required that ferredoxin be maintained in a predominantly reduced steady state and that, simultaneously, overreduction of the chloroplast system be avoided by an attenuation of electron pressure from water. An unexpected finding was the unusually high affinity of ferredoxin for electrons from water, which explained why the few

*Abbreviations: DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (diuron); EPR, electron paramagnetic resonance

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reducing equivalents that could still be liberated from water by chloroplasts under far-red (715nm) illumination (or under 554nm illumination, in the presence of diuron*) were sufficient to maintain ferredoxin in a predominantly reduced state. When the steady state reduction level of ferredoxin was diminished, cyclic photophosphorylation was severely inhibited.

METHODS

The chloroplast preparations used were 'broken' spinach chloroplasts depleted of soluble components but retaining the integrity of thylakoid membrane structure needed for complete electron transport from water to $NADP^+$ and for ferredoxin-catalyzed photophosphorylations (1). Procedures described earlier were used for the isolation and purification of ferredoxin (10), the determination of NADPH (11) and of chlorophyll (12); the ATP formed was measured by the increase in organic phosphate (1,13).

Measurement of photoreduced ferredoxin at different levels of electron pressure from water were made possible by the use of EPR* spectroscopy (see Discussion). The EPR measurements were carried out at 60K, a temperature at which the EPR signals of the membrane-bound iron-sulfur centers broaden and are barely detectable but at which the characteristic EPR spectrum of soluble ferredoxin is readily observed (14). Photoreduced ferredoxin was measured on duplicates of the same preparation of chloroplasts and reaction mixture (except for the ^{32}P marker) that was used for measurements of ferredoxin-catalyzed cyclic photophosphorylation. Samples were placed in quartz EPR tubes (3-mm i.d.) and illuminated by 554 nm or 715nm monochromatic light at room temperature for 30 sec. Illumination was continued without interruption for another 30 sec through the window of a silvered dewar containing liquid nitrogen in which the samples were quickly immersed for freezing. Monochromatic illumination was provided by a quartz-halogen lamp with the light passing through an assembly of heat, neutral density, and appropriate interference filters (Baird-Atomic Co.).

After illumination, first-derivative EPR spectra of the frozen samples were recorded on a Bruker Instruments Co. EPR spectrometer (model ER200tt) equipped with a 20-cm ("8 inch") magnet (14). The samples were further cooled to 60K in the cavity with liquid helium by an Oxford Instruments Co. temperature controller (model DTC) and cryostat (model ESR9) equipped with a quartz dewar cell (J. F. Scanlon Co., Solvang, Calif.).

RESULTS

The extent to which electron pressure from water was restricted to insure optimal poising for ferredoxin-catalyzed cyclic photophosphorylation is shown in Fig. 1. Cyclic photophosphorylation proceeded at optimal rates either under 554nm illumination in combination with 10^{-6} M diuron or under 715nm illumination without diuron. Under these conditions, the light-induced electron pressure from water (proportional to the rate of $NADP^+$ reduction) was not totally abolished but was drastically lower than that in the 554nm, no-diuron control treatment (Fig. 1). Concentrations of diuron that virtually stopped electron flow from water (Fig. 1) were detrimental to cyclic photophosphorylation (see below).

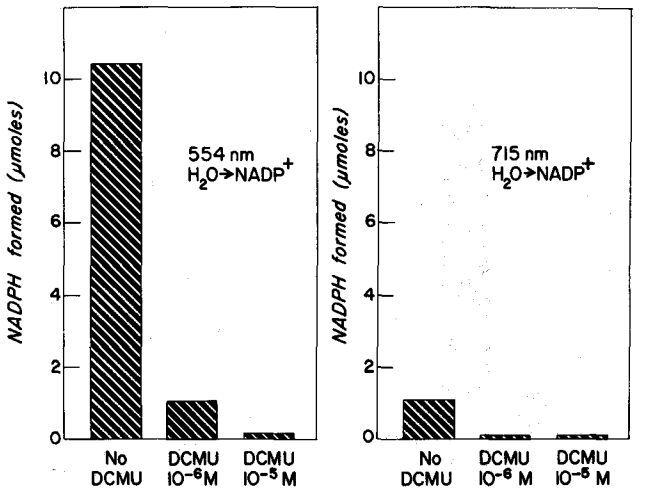

Fig. 1. Influence of monochromatic light and diuron (DCMU) concentration on $NADP^+$ reduction by water. Reaction mixtures, containing spinach chloroplasts (equivalent to 0.3 mg chlorophyll per ml), 0.01 mM spinach ferredoxin, 100 mM Tricine buffer (pH 8.2), 10 mM $MgCl_2$, 5 mM $NADP^+$, 5 mM ADP, 5 mM K_2HPO_4 and DCMU as indicated, were illuminated at room temperature in cuvetts (1-mm light path) for 1 min. Monochromatic illumination: 2 x 10^4 ergs·cm^{-2}·sec^{-1} at 715nm and 2 x 10^4 ergs·cm^{-2}·sec^{-1} at 554nm. The NADPH formed, measured by the absorbance change at 340nm, was calculated for a 30-min illumination period. Gas phase, air.

Experiments with 554nm illumination and the same concentrations of diuron as those in Fig. 1 yielded evidence that a predominantly reduced steady state of ferredoxin, in combination with an attenuated electron pressure from water, are needed for optimal poising of cyclic photophosphorylation. As shown in Fig. 2 by the amplitudes of its characteristic main signal, g_y = 1.96, and those of the related signals, g_x = 1.89 and g_z = 2.05, ferredoxin was fully photoreduced both in the minus-diuron control treatment, where electron pressure from water was not restricted, and at 10^{-6} M diuron, a concentration that inhibited severely electron flow from water (see Fig. 1). By contrast, photophosphorylation proceeded optimally only at 10^{-6} M diuron; it was markedly impaired in the minus-diuron treatment (Fig. 2). At a concentration of diuron (10^{-5} M) that virtually suppressed electron flow from water (Fig. 1), both the steady state reduction level of ferredoxin and the rate of cyclic photophosphorylation were very low (Fig. 2).

The positive correlation between a predominantly reduced steady state of fer redoxin and cyclic photophosphorylation is shown in another way in Fig. 3. Here,

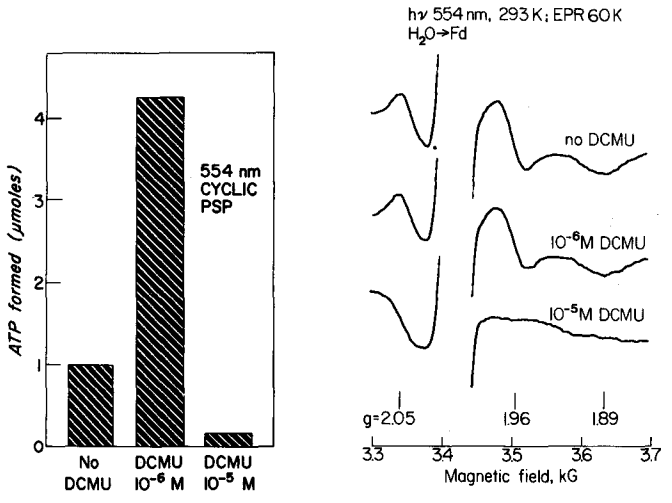

Fig. 2. Cyclic photophosphorylation (left) and EPR signals of ferredoxin (right) photoreduced by water in the presence and absence of diuron. Experimental conditions as in Fig. 1 except that $NADP^+$ was omitted and a ^{32}P marker was added in the photophosphorylation experiment. Gas phase, air (see ref. 1). Photophosphorylation illumination at 554nm was for 30 min at room temperature; for EPR illumination, see Methods. EPR spectra were recorded at 60K; instrument field setting, 3450 ± 200 G; microwave power, 10 mW; modulation amplitude, 10 G.

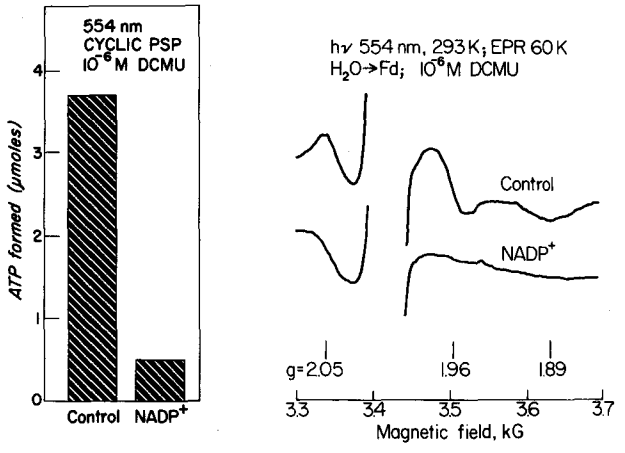

Fig. 3. Effect of $NADP^+$ on cyclic photophosphorylation (left) and EPR signals of ferredoxin (right) photoreduced by water in the presence of diuron. Experimental conditions as in Fig. 2 except that 1 x 10^{-6} M DCMU was present throughout. Where indicated, 5 mM $NADP^+$ was present.

photophosphorylation was impeded when the steady state reduction level of ferredoxin was diminished by the addition of $NADP^+$. In the presence of $NADP^+$, ferredoxin no longer remained in a predominantly reduced state because the rate of electron outflow from reduced ferredoxin to $NADP^+$ (catalyzed by the membrane-

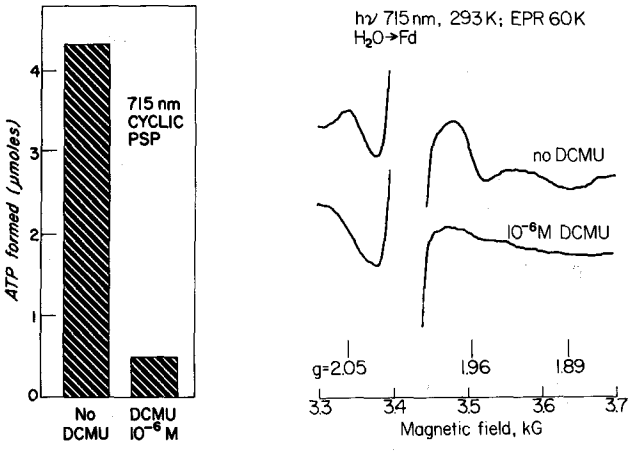

Fig. 4. Ferredoxin-catalyzed cyclic photophosphorylation (left) and EPR signals of ferredoxin (right) photoreduced by water under 715nm illumination in the presence and absence of diuron. Experimental conditions as in Fig. 2 except that monochromatic illumination was at 715nm.

bound ferredoxin-$NADP^+$ reductase) was greater than the low rate of electron inflow from water to ferredoxin that could be sustained by 554nm illumination, in the presence of 10^{-6} M diuron.

Fig. 4 shows that far-red (715nm) illumination that can support only a low rate of electron flow from water (Fig. 1), supported a high level of cyclic photo phosphorylation and also maintained ferredoxin in a predominantly reduced steady state. Ferredoxin was kept in the reduced state by the trickle of electrons from water that 715nm illumination could generate in the absence of diuron. When electron flow from water was further restricted by the addition of diuron, so that ferredoxin could no longer be maintained in a predominantly reduced state, photophosphorylation was severely inhibited (Fig. 4).

Fig. 5 shows that under 715nm illumination the addition of $NADP^+$ had the same effect as it did under 554nm illumination in the presence of 10^{-6} M diuron (Fig. 3). Here again, $NADP^+$ trapped electrons from reduced ferredoxin at a faster rate than they could be supplied by water; ferredoxin could not longer be maintained in a predominantly reduced steady state, and cyclic photophosphorylation was severely inhibited.

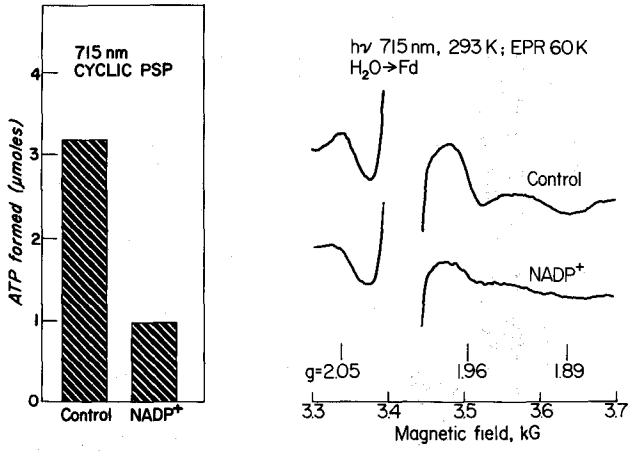

Fig. 5. Effect of $NADP^+$ on cyclic photophosphorylation (left) and EPR signals of ferredoxin (right) photoreduced by water under 715nm illumination. Experimental conditions as in Fig. 4. Where indicated, 5 mM $NADP^+$ was present.

For reasons discussed elsewhere (1,2), all of the photophosphorylation experiments were performed with high concentrations of chlorophyll and with light well below saturation, i.e., under conditions not suitable for the usual calculations of maximum activity rates. However, the chloroplast preparations used had a high capacity for photophosphorylation and electron transport, as evidenced by the high Q values for the ATP and NADPH formed (in μmol/mg of chlorophyll per hr) that were obtained in earlier experiments with low concentrations of the same chloroplast preparations and saturating light.

DISCUSSION

The importance of poising for the optimal operation of ferredoxin-catalyzed cyclic photophosphorylation became apparent (15,16) soon after ferredoxin was recognized as the physiological catalyst of that process (17,18). The general importance of poising for cyclic photophosphorylation in both chloroplasts and bacterial chromatophores was reviewed and stressed by Avron and Neumann (19). Until recently, however, little was known about specific poising mechanisms and the optimal redox state of individual electron carriers native to chloroplasts.

The present evidence supports the view that optimal poising of cyclic photophosphorylation in chloroplasts depends on maintaining ferredoxin in a predominately reduced state by electrons from water and simultaneously sharply attenuat-

ing electron pressure from water to protect the chloroplast system from overreduction. These conditions are not mutually exclusive because ferredoxin was found to have an unusually high affinity for electrons from water: a mere trickle of electrons generated by photosystem II under far-red illumination and superimposed on the main concurrent cyclic electron flow, driven by photosystem I, was sufficient to keep ferredoxin fully reduced without any apparent unfavorable overreduction of other carriers.

The observation that a predominantly reduced state of ferredoxin favors cyclic photophosphorylation is consistent with recent findings (2,5,8) that ferredoxin reduced by NADPH will, in turn, close reaction centers of photosystem II by reducing in the dark its primary electron acceptor, C-550 (2). As a consequence, the light-induced electron flow from water is restricted to a level optimal for cyclic photophosphorylation. This 'reverse' dark electron flow provides chloroplasts with a mechanism for poising cyclic photophosphorylation when NADPH accumulates, i.e., under conditions when chloroplasts do not use reducing power and need only cyclic photophosphorylation to produce ATP (1,2).

More frequently, the biosynthetic reactions of chloroplasts consume both the ATP and NADPH that are generated by the concurrent operation of cyclic and noncyclic photophosphorylation (1,2). Under such conditions, $NADP^+$ reduction and turnover drain off electrons from water and provide automatically the attenuation of electron pressure from water and poising that favor the concurrent operation of cyclic photophosphorylation (1,2). This normal poising effect of $NADP^+$ is in marked contrast to the inhibitory effect of $NADP^+$ on cyclic photophosphorylation demonstrated here experimentally when electron pressure from water was already greatly restricted either by diuron or by far-red illumination.

The specificity and sensitivity of EPR spectroscopy for the detection of reduced ferredoxin deserve special mention. So far as we are aware, no other method would have given equally reliable measurements of the steady state of photoreduced ferredoxin under the conditions of our experiments, i.e., when 10 μM ferredoxin was being photoreduced by chloroplast membranes (300 μM chlorophyll) under aerobic conditions.

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REFERENCES

1. Arnon, D.I. & Chain, R.K. (1975) Proc. Nat. Acad. Sci. USA 72, 4961-4965
2. Arnon, D. I.& Chain, R. K (1977) Plant & Cell Physiol. Spec. Is. 3, 129-147
3. Chain, R. K. & Arnon, D. I. (1977) Proc. Nat. Acad. Sci. USA 74, 3377-3381
4. Arnon, D.I. & Chain, R. I. (1977) FEBS Lett. 82, 297-302
5. Arnon, D. I. & Chain, R. K. (1979) FEBS Lett. 102, 133-138
6. Kaiser, W. & Urbach, W. (1976) Biochim. Biophys. Acta 423, 91-102
7. Hind, G., Mills, J. D. & Slovacek, R. E. (1977) In: Photosynthesis 77: Proc. 4th Int. Cong. Photosynthesis (Hall, D. O., et al., eds.), pp. 591-600, The Biochemical Society, London
8. Mills, J. D., Crowther, D., Slovacek, R. E., Hind, G. & McCarty, R. E. (1979) Biochim. Biophys. Acta 547, 127-137
9. Heber, U., Egneus, H., Hanck, U., Jensen, M. & Köster, S. (1978) Planta 143, 41-49
10. Losada, M. & Arnon, D.I. (1967) In: Modern Methods of Plant Analysis (Linskens, et al., eds.), v. 7, pp. 569-615, Springer-Verlag, Berlin
11. McSwain, B. D. & Arnon, D. I. (1968) Proc. Nat. Acad. Sci. USA 61, 989-996
12. Arnon, D. I.(1949) Plant Physiol. 24, 1-15
13. Hagihara, B. & Lardy, H. A. (1960) J. Biol. Chem. 235, 889-894
14. Arnon, D.I., Tsujimoto, H. Y. & Hiyama, T. (1977) Proc. Nat. Acad. Sci. USA 74, 3826-3830
15. Tagawa, K., Tsujimoto, H.Y. & Arnon, D.I. (1963) Nature 199, 1247-1252
16. Grant, B. R. & Whatley, F. R. (1967) In: Biochemistry of Chloroplasts (Goodwin, T. W., ed.), v. 2, pp. 505-521, Academic Press, NY
17. Tagawa, K., Tsujimoto, H. Y. & Arnon, D. I. (1963) Proc. Nat. Acad. Sci. USA 49, 567-572
18. Tagawa, K., Tsujimoto, H. Y. & Arnon, D .I. (1963) Proc. Nat. Acad. Sci. USA 50, 544-549
19. Avron, M. & Neumann, J. (1968) Annu. Rev. Plant Physiol. 19, 137-166