ON THE MECHANISM OF CONTROL OF PHOTOSYNTHETIC ELECTRON TRANSPORT BY PHOSPHORYLATION

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1. Introduction

It is well established that photosynthetic electron transport can be coupled to energy-requiring reactions such as the phosphorylation of ADP such that the coupling mechanism controls the rate of electron flux. The explanations of this lowering of the electron transport rate which are most commonly encountered deal with the well-documented acidification of the thylakoid's inner aqueous phase due to electron transport-dependent hydrogen ion accumulations. Thus Rumberg and Siggel [1] suggested that the rate of electron flow is controlled by the effect of internal acidification on the rate of plastohydroquinone oxidation. Bamberger et al. [2] suggested a somewhat more complex dependence of electron transport on the size of the transmembrane pH gradient and the pH of the membrane itself, both of which are combined functions of the internal and external pH values. Bamberger et al. also feel that control is exerted close to photosystem II. A more complete listing of works dealing with the question of electron transport 'control' is provided in a recent review by Trebst [3]. One feature which is common to all previous models of photosynthetic electron transport control is the role of the proton activity in the thylakoid's inner aqueous phase.

In this communication we present data which suggest that the pH of the inner aqueous phase has nothing whatever to do with the *primary* control of electron transport. Recently in this laboratory we have been investigating the characteristics of photophosphorylation during the formation of the 'high energy state' responsible for photophosphorylation [4,5]. During the course of the earlier investigations we noted that electron transport is initially very rapid but that it slows at about the same time that the capacity of the membranes to phosphorylation ADP develops. We concluded from these earlier studies that photophosphorylation can occur in the absence of any significant differences between the pH values of the inner and outer aqueous phases of the thylakoid vesicles and in the absence of any appreciable transmembrane electrical potential. In the study reported here, we have looked more closely at the electron transport resulting from very brief illumination times (> 2 ms) in the hope of defining more clearly the primary events resulting in electron transport control.

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2. Methods

Most of the procedures used in this study are described in detail in the two previous publications [4,5]. The spectrofluorometric assay for hydrogen peroxide is described in the legend of fig.1.

The accurate determination of the very small amounts of photochemically reduced acceptor and of ATP resulting from millisecond flashes of light is not a trivial problem. The required accuracy was attained by summing the yields from 40 sequential flashes, each separated from the next by a 15 s dark period to allow relaxation of the light-induced pH gradient and of any other membrane energization. The validity of this procedure was established in the earlier work by showing that the yield of ATP and the yield of reduced acceptor per flash was not altered by increasing the

dark time several-fold and, in a few instances, by using single flashes [5].

3. Results and discussion

In carefully prepared chloroplasts, the capacity for phosphorylation develops abruptly as soon as the illumination time exceeds about 4 ms, if the illumination is sufficiently intense (fig.1). Electron transport

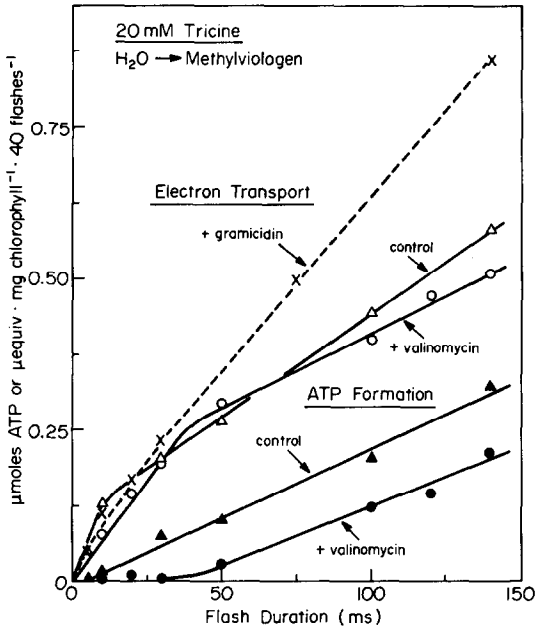

Fig.1. The relationship between electron transport and ATP formation during brief illumination periods in the presence of a nonpermeant buffer. When they were used the concentration of valinomycin was 0.2 µM and the concentration of gramicidin was 2 µM. The stirred 2 ml reaction mixture contained 0.1 M sucrose, 20 mM Tricine–KOH (8.0), 15 mM KCl, 0.75 mM $Na_2H^{32}PO_4$ (approx. 0.03 mCi), 50 µM methylviologen, 1 mM ADP, 5 mM NaN_3 and chloroplasts containing 80 µg chlorophyll. The reaction temperature was 16°C and the light intensity was 530 Kergs/cm^2/s of heat filter white light. The amount of H_2O_2 formed due to the autooxidation of reduced methylviologen was determined from the fluorescence of dichlorofluorescin which is formed by the oxidation of dichlorofluorescin by H_2O_2 and catalyzed by horseradish peroxidase. 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (0.1 ml of 10 mM) was injected into each sample immediately after the last flash. Chloroplasts were then removed by forcing the reaction medium through a Millipore filter (0.22 µM pore size) and a 0.1 ml sample of the medium was assayed for H_2O_2 concentration. The spectrofluorometric assay for H_2O_2 is described by Black and Brandt [14].

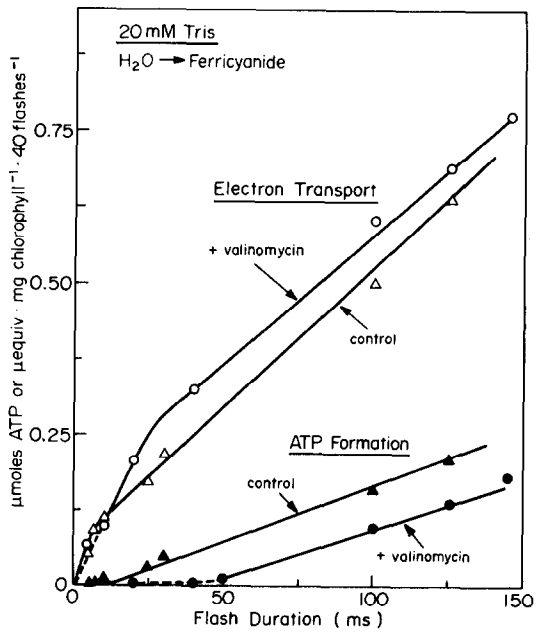

Fig.2. The relationship between electron transport and ATP formation during brief illumination periods in the presence of a permeant buffer. The 2 ml reaction mixture was composed of: 20 mM tris (hydromethyl) aminomethane–HCl (pH 8.0), 0.1 M sucrose, 15 mM KCl, 2 mM $MgCl_2$, 1 mM ADP, 1 mM $Na_2H^{32}PO_4$, 0.1 mM potassium ferricyanide. All other conditions are as in fig.1. The amount of ferrocyanide formed due to the photochemical reduction of ferricyanide was determined by reacting ferrocyanide ions with bathophenanthroline sulfonate as described in ref. [5].

is very rapid while ATP formation is in abeyance and it remains rapid as long as ATP formation is held in abeyance by an uncoupler such as gramicidin. These data suggest that phosphorylation capacity and a reduced rate of electron flux are interdependent, a notion which fits nearly everyone's model of control (e.g. [1,2]). However because we are able to make measurements during the formation of the high energy state, we can better define the interdependency and better relate it to the simultaneous pH changes. Thus, tris (hydroxymethyl)-aminomethane (p*K*a 8.3) rapidly equilibrates across the thylakoid membrane, establishing in the dark nearly equal concentration of the amine in the inner and outer aqueous phases (table 1; see also fig.2 of ref. [5]). The presence of tris(hydroxymethyl)aminomethane (Tris) buffer must delay the acidification of the inside of the thylakoid

Table 1
Measurement of Tris equilibration across the thylakoid membrane in the dark

Determination	Internal water volume	Tris inside thylakoid	conc. Tris inside thylakoid	Minimum illumination time required to overcome Tris buffering ($\Delta pH < 1$)
	($\mu l\ mg{\cdot}chl^{-1}$)	(nmoles·mg chl^{-1})	(mM)	(ms)
1	27	630	23	340
2	32	680	21	400
3	30	660	22	380

Internal volume was measured by centrifugation through silicon fluids as described by Gaensslen and McCarty [13] and in Methods of ref. [4]. The 0.1 ml samples, which were layered on top of the silicon fluid, consisted of: chloroplasts containing 100–160 μg chlorophyll, 100 mM sucrose, 0.75 mM P_i, 1 mM ADP, 2 mM $MgCl_2$, 15 mM KCl and 20 mM Tris (pH 8.0, 1.0 μCi). The chloroplast suspensions were allowed to equilibrate for 60 min at 4°C in the dark. The internal volume was calculated from the uptake of ^3H_2O (1.0 μCi/0.1 ml). The amount of ^3H_2O 'trapped' by the chloroplast membranes and not part of the internal volume was assumed to be the same as the volume occupied by the 'trapped' [^{14}C]inulin (0.5 μCi/ml). The uptake of Tris was measured by the same procedure employing [^{14}C]hydroxymethyl labeled Tris (see ref. [5] for details). The lower aqueous phase (0.1 ml) contained 10% sucrose and 1% Triton X-100. The silicone fluid used was a mixture of Versilube F-50 and SF-96 (50) in a 4.5 to 0.50 ratio (w/w). After centrifugation the tubes were frozen in dry ice and then sliced to separate the upper and lower aqueous phases. The lower sample was bleached for 18 to 24 h at 45° in 1 ml of 30% H_2O_2 and 5% sodium lauryl sulfate. Its radioactivity was then determined by liquid scintillation counting using external standard ratios to monitor quenching.

substantially yet it causes no commensurate delay in either the onset of phosphorylation or the development of electron transport control.

The magnitude of the discrepancy between the time of acidification and the time of development of the phosphorylation-related control is very great. Assuming that the concentration of unprotonated Tris inside the thylakoid is the same as in the suspending medium (see table 1), approximately 210 nmol of hydrogen ions·mg $chlorophyll^{-1}$ would have to be accumulated before there could be much change in the internal pH. Accepting an H^+/e^- ratio of 2.0 [6,7], and computing from the initial rapid rates of electron flux shown in figs.1 and 2 (about 1000 μequiv·mg $chlorophyll^{-1}\cdot h^{-1}$) a minimum of 380 ms of illumination would be required to overcome the buffering. Yet despite the fact that the pH of the internal space cannot drop much for more than 380 ms, the lowering of the rate of electron flux and the onset of phosphorylation occur as soon as the illumination time exceeds about 10 ms.

Clearly, acidification of the bulk of the inner aqueous phase of the thylakoid vesicle is not involved at all in the phosphorylation-related control of electron transport. On the other hand, transmembrane or intra-membrane electrical potential differences seem to influence the length of the delay in the onset of phosphorylation and the phosphorylation associated control of electron transport. It is apparent from figs.1 and 2 that valinomycin plus KCl delays the onset of ATP synthesis for about 50 ms and that the initial rapid rate of electron flux persists for about the same time. This is true whether or not a permeant buffer is present (cf. fig.1 and fig.2). The nature of this effect of valinomycin and potassium ions (which is also caused by other permeant ions such as SCN^-)

is obscure since 50 ms is far too short of an illumination time for a redistribution of ions to reestablish any transmembrane potential which may have been destroyed by the permeant ions (see ref. [4]) and, in any event, there is ample evidence that a steady state phosphorylation normally occurs with very little such potential.

The transport of electrons from water to lipophilic strong oxidants, which involves only photosystem II, seems not to be subject to control by the phosphorylation system even though ATP is formed [8–10]. That is to say, the rate of electron transport is independent of the presence of uncouplers and of ADP plus P_i [8,10]. It seemed to us that a comparison of the early electron transport and phosphorylation events in systems subject and not subject to control might be instructive. Therefore we examined the reduction of 2,5-dimethyl-*p*-benzoquinone as a function of the illumination time. To be certain that the electron transport had no photosystem I component, the inhibitor of plastohydroquinone oxidation, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone [11] was added. Since the reaction was carried out in the presence of ferricyanide, which rapidly reoxidized reduced dimethylquinone, it was possible to follow the electron transport by measuring the amount of ferrocyanide formed as in fig.2. Again electron transport was initially rapid and then slowed (fig.3). However the cause of the ultimate decrease in the rate of reduction is not the same as when ferricyanide or methylviologen are being reduced. Although valinomycin and KCl delay the onset of phosphorylation in the photosystem II reaction just as they do in the overall transport through both photosystems, they have no effect on the duration of the rapid phase of reduction of dimethylquinone which is in any event much more prolonged in the photosystem II reaction than in the overall system. Apparently in the absence of phosphorylation control the initial high rate of transport persists until some process unrelated to phosphorylation becomes limiting – perhaps the availability of the exogenous oxidant.

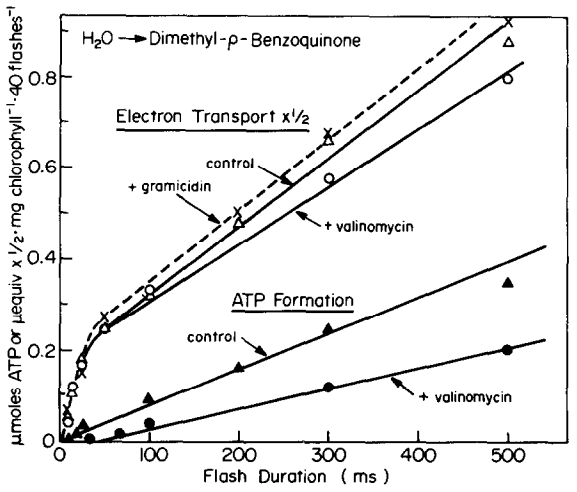

Fig.3. The relationship between Photosystem II-dependent electron transport and ATP formation during short illumination periods. Chloroplasts containing 86 µg of chlorophyll were illuminated for the indicated amount of time in a continuously stirred reaction mixture (2 ml) containing 50 mM sucrose, 20 mM KCl, 10 mM Tricine–KOH (pH 7.8), 2 mM $MgCl_2$, 1 mM ADP, 0.75 mM $Na_2H^{32}PO_4$ 1 µM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, 0.1 mM potassium ferricyanide, 0.2 mM 2,5-dimethyl-*p*-benzoquinone and when added, 0.2 µM valinomycin. All other conditions and procedures are as in fig.2.

4. Conclusions

The control of electron transport associated with the phosphorylation mechanism cannot involve changes in the pH of the bulk of the inner aqueous phase of the thylakoids since electron transport slows at the same time whether or not the internal pH has been stabilized with buffer and long before there could be much change of the internal pH. Rather, the control of electron transport seems to be a response to some other aspect of the high energy state. Thus, the lowering of the initially high electron transport rate coincides with the development of the capacity of the membranes to make ATP even when this is delayed by valinomycin and potassium ions. The nature of the high energy state responsible for the control of the electron transport reaction and, indeed, the precise identity of the controlled reaction remain to be discovered. The data of fig.3 and much previously published information (e.g. [3,10]) suggest that the controlled reaction is *not* directly associated with photosystem II. We have no reason to doubt that the phosphorylation-controlled reaction is the photosystem I rate-limitation postulated by Kok et al. [12]

and Avron and Chance [15]. We think it probable that the influence on electron transport rate by pH reported by Rumberg and Siggel [1], Bamberger et al. [2] and others [3] is secondary and not acting directly on the phosphorylation-controlled reaction.

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