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LACK OF SITE-SPECIFICITY OF SPINACH CHLOROPLAST COUPLING FACTOR 1

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SUMMARY

The irreversible inhibition of chloroplast phosphorylation by either sulfate anions, or *N*-ethylmaleimide, is energy dependent. Chloroplasts must first be illuminated in the presence of the inhibitors and a mediator of electron flow, for the subsequent phosphorylation to show any inhibition. Both inhibitors affect the chloroplast coupling factor 1.

Electron transport only through Photosystem I can be used to activate either of these inhibitions. The subsequent inhibition in a second light reaction is the same whether ATP synthesis is supported by Photosystem I, or by Photosystem II electron transport. The reverse experiment, activating inhibition by electron transport only through Photosystem II, is possible in the case of sulfate. Again, the inhibition is expressed whether Photosystem II or Photosystem I electron flow supports ATP synthesis. We conclude that the two electron transport regions probably generate the same high energy state which is able to activate all members of a functionally uniform coupling factor population. These enzyme molecules must catalyze phosphorylation coupled to electron transport through either region of the chain. The results tend to discredit models requiring a separate group of coupling factor molecules unique to each part of the chain.

INTRODUCTION

The electron transport chain in chloroplasts has been divided experimentally into two portions, each of which is capable of supporting ATP synthesis. Electron transport can be restricted to the Photosystem II region by using hydrophobic compounds as electron acceptors, and inhibitors such as dibromothymoquinone (DBMIB) [1] or KCN [2, 3] to prevent electrons from moving through to Photosystem I.

Abbreviations: CF_1, chloroplast coupling factor 1; DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; tricine, tris(hydroxymethylmethyl)glycine.

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Formation of ATP can be driven by this restricted pathway [2, 4, 5] as well as by the more familiar electron flow patterns restricted to the Photosystem I region [6, 7]. It has been suggested that the phosphorylation sites are those regions of electron transport coupled to proton translocation across the thylakoid membrane [8]. In chloroplasts the two native coupling sites are those of the water-splitting reaction, and of the plastoquinone membrane shuttle [9]. When phosphorylation depends on electron transport through Photosystem I in the presence of DBMIB which prevents electrons from leaving plastoquinone, it is proposed that an artificial coupling site has been created in the sense of proton movement into the thylakoid associated with the reduced form of the electron donor such as dichloroindophenol or phenazine methosulfate [9]. Alternatively, Gould and Izawa [10] suggested that electron transport from reduced dichlorophenolindophenol to methyl viologen through Photosystem I includes one of the two native coupling sites. These workers designated the phosphorylation "site" associated with Photosystem I, Site I, and that associated with Photosystem II, Site II [9]. For the purposes of the work to be described here, this terminology will be followed.

An unresolved and important question is the proper definition of the term "site". It could mean simply that portion of the electron transport chain generating a proton electrochemical activity gradient or other high energy intermediate. In this model, consistent with Mitchell's chemiosmotic concept for oxidative and photosynthetic phosphorylation [8], the high energy intermediate would be identical for the two regions of the electron transport chain. No difference in the specific coupling factor molecules activated by either site would be predicted, as long as they were all on the same thylakoid vesicle.

Alternatively, "site" could imply a population of high energy intermediates and of coupling factor molecules specific to each part of the electron transport chain. In this case, one might expect that electron transport in one region of the chain would activate only its particular coupling factor, and not those associated with other regions in electron transport.

Some differences with respect to phosphorylation parameters between Site I and Site II have been reported with spinach chloroplasts but many of these are still controversial. The P/e_2 ratio was reported as 0.3–0.4 at Site II and 0.5–0.6 at Site I (4, 9) but also as 0.8–0.9 at both sites as long as integrity of the chloroplasts and concentrations of DBMIB and of electron acceptors were controlled carefully [11]. At Site II, "photosynthetic control" of electron transport has been reported to be missing [12] and present at low levels [11]. The P/e_2 and H^+/ATP ratios at Site II, but not those at Site I, were reported to be independent of pH in the range from 6.5 to 9.0 [9, 13] or to show a pH optimum at 7.5 [14]. It should be noted that most of these differences in results are associated with the use of somewhat different techniques for isolating chloroplasts.

A more critical difference might be in sensitivity to the energy transfer inhibitor $HgCl_2$. Site I phosphorylation is inhibited by about 1/50 th of the concentration required for Site II. A set of functional hydrophobic proteins specific to each phosphorylation site, one of them more exposed to $HgCl_2$ attack than the other, was postulated to explain these results [15, 16]. Exposure of the coupling factor 1 (CF_1) at each site was considered similar, in view of equal sensitivity of both to inhibition by antibodies against CF_1 [16]. However, the particular CF_1 molecules at each site

would be distinguishable by their topographical and functional association with the underlying elements specific to the particular electron transport enzymes.

In the present work, the light-dependent inhibitions of photophosphorylation by sulfate [17] and by *N*-ethylmaleimide [18] were used to test the idea that there are separate populations of CF_1 catalyzing ATP synthesis at Sites I and II. With both of these, inhibition requires the high energy state of the chloroplasts, generated by light and electron flow. *N*-Ethylmaleimide binds covalently to a functional group of CF_1 exposed during a conformational change brought about by the high energy state [19]. Sulfate inhibition is localized to CF_1 or the CF_1-membrane-interaction point, although the mechanism of inhibition is not fully understood [20]. The rationale was to see if the high energy state, generated by electron flow through one site, could bring about inhibitions at the other site as measured by subsequent ATP synthesis. Reciprocal inhibitions would occur if there were only one general population of CF_1 serving both sites, activated by a single high energy state generated by electron transport at either site. The reciprocal inhibition would not be expected if all CF_1 molecules are associated specifically with one site or the other; in this case activation and inhibition of CF_1 ought to be localized entirely to the site used in the first illumination.

METHODS

Type *c* chloroplasts [21] were prepared from market spinach by grinding leaves in a Waring Blendor for 5 s in 0.4 M sorbitol, 0.05 M tricine/NaOH (pH 8.0), and 0.01 M NaCl. The homogenate was strained, chloroplasts sedimented by centrifuging at 3500 rev./min in a Sorvall SS-34 rotor, resuspended in the same medium and washed once. Chlorophyll was determined by the method of Arnon [22].

Chloroplasts equivalent to 100 μg of chlorophyll were preilluminated with $2 \cdot 10^6$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} of white light for 1 min at 23 °C in a 2 ml mixture which contained: 50 mM tricine/NaOH (pH 8.0), 50 mM NaCl, 25 μM pyocyanine, 1.5 mM $MgCl_2$, 0.5 mM ADP, and 10 mM K_2SO_4. ADP was omitted from the controls. After illumination, 8 ml of the sorbitol/tricine/NaCl medium was added to each 2 ml sample, and the chloroplasts collected by centrifuging at 5000 rev./min for 10 min in a Sorvall SE-12 rotor. Each pellet was resuspended in 0.55 ml of the sorbitol/tricine/NaCl medium. Duplicate phosphorylation measurements were made using 0.2 ml of the suspension for each. Separate chlorophyll determinations were made on each resuspended sample.

1-ml assay mixtures for phosphorylation contained: 50 mM tricine/NaOH (pH 8.0), 50 mM NaCl, 25 μM pyocyanine, 2.5 mM $MgCl_2$, 1 mM ADP, 2.5 mM K_2HPO_4, $2 \cdot 10^5$ cpm $^{32}PO_4$, and chloroplasts containing 20–25 μg chlorophyll. Illumination was for 2 min with $2 \cdot 10^6$ ergs $\cdot$ cm$^{-2} \cdot$ sec^{-1} of white light. Electron transport through Photosystem I only was generated by using pyocyanine, or with *N*-methyl phenazonium methosulfate, 30 μM; or with sodium ascorbate 2.5 mM and dichloroindophenol 0.4 mM (donor system) plus methyl viologen, 50 μM (reoxidized by O_2 as the acceptor system). In these cases, DCMU was present at 5 μM to prevent Photosystem II operation. For exclusively Photosystem II electron flow, either phenylenediamine, 0.5 mM or the oxidized form of diaminodurene, 0.5 mM, was used together with potassium ferricyanide at 2.5 mM as net electron acceptor and DBMIB at 1 μM to block electrons from continuing on to Photosystem I.

Incorporation of $^{32}PO_4$ into ATP was measured by the method of Avron [23]. Radioactivity was counted in a Nuclear-Chicago gas flow counter.

RESULTS

Sulfate inhibition

Electron transport cofactors and appropriate inhibitors were added to the preillumination medium so that electron transport would go through only Site I or only Site II, but not both. Electron transport during the subsequent phosphorylation assay was also through only one or the other site. Several different cofactors were used, since the possible effects of various components added to the preillumination steps on the phosphorylation assays were not known. Although there was a wash step between the preillumination and the assay, not all of the reagents can be washed out easily.

The results of an experiment in which electron transport through Site I was used to generate the inhibition are shown in Table I, and those in which electron transport through Site II was used are shown in Table II. Samples to which no cofactors were added for the phosphorylation assay were included in each set to estimate the extent to which residual cofactors from the preillumination might have contributed to ATP synthesis.

TABLE I

INHIBITION BY SULFATE, ACTIVATED BY PHOTOSYSTEM I ELECTRON TRANSPORT

Chloroplasts were preilluminated with sulfate and one of the two redox systems indicated, as described in Methods. Controls were treated identically, except that ADP was omitted from the preillumination. After dilution and washing, chloroplasts were assayed with the variety of redox systems shown; the top two lines in each experiment show Site II phosphorylations, the next three lines Site I. Rates are given in $\mu mol \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$. Abbreviations for redox components are as follows: PD_{ox} for the oxidized form of phenylenediamine; DAD_{ox} for the oxidized form of diaminodurene, DAD for its reduced form; $DCIPH_2$ for the reduced form of dichlorophenol indophenol dye; MV for methyl viologen, and PMS for *N*-methylphenazonium methosulfate. Reactions in which H_2O is the electron donor require Photosystem II and activate Site II; all others require only Photosystem I and activate Site I.

Expt.	Electron flow pathway				Phosphorylation rate		Inhibition (%)
	Pre-illumination		Phosphorylation		Control	Inhibited	
	Donor	Acceptor	Donor	Acceptor			
1.	PMS cycle		H_2O	PD_{ox}	163	80	51
			H_2O	DAD_{ox}	155	50	68
			DADH	MV	675	473	30
			$DCIPH_2$	MV	61	13	78
			PMS cycle		554	323	42
			no addition		33	8	76
2.	$DCIPH_2$	MV	H_2O	PD_{ox}	73	21	71
			H_2O	DAD_{ox}	70	55	21
			DAD	MV	821	569	31
			$DCIPH_2$	MV	100	50	50
			PMS cycle		995	686	31
			no addition		64	30	54

TABLE II

INHIBITION BY SULFATE, ACTIVATED BY PHOTOSYSTEM II ELECTRON TRANSPORT

All methods and abbreviations as noted in Table I.

Expt.	Electron flow pathway				Phosphorylation rate		Inhibition (%)
	Pre-illumination		Phosphorylation		Control	Inhibited	
	Donor	Acceptor	Donor	Acceptor			
1.	H_2O	PD_{ox}	H_2O	PD_{ox}	35	3	90
			H_2O	DAD_{ox}	138	37	73
			DAD	MV	408	300	27
			$DCIPH_2$	MV	30	12	59
			PMS cycle		272	205	25
			no addition		10	4	60
2.	H_2O	DAD_{ox}	H_2O	PD_{ox}	59	27	53
			H_2O	DAD_{ox}	99	63	36
			DAD	MV	687	615	10
			$DCIPH_2$	MV	56	33	41
			PMS cycle		299	261	13
			no addition		29	16	44

TABLE III

COMPARATIVE INHIBITION IN INDEPENDENT EXPERIMENTS

The methods and abbreviations are the same as those noted in Table I. Each line across shows results from a separate experiment. The inhibitions noted on line 4 are taken from Experiment 2 of Table I; those on line 7 are from Experiment 1 of Table II; and those on line 10 are from Experiment 2 of Table II.

Expt.	Preillumination		Inhibition of photophosphorylation (%)					
	Donor	Acceptor	Donor	Acceptor	Donor	Acceptor	Donor	Acceptor
			H_2O	PD_{ox}	H_2O	DAD_{ox}	$DCIPH_2$	MV
1	$DCIPH_2$	MV	52		51		53	
2			39		54		40	
3			22		21		17	
4			71		21		50	
5	H_2O	PD_{ox}	85		79		78	
6			78		65		80	
7			90		73		59	
8	H_2O	DAD_{ox}	61		64		64	
9			58		51		48	
10			36		36		41	

TABLE IV

DEPENDENCE OF SO_4 INHIBITION EXTENT ON LIGHT INTENSITY DURING PHOSPHORYLATION

Conditions as in Table I, with pyocyanine in both the preillumination and the phosphorylation assay. Light intensities were varied by means of calibrated wire screens.

Light intensity ($ergs \cdot cm^{-2} \cdot s^{-1} \cdot 10^5$)	Phosphorylation rate		
	Control	Inhibited	Inhibition (%)
1.2	275	67	76
2.6	447	138	69
5.2	724	270	63
8.8	880	406	54
20.0	942	497	47

No matter which electron transport site was used during the preillumination, inhibition of both Site I and Site II photophosphorylation was apparent. While the extent of inhibition varied with the electron transport system, in each case there is at least one phosphorylation assay using the opposite electron transport site where inhibition is highly significant. A better idea of the reproducibility of these results may be found by comparing the inhibition observed in various experiments when using the same electron transport couples in preillumination and in photophosphorylation. Results from 7 different experiments in addition to those in Tables I and II, in all of which the dichloroindophenol to methyl viologen pair was used for Photosystem I and H_2O to either diaminodurene or phenylenediamine for Photosystem II, are shown in Table III. These results are taken from independent experiments, each using a different chloroplast preparation. It is apparent that in any one experiment, the inhibition of the heterologous is very little different from that of the homologous electron transport system phosphorylation.

The results shown in Table III were taken from experiments in which the rates of electron transport and photophosphorylation were similar (and low) in the Site I and Site II regions. A large proportion of the apparent variation in inhibition seen in Tables I and II arises from the fact that the electron transport and phosphorylation rates vary with the electron transport mediators in use. However, the expression of uncoupling by sulfate is more severe at lower rates of phosphorylation [20]. This is illustrated in Table IV, in which the degree of inhibition is seen to decrease as the light intensity becomes stronger. In general, the intrinsically slower Photosystem II-dependent phosphorylations were inhibited more severely than were the faster Photosystem I-dependent phosphorylations, no matter which electron transport region was used for activation.

Inhibition by N-ethylmaleimide

Similar experiments were attempted using the inhibition of photophosphorylation by *N*-ethylmaleimide [18]. No inhibition could be generated by non-cyclic electron transport, whether through Site I or Site II alone. These systems, which do not support high rates of phosphorylation, probably do not generate a sufficiently high protonmotive force differential for the inhibition by *N*-ethylmaleimide to develop [24].

TABLE V

N-ETHYLMALEIMIDE INHIBITION AT PHOTOPHOSPHORYLATION SITES I AND II

Pre-illumination with *N*-ethylmaleimide and pyocyanin was performed as described in Materials and Methods. Notation for the electron transport cofactors as in Table I. Rate are $\mu mol \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$.

Electron transport system in phosphorylation assay		Phosphorylation rate		
Donor	Acceptor	Control	Inhibited	Inhibition (%)
H_2O	DAD_{ox}	89	53	41
$DCIPH_2$	MV	33	16	50
No addition		13	9.3	28

Cyclic electron transport through Site I supported by *N*-methylphenazonium methosulfate, however, could be used for this purpose.

Table IV shows results of an experiment in which *N*-methylphenazonium methosulfate was present in the preillumination, and phosphorylation was measured at Sites I and II separately, using non-cyclic electron transport which supported comparable phosphorylation rates. As with sulfate, inhibition was seen at both sites. Essentially the same results were obtained in four other independent experiments.

DISCUSSION

For evaluation of the results, it is important to know whether electron transport during the preillumination period was entirely restricted to one site. A restricted pathway seems certain when the lipophilic acceptors phenylenediamine or diaminodurene were used in the presence of DBMIB [4, 5] and no electron donors to Photosystem I were provided. With the Site I reactions, DBMIB, which was added to block electron flow from water at the level of plastoquinone, might itself have been reduced [25]. Phosphorylation is coupled to the reduction of dibromothymoquinone, but high concentrations (10–20 μM) are required [25]. However, 1 μM DBMIB blocks electron flow from water to methyl viologen or $K_3Fe(CN)_6$, but does not support appreciable net transport in its own right. Thus the specificity of electron flow during the preillumination seems assured.

Another question is that of carry-over of electron donors or acceptors from the preillumination to the assay step, in spite of the intermediate wash. However DCMU was added to Site I phosphorylation assays, so even if Photosystem II electron transport mediators had been carried over they would not have been able to contribute to Site I phosphorylation. DCMU could not be used in the preillumination step because of the difficulty in removing it by washing.

No equivalent inhibitor for Site I was available for use during Site II phosphorylation. Controls were therefore included in which no electron transport cofactor was added, to evaluate the possible extent of carry-over of Photosystem I electron transport mediators. When *N*-methylphenazonium methosulfate was used in the preillumination, essentially no carryover could be detected. In this case, the demonstration of inhibition at Site II due to prior activation by electron flow at Site I seems

unequivocal. While carryover is apparent after using reduced indophenol dye in the preillumination (Table I bottom), its interference with the Photosystem II electron flow can be discounted because during phosphorylation an excess of ferricyanide was present (see Methods). It was shown some time ago [26] that no cyclic electron flow around Photosystem I is possible under these conditions, because residual reduced indophenol dye would have been oxidized rapidly by the ferricyanide. Thus, with both systems for activating Photosystem I electron transport, the subsequent inhibition is detected at phosphorylation Site II.

Cross-site inhibition by *N*-ethylmaleimide gives the clearest evidence for a single functional population of CF_1 molecules. *N*-Ethylmaleimide inhibition is clearly correlated with its binding to a specific group on the CF_1 molecule [19].

Sulfate is more complex, in that the sulfate causes an uncoupling of electron transport from photophosphorylation. However, this uncoupling is the result of a specific proton leak, probably associated with the place where CF_1 interacts with the chloroplast membrane [20]. Since this is the case, the larger definition of "site", involving a clearly delimited complex of electron transport molecules, high energy intermediates, hydrophobic membrane-located proteins and CF_1 molecules, would still predict that sulfate uncoupling should be restricted to the point at which electron transport caused the initial activation of that complex. The fact that uncoupling was not so restricted means that the two phosphorylation sites must share at least a common pool of internal protons which is dissipated by this sort of uncoupling. This conclusion applies whether one considers the internal proton-storing compartment in question to be a space inside a membrane vesicle, or a region in the membrane structure proper. Thus, the results of the sulfate experiment are consistent with those of the *N*-ethylmaleimide experiment, providing in this case information related to the CF_1-membrane interaction as well as to CF_1 itself. Both experiments fail to support the concept [16] of two kinds or groups of CF_1 molecules and associated membrane components, each transducing energy from only one specific site in the electron transport chain. The results must also cast serious doubt on proposals (27–29) for energy transmission which require direct contact between an electron transport enzyme and a particular phosphorylation enzyme. Instead, these data point to the more limited definition in which an energy-conserving "site" is a part of the electron transport chain able to generate a protonmotive force or other high energy intermediate, in turn capable of activating the total population of CF_1 molecules on the vesicle more or less uniformly.

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