THE STOICHIOMETRY OF NADP DEPENDENT PHOTOSYNTHETIC PHOSPHORYLATION

Giorgio Forti

Istituto di Scienze Botaniche dell'Università,Milano
and
Istituto di Fisiologia Vegetale dell'Università di Bari
Via Amendola 175,Bari ,Italy.

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The ratio of ATP formed to electron pair transferred to an acceptor in photosynthetic phosphorylation has been considered to be 1 for many years,whether the physiological acceptor NADP or the artificial acceptor ferricyanide are used (Arnon et al. 1958; Jagendorf and Avron,1958;Stiller and Vennesland,1962; Turner et al.,1962).More recently,Winget et al. (1965) found this ratio to be up to 1.3 with ferricyanide as acceptor, and the same authors considered that the real ratio ATP/$2e^-$ during the transport of electrons through the phosphorylating pathway might be 2,on the basis of studies with the inhibitor phloridzin (Izawa et al.,1966).Furthermore,Lynn and Brown (1967,a and b) reported ATP/$2e^-$ ratios up to 4,with non -physiological acceptors,while they found the traditional ratio of 1 with NADP as the acceptor. These conclusions have been challenged by Del Campo et al. (1968).

All the above mentioned studies were done on chloroplasts devoid of their outer enveloppe,capable of only a very small residual photosynthetic activity.Recently,Jensen and Bassham (1966) developped a method to prepare chloroplasts retaining intact double membrane,capable of high rates of photosynthesis.The stoichiometry of ATP formation coupled to NADP photoreduction has now been reinvestigated utilizing the Jensen and Bassham's preparative procedure;the results reported here prove that the ratio ATP/NADPH is 2.

METHODS

Chloroplasts were prepared from freshly-harvested spinach leaves as described by Jensen and Bassham (1966). To measure NADP photoreduction, a small volume (not more than 25 to 30 microliters) of the chloroplasts in the resuspending medium (Jensen and Bassham 1966) were incubated with the chemicals specified in the tables, in a final volume of 2 ml. The absorbance at 340 nm was measured before and after a one-minute exposure of the cuvettes to red light, provided by a slide projector and filtered through 5 cm of water and a sharp cut-off filter, transmitting light of more than 600 nm. Light intensity was 120000 ergs $xsec^{-1}/cm^2$. at the level of the cuvettes. A non-illuminated complete reaction mixture served as blank. ATP was measured on a perchloric acid-deproteinized sample taken after the absorbance reading, either as the incorporation of ^{32}P-labelled orthophosphate into organic phosphate (Avron, 1960), or enzymatically, in a system containing glucose, hexokinase, glucose$_6$-phosphate dehydrogenase and NADP, after separation of $HClO_4$ as the potassium salt. The two methods were in complete agreement, within 5%. Chlorophyll was determined as by Arnon (1949). Ferredoxin was purified from spinach according to Böger et al. (1966).

RESULTS AND DISCUSSION

The chloroplasts prepared as described and used immediately after the preparation display a strong stimulation of electron transport by the phosphorylating reagents (table 1). The ratio of ATP produced to NADP reduced is between 2 and 3 usually, and often exceeds 3 in the complete reaction mixture. If NADP is omitted, the chloroplasts produce ATP by means of an electron transport system probably involving the photoreduction of ferredoxin and its reoxidation by O_2. Though it is doubtful whether this system works fully in the presence

Table I

NADP photoreduction and photophosphorylation.

Reaction mixture	ΔA_{340}	NADPH	ATP	ATP/NADPH	ATP/NADPH corrected[a]
		μ moles/mg Chl.x hour			
ADP, PO_4^{3-}, $MgCl_2$ and hexokinase omitted	0.120	68.4	-	-	-
Complete	0.330	190	585	3.08	2.16
NADP omitted	-0.031	(-17)	164	-	-
Ferredoxin omitted	0.125	57.2	152	2.68	1.46
NADP and ferredoxin omitted	-	-	43	-	-

[a] See text.

Conditions: complete reaction mixture (2 ml) contained tris buffer 0.03 M, pH 8.2; ADP 2mM; orthophosphate 2.5 mM, containing 10^5 cpm of ^{32}P; Mg Cl_2 3.5 mM; glucose 20 mM; hexokinase in large excess; NADP 1 mM; spinach ferredoxin 1.9 μM; chloroplasts with 34 γ of chlorophyll. Illumination for 1 min., in air. Temp.: 22°C.

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of NADP, owing to the much higher affinity of ferredoxin for the NADP reductase system than for O_2, the value of ATP formed in the absence of NADP has been subtracted from the value in the complete system, yielding the ratio ATP/NADPH of 2.16 reported in the last column, line 1 of table 1. Table 1 also reports the rates of NADP reduction and ATP formation in the absence of added ferredoxin: it can be seen that the endogenous ferredoxin left in the chloroplasts prepared as described is quite adequate for relevant activity. This finding is noteworth, considering that the chloroplasts prepared in the traditional way do not retain appreciable amounts of ferredoxin. In this case, if one corrects the amount of NADP produced for the small decrease in absorbance observed in

Table 2.

Stoichiometry of NADP photoreduction and ATP formation in chloroplasts.

Experiment No.	Conditions Ferredoxin μM	Conditions Chlorophyll μM	NADPH μmoles/mg Chl.hr.	ATP μmoles/mg Chl.hr.	ATP/NADPH
1	2	23	146	263	1.8
2	4	20	410	6I6	1.5
3	2	13	167	257	1.5
4	1.6	28	206	380	1.9
5	4	15	313	653	2.1

Conditions:as in table 1.

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the absence of NADP (table 1,line 3),and if the ATP made in the absence of both NADP and ferredoxin (table 1,last line) is subtracted,the corrected ratio ATP/NADPH of 1.46 (table 1,last column,line 4) is found.In a number of experiments, this last value has been found to oscillate from a minimum of 1.4 to 2.2.Table 2 shows the rates of NADP reduction,of ATP formation and of ATP/NADPH ratios in several experiments.

On the basis of the observations reported above,it is possible to conclude that the chloroplasts produce 2 molecules of ATP per molecule of NADP photoreduced.It is worthy to note that to obtain this result the chloroplasts need to be prepared in such a way that their double membrane is preserved,but the isotonicity during the reaction is not required.In fact,in the experiments reported above the intact chloroplasts have been diluted in low-tonicity medium during the phosphorylation reaction(see legends to the tables).

It was previously reported from this laboratory (Forti and Zanetti,1968) that the chloroplasts prepared as described

here are capable of relevant rates of cyclic photophosphorylation without the addition of any cofactor.work is now in progress to the aim of identifying a possible factor(s) of photophosphorylation released from the chloroplasts when their outer enveloppe is broken.

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