Existence and Localization of Two Phosphorylation Sites in Photophosphorylation of Isolated Spinach Chloroplasts

HERRICK BALTSCHEFFSKY and DOROTHY Y. de KIEWIET

Wenner-Gren Institute and Department of Biochemistry, University of Stockholm, Stockholm, Sweden

An investigation of photophosphorylation coupled to five different electron transport pathways in isolated spinach chloroplasts has revealed an unusual pattern of responses to desaspidin, which is an agent known to uncouple phosphorylation from electron transport in oxidative phosphorylation of animal mitochondria.[1] The results may be explained on the basis of known characteristics of these five pathways and appear to show conclusively that at least two phosphorylation or coupling sites exist in photophosphorylating spinach chloroplasts and that only the energy transfer reactions coupled to one of the two sites may be uncoupled from electron transport by low concentrations of desaspidin. The gross localization of the phosphorylation sites is given in the scheme presented below.

The five different electron transport pathways are (experimental conditions were essentially as in the references, unless otherwise mentioned): (1) the cyclic pathway operating in the presence of added phenazine methosulfate;[2] (2) the "pseudocyclic"[3] pathway operating in the presence of added flavin mononucleotide (FMN);[3] (3) the non-cyclic pathway from water to ferricyanide;[4] (4) the non-cyclic pathway from water to NADP, in the presence of added ferredoxin;[5] and (5) the pathway from ascorbate to NADP, in the presence of added ferrodoxin, 2,6-dichlorophenol indophenol (DCPIP),[6] and *p*-chlorophenyl-1,1-dimethylurea (CMU), which inhibits electron transport from the accessory, water-splitting light reaction.[7,8] The systems (3)–(5) were investigated with the Warburg-technique under anaerobic conditions, with a light intensity of about 10 000 lux at the borttom of the Warburg-vessels.

In the cases of cyclic (1) and "pseudocyclic" (2) electron transport only the rate of phosphate esterification can be determined, but in the systems (3), (4), and (5) not only phosphorylation but also electron transport may be measured, allowing one to differentiate between an *uncoupling* and a general *inhibitory* action of desaspidin. This agent decreased the rate of phosphorylation by half at about 10^{-7} M concentration in the systems (1)[9] and (5),[10] while in the systems (2),[9] (3),[10] and (4)[10] a concentration as high as about 10^{-5} M was required. These differences enabled us to stop photophosphorylation in a sensitive system (1) by addition of desaspidin and then start it again in the same chloroplast suspension by adding a suitable agent to allow the functioning of a less sensitive system.[11] Furthermore, it could be shown that desaspin *uncoupled* phosphorylation from electron transport, which was not inhibited, in system (5),[10] but *inhibited* both phosphorylation and electron transport in systems (3) and (4),[10] which both required about 100-fold higher concentrations for half-maximal effect than system (5), as was indicated above. These results also strengthen by analogy the reasonable assumption that system (1) might have been *uncoupled* by the low concentrations of desaspidin required and system (2) *inhibited* by the high concentrations required.[1]

Various general schemes for the five patways and how some of them are interconnected exist.[12-14] The following details are of particular significance for the interpretation of the results: a) system (2), in contrast to system (1), is connected with a rapid ^{18}O-exchange reaction between O_2 and H_2O,[15] and is under our experimental conditions inhibited by CMU;[10,15] b) DCPIP acts both as donor and acceptor of electrons in photophosphorylating systems and thus may allow electrons originating from ascorbate to complete a number of turns in a cyclic pathway (see Fig. 2 b in Ref.[16]) before being accepted by NADP.[17-21] From a) it is evident that system (2) shares part of the non-cyclic electron transport pathway originating from the accessory light reaction with systems (3) and (4). This part is not shared by systems (1) and (5). From b) it is evident that a cyclic pathway may operate in system (5) which also explains the high P/2 e^- ratios that can be obtained in this system.[17-21,10]

According to our interpretation of the results one phosphorylation site is shared by the systems (1) and (5) and another site by the systems (2), (3), and (4). The energy transfer reactions which are

coupled to the former site are *uncoupled* by low concentrations of desaspidin in contrast to those which are coupled to the latter site. The five different electron transport systems may be incorporated into a common pattern of electron transport reactions in photophosphorylation of spinach chloroplasts where the localization of the two phosphorylation sites is indicated. Each system in Fig. 1 contains the phosphorylation site expected from the results obtained with desaspidin. The results show that one site must exist between water as the electron donor and the point where electrons from ascorbate enter the system, in disagreement with an earlier claim [6] but in agreement with very recent ones.[19-21] In this minimum scheme various details, such as carriers participating in electron transport [22] from the primary acceptor at the B-level to NADP are not included.

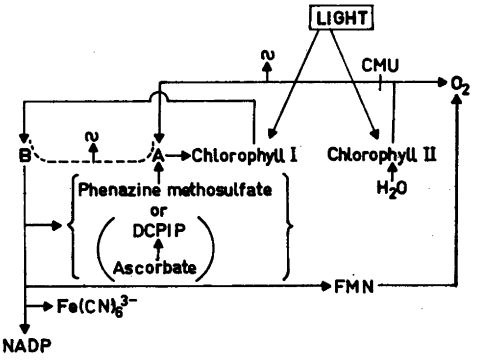

Fig. 1. Minimum scheme for five electron transport pathways and localization of two phosphorylation sites (∼). One site is coupled to the span Chlorophyll II → A and another site to the span B → added carrier (phenazine methosulfate or DCPIP) → A. The energy transfer reactions coupled to the latter site are uncoupled by desaspidin in contrast to those coupled to the former. (A and B are electron carriers.)

In bacterial photophosphorylation the "physiological", cyclic electron transport chain appears to contain two phosphorylation sites.[23-26] The fact that added electron carriers are required in order to obtain cyclic electron transport in isolated chloroplasts from green plants and the existence of an accessory light reaction system have been sources of experimental difficulty in this material, and evidence both for [27,28] and indirectly, against [29] multiple phosphorylation sites has been presented. The data obtained with, desaspidin very strongly indicate that the photophosphorylation system in chloroplasts also contains more than one site of ATP formation.

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Further Purification of Cholecystokinin and Pancreozymin

ERIK JORPES, VIKTOR MUTT and KAZIMIERZ TOCZKO

Department of Chemistry II, Karolinska Institutet, Stockholm 60, Sweden

In 1961 Jorpes and Mutt [1] described the preparation of cholecystokinin-pancreozymin material with about 100 Ivy cholecystokinin and 600 Crick, Harper and Raper units of pancreozymin per mg. In all the purification steps leading to this preparation from the crude starting material [2] the activities of cholecystokinin and of pancreozymin did not separate. Subsequently Dhariwal *et al.*[3] succeeded in pyrifying pancreozymin to an activity of 3750 pancreozymin units per mg.

By minor modifications of the technique described in 1961 we have routinely obtained preparations with 250 cholecystokinin and 1500 pancreozymin units per mg. This has been our starting material for further work. First we attempted purification by counter current distribution. Of the systems tried an aqueous acetic acid-sodium chloride-butanol system [4] and especially, an aqueous acetic acid-pyridine-butanol system [5,6] gave encouraging results. Again both cholecystokinin and pancreozymin were purified to the same extent. However, because there were disconcerting losses in activity during recovery of the material from the solvents after distribution and also because the distribution patterns showed that the starting material was still quite impure simpler methods for purification were tried. Dhariwal *et al.*[3] had used filtration through Sephadex, G-50 in 0.2 M AcOH for the purification of pancreozymin. We tried this technique on our starting material with some success. However, distinctly better separations were obtained on filtration at higher pH values. Filtration in 0.25 M Na_2HPO_4 adjusted to pH about 8 was uniformly successful. After recovery in lyophilized form from the buffer solution the material from the active fractions was found to be about six times purer on a weight basis with respect to both cholecystokinin and pancreozymin. This material was further purified by a factor of about 2 by chromatography on the ion exchange resin Amberlite XE—64 (Rohm and Haas Co.), again with respect to both cholecystokinin and pancreozymin. This material consequently assayed at 3000 cholecystokinin and 18 000 pancreozymin units per mg.

In acid hydrolysates of the most purified material the following amino acids were identified by the two dimensional system of Redfield:[7] alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, lysine, proline, leucine, methionine, phenylalanine, serine, tyrosine and valine. Only traces of threonine were present. After performic acid oxidation no cysteic acid could be demonstrated.[8] Analysis for tryptophan by the Voisenet-Rhode *p*-dimethylaminobenzaldehyde method [9] showed that this amino acid was present.

Experimental. The starting material was prepared as described by Jorpes and Mutt.[1] It was recovered from the eluate from the TEAE-cellulose column by adsorption on alginic acid at pH 3, elution with 0.2 M HCl, exchange of the chloride for acetate on a column of DEAE Sephadex and lyophilization from dilute aqueous acetic acid. The lyophilized material had an activity of 250 Ivy dog units of cholecystokinin and 1500 Crick, Harper and Raper units of pancreozymin per mg.

First purification step. 100 mg of the starting material was dissolved in 1 ml of 0.25 M Na_2HPO_4, which had been preadjusted to pH 8.0 $\pm$ 0.1 with M H_3PO_4 and allowed to sink into a column, 1.5 $\times$ 90 cm of Sephadex, G-50, fine, bead form (Pharmacia, Uppsala). The column had been equilibrated with the