GENERATION OF ATP BY CHLOROPLASTS THROUGH SOLVENT PERTURBATION

Jui H. Wang and Man-chiu Yang

Bioenergetics Laboratory, Acheson Hall, State University of New York, Buffalo, New York 14214

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SUMMARY:

A two-stage method was discovered for generating ATP by chloroplasts in the dark at constant pH through solvent perturbation. With cold acetone as the perturbing solvent, the yield of ATP was found to increase with the volume percent of acetone in the first stage medium. The results are difficult to explain in term of the proton gradient model, but is consistent with the conventional model of prior water formation and subsequent ATP generation.

INTRODUCTION

The development of two-stage processes for generating ATP by chloroplasts has broadened considerably our knowledge on the mechanism of photosynthetic energy transduction. The results of two-stage photophosphorylation experiments show that light energy is stored in a certain form which can subsequently be used to generate ATP from ADP and inorganic phosphate (P_i) in the dark (1,2,3,4). The data of acid-base transition phosphorylation experiments (5,6), show that the free energy stored in a medium of high acid concentration can be utilized to generate ATP subsequently in a medium of much lower acid concentration. These observations have been interpreted in terms of the proton gradient (7) or proton concentration hypothesis (8), but they are also consistent with the classical chemical model of water formation during the first stage, followed by the generation of ATP from ADP and P_i during the second stage of the process.

In the experiments described in the present work, ATP was generated from ADP and P_i by chloroplasts through solvent perturbation in the absence of either light or proton concentration gradient. Acetone was chosen as the perturbing solvent, because it is miscible with water and chemically inert under

our experimental conditions, has a static dielectric constant much lower than that of water, and does not completely denature the energy transducing enzymes at 0°C.

MATERIALS AND METHODS

Materials: Chloroplasts were prepared from fresh spinach leaves as described previously (9), kept at near 0°C and used within two hours after preparation. ATP (disodium salt, Sigma Grade), ADP (disodium salt, fermentation grade) and Tricine (N-tris [hydroxymethyl]-methylglycine) were from Sigma Chemical Co. All other reagents used were of the highest grade available.

Phosphorylation through Solvent Perturbation: In a typical experiment, freshly prepared spinach chloroplasts suspended in a preparation medium containing 0.25M sucrose, 10mM NaCl, 20mM Tricine at pH 7.9 (0°C) and approximately 2 mg chlorophyll/ml were kept in the dark for 30 minutes or longer. 0.2 ml aliquots of the chloroplast suspension were placed in dry 30-ml centrifuge tubes in the dark at 0°C. To each tube was added 0.2 ml of cold acetone, followed by 3.6 ml of cold acetone-aqueous buffer mixture of calculated volume ratio to give a final mixture of predetermined composition. The content of each tube was then mixed, left standing in the dark for 10 minutes at 0°C, and subsequently centrifuged at 12,000 x g for 3 minutes. After removal of the supernatant, the pellet in each tube was triturated with 0.2 ml of a second buffer containing 0.1M Tricine at pH 7.9 (0°C), 10mM $MgCl_2$, 5mM ADP and $[^{32}P]P_i$. The mixture was left standing in the dark at 0°C for a predetermined length of time, and then 25 µl of 40% TCA were added to each tube to terminate the reaction. After centrifugation, the radioactive ATP and ADP in the supernatant were determined by paper chromatography.

Paper Chromatography: The separation of ATP, ADP and P_i was performed expediently by chromatography on polyethyleneimine-paper made in our laboratory (10). With PEI-paper made from Whatman No. 1 chromatographic paper and 2.5% polyethyleneimine (from Badische Anilin- und Sodafabrick, Germany) and a developing solution which contained 0.25M Trizma acetate, 0.25M acetic acid and 1.0M NaCl (pH 4.75), effective separation of ATP, ADP and P_i by ascending chromatography was achieved within 4 hours. Before the application of the assay samples, 0.5 µmole of P_i, 0.1 µmole of ADP and 0.1 µmole of ATP were applied to the paper at the origin of each sample as carriers for easy location of the separated spots under ultraviolet light and for improvement of the separation. After drying, the developed chromatogram was cut into 1.0 cm strips, transferred into plastic counting vials and assayed with a Beckman-233 Liquid Scintillation Counter.

The quality and efficiency of this separation is illustrated by the chromatogram in Fig. 1. Since all radioactive components in the sample are quantitatively displayed in this chromatogram, the concentration of each component can be computed by multiplying the total rapidly exchangeable phosphate concentration (i.e., total P_i concentration) by the corresponding fraction of total radioactivity of that component. If carrier-free $[^{32}P]P_i$ was used in the experiment, the total P_i concentration should be equal to the endogenous phosphate concentration.

Determination of Endogenous Phosphate: The total endogenous inorganic orthophosphate in chloroplasts was determined by the Malachite Green method described by Hess and Derr (11). This was found to vary from 80 to 240 nmole/mg chlorophyll. Absorption spectra were taken with an Aminco DW-2 spectrophotometer. Chlorophyll and proteins in each sample were removed by prior TCA (4%)

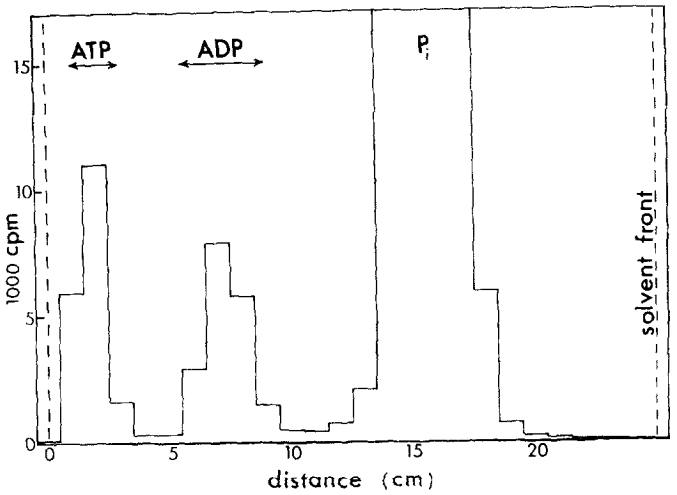


Figure 1 Assay of a 10 µl-sample of reaction mixture for ATP, ADP and inorganic orthophosphate (P_i) by ascending chromatography on polyethyleneimine paper. The double-headed arrows represent the positions of ATP and ADP respectively as located under UV-illumination due to the non-radioactive ATP and ADP added as carriers to the sample. The total radioactivity due to $[^{32}P]P_i$ band is 379 x 10^3 cpm. Total development time, 4 hrs.

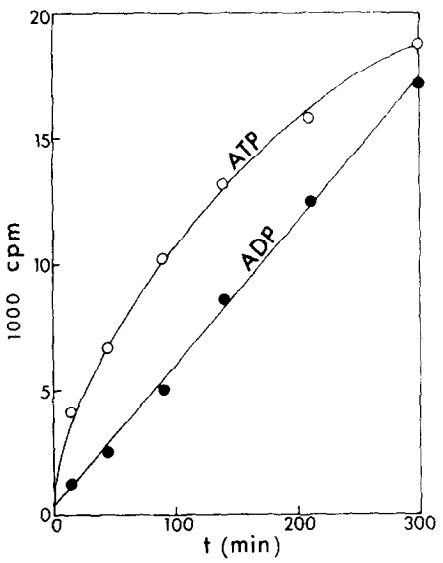


Figure 2 Rate of generation of ATP and labeling of ADP by chloroplasts in the dark at pH 7.9 and 0°C through solvent perturbation as a function of incubation time with the second stage buffer. A 0.5 ml chloroplast sample was used which contained 2.13 mg chlorophyll and 183 nmoles endogenous P_i. Acetone content of the medium at the perturbation stage was 95% by volume. Composition of the incubation medium for the second stage: 0.10M Tricine buffer (Cl^-) at pH 7.9, 5mM ADP, 10mM $MgCl_2$ and carrier-free $[^{32}P]P_i$.

precipitation. To ensure that the error due to P_i trapped in the precipitate was negligible, a measured amount of carrier-free $[^{32}P]P_i$ was added to a sample before the addition of TCA. The concentration of radioactivity in the supernatant of the subsequently centrifuged mixture was found to be equal to that of a control sample of the medium without chloroplasts which had been treated similarly.

RESULTS AND DISCUSSION

The yield of ATP produced in the dark at pH 7.9 and 0°C through perturbation of 95 volume percent acetone is shown in Fig. 2 as a function of subsequent incubation time of the triturated pellet with the second buffer containing $[^{32}P]P_i$ and ADP at 7.9. The linear increase in the concentration of radioactive ADP is probably due to the following exchange reaction between the newly generated $[^{32}P]$ATP and ADP as catalyzed by adenylate kinase:

$$2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \qquad (1)$$

$$[^{32}P]\text{ATP} + \text{AMP} \rightleftharpoons \text{ADP} + [^{32}P]\text{ADP} \qquad (2)$$

The results of a typical series of experiments on the dependence of ATP yield on the composition of the perturbing solvent during the first stage of the process are summarized in TABLE I. The data show that as the volume percent of acetone in the perturbing solvent during the first stage of the process was raised from 5 to 95%, the yield of ATP increased by a large factor. At acetone concentrations below 50%, only very small amounts of $[^{32}P]$ATP was formed. The last observation also indicates that under our experimental conditions most of the $[^{32}P]$ATP was produced by net synthesis rather than through phosphate exchange between $[^{32}P]P_i$ and endogenous ATP.

Since both the original chloroplast preparation and the Tricine buffer containing ADP and $[^{32}P]P_i$ for the second stage of the process were at pH 7.9 (0°C), these results are difficult to explain in terms of the proton gradient model of phosphorylation by chloroplasts. But these data are still consistent

TABLE I. Generation of ATP by Chloroplasts through Perturbation by Acetone-Water Mixtures[a]

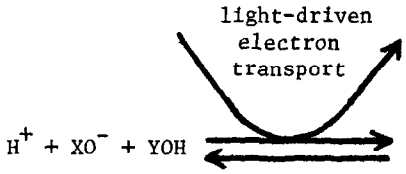
Volume % of Acetone in the Perturbing Mixture[b]	ATP generated			ADP labeled		
	10^3 cpm	% of total radioactivity	nmole/mg of chlorophyll	10^3 cpm	% of total radioactivity	nmole/mg of chlorophyll
5	0.6	0.12	0.10	0.7	0.14	0.12
10	1.4	0.26	0.22	1.4	0.25	0.22
50	2.0	0.31	0.27	1.8	0.27	0.23
80	6.1	0.86	0.74	4.0	0.57	0.49
90	13	1.6	1.4	12	1.5	1.3
95[c]	19	4.4	3.8	17	4.0	3.5

[a]All operations were performed in the dark at 0°C. The chloroplast preparation for the listed experiments contained 4.25 mg chlorophyll/ml and 86 nmole endogeneous P_i per mg chlorophyll.

[b]Auxiliary experiments showed that even a brief treatment with cold acetone destroyed the photophosphorylation properties of chloroplasts, inhibited phosphate exchange between P_i and ATP, but apparently did not destroy the phosphoryl transfer enzymes.

[c]Incubation time of the triturated pellet with the second buffer containing ADP and [^{32}P]P_i at 0°C was 5 hours for this particular sample, but 3 hours for all the other samples.

with the conventional two-step mechanism in which the endergonic water formation from unidentified groups XO^- and YOH



(3)

is followed by the reaction of the unidentified intermediate XOY with ADP and P_i to form ATP

$$XOY + P_i + ADP \rightleftharpoons XO^- + YOH + ATP \qquad (4)$$

The perturbing solvent acetone could make reaction 3 proceed farther to the right by lowering the dielectric constant of the medium as well as by diluting the water formed. Presumably reaction 3 can be driven by either electron transport or high concentration of protons or perturbation by a suitable solvent.

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