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Co-immobilization of CO_2 Reductase from Potato Tuber Chloroplasts with Thylakoid Membrane for the Production of Formic Acid

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Illumination of isolated choroplasts from green potato tuber, in absence of HCO_3^- in the medium and in dim 30 lux light, produced a low potential reductant. The generation of this reductant was detected by the characteristic difference spectrum of dark minus light incubated choroplasts, which showed troughs having maximum at 664, 650, 463 and 392 nm. The spectrum was stable for 1 hr after incubation in dark. But addition of HCO_3^- and incubation at 30 lux light intensity for 30 min abolished the spectrum with concomitant production of formate. Envelope free chloroplasts did not exhibit this difference spectrum under identical conditions. Substitution of envelope free chloroplasts (thylakoid membrane) in the CO_2 reductase assay mixture in place of dithionite showed about 3 fold activation of formate production. Prior treatment of Thylakoid with either DCMU or NADP (10μM) inhibited formate formation considerably. Thyakoid membrane and CO_2 reductase protein were Co-immobilized in 2% agarose to give a stable system for the biophotolytic production of formate using H_2O and CO_2.

Key Words: Green potato tuber chloroplasts, CO_2 reductase, Thylakoid membrane, Co-immobilization, Bio-photolytic production of formic acid

Introduction

While studies on the production of hydrogen in immobilized systems are in progress, there are some reports on formic acid production by photochemical reduction of CO_2 using synthetic systems. One of such systems where electron transfer from a singlet excited state of aromatic hydrocarbon to another acceptor molecule which in turn reduced CO_2 to formate has been reported by Tazuku and Kitamura (1978). In another system Parkinson and Weaver (1984) achieved the reduction of CO_2 to formate by photogenerated electron in a semiconductor photoelectrode (Indium phosphate) coupled to the enzyme formate dehydrogenase through a mediator methylviologen. Apart from some reports like this, attempts to develop a system producing formate by direct reduction of CO_2 using light energy and water are not many.

Our discovery on the existence of a novel pathway for the direct reduction of CO_2 to formate in green potato tuber chloroplasts, utilizing light energy and water gave a new impetus to this project. Potatoes when exposed to cold temperature (0–4°C) and dim light (30 lux) become green after storage for 8 weeks. The greening was due to the synthesis of solanine, a toxic alkaloid. We were able to isolate intact chloroplasts from peel tissue which had the complete system for the synthesis of solanidine, the aglycone of the alkaloid (Ramaswamy et al. 1976, Ramaswamy & Nair 1984). The pathway for solanidine formation was established and the primary product of the reaction was identified as formate (Ramaswamy et al. 1983, Nair et al. 1981). Subsequently the CO_2 reductase was isolated and partially purified from these chloroplasts (Arora et al. 1985). These studies proved the way to the development of an immobilized system for the production of formic acid using light energy CO_2 and water which is reported here.

Materials and Methods

Potatoes used for these studies were *C.V. Kufri chandramukhi*. Fresh tubers were brought from local market, cleaned and stored in cold room at 0–4°C under light intensity of 30 lux for 6 to 8 weeks for greening. The green peelings were used for the isolation of chloroplasts.

Chloroplasts were isolated by a modified method of Cockburn et al. (1986) as described by Ramaswamy et al. (1976). All operations were done at 0–4°C and dim light unless otherwise stated.

Activation of CO_2 Reductase

The method of activation of latent CO_2 reductase present in the potato tuber chloroplasts was the same as described in Arora et al. (1985).

Preparation of Envelope-free Chloroplasts

Envelope free chloroplasts i.e. the thylakoid membrane, were prepared by osmotic shock treatment to chloroplasts as described by Ramaswamy and Nair (1984) following Walker's procedure (Walker 1971).

Spectral Evidence for the Formation of a Reductant during Light Irradiation of Chloroplasts

Chloroplasts were suspended in 6 ml of suspension medium (330 mM, sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 50 mM N-2-hydroxy ethylpiperazine-N-2-ethane sulfonic acid, Hepes adjusted to *p*H 7.6 at 20°C with NaOH) to give 200–250μg chlorophyll/ml. Three ml of this chlorophyll suspension was taken in matched cuvettes of Shimadzu UV 240 spectrophotometer. For taking difference spectrum the absorbance range was adjusted to −0.2 to +0.2 and wave length from 700 nm to 350 nm. The base line was drawn and the chloroplast suspension in the sample compartment was then illuminated for 30 min at 15°C in the presence of 30 lux light under anaerobic condition by passing repurified nitrogen. After incubation the spectrum was recorded against the dark incubated sample. The illuminated sample was then kept in the dark for different time intervals from 1 to 2 hr and then difference spectrum was recorded in between. To the illuminated sample after 1 hr incubation in the dark 7.5 mM $NaHCO_3$ was added and then incubated for 30 min at 15°C under 30 lux light and anaerobic condition. After the incubation the difference spectrum was recorded. These experiments were repeated with envelop free chloroplasts also.

The chlorophyll content was determined according to the method of Whatley and Arnon (1963).

Reconstitution of CO_2 Reductase to the Thylakoid Membrane

Potato tuber chloroplast CO_2 reductase was activated and then precipitated with acetone. The precipitated proteins were washed with 0.02M Hepes buffer *p*H 7.5 containing 0.1 M NaCl to remove soluble enzymes and glyoxylate synthetase (Ramaswamy et al. 1983) and suspended in 4 ml of the same buffer. An aliquot of 1 ml was kept for assay of CO_2 reductase. The remaining 3 ml suspension was mixed with washed envelope free chloroplast pellet obtained from 50 g peel tissue to give the reconstituted system. CO_2 reductase activity of this mixture was also determined under same experimental conditions.

Formate formed was estimated by the colorimetric method of Wood and Gest (1957).

Protein concentration was determined by the method of Bradford (1976) using serum albumin as standard protein.

Immobilization of thylakoid and CO_2 Reductase on Agarose to produce Formic Acid Continuously

Agarose was used as the support matrics for the immobilization of CO_2 reductase and thylakoid membrane. Before use, the agarose was sterilized at 15 psi for 15 min. The gels, 2% and 4% were made in 0.02 M Hepes buffer *p*H 7.5 by heating in a boiling water bath and then cooling to 40°C. The CO_2 reductase preparation and thylakoid membrane preparations from 50 g peel tissue were mixed together, added to the agarose solution and the volume was made up to 10 ml. During addition the solution was stirred well and agarose was allowed to solidify. These gels were packed into two different columns of dimension 9 × 1 cm after cutting into small pieces with scissor. The packed gel was washed thoroughly with 0.02 M Hepes deoxygenated buffer *p*H 7.5 containing 5/μ g streptomycin/ml till the washings were colourless. All these operations were done at 15°C and 30 lux light intensity.

At this stage, 10 ml 0.02 M Hepes buffer *p*H 7.5 containing $NaHCO_3$, 9mM and 50μg streptomycin was allowed to flow through this column at 12 ml/hr. After collection of 10 ml from the column formate concentration of this eluate was estimated and the column was again washed with $NaHCO_3$ free buffer. These operations were repeated for number of days.

Results and Discussions

Demonstration of the Formation of Low Potential Reductant by Illumination of Potato Tuber Chloroplasts

The latency of potato tuber CO_2 reductase was established in our earlier studies (Arora et al. 1985). For obtaining an active soluble CO_2 reductase, it was essential to illuminate the chloroplast in the absence of substrate, CO_2, at 30 lux light intensity, which was found to be optimal for the photosynthetic function of these chloroplasts. Absence of the final acceptor of electron, in this case CO_2, may allow accumulation of reducing equivalents and the enzyme CO_2 reductase

may function as a charge accmulator. Formation of a stable low potential reductant in potato tuber chloroplasts upon illumination in the absence of CO_2 was demonstrated by spectral studies. A difference spectrum of dark minus light illuminated chloroplast is shown in figure 1. At 30 min after illumination these chloroplasts displayed a difference spectrum with troughs having maxima at 664, 650, 572, 463, and 392 nm. The light induced spectrum persisted even after 1 hr incubation in the dark. Now the addition of $NaHCO_3$ and incubation for 30 min, the spectral characterisitcs disappeared indicating the utilization of accumulated reductant for the reduction of CO_2. Formate formed could be estimated in this case. The reductant developed during the illumination of the chloroplast in the absence of HCO_3^- was stable even for 2 hr incubation in the dark at 15°C (figure 2). Incubation of chloroplast for such a prolonged period impaired the capacity of HCO^-_3 to change the spectrum (figure 2). Similarly removal of CO_2 reductase from chloroplast by preparing envelope free chloroplast did not exhibit characteristic spectrum (figure 3). In this case addition of $NaHCO_3$ did not affect the spectrum on the other hand an enhancement was observed. These observations are only an indication that some type of reductant accumulates in the chloroplasts altering its

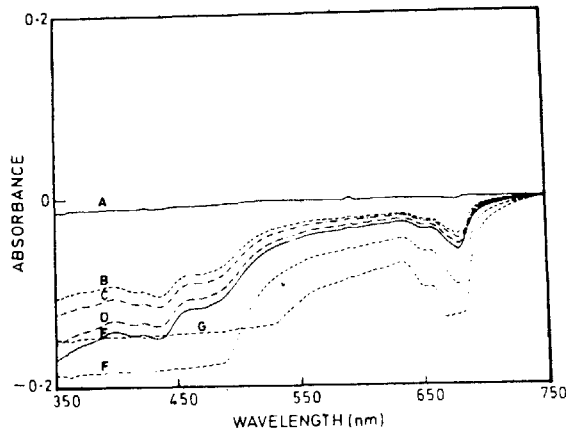

Figure 2 Difference spectra of dark minus light illuminated chloroplast at different time intervals of dark incubation. *Curve A* Base line; *Curve B* 1/2 hr in light; *Curve C*: 1 hr incubation in dark; *Curve D*, 2 hr incubation in dark; *Curve E*, bicarbonate added at 0 time after 2 hr dark incubation. *Curve F* 1/2 hr after adding bicarbonate in light. *Curve G* 1 hr after adding bicarbonate

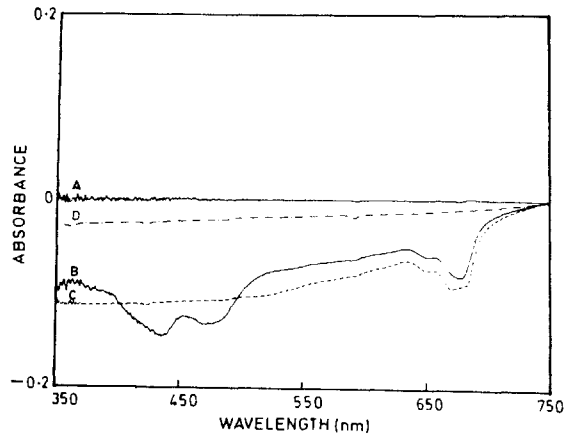

Figure 1 Difference spectra of dark minus light illuminated chloroplasts: *Curve A*, base lines; *curve B* after 1/2 hr incubation in light; *curve C*, after 1 hr incubation in dark; *curve D*, 1/2 hr incubation after addition of bicarbonate in light

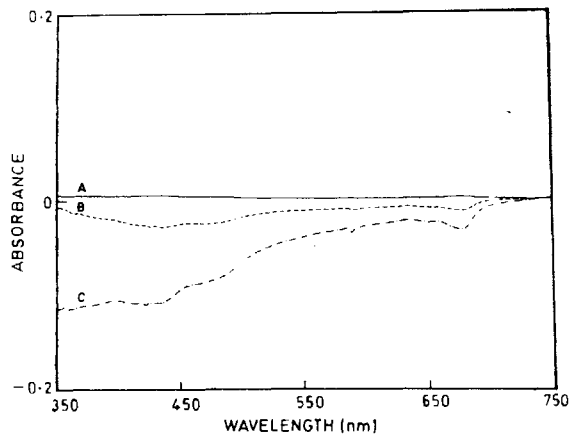

Figure 3 Difference spectra of dark minus light illuminated envelope free chloroplast. *Curve A*, base line; *Curve B*, after exposure to light for 1/2 hr; *Curve C*, after adding bicarbonate

normal absorption spectrum. Significance of these findings in relation to the activation of CO_2 reductase is not well understood at present. Confirmatory proof needs further experimentation and identification of the charge accumulator in the chloroplast. Formation of this type of stable reductant is not observed in any other chloroplasts (Malkin 1976).

Reconstitution of CO_2 Reductase into Isolated Thylakoid Membrane

Isolated CO_2 reductase preparation showed a 3-fold activation by supplementing dithionite in the reaction mixture suggesting that for the optimal functioning of the enzyme there is additional requirement for reducing equivalents (table 1). In the next experiment we have examined whether this requirement for electron donor can be met by supplementing thylakoid membrane free from all soluble enzyme in the reaction system. The thylakoid membrane prepared by osmotic shock did not show any CO_2 reductase activity (table 1). The washed membrane, however, was capable of photosynthetic activity using ferricyanide as electron acceptor (Ramaswamy & Nair 1984). The supplementation of thylakoid preparation in the CO_2 reductase reaction mixture resulted in about 2.7 fold activation of reductase activity (table 1) which showed that CO_2 reductase is reconstituted into thylakoid membrane coupling to electron transfer to CO_2 reduction. Addition of DCMU or NADP strong inhibitors of photolysis of water and photosystem II function in these chloroplasts (Ramaswamy & Nair 1979), inhibited the formate formation. This is suggestive that reducing equivalents derived from water splitting reactions of photosystem II are involved in the reduction of CO_2. The requirement for thylakoid membrane was specific because spinach thylakoids could not replace potato tuber thylakoids.

Table 1 *Reconstitution studies on CO_2 reductase with thylakoid membrane*

Experiment	Activity (/μmoles of formate formed per mg protein)
CO_2 reductase alone	16.3
CO_2 reductase plus Na dithionite	48.3
Thylakoid membrane alone	0.0
CO_2 reductase plus thylakoid membrane	47.5
CO_2 reductase plus 10μM DCMU treated thylakoid	15.0
CO_2 reductase plus 10μM NADP treated thylakoid	0.8

Immobilized System for Formate Production

Since it was possible to reconstitute isolated CO_2 reductase to the washed thylakoid membrane preparation to generate formic acid production, this was thought to be an ideal system for immobilization. The commonly used matrics for immobilizing photosynthetic system are alginate and agarose (Rao & Hall 1984), because they form translucent or transparent gels specially required for light penetration. In this case we have choosen agarose because of its advantage over the other support materials. Here, the thylakoid membrane preparation and CO_2 reductase were Co-immobilized in agarose gel. Two concentration of agarose, 2% and 4% were employed. The efficiency of these two different concentration of gels for the reduction of CO_2 was determined by estimation of formate produced during each operation. The life of the column was determined by continuing the operations till about 40 days. The results of these studies are given in (figure 4). It is evident that 2% gel gave a better yield of formate and its production declined after about 20 days. In both gels maximum activity was obtained after seven days and the maximum yield was retained after for about 2 weeks. Thereafter, the rate of production of formate reduced considerably after 20 days. Continous illumination of the column at 30 lux light intensity was required for the retention of CO_2 reduction. The design and experimental conditions of a reactor for continuous production of formate using 2% gel for immobilization were described by Arora and Nair (1986).

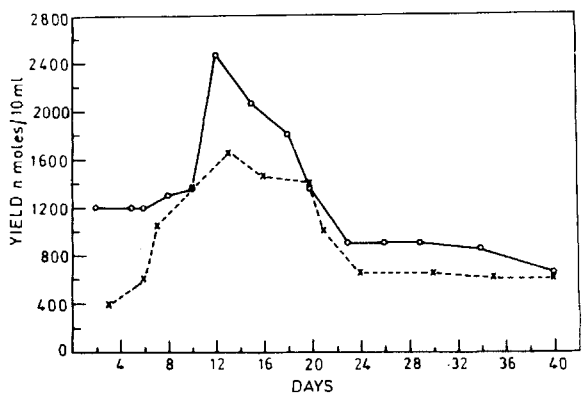

Figure 4 Formic acid production on different days after immobilizing CO_2 reductase and thylakoid preparation in O—O 2% agarose and X—X 4% agarose

The novelty of this system is that it can produce formic acid continuously for about 20 days without any inactivation of the system, if it is washed alternatively after each cycle to remove formic acid completely. Formate trapped in the column may inhibit the binding of CO_2 to the enzyme. The photo-inhibition observed in the case of higher plant chloroplasts is absent here. This chloroplast system also has an additional advantage as both hydrogen storage and production of organic chemicals from H_2O and CO_2 using light energy.

References

Arora S, Ramaswamy N K and Nair P M 1985 Partial purification and some properties of a latent CO_2 reductase from green potato tuber chloroplasts; *Eur. J. Biochem.* **153** 509–514

—— and Nair P M 1986 An immobilized system for continuous production of formic acid using thylakoid membranes and CO_2 reductase from potato tuber chloroplasts; *Photobiochem. Photobiophys.* **13** 73–84

Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding; *Anal. Biochem.* **72** 248–254

Cockburn W, Walker D A and Baldry C W 1968. The isolation of spinach chloroplasts in pyrophosphate media; *Pl. Physiol.* **43** 1415–1418

Malkin R 1976 Photosystem I; *A. Rev. Pl. Physiol.* **33** 455–479

Nair P M, Behere A G and Ramasway N K 1981 Glycoalkaloids of solanum tuberosum—a review; *J. Sci. Indian Res.* **40** 529–532

Parkinson B A and Weaver P F 1984; *Nature* **309** 148–149

Ramaswamy N K, Behere A G and Nair P M 1976 Novel pathway for the synthesis of solanidine in the isolated chloroplasts from greening potatoes; *Eur. J. Biochem.* **67** 275–282

—— and Nair P M 1979 Inhibition of CO_2 evolution by NADP in isolated potato tuber chloroplasts and its identity with DCMU inhibited site; *Arch. Biochem. Biophys.* **193** 56–62

——, Sangeetha G J, Behere A G and Nair P M 1983 Enzymatic synthesis of glyoxylate from formate catlysed by glyoxylate synthetase from green potato tuber chloroplasts; *Pl. sci. lett.* **32** 213–220

—— and Nair P M 1984 Evidence for the operation of a C_1 pathway for the fixation of CO_2 in isolated intact chloroplasts from green potato tuber; *Pl. sci. lett.* **34** 261–267

Tazuku S and Kitamura N 1978 Photofixation of carbondioxide to formic acid *in vitro* using water as hydrogen source; *Nature* **275** 301–302

Walker D A 1971 Photosynthesis; *Method Enzymol.* **23** 211–221

Whatley F R and Arnon D I 1963 Photosynthetic phosphorylation in plants; *Method. Enzymol.* **6** 308–313

Wood H G and Gest H 1957 Determination of formate; *Method Enzymol.* **3** 285–292