Photosynthetic Activities of Chloroplast Coupling Facor 1 after Modification by Pyridoxal Phosphate

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ABBREVIATIONS

ADP: adenosine-5'-diphosphate

AMP: adenosine-5'-monophosphate

ATP: adenosine-5'-triphosphate

CD: circular dichroism

CF_o: coupling factor o

CF_1: chloroplast coupling factor 1

DEAE: diethyaminoethyl-

DMQ: 2,5-dimethyl-*p*-benzoquinone

DTT: dithiothreitol

EDTA: ethylenediamine tetraacetic acid

F_1: coupling factor 1

Fecy: potassium ferricyanide

MV: methyl viologen

NBD-Cl: 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole

NEM: N-ethylmaleimide

PAGE: polyacrylamide disc gel electrophoresis

PCA: perchloric acid

Pi: inorganic phosphate

PLP: pyridoxal-5'-phosphate

PMS: phenazine methosulfate

SDS: sodium dodecyl sulfate

TCA: trichloroacetic acid

tricine: tris(hydroxymethyl)methylglycine

Tris: tris(hydroxymethyl)aminomethane

I INTRODUCTION

Detailed understanding of the energy transfer process leading to ATP formation in chloroplasts requires the investigation of coupling device (coupling factors) of thylakoid membrane.

Chloroplast coupling factor 1 (CF_1) (1) is the only enzyme purified from the membrane. Other factors such as the membrane counterpart of CF_1 (i.e., CF_o) (2) may play a role in the process of energy transfer from proton gradient (3) to CF_1, but not so much has been known about them.

The photophosphorylation activity of chloroplasts which were suspended in 1 mM EDTA then collected by centrifugation was lost but could be restored on association with the supernatant (4). This was the first report describing the coupling factor activity in chloroplasts. This factor protein was then purified and termed "chloroplast coupling factor 1" (1). CF_1 has the molecular weight of 325,000 determined by the sedimentation equilibrium method (5) and is a latent ATPase which exhibits hydrolytic activity of ATP after treatment by heat or trypsin (1,6). Specific antibody for CF_1 inhibited trypsin-activated Ca^{2+}-ATPase, phosphorylation and light-DTT-activated Mg^{2+}-ATPase (7). This demonstrated that CF_1 is involved in the terminal steps of photophosphorylation.

ADP is an allosteric inhibitor of the heat-activated Ca^{2+}-ATPase of isolated CF_1 (8) and trypsin-activated Ca^{2+}-ATPase of chloroplasts (9). From the Hill plot (10) of the activity against ATP concentration, the apparent reaction order of 1 in the absence of ADP changed to 1.9 in the presence of ADP. This suggested that CF_1 is an allosteric enzyme that contains at least two active sites acting cooperatively (11). The binding sites for nucleotide on CF_1 were studied with the ultrafiltration method (12). Two apparently identical tight sites for ADP and ADP-analogs with dissociation constant of less than 10 μM were detected. The slow rate of association for ADP (12) seemingly ruled out the possibility that these tight sites are the catalytic site for ATPase of isolated CF_1. There exist also the tight binding sites for ADP or ATP on membrane-bound CF_1 with a dissociation constant in the similar range (13,14). The nucleotides bound to these sites stimulated (13) the extent of light-induced proton uptake by blocking the leakage of protons through CF_1, resulting in the partial inhibition of electron transport (14,15).

However, it has not yet been known how these tight sites interact with the catalytic site in the process of ATP synthesis or hydrolysis.

When CF_1 was electrophoresed on a polyacrylamide disc gel in the presence of SDS (16), it was separated into five different polypeptide bands (17). The polypeptides were named

α, β, γ, δ and ε subunits according to the order of their decreasing molecular weight of 59,000, 56,000, 37,000, 17,500 and 13,000, respectively (18). The stoichiometry of these subunits was determined by the relative staining intensity of SDS gels (19) and by the relative amounts of radioactivity incorporated into each subunit of CF_1 isolated from the chloroplasts of pea grown in an atomosphere of $[^{14}C]\,CO_2$ (8). CF_1 includes 2α, 2β, 1γ, 1δ and 2ε subunits (8,19).

These five individual subunits were isolated (18) and their function was investigated by anti-serum for each subunit (20). The anti-α or anti-γ-antibody inhibited photophosphorylation and light-DTT-activated Mg^{2+}-ATPase of chloroplasts (20), suggesting that the α and γ subunis might be involved in phosphorylation. The stimulation of light-induced proton uptake by ATP (13) was inhibited by anti-α, indicating that tight (regulatory) sites for ATP are located on the α subunit.

CF_1 composed of only α and β subunits, which obtained after trypsin digestion of CF_1 (18), exhibited normal ATPase activity, indicating that the active site for ATPase is present on the α and/or β subunits. CF_1 depleted δ subunit was obtained by chloroform extraction followed by DEAE-Sephadex A-50 column chromatography (21), which could not be recombined with CF_1 depleted membrane. This would indicate that the δ subunit is a link for binding CF_1 to the membrane (21). Purified ε subunit inhibited the ATPase activity of heat-activated CF_1 (18).

However, it has not yet been known how these subunits function in the process of ATP synthesis (or hydrolysis), in collaboration with others, or whether the active site of membrane-bound CF_1 for ATP synthesis is identical to that for ATP hydrolysis of CF_1 activated as ATPase.

Chemical modification (22) sometimes leads to useful information about the active site of an enzyme. Modification of isolated CF_1 by N-ethylmaleimide (NEM) or iodoacetamide (23) did not inhibit the ATPase activity, suggesting that SH-groups are not involved in the active site. 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (24) modified the β subunit of isolated CF_1 and inhibited the ATPase activity (25). This suggested that a tyrosyl residue of the β subunit is located within or very close to the active site of ATPase.

On the other hand, the chemical modification of membrane-bound CF_1 was confronted with difficulties since many different proteins are present in thylakoid membrane, therefore, the CF_1-specific modifier has been required. Successful modifier of membrane-bound CF_1 was NEM which modified the SH group of the γ subunit in the light (26) and inhibited photophosphorylation by 50% (27). Although the results suggested that the conformation of CF_1 would be changed by energization (28) of thylakoid membrane (by illumination or acid-base transition (29)), they did not give any information of the active site for ATP synthesis.

Pyridoxal phosphate (PLP) has been used for several enzymes interacting with phosphate-containing ligands (30,31) in order to detect lysyl residue(s) in the active site or its vicinity.

In this paper, the results of PLP modification of both membrane-bound and isolated CF_1 are described and possible changes in the subunit architecture of CF_1 by detachment from the membrane and by an addition of divalent cation are postulated.

II EXPERIMENTAL

II-1 Materials

Spinach chloroplasts were prepared according to the method of Mukohata et al. (32) (Diagram 1).

Chlorophyll was determined according to the method of Arnon (33) (Diagram 2).

Chloroplast coupling factor 1 (CF_1) was prepared according to the method of Lien and Racker (34) with slight modification (sucrose density gradient centrifugation was omitted) (Diagrams 3A, B and C).

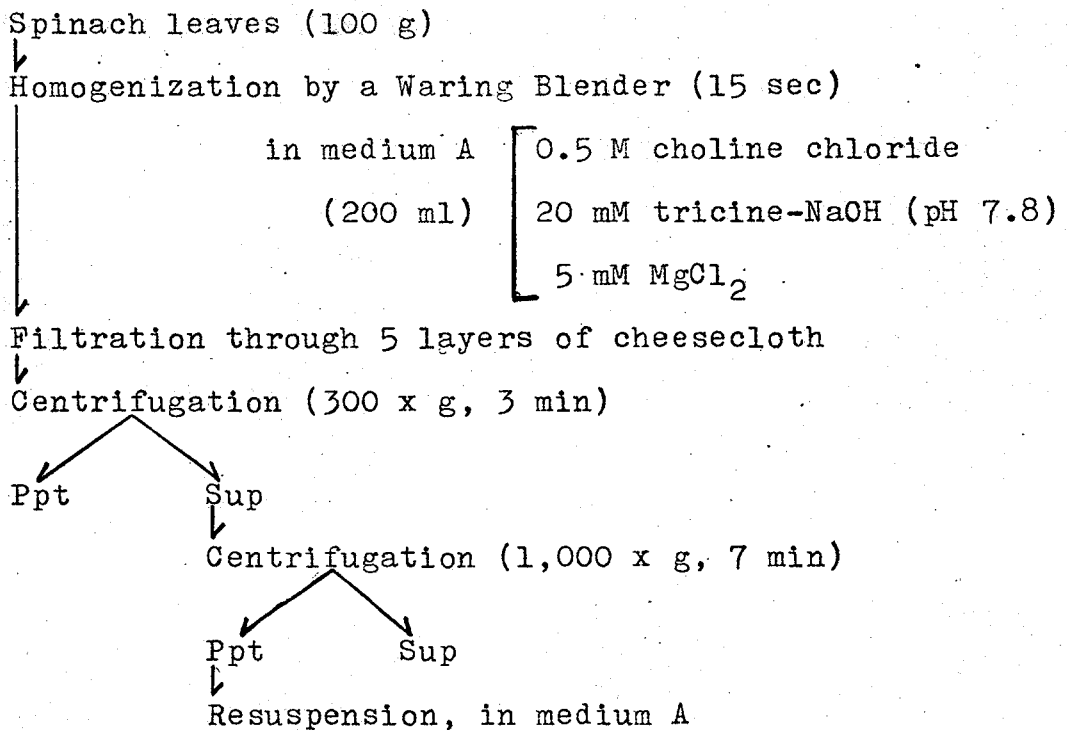
[^{3}H] labeled pyridoxal phosphate was synthesized according to the method of Stock et al. (35) (Diagram 4). Fig. 1 shows the elution profiles of Dowex 1 x 4 column chromatography of an oxidized PLP mixture. The eluate was monitored by absorbance at 330 nm for pyridoxine phosphate and PLP and at 390 nm for PLP (not for pyridoxine phosphate). The first peak was thus assigned for pyridoxine phosphate and the second one for PLP. The profiles of radioactivity and the absorbance of PLP only partly overlapped, which had been reported and explained (36) that [^{3}H] PLP differed from cold PLP in the migration rate through this column but other chemical and biological properties of [^{3}H] PLP were same as PLP. Therefore, the fractions of 20-27 were pooled and condensed by evaporation. The residue was then dissolved in a minimum volume of 10 mM tricine-NaOH (pH 7.5), frozed and stored at

-20 °C. The concentration of PLP was determined by the absorbance at 388 nm with the molar extinction coefficient of 6,600 M^{-1} cm^{-1} in 0.1 N NaOH (37). Specific radioactivity of synthesized [^{3}H] PLP was 5 x 10^{7} cpm/umole.

Reagents: ADP, ATP, dithiothreitol (DTT), pyridoxal phosphate (PLP), tris(hydroxymethyl)methyl-glycine (tricine) were purchased from Sigma Chemical Co.. Bovine serum albumin (fatty acid free) was purchased from Miles Laboratory Inc.. Trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone) was purchased from Worthington Biochemical Co.. [^{3}H] $NaBH_4$ and [^{32}P] Pi were purchased from New England Nuclear.

All other chemicals were reagent grade and used without further purification.

Diagram 1 Preparation of Spinach Chloroplasts



All operations were carried out in a cold room at 4 °C.

Diagram 2 Determination of Chlorophyll Content

Chloroplast Suspension (0.1 ml)

↓

Mixing into 80% aqueous acetone (20 ml)

↓

Filtration through a filter paper (Toyo No 3)

↓

Spectrophotometry at 652 nm (OD_{652})

Chlorophyll concentration (mg/ml) = $5.8 \times OD_{652}$

Diagram 3A Preparation of Crude Extract of CF_1

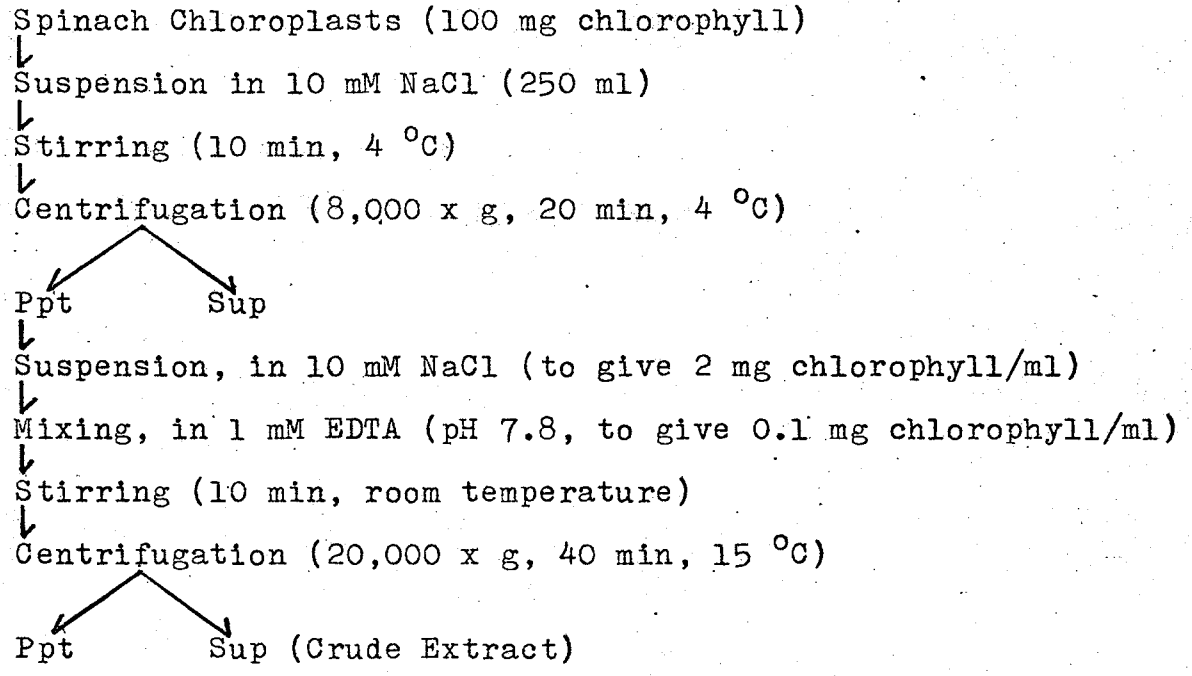


Diagram 3B Partial Purification of CF_1

Crude Extract (1 liter)

↓

Addition of 1 M Tris-SO_4 (pH 7.1) (20 ml), 50 mM ATP (2 ml)

$(NH_4)_2SO_4$ (10.56 g)

↓

Applying to DEAE-Sephadex A-50 (3 x 13 cm) column

equilibrated with medium A [20 mM Tris-SO_4 (pH 7.1)
1 mM EDTA
0.1 mM ATP
80 mM $(NH_4)_2SO_4$]

↓

Washing

with medium B (200 ml) [20 mM Tris-SO_4 (pH 7.1)
1 mM EDTA
0.1 mM ATP
0.1 M $(NH_4)_2SO_4$]

↓

Elution (9 ml fraction)

with medium C (200 ml) [20 mM Tris-SO_4 (pH 7.1)
1 mM EDTA
0.1 mM ATP
0.28 M $(NH_4)_2SO_4$]

↓

Addition of saturated $(NH_4)_2SO_4$ (9 ml)

↓

Centrifugation (10,000 x g, 10 min, 4 °C)

Sup Ppt

Dissolution

in medium D [20 mM Tris-SO_4 (pH 7.1)
1 mM EDTA]

↓

Desalting by passing through Sephadex G-25 (2 x 15 cm)

↓

Crude CF_1

Diagram 3C Purification of CF_1

Crude CF_1

↓

Addition of $(NH_4)_2SO_4$ (final 80 mM)

↓

Applying to DEAE Sephadex A-50 (2 x 15 cm) column equilibrated with medium A

↓

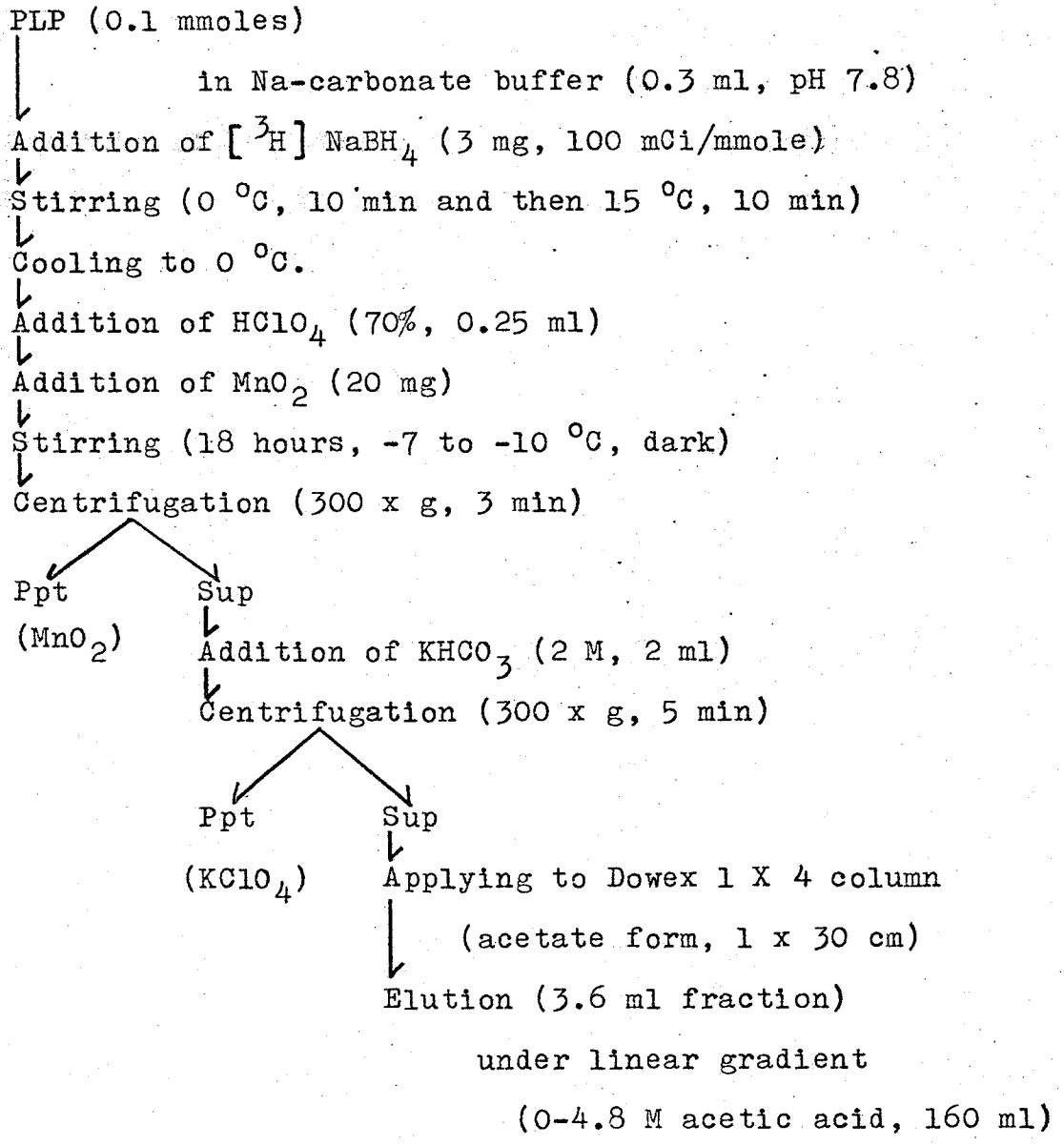
Washing with medium E [20 mM Tris-SO_4 (pH 7.1), 1 mM EDTA, 0.1 mM ATP, 0.12 M $(NH_4)_2SO_4$]

↓

Elution (5 ml fraction) under linear gradient 0.12-0.3 M $(NH_4)_2SO_4$ (400 ml)

Column chromatography in Diagrams 3B and C was carried out at room temperature. Fractions with the fluorescence emission ratio (305 nm : 340 nm, by 280 nm excitation) greater than 1.8 (12) were pooled and stocked as ammonium sulfate precipitate (50% saturation) in a medium containing 20 mM Tris-SO_4 (pH 7.1), 1 mM EDTA and 1 mM ATP at 4 °C.

Diagram 4 Synthesis of 4'-[^{3}H]-Pyridoxal-5'-phosphate (PLP)



Column chromatography was carried out in the dark at room temperature.

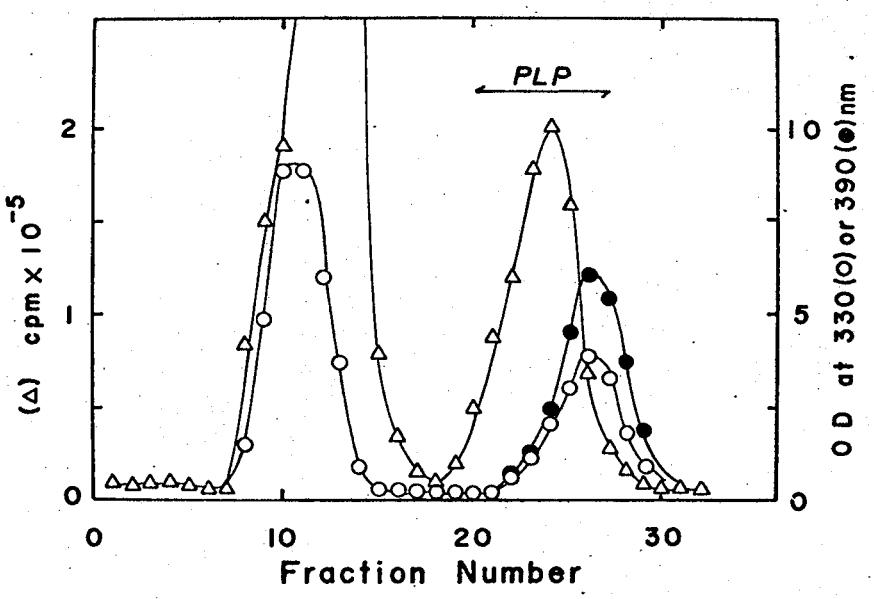


Fig. 1. Separation of [^{3}H] PLP from [^{3}H] Pyridoxine phosphate in a Dowex 1 x 4 column. Chromatography was carried out by the method in Diagram 4. Absorbance at 330 nm (O) or 390 nm (●) was determined after 60 fold dilution of the eluate with 0.1 M sodium phosphate buffer (pH 7.8). Relative radioactivity (Δ) was measured in Bray's cocktail.

II-2 Methods

Protein was determined according to the method of Lowry at al. (37) (Diagram 5) with bovine serum albumin (fatty acid free) as standard. The concentration of CF_1 was determined by using a value of 325,000 (5) for the molecular weight of CF_1.

Modification of chloroplasts and isolated CF_1 by PLP was carried out by the method shown in Diagrams 6 and 7, respectively.

Polyacrylamide disc gel electrophoresis in the presence of SDS (SDS-PAGE) was carried out according to the method of Weber and Osborn (16) (Diagram 8).

The distribution of [^{3}H] PLP among the subunits of membrane-bound and isolated CF_1 was determined by the procedure in Diagrams 9 and 10, respectively.

The circular dichroism (CD) spectra of CF_1 and PLP modified CF_1 were measured with scan speed of 2 nm/min and instrument time constant of 4 sec at 25 ^{o}C by a Jasco spectropolarimeter (model J-20) with a CD attachment. The optical path length used was 0.5 cm for a region between 250-300 nm and 0.1 cm for 205-250 nm. The mean residue ellipticity $[\theta]$ was calculated from the relation of

$$[\theta] = \frac{100 \times \theta}{C \times L}$$

where θ is the observed ellipticity in degrees, C is the mean

residue molar concentration of CF_1 and L is the optical path length in centimeter. Mean residue weight of CF_1 used was 108.

Difference spectra of CF_1-PLP complex were measured by Cary 118 spectrophotometer at 25 °C. Two pairs of matched quartz cells of 1 cm path length were used. In the sample beam side, the first cell contained 2.7 μM CF_1, 20 mM tricine-NaOH (pH 8.0), 1 mM EDTA and 150 μM PLP and the second cell only buffer, whereas in the reference beam side, the first cell contained CF_1 and the second cell PLP. The difference spectra were measured before and after addition of PLP to allow for appropriate base-line correction. $MgCl_2$ and ATP were added as required.

Diagram 5 Determination of Protein Concentration

Protein Solution (0.5 ml)

↓

Mixing, in medium A (3 ml)
- 0.01% $CuSO_4$
- 0.02% sodium citrate
- 2% Na_2CO_3
- 0.01 N NaOH

↓

Incubation (10 min, at room temperature)

↓

Addition of phenol reagent (0.3 ml)

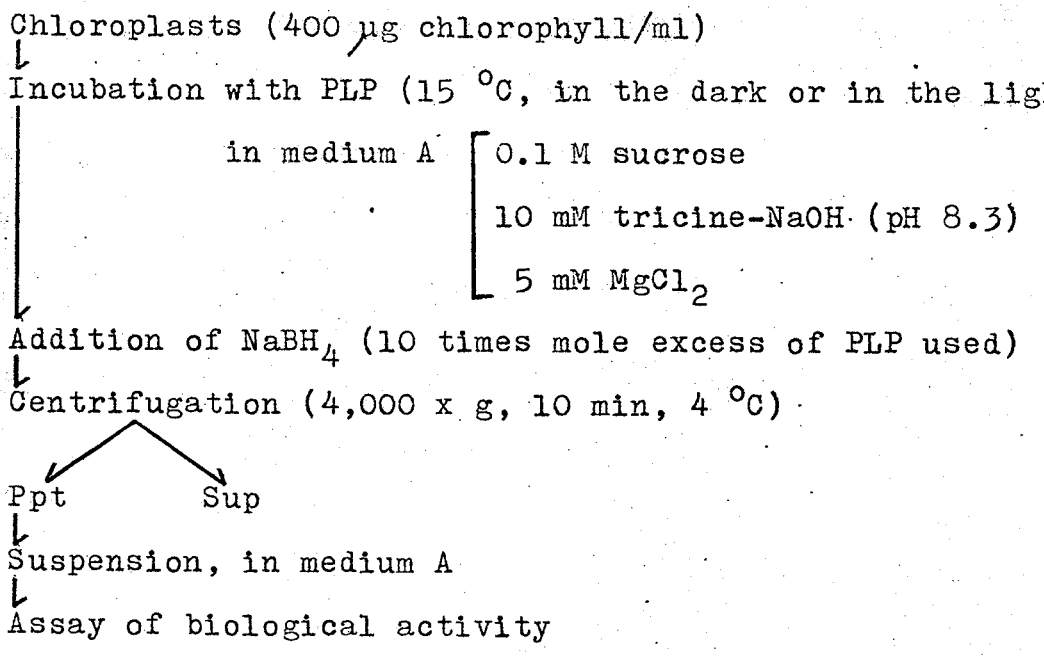
↓

Incubation (30 min, at room temperature)

↓

Spectrophotometry (500 nm or 750 nm)

Diagram 6 PLP Modification of Chloroplasts



In the case of PLP modification in the light, the incubation mixture was illuminated with a 750 W slide projector

Diagram 7 PLP Modification of Isolated CF_1

Isolated CF_1 (200-300 μg/ml)

↓

Incubation with PLP (15 °C, in the dark)

in medium A [20 mM tricine-NaOH (pH 8.0)
1 mM EDTA

↓

Addition of $NaBH_4$ (50 times mole excess of PLP used)

↓

Gel Filtration (Sephadex G-25, 1 x 13 cm)

1) Assay of heat-activated Ca^{2+}-ATPase; see Diagram 13

2) Determination of PLP/CF_1

The mole ratio of bonded PLP to CF_1 (mole PLP/mole CF_1) was determined with the molar extinction coefficient of 1×10^4 for N^{ϵ}-phosphopyridoxyl lysine (39).

Diagram 8 Polyacrylamide Gel Electrophoresis in the presence of Sodium Dodecyl Sulfate (SDS-PAGE)

Protein Solution (1-2 mg/ml, 0.1 ml)
in medium A [0.1 M Tris-acetate (pH 9)
1 mM EDTA
2% SDS

↓

Addition
medium B (0.1 ml) [50% glycerol
20 mM sodium phosphate (pH 7.0)
2% SDS
0.05% Brome phenol blue

↓

Applying on a polyacrylamide gel
10-50 μl, gel tube (0.6 x 8 cm)

↓

Electrophoresis (5 mA/tube, 3 hours)

↓

Staining in medium C (45 °C, 1 hour) [50% methanol
15% acetic acid
0.2% Coomassie brilliant blue

↓

Destaining in medium D (45 °C, 20 hours) [8% methanol
12% acetic acid

Polyacrylamide gel (10%) was composed of 10% acrylamide, 2.7% N,N'-methylenebisacrylamide, 0.15% ammonium persulfate, 50 mM sodium phosphate (pH 7.0), 0.15% N,N,N',N'-tetramethylenediamine and 0.1% SDS.

Diagram 9 Determination of PLP Distribution among Membrane-bound CF_1 Subunits

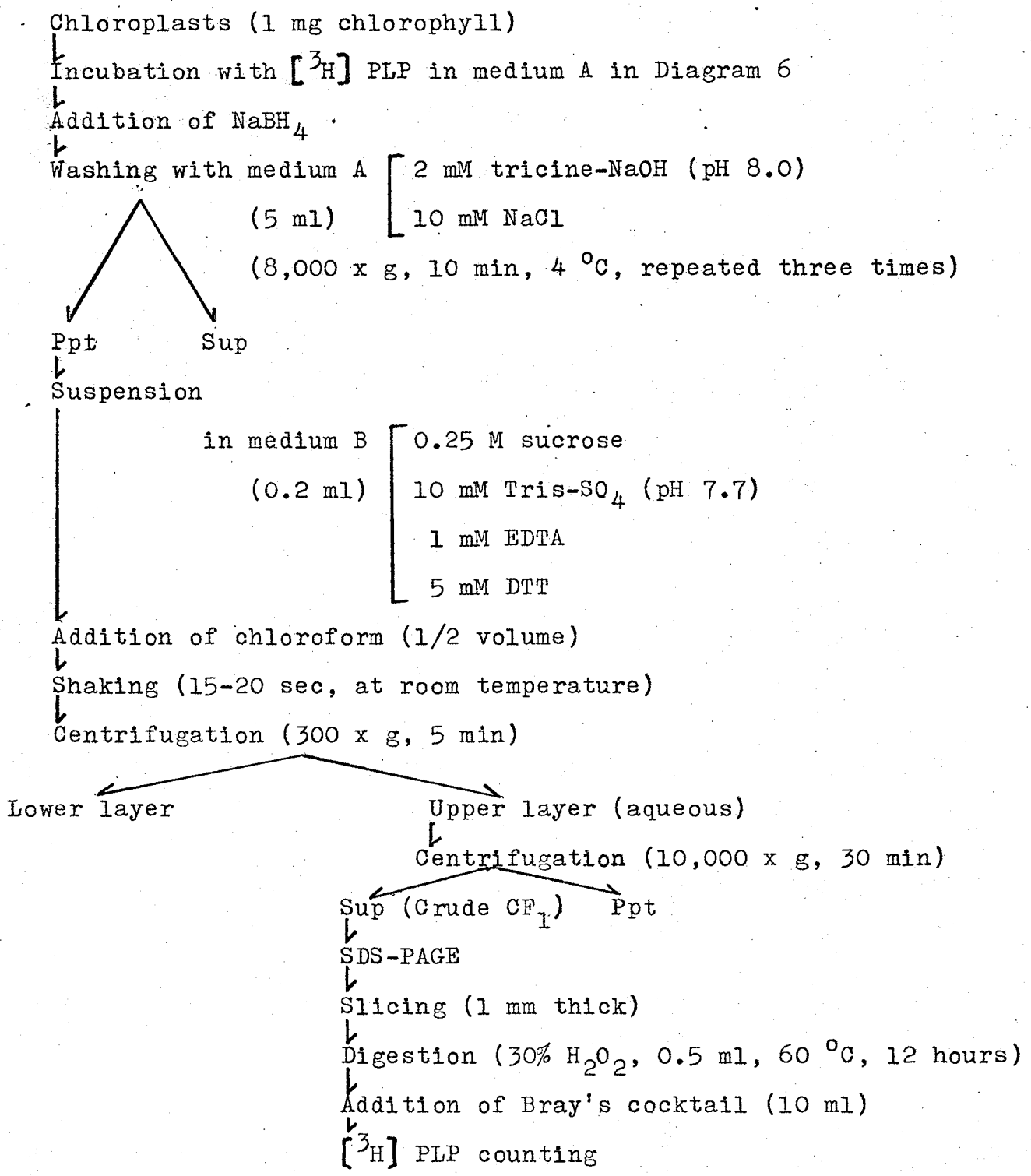


Diagram 10 Determination of PLP Distribution among Isolated CF_1 Subunits

Isolated CF_1 (300 μg/ml)
↓
Incubation with [^{3}H] PLP in medium A (0.5 ml) in Diagram 7
↓
Addition of $NaBH_4$.
↓
Washing with 5% TCA (5 ml, 1,000 x g, 5 min, three times)
↓
Washing with acetone (5 ml, 1,000 x g, 5 min)
↓
Solubilization
with medium A (0.1 ml) [0.1 M Tris-acetate (pH 9.0), 1 mM EDTA, 2% SDS]
↓
SDS-PAGE
↓
Slicing (1 mm thick)
↓
Digestion (30% H_2O_2, 0.5 ml, 60 °C, 12 hours)
↓
Addition of Bray's cocktail (40) (10 ml)
↓
[^{3}H] PLP counting

Bray's cocktail contains 100 g naphthalene, 6 g 2,5-diphenyloxazole, 0.275 g 2,2'-p-phenylene-bis(5-phenyl-oxazole) in 1 liter of dioxane.

---Measurements of Biological Activities---

Fecy reduction of chloroplasts was assayed by the method in Diagram 11.

The activities of other electron transport systems (H_2O-DMQ, H_2O-MV) were measured polarographically by an oxygen electrode (Field oxygen analyzer, Beckman) at 15 °C. The rates of electron transport were determined from the recorded slopes of oxygen evolution for H_2O-DMQ and oxygen uptake for H_2O-MV. In the latter case, reduced MV is auto-oxidizable and thus oxygen is taken up when the reaction is carried out under aerobic condition (Mehler reaction (41)). The reaction mixture contained 0.1 M sucrose, 10 mM tricine-NaOH (pH 8.3), 5 mM $MgCl_2$ and chloroplasts equivalent to 125 μg chlorophyll and additional 500 μM DMQ or 50 μM MV in a final volume of 5.75 ml. ATP was added as required.

The light-induced proton uptake (42) of chloroplasts was measured in a medium containing 50 mM NaCl, 1 mM tricine, 5 mM $MgCl_2$, 20 μM PMS and chloroplasts equilvalent to 700 μg chlorophyll. ATP was added as required. The measurements were run in a water-jaketted cell at 15 °C. Actinic light was provided by a 750 W slide projector with a cut-off filter at 580 nm to eliminate the light of shorter wavelength on Ag-AgCl electrodes in the combination pH electrode used. The pH changes were continuously recorded by a combination of a pH meter (Hitachi-Horiba, F-7) and a recorder (Toa

Electric Co., EPR 10A).

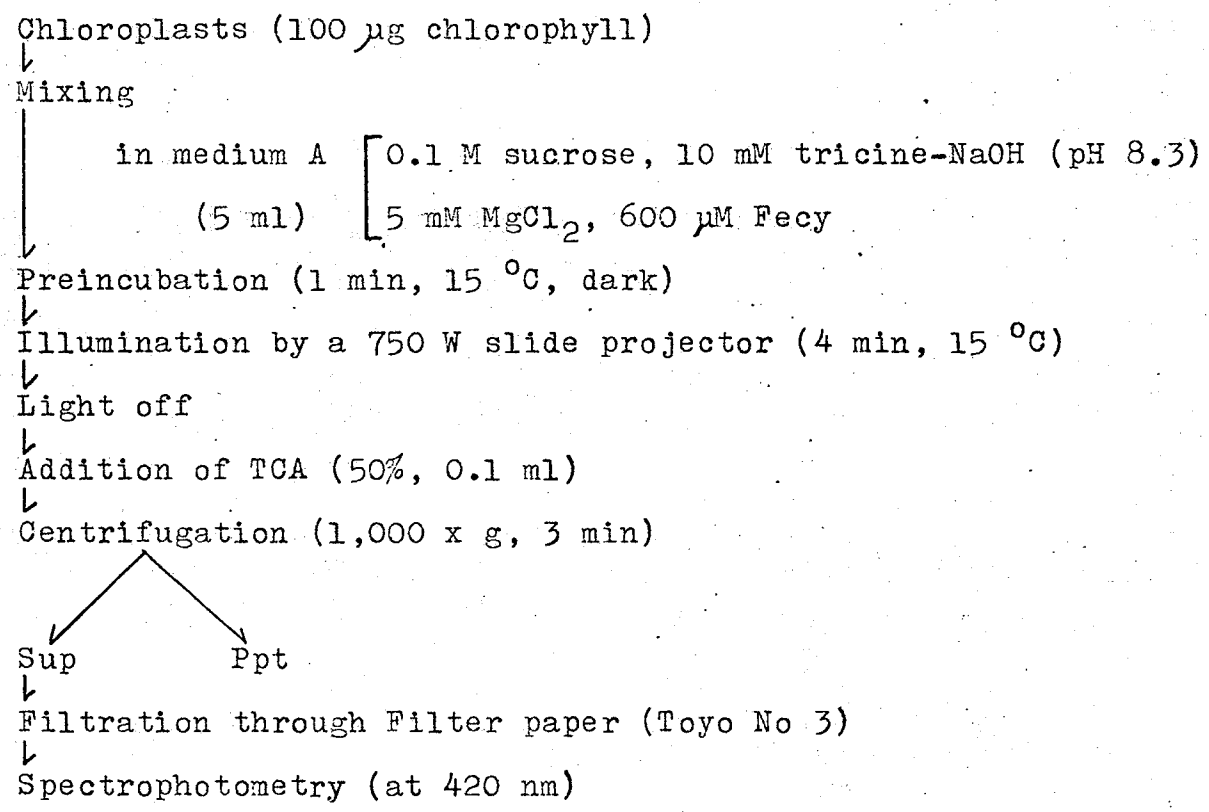
The activity of photophosphorylation was measured according to the method of Asada et al. (43) with slight modification (Diagram 12).

Light-DTT-activated Mg^{2+}-ATPase and trypsin-activated Ca^{2+}-ATPase of chloroplasts were assayed according to the method of Datta et al. (9) (Diagrams 13A and B).

Heat-activated Ca^{2+}-ATPase of isolated CF_1 was assayed by the method of Farron and Racker (23) (Diagram 13C).

For the ATPase assays liberated inorganic phosphate in the final supernatant (Diagrams 13A, B and C) was determined by the method of Taussky and Shorr (44) (Diagram 13D).

Diagram 11 Fecy Reduction



The amounts of Fecy reduced were determined by using a molar extinction coefficient of 1,060 M^{-1} cm^{-1} for Fecy, before and after the illumination. ADP, ATP and Pi (or arsenate) were added to medium A as required.

Diagram 12 Photophosphorylation

Chloroplasts (40 µg chlorophyll)

↓

Mixing

in medium A (2 ml) [
- 0.1 M sucrose, 10 mM tricine-NaOH (pH 8.3)
- 5 mM $MgCl_2$, 600 µM Fecy or 20 µM PMS
- 0.5 mM ADP, 1 mM [^{32}P] Pi

↓

Preincubation (1 min, 15 °C, dark)

↓

Illumination by a 750 W slide projector (4 min, 15 °C)

↓

Light off

↓

Addition of [
- PCA (10%, 5 ml), acetone (2 ml), aqueous ammonium molybdate (10%, 1 ml), 1-butanol-benzene (4 ml) (water saturated, 1:1, V/V)

↓

Shaking by a vertical mixer (30 sec)

↓ Lower layer (aqueous) → Upper layer (organic)

↓

Addition of [
- cold Pi (0.1 M, 0.1 ml), ammonium molybdate (10%, 0.1 ml), butanol-benzene (4 ml)

↓

Shaking (repeated two times)

↓ Lower layer (5 ml) → Upper layer

↓

Addition of water (5 ml)

↓

Addition of aqueous ammonia (28%, 2 ml)

↓

[^{32}P] counting (Cerenkov radiation)

Diagram 13 ATPase Assay

A) Light-DTT activated Mg^{2+}-ATPase

Chloroplasts (20 µg chlorophyll)

↓

Illumination by a 750 W slide projector (10 min, 15 °C)

in medium A (0.2 ml):
- 50 mM Tris-Cl (pH 8.0)
- 50 mM NaCl
- 5 mM $MgCl_2$
- 5 mM DTT
- 20 µM PMS

↓

Mixing

in medium B (1.0 ml):
- 50 mM Tris-Cl (pH 8.0)
- 5 mM $MgCl_2$
- 5 mM ATP

↓

Incubation (37 °C, 10 min, dark)

↓

Addition of TCA (15%, 3 ml)

↓

Centrifugation (1,000 x g, 3 min)

↙ ↘

Ppt Sup I

B) Trypsin-activated Ca^{2+}-ATPase of Chloroplasts

Chloroplasts (10 µg chlorophyll)

↓

Incubation (10 min, 20 °C)

in medium C (0.15 ml):
- 20 mM Tris-Cl (pH 8.0)
- 2 mM EDTA
- 2 mM ATP
- 100 µg trypsin

↓

Addition of bovine serum albumin (1%, 50 µl)

↓

Mixing

in medium D (1 ml):
- 50 mM Tris-Cl (pH 8.0)
- 5 mM $CaCl_2$
- 6 mM ATP

↓

Incubation (37 °C, 10 min)

↓

Addition of TCA (15%, 3 ml)

↓

Centrifugation (1,000 x g, 3 min)

↙ ↘

Ppt Sup II

C) Heat-activated Ca^{2+}-ATPase of isolated CF_1

CF_1 (3-5 μg)

↓

Heating (62 °C, 2.5 min)

in medium E (50 μl):
- 20 mM tricine-NaOH (pH 8.5)
- 1 mM EDTA
- 25 mM ATP
- 2 mM DTT

↓

Mixing

in medium F (0.45 ml):
- 20 mM tricine-NaOH (pH 8.5)
- 10 mM $CaCl_2$
- 10 mM ATP

↓

Incubation (37 °C, 10 min)

↓

Addition of TCA (50%, 0.1 ml)

↓

Centrifugation (1,000 x g, 3 min)

↙ Ppt ↘ Sup III

D) Determination of Liberated Inorganic Phosphate

Supernatant (3 ml of Sup I, II, 0.4 ml of Sup III)

↓

Mixing

in medium G

(2 ml for Sup I, II, 3 ml for Sup III)

- 1% ammonium molybdate
- 5% $FeSO_4$ 7 H_2O
- 1 N H_2SO_4

↓

Incubation (10 min, at room temperature)

↓

Spectrophotometry (at 660 nm)

The standard curve was obtained by the same procedure except that varying concentrations of inorganic phosphate were added into medium B, D or F instead of ATP and enzymes.

III RESULTS

III-1 PLP Modification of Chloroplasts

i) Effects of PLP modification on the activities of chloroplasts

Chloroplasts were incubated with an indicated concentration of PLP for 10 min in the dark or in the light and collected by centrifugation after the addition of $NaBH_4$, then assayed for electron transport and phosphorylation.

Fig. 2A shows that PLP modification of chloroplasts in the dark depressed the activity of Fecy reduction coupled to phosphorylation (in the presence of ADP and Pi) in parallel with the inhibition of phosphorylation, but gave no effect on the basal activity of Fecy reduction (in the presence of ATP and Pi). Fig. 2B shows that PLP modification of chloroplasts in the light (5×10^4 lux) stimulated the basal activity of Fecy reduction (ATP + Pi) and inhibited phosphorylation.

When the electron transport activity with other electron acceptors was tested, the results were identical to those of Fecy reduction (Table 1). Table 1 also shows that the oxygen evolution was not inhibited by PLP modification.

The extent of the light-induced proton uptake and its stimulation by ATP were not affected by PLP modification in the dark but slightly depressed by the modification in the light (Table 2).

Membrane-bound CF_1 is latent ATPase which is activated as Mg^{2+}-ATPase by the illumination in the presence of DTT or as Ca^{2+}-ATPase by the trypsin digestion.

Fig. 3 shows that the activities of Mg^{2+}-ATPase and Ca^{2+}-ATPase of chloroplasts modified with an indicated concentration of PLP in the dark for 10 min were decreased with an increase of PLP concentration. Mg^{2+}-ATPase was inactivated at lower concentrations of PLP than Ca^{2+}-ATPase. The degree of Mg^{2+}-ATPase inactivation was seemingly larger than that of Ca^{2+}-ATPase.

ii) Effect of adenine nucleotide on the PLP modification of chloroplasts

Fig. 4 shows that ATP (at 400 μM) in the incubation mixture decreased the rate of phosphorylation inactivation due to PLP modification in the dark. ADP showed similar effect (data not shown). In the PLP modification in the light, ATP showed similar effect.

Fig. 5 shows that low concentrations of ATP (e.g., at 20 μM) in the incubation mixture depressed the stimulation of basal Fecy reduction (ATP + Pi) caused by PLP modification in the light, while ATP at the same concentration did not protect chloroplasts from inactivation of phosphorylation so largely.

Table 3 shows that ATP could be replaced by PPi, AMP and ADP but not by Pi or arsenate.

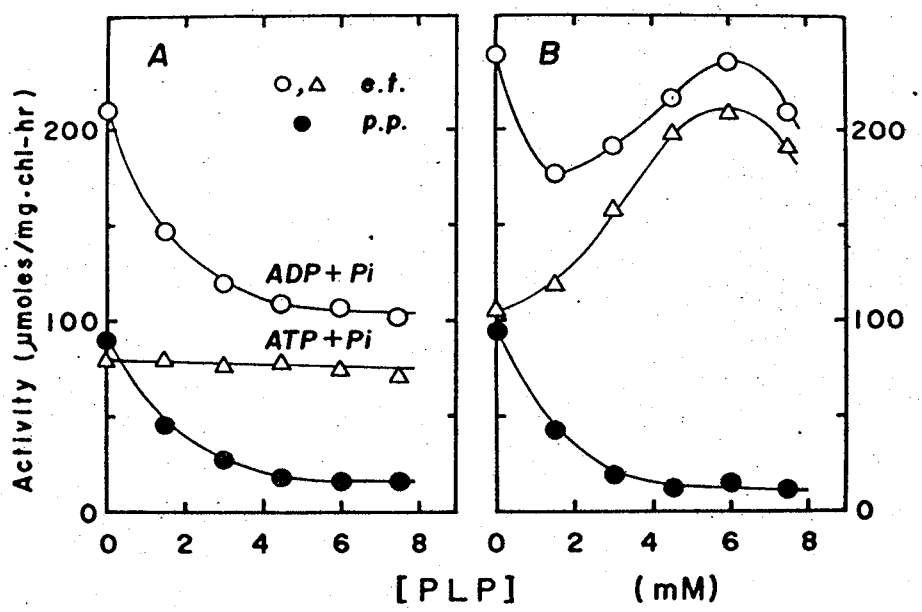


Fig. 2. Effects of PLP modification on the photosynthetic activities in chloroplast. Chloroplasts were modified with an indicated concentration of PLP for 10 min by the method in Diagram 6; A, in the dark or B, in the light. Fecy reduction (e.t.) was measured in the presence of 500 μM ADP and 1 mM Pi (○) or 100 μM ATP and 1 mM Pi (△). Phosphorylation (P.P.) coupled with Fecy reduction was measured in the presence of 500 μM ADP and 1 mM [^{32}P] Pi (●).

TABLE 1

Effects of the PLP Modification on the Activities of Electron Transport

Chloroplasts were modified with 4 mM PLP in the dark or in the light for 10 min. The electron transport activities were measured in the presence or absence of 100 μM ATP.

MODIFICATION	ELECTRON TRANSPORT[a]			
	H_2O--DMQ		H_2O--MV	
	no addition	ATP + Pi	no addition	ATP + Pi
control, light	159	113	40	26
4 mM PLP, light	138	141	62	59
control, dark	131	115	39	21
4 mM PLP, dark	120	81	41	28

a: μmoles O_2 liberated or consumed/mg·chl-hr

TABLE 2

Effects of the PLP Modification on the Light-induced Proton Uptake and Cyclic Phosphorylation

Chloroplasts were modified with 4 mM PLP in the dark or in the light for 10 min. Light-induced proton uptake by chloroplasts was measured at initial pH 7.8 in the presence or absence of 110 μM ATP. Cyclic photophosphorylation was measured with 500 μM ADP and 1 mM [^{32}P] Pi in the presence of 20 μM PMS.

MODIFICATION	ACTIVITY		
	proton uptake[a]		PMS-phospho-rylation[b]
	no addition	+ATP	
control, light	0.52	1.00	366
4 mM PLP, light	0.23	0.35	45
control, dark	0.55	1.00	370
4 mM PLP, dark	0.36	0.81	106

a: μeq. H^+/mg·chl b: μmoles ATP /mg·chl-hr

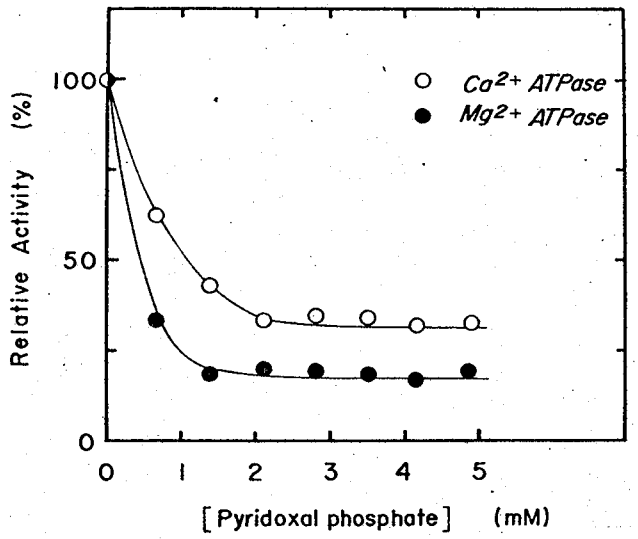


Fig. 3. Effects of PLP modification on the activities of trypsin-activated Ca^{2+}-ATPase (O) and light-DTT-activated Mg^{2+}-ATPase (●). PLP modification of chloroplasts was carried out in the dark with an indicated concentration of PLP. The rates of Ca^{2+}-ATPase and Mg^{2+}-ATPase of the control (100%) were 303 and 481 μmoles Pi liberated/mg chl-hr, respectively.

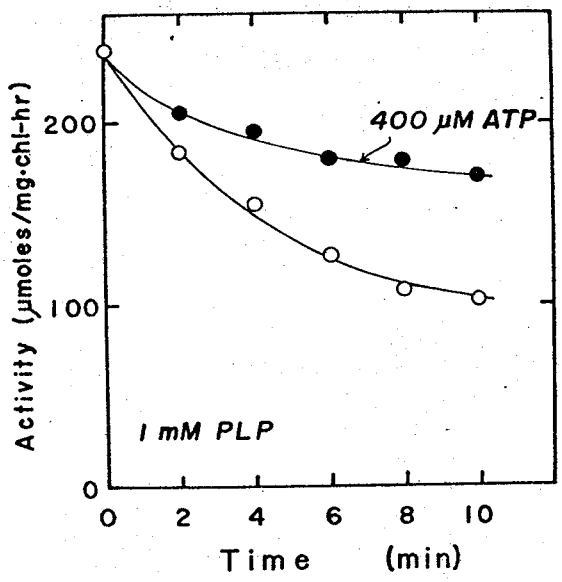


Fig. 4. Time course of phosphorylation inactivation by PLP modification. Chloroplasts were modified with 1 mM PLP for a given time in the dark in the presence (●) or absence (○) of 400 μM ATP. Cyclic phosphorylation was measured as in Table 2.

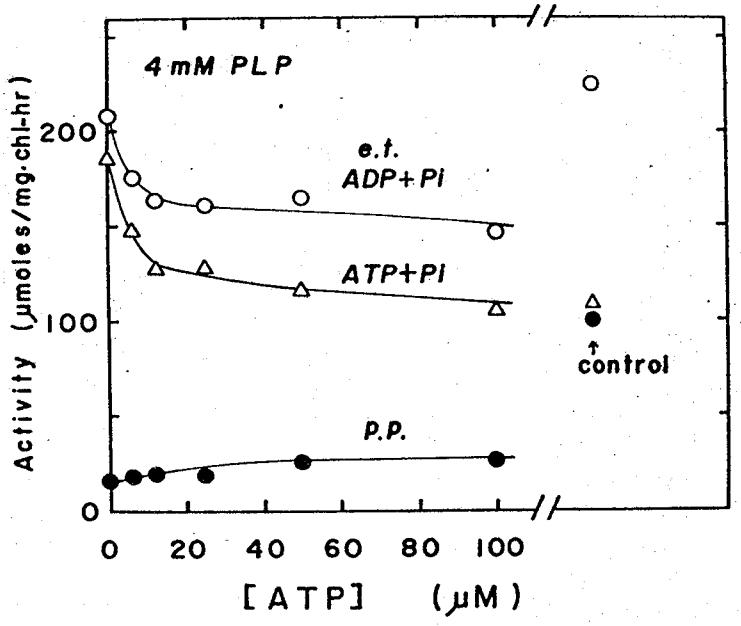


Fig. 5. Protection by ATP from the uncoupling due to the PLP modification in the light. Chloroplasts were modified with 4 mM PLP in the presence of an indicated concentration of ATP for 10 min in the light. Then, basal and coupled Fecy reduction (e.t.) and phosphorylation (P.P.) were measured as in Fig. 2. Control chloroplasts were illuminated for 10 min in the absence of PLP.

TABLE 3

Effects of Adenylates on the Uncoupling due to the PLP Modification in the Light

Chloroplasts were modified with 4 mM PLP in the presence of a given concentration of reagents indicated in the left column. Fecy reduction and phosphorylation were measured as in Fig. 2.

MODIFICATION	REMAINING ACTIVITY		
	Fecy reduction[a]		Fecy phosphorylation[b]
	ATP + Pi	ADP + Pi	
control, light	95	234	110
4 mM PLP, light	155	181	20
+ 2 mM Pi	153	187	22
+ 2 mM As	146	180	20
+ 2 mM PPi	117	161	29
+ 0.2 mM AMP	113	160	32
+ 0.2 mM ADP	103	169	48
+ 0.2 mM ATP	102	173	47

a: μmoles Fecy reduced/mg·chl-hr

b: μmoles ATP formed/mg·chl-hr

iii) Reversibility of the PLP modification

After the incubation of chloroplasts with 4 mM PLP in the dark or in the light for 10 min, lysine was added to the incubation mixture (final concentration of 50 mM), then incubated again for 10 min. Then, the chloroplasts were collected by centrifugation and assayed for the activities.

In the incubation in the dark, an addition of lysine restored the phosphorylation activity up to 94% of the control (Table 4). On the other hand, in the incubation in the light, an addition of lysine restored the phosphorylation activity only up to 40% of the control and did not depress the stimulation of basal Fecy reduction (ATP + Pi) at all.

Table 5 shows that other aldehydes tested so far did not have any effect on the activities of both electron transport and phosphorylation.

TABLE 4

Effects of an Addition of Lysine on the Activities of Chloroplasts Incubated with PLP

Chloroplasts were first incubated with 4 mM PLP for 10 min in the dark or in the light. Then a lysine solution (final concentration of 50 mM) or $NaBH_4$ solution (final concentration of 40 mM) was added to the incubation mixture which was again incubated for 10 min and centrifuged. Fecy reduction and phosphorylation were measured as in Fig. 2.

INCUBATION		REMAINING ACTIVITY		
		Fecy reduction[a]		Fecy phosphorylation[b]
1st	2nd	ATP + Pi	ADP + Pi	
control, dark	no addition	102	275	113
4 mM PLP, dark	+ $NaBH_4$	109	168	33
	+ lysine	83	240	106
control, light	no addition	102	221	98
4 mM PLP, light	+ $NaBH_4$	221	234	11
	+ lysine	185	230	36

a: μmoles Fecy reduced/mg·chl-hr

b: μmoles ATP formed/mg·chl-hr

TABLE 5

Effects of Aldehyde on the Activities of Chloroplasts

Chloroplasts were incubated with pyridoxal (4 mM) or piperonal (3.2 mM) or salicylaldehyde (4 mM) or helicin (6 mM) in the dark or in the light for 10 min and $NaBH_4$ was added. Fecy reduction and phosphorylation were measured as in Fig. 2.

REAGENTS	REMAINING ACTIVITY		
	Fecy reduction[a]		Fecy phosphorylation[b]
	ATP + Pi	ADP + Pi	
Exp. 1			
control	119	253	111
pyridoxal, dark	137	283	124
pyridoxal, light	132	266	107
Exp. 2			
control	102	241	111
piperonal, dark	103	247	105
piperonal, light	109	252	112
salicyl-aldehyde, dark	132	272	118
salicyl-aldehyde, light	120	270	115
Exp. 3			
control	83	221	102
helicin, dark	80	216	101
helicin, light	88	219	93

a: μmoles Fecy reduced/mg·chl-hr

b: μmoles ATP formed/mg·chl-hr

iv) Time courses of the inactivation of phosphorylation and light-DTT-activated Mg^{2+}-ATPase due to PLP modification

Figs. 6A and 7A give semiligarithmic plots of the remaining activity of phosphorylation and Mg^{2+}-ATPase versus the incubation time, showing that the process of inactivation of phosphorylation and Mg^{2+}-ATPase followed pseudo-first order kinetics. From the plots of pseudo-first order rate constant (k') against PLP concentration shown in Figs. 6B and 7B, second order rate constants of the process of phosphorylation and Mg^{2+}-ATPase inactivation were obtained to be 0.75×10^2 and 2.6×10^2 M^{-1} min^{-1}, respectively.

v) Effect of adenine nucleotide on Fecy reduction of PLP-modified chloroplasts

Fig. 8A shows the effect of ATP on Fecy reduction of the chloroplasts modified with 1 mM PLP in the dark. The maximum inhibition level of electron transport by ATP (i.e., the level of basal electron transport) was not changed by the PLP modification in the dark. Fig. 8B is a Hill plot of the data in Fig. 8A, which shows that an apparent Hill constant and an apparent binding constant of ATP were decreased by PLP modification from 1.2 to 0.8 and from 3×10^7 to 4.5×10^4 M^{-1}, respectively.

Fig. 9A shows the rate of Fecy reduction versus ADP concentration under arsenylation conditions (ADP + arsenate) in chloroplasts partially modified by PLP (controlled by the PLP

concentration in the incubation mixture). Double reciprocal plots of the arsenylation-coupled Fecy reduction activity [$V-V_o$, V_o is the basal electron transport (ATP + Pi)] versus ADP concentration (Fig. 9B) reveal that the apparent K_m value (20 μM) for ADP was not changed by PLP modification but the apparent V_{max} value was lowered.

Fig. 10A shows the rate of Fecy reduction against arsenate concentration in the same chloroplasts as in Fig. 7. Double reciprocal plots of coupled electron transport versus arsenate concentration (Fig. 10B) show that the apparent K_m value for arsenate (280 μM) was not changed by PLP modification.

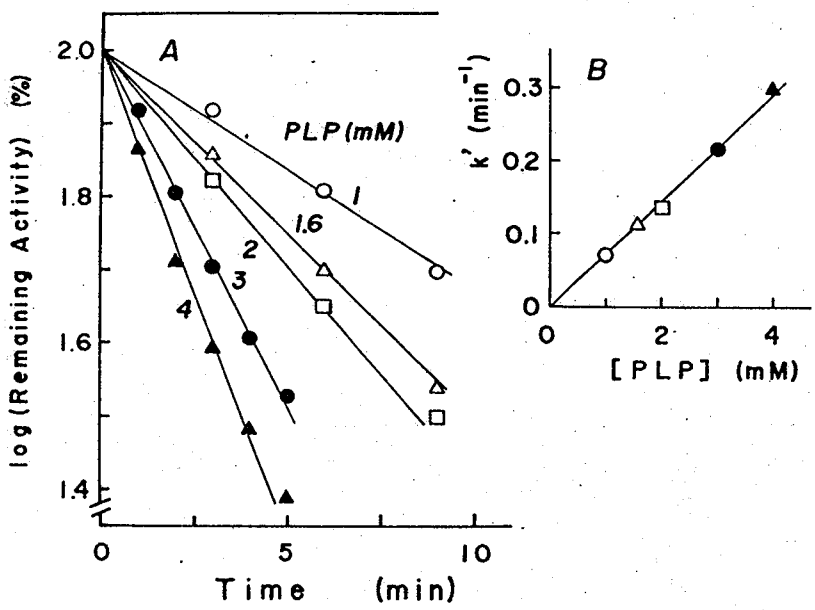


Fig. 6. Inactivation kinetics of the phosphorylation by PLP modification of chloroplasts. (A) Semilogarithmic plots of the remaining activity of PMS-mediated phosphorylation versus incubation time of chloroplasts with an indicated concentration of PLP. The rate of phosphorylation (100%) was 130 μmoles ATP formed/mg·chl-hr. (B) Plots of the rate constants (k') obtained from the slopes in Fig. 6A versus PLP concentration. The slope indicates the second order rate constant.

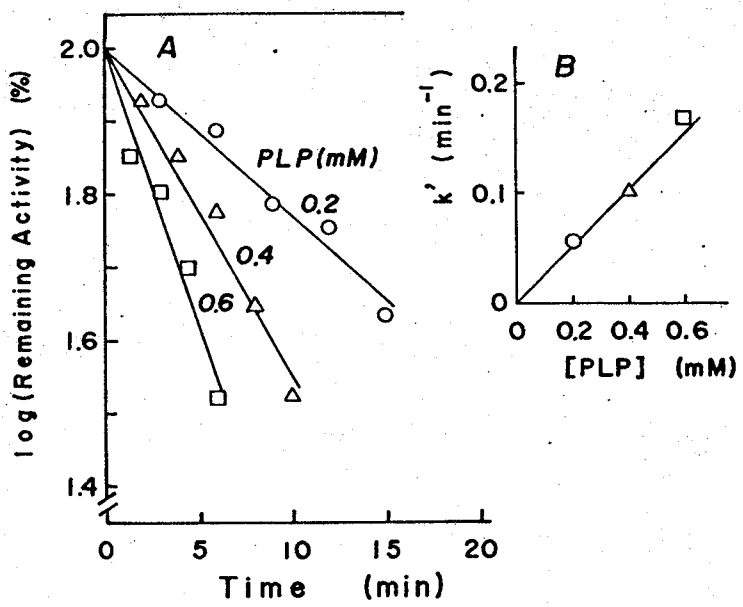


Fig. 7. Inactivation kinetics of the light-DTT-activated Mg^{2+}-ATPase by PLP modification of chloroplasts. (A) Semi-logarithmic plots of the remaining activity of Mg^{2+}-ATPase versus incubation time with an indicated concentration of PLP. The rate of Mg^{2+}-ATPase of the control (100%) was 385 μmoles Pi liberated/mg·chl-hr. (B) Plots of the rate constants (k') obtained from the slopes in Fig. 7A versus PLP concentration.

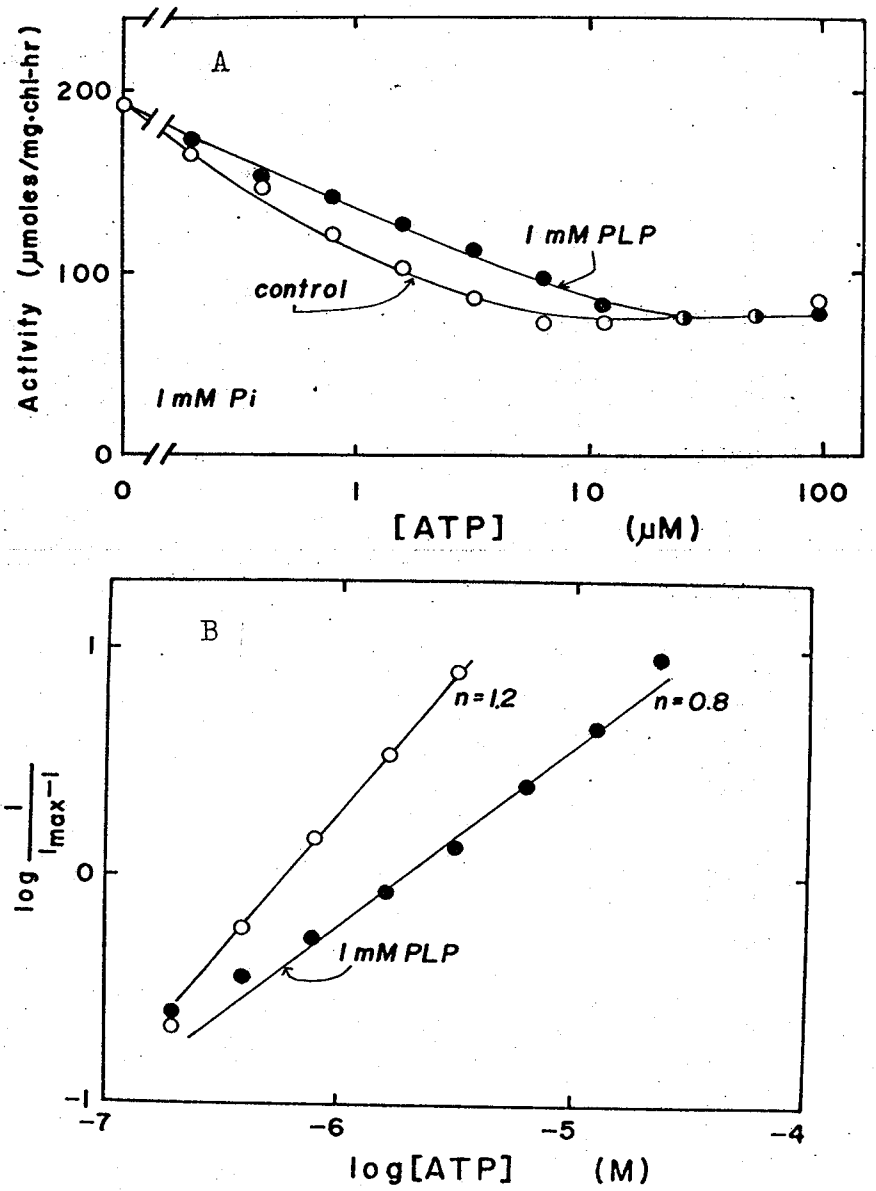


Fig. 8. (A) Effects of PLP modification on the inhibition of Fecy reduction by ATP. Chloroplasts were modified with 1 mM PLP for 10 min in the dark. Then, Fecy reduction was measured in the presence of an indicated concentration of ATP and 1 mM Pi. (B) Apparent Hill plot of the inhibition process of Fecy reduction caused by ATP. I_{max} is set to be the difference in Fecy reduction activity at 0 μM ATP and that at 100 μM ATP. I is the extent of Fecy reduction inhibition from the activity at 0 μM ATP.

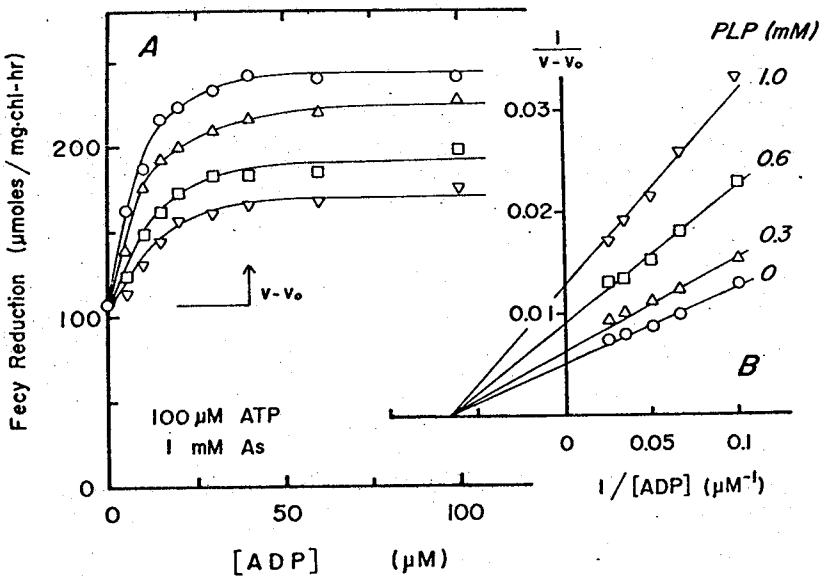


Fig. 9. (A) Effects of PLP modification on the arsenylation-coupled stimulation of Fecy reduction. Chloroplasts were modified for 10 min in the dark with 0.3, 0.6 or 1 mM PLP. (B) Double reciprocal plots of arsenylation-coupled electron transport activity ($V-V_o$) versus ADP concentration. V_o is the activity of Fecy reduction in the presence of 100 μM ATP and 1 mM arsenate (basal electron transport activity).

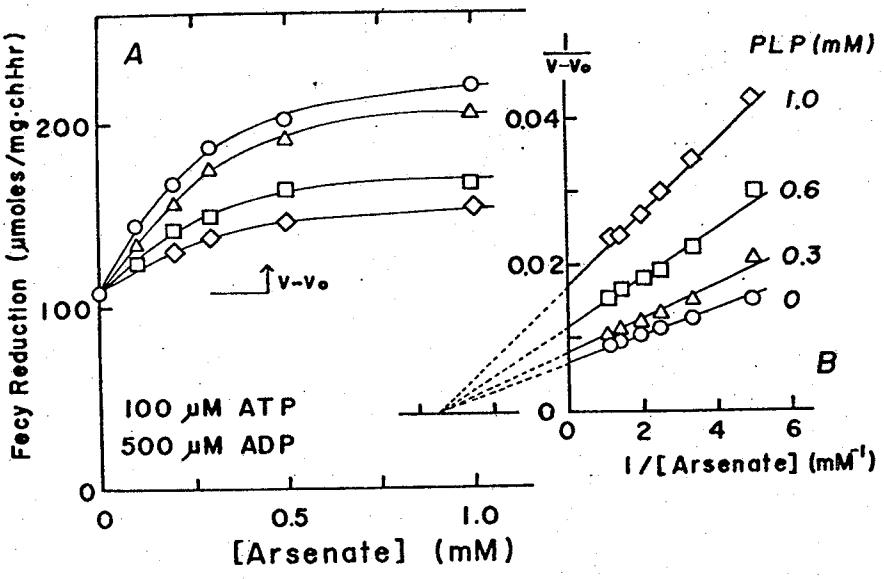


Fig. 10. (A) Dependence of the arsenylation-coupled stimulation of Fecy reduction on the arsenate concentration. Experimental procedures were as in Fig. 9 except that the arsenate concentration was varied instead of ADP. (B) Double reciprocal plots of arsenylation-coupled electron transport activity ($V-V_o$) versus arsenate concentration. V_o is the activity of Fecy reduction in the presence of 100 μM ATP and 500 μM ADP.

vi) Relation between the incorporation of PLP into CF_1 and the inactivation of phosphorylation by PLP modification

Fig. 11 shows the plot of the remaining activity of phosphorylation versus the amounts of PLP incorporated into the membrane-bound CF_1. CF_1 was extracted in 1 mM EDTA from the chloroplasts which had been modified with PLP at an indicated concentration for 10 min in the dark, then purified by passing through a DEAE Sephadex A-50 column.

The inactivation of phosphorylation increased up to 50% in parallel with an increase of the amounts of PLP incorporated into CF_1. By extrapolation from the linear portion, 6 moles of PLP per mole CF_1 were found to be required for complete inactivation of phosphorylation.

vii) Incorporation of [^{3}H] PLP into membrane proteins and CF_1

Table 6 shows that [^{3}H] PLP was incorporated into membrane protein and CF_1 fraction. On the mg protein basis, the CF_1 fraction incorporated [^{3}H] PLP 1.5 times more than the membrane protein fraction.

The amounts of PLP incorporated into both membrane protein and CF_1 fractions were not affected by illumination during modification. Table 6 also shows that incorporation of [^{3}H] PLP into both fractions became negligible by an addition of lysine after incubation of chloroplasts with [^{3}H] PLP,

irrespective of whether incubation was carried out in the dark or in the light.

Fig. 12 shows the distribution of radioactivity among the membrane proteins lacking CF_1. Densitometric trace shows the presence of many peptides with a wide range of molecular weights. Radioactivity was mainly found in the slices which involved proteins with the molecular weight between 28,000 and 18,000. Since the modification of these membrane proteins had no effects on the chloroplast actvities of present interest, further analysis was not made.

Fig. 13 shows the distribution of radioactivity among subunits of CF_1 separated by SDS-PAGE (10% gel). CF_1 was isolated from the chloroplasts modified with [^{3}H] PLP in the presence of 5 mM $MgCl_2$, which inhibited about 50% of the phosphorylation activity. About 3 moles of PLP were found per mole of CF_1. The labeling pattern shows that radioactivity distributed mostly among the α, β and γ subunits with a ratio of $(\alpha + \beta) : \gamma = 2 : 1$ but negligible in the δ and ε subunits.

Fig. 14 shows the distribution of [^{3}H] PLP among the α and β subunits separated by SDS-PAGE on 12% gel. Radioactivity was almost equally found in both subunit fractions.

The distribution pattern of PLP among CF_1 subunits was not affected by whether PLP modification was carried out in the dark or in the light. Furthermore, when chloroplasts were

modified by [^{3}H] PLP in the absence of $MgCl_2$, the total amounts of PLP incorporated into CF_1 were decreased to 70% of those in the presence of 5 mM $MgCl_2$ but the distribution pattern of PLP among CF_1 subunits was not affected by the absence of $MgCl_2$.

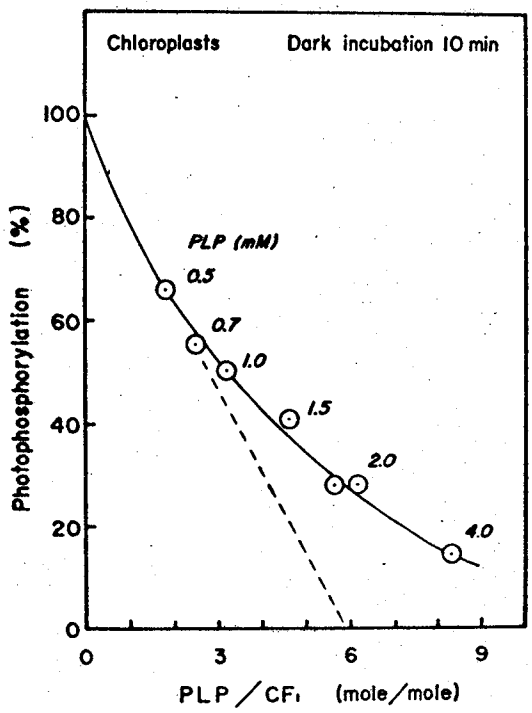


Fig. 11. Relationship between phosphorylation inactivation by the PLP modification and the amounts of PLP incorporated into membrane-bound CF_1. Chloroplasts were modified with an indicated concentration of PLP for 10 min in the dark then phosphorylation was assayed and also CF_1 was isolated to determine the amounts of incorporated PLP.

TABLE 6

Incorporation of [^{3}H] PLP into Chloroplast Membrane Proteins

Chloroplasts were modified with 1 mM [^{3}H] PLP for 10 min in the dark or in the light. CF_1 and membrane protein fractions were separated each other by the method in Diagram 9. Then, the amounts of [^{3}H] PLP incorporation and the protein concentration were determined (A). Chloroplasts were incubated with 1 mM [^{3}H] PLP for 10 min in the dark or in the light, then a lysine solution (final concentration 50 mM) was added, and incubated again for 10 min then centrifuged. The amounts of [^{3}H] PLP incorporated into CF_1 and membrane protein fractions were determined (B).

(A)

FRACTION	PROTEIN	[^{3}H] PLP	
	mg/mg chl	nmol/mg·chl	nmol/mg·protein
membrane protein	1.7	12	7.0
CF_1	0.4	4.5	11.2

(B)

FRACTION	[^{3}H] PLP (nmole/mg protein)			
	dark + $NaBH_4$	dark + lysine	light + $NaBH_4$	light + lysine
membrane protein	7.5	0.4	7.3	0.4
CF_1	10.6	0.3	11	0.3

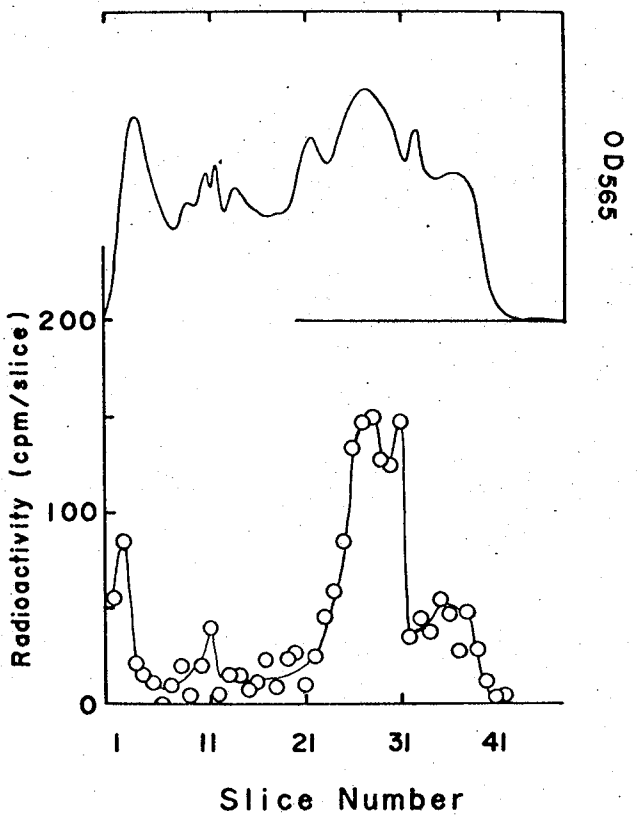


Fig. 12. Labeling of membrane proteins by [^{3}H] PLP. Chloroplasts were modified with 1 mM [^{3}H] PLP for 10 min in the dark. After the removal of CF_1 by chloroform extraction, membrane protein fraction was freed from chlorophylls and lipids by washing with acetone, then solubilized with SDS. An aliquot (50 μl) containing 50 μg protein was applied to 10% polyacrylamide gel for SDS-PAGE.

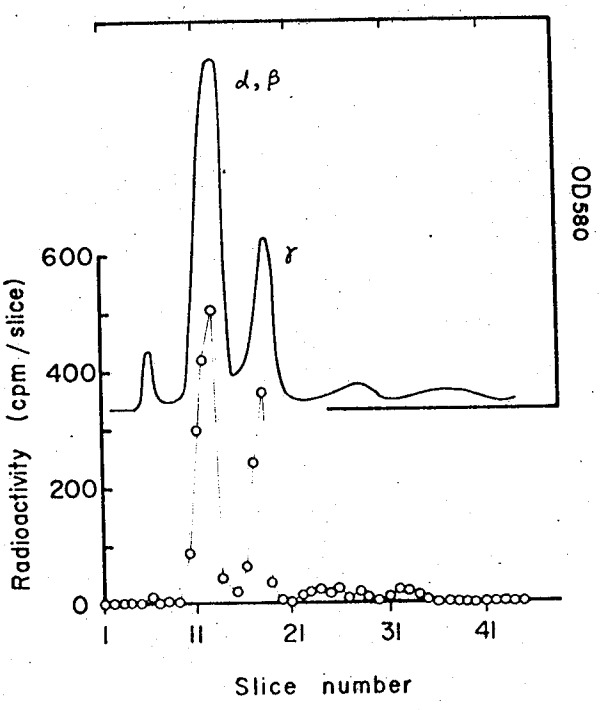


Fig. 13. Labeling of CF_1 subunits by [^{3}H] PLP. CF_1 was isolated from chloroplasts modified with 1 mM [^{3}H] PLP for 10 min in the dark. Distribution of [^{3}H] PLP among CF_1 subunits was determined by the method in Diagram 9. CF_1 solution (40 μl) containing 40 μg protein was applied to 10% polyacrylamide gel for electrophoresis. Subunits α and β ran together and the distribution of radioactivity between them could not be determined under this condition.

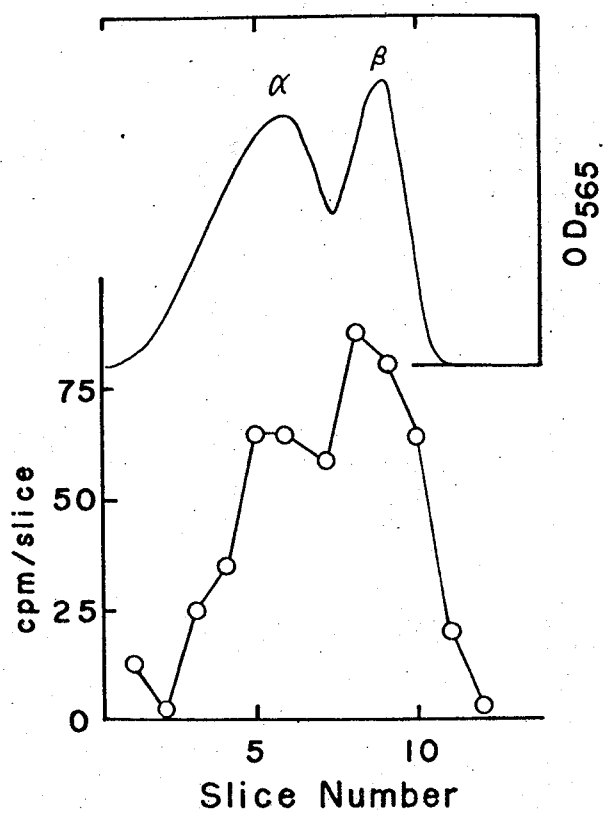


Fig. 14. Distribution of [^{3}H] PLP among the α and β subunits of membrane-bound CF_1. The same CF_1 sample (30 μl) as in Fig. 13 was separated into these subunits by SDS-PAGE with 12% polyacrylamide for 18 hours.

III-2 PLP Modification of Isolated CF_1

i) Time courses of PLP modification of isolated CF_1

CF_1 was incubated with PLP for a given time, modified by PLP through reduction by $NaBH_4$, then thermally activated to be Ca^{2+}-ATPase. Fig. 15 shows the incubation-time dependence of the amounts of bonded PLP and the extent of ATPase inactivation. Both saturate after incubation for longer than 30 min, depending on the concentration of PLP in the incubation mixture.

ii) Divalent cation requirement for the PLP modification

Figs. 16A, B and C show the effects of divalent cation on the PLP modification of CF_1. In the absence of divalent cation, both the degree of ATPase inactivation and the amounts of bonded PLP to CF_1 were very small, which increased depending on the concentration of divalent cation in the incubation mixture. The half maximum effect was obtained at 1 mM $MnCl_2$, 2 mM $MgCl_2$ and 4 mM $CaCl_2$.

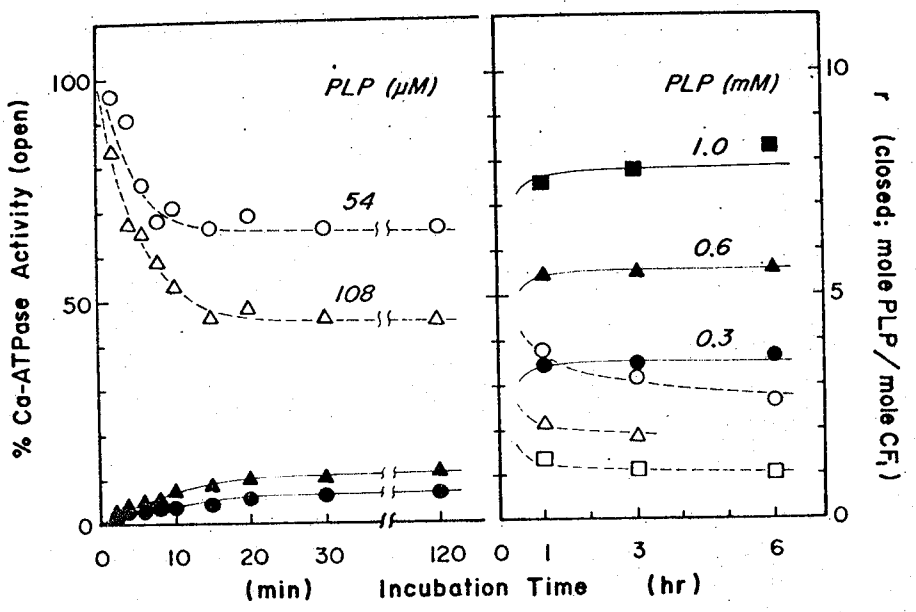


Fig. 15. Incubation time dependence of Ca^{2+}-ATPase activity and the mole ratio of bonded PLP to CF_1 at varying concentrations of PLP. CF_1 was modified with an indicated concentration of PLP in the presence of 10 mM $MgCl_2$. Then, the amounts of PLP bonded to CF_1 and heat-activated Ca^{2+}-ATPase were measured. r is the mole ratio of bonded PLP to CF_1 determined by the absorbance at 325 nm. The Ca^{2+}-ATPase activity of the control {(PLP)=0} was 12.5 μmoles Pi liberated /mg·min.

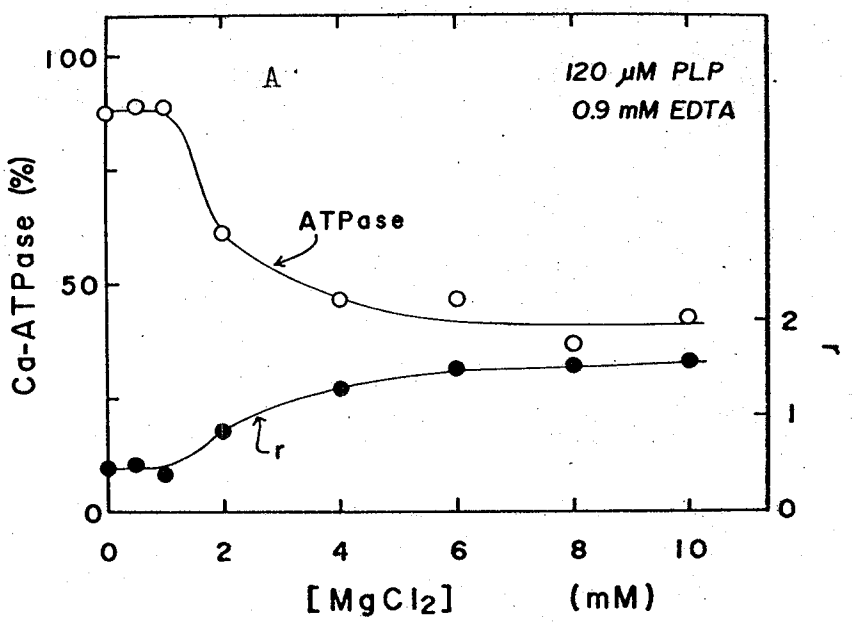
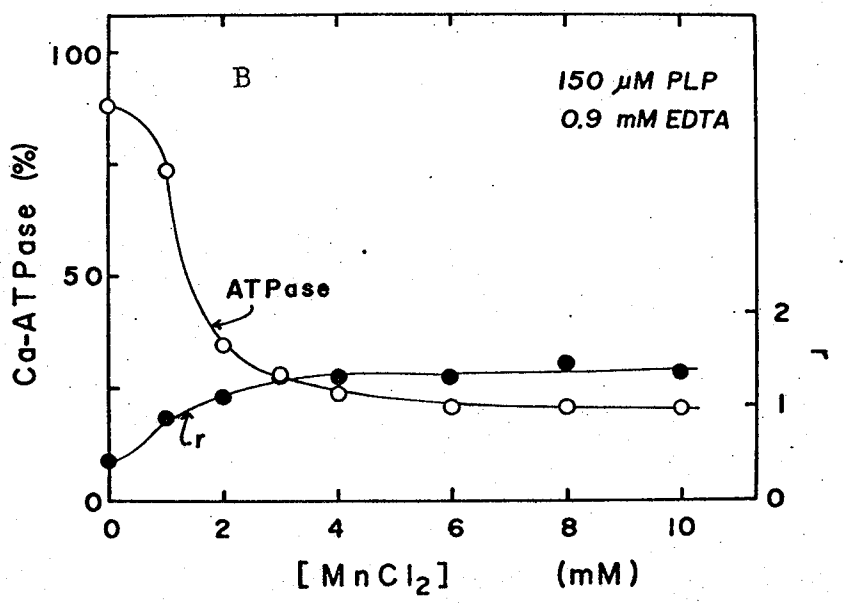
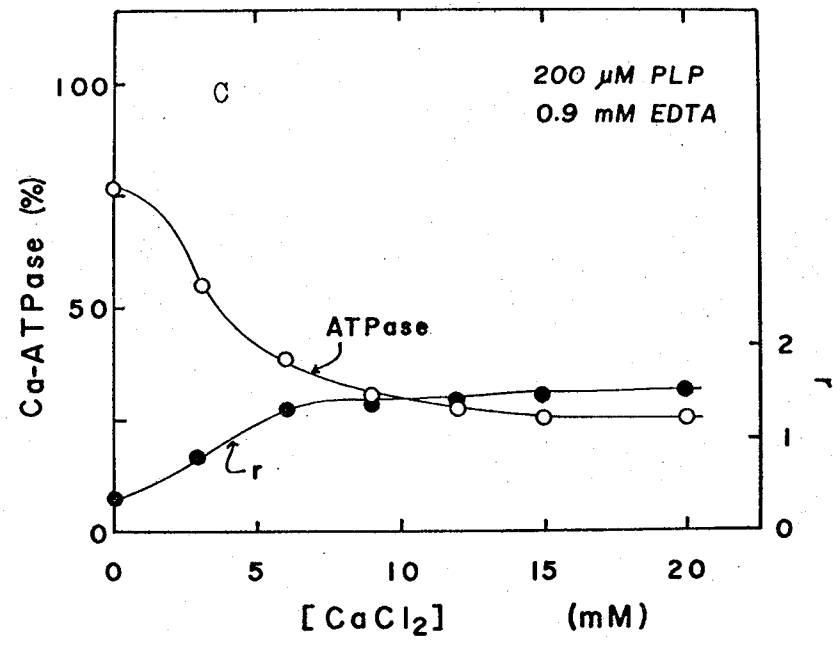


Fig. 16. Dependence of the PLP modification of isolated CF_1 on divalent cation concentration. Isolated CF_1 was modified with PLP in the presence of an indicated concentration of (A) $MgCl_2$ or (B) $MnCl_2$ or (C) $CaCl_2$ for 60 min. The Ca^{2+}-ATPase activity of the control (100%) was 13 μmoles Pi liberated/mg·min. The ratio (r) was determined as in Fig. 15.


B
150 μM PLP
0.9 mM EDTA
Ca-ATPase (%)
ATPase
r
100
50
0
2
1
0
0
2
4
6
8
10
[MnCl2] (mM)


C
200 μM PLP
0.9 mM EDTA
Ca-ATPase (%)
ATPase
r
100
50
0
2
1
0
0
5
10
15
20
[CaCl2] (mM)

iii) Effect of adenine nucleotide on the PLP modification

Fig. 17 shows that ADP and ATP protected isolated CF_1 from inactivation of Ca^{2+}-ATPase by PLP modification. Half maximum protection was obtained at 0.3 mM ADP and 0.4 mM ATP. On the other hand, the amounts of bonded PLP to CF_1 were not remarkably decreased by ADP or ATP.

iv) Dependence of Ca^{2+}-ATPase inactivation on the concentration of PLP

Fig. 18 shows the amounts of bonded PLP and the ATPase activity against PLP concentration in the incubation mixture which was incubated for 60 min in the presence or absence of 2 mM ATP.

Inactivation of Ca^{2+}-ATPase in PLP modified CF_1 increased with an increase of bonded PLP. The degree of ATPase inactivation was almost saturated at 400 μM PLP but the amounts of the bonded PLP still increased at 800 μM PLP. When CF_1 was first thermally activated as Ca^{2+}-ATPase, then modified by PLP, the results were identical with those in Fig. 18. ATP in the incubation mixture depressed the inactivation of Ca^{2+}-ATPase due to PLP modification, but not the amounts of bonded PLP so largely.

v) Two step PLP modification of CF_1

Isolated CF_1 was first modified with 250 μM PLP in the

presence of 2 mM ATP and collected by ammonium sulfate (final 50% saturation) precipitation. This PLP modified CF_1 bonded 2.2 moles of PLP per mole of CF_1 and retained 70% of the Ca^{2+}-ATPase activity. After the second modification with fairly low concentrations of PLP in the absence of ATP, CF_1 largely lost its Ca^{2+}-ATPase activity but the amounts of PLP bonded to CF_1 increased by not more than one (Fig. 19A). Fig. 19B shows the plots of the remaining Ca^{2+}-ATPase activity after the second modification against the increment of bonded PLP due to the second modification (Δr), indicating that one mole of PLP is bound per mole of CF_1 at 100% inactivation of Ca^{2+}-ATPase.

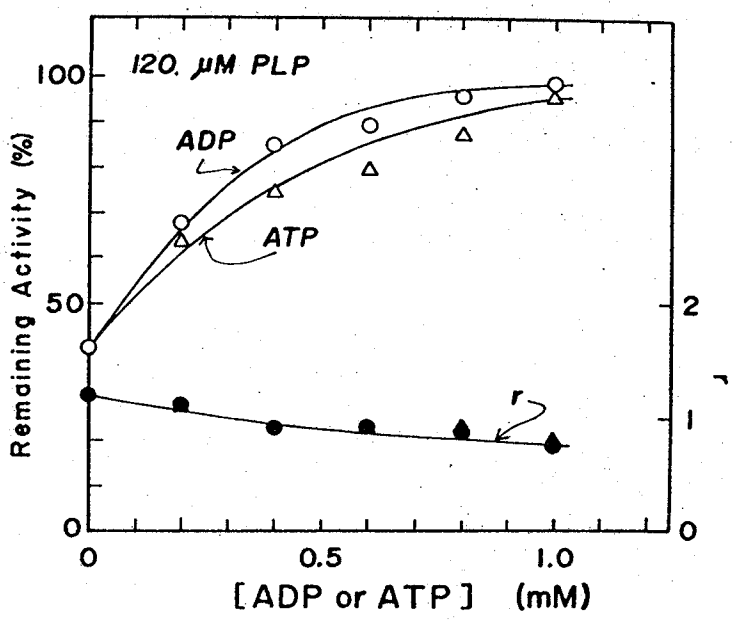


Fig. 17. Effect of adenine nucleotide on the PLP modification of isolated CF_1. CF_1 was modified with 120 μM PLP for 60 min in the presence of an indicated concentration of ADP (O) or ATP (Δ). The Ca^{2+}-ATPase activity of the control (100%) was 12 μmoles Pi liberated/mg·min. The ratio (r) was determined as in Fig. 15.

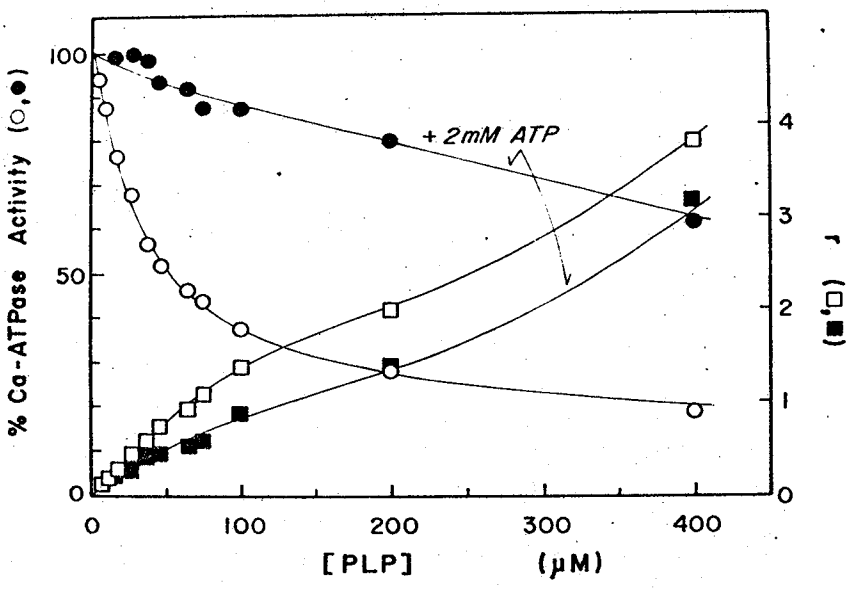


Fig. 18. Remaining Ca^{2+}-ATPase activity and the mole ratio of bonded PLP to CF_1 after modification with varying concentrations of PLP in the presence or absence of ATP. CF_1 was modified for 1 hour with an indicated concentration of PLP in the presence (closed marks) or absence (open marks) of 2 mM ATP.

vi) ATP concentration dependence of the Ca^{2+}-ATPase activity in CF_1 partially modified by PLP

CF_1 was first thermally activated and partially modified by PLP (controlled by PLP concentration in the incubation mixture) for 30 min. Double reciprocal plots of the Ca^{2+}-ATPase activity versus ATP concentration (Fig. 20) revealed that in PLP-modified CF_1, the apparent K_m value for ATP (1.6 mM) was identical with that of the control (0 μM PLP) but the apparent V_{max} value was lowered.

vii) Reversibility of the formation of Schiff base between isolated CF_1 and PLP

After incubation of CF_1 with PLP (80 μM) for 2 hours, the incubation mixture was diluted ten fold with the buffer then $NaBH_4$ was added. Fig. 21 shows that the amounts of bonded PLP decreased after dilution, following the first order kinetics (inset), and became constant after 60 min.

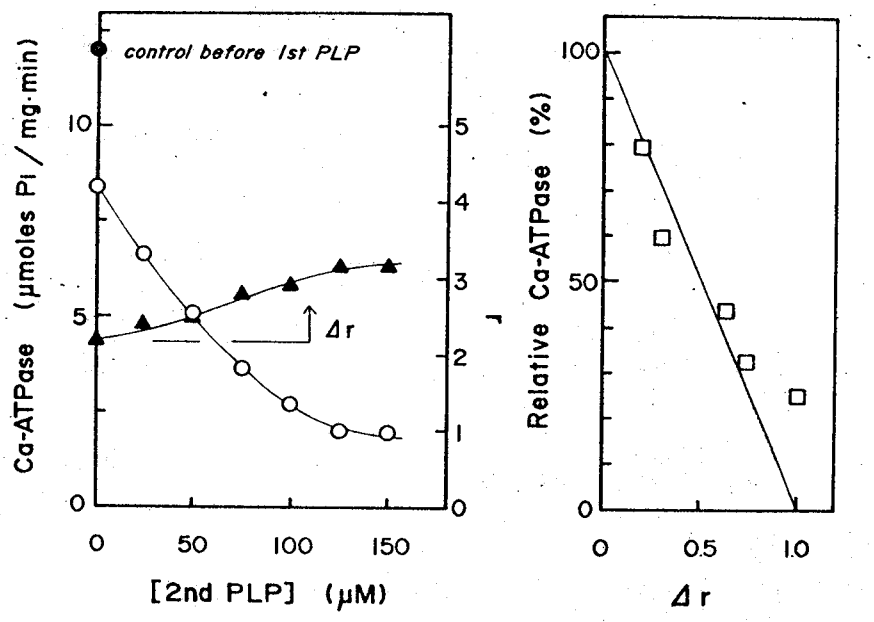


Fig. 19. Two-step PLP modification of CF_1. (A) Changes in the Ca^{2+}-ATPase activity and the mole ratio of PLP per CF_1 by the second modification. CF_1 was modified with 250 μM PLP in the presence of 2 mM ATP and 10 mM $MgCl_2$ for 60 min. After the removal of unreacted PLP and ATP, CF_1 was secondly modified with an indicated concentration of PLP without ATP for 60 min. Then the ATPase activity and the ratio (r) were determined. (B) Plots of the Ca^{2+}-ATPase activity (%) against the increment of the ratio (Δ r) due to the second modification.

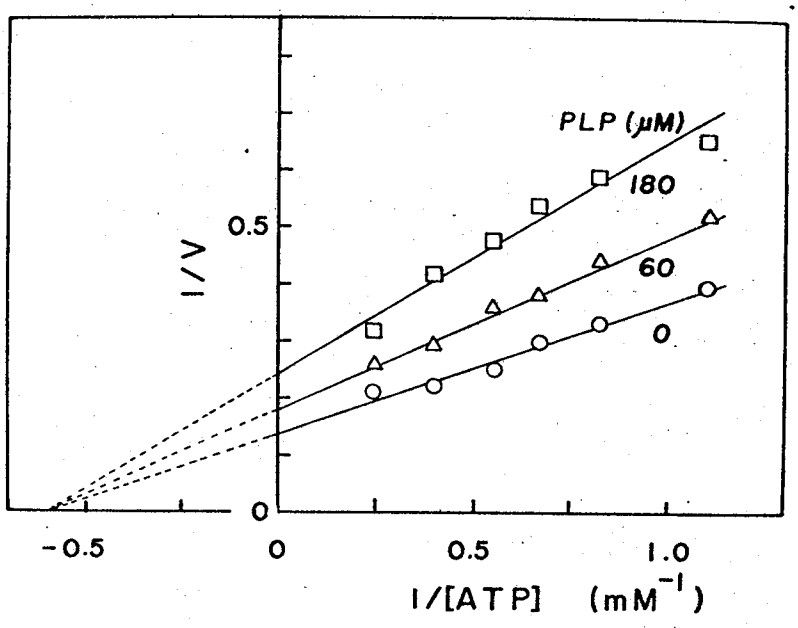


Fig. 20. Double reciprocal plots of the Ca^{2+}-ATPase activity versus ATP concentration. CF_1 was thermally activated as Ca^{2+}-ATPase then modified with 60 or 180 μM PLP for 30 min, then the Ca^{2+}-ATPase was assayed. The Ca^{2+}-ATPase activity (V) is given in μmoles Pi liberated/mg·min.

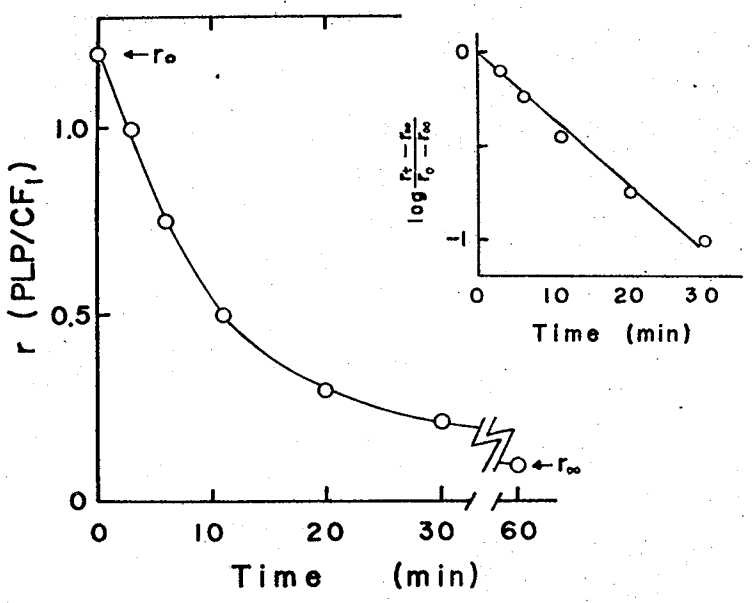


Fig. 21. Time course of the dilution effect on the mole ratio of bonded PLP to CF_1. r_o is the ratio after the modification with 80 μM PLP for 2 hours (at time 0 on the abscissa, incubation mixture was diluted 10 fold with the buffer). r_∞ is the ratio at 60 min after dilution. The inset is the semilogarithmic plots of $(r_t-r_\infty)/(r_o-r_\infty)$ versus the dilution time.

viii) Distribution of PLP among the subunits of CF_1 modified in a solution

Fig. 22 shows the distribution of [^{3}H] PLP among the subunits of CF_1 separated by SDS-PAGE (10% gel). Radioactivity was found in the α and β subunits but negligible in the γ, δ and ε subunits. This CF_1 sample contained 1.5 moles of PLP per mole CF_1 and retained 35% of the Ca^{2+}-ATPase activity. When the α and β subunits were separated by SDS-PAGE (12.5% gel), the radioactivity was equally found in both subunits. Only a fractional mole (0.7) of PLP was found in the γ subunit even when a total of 7 moles of PLP were found per mole CF_1.

ix) Time course of PLP modification of the α and β subunits

CF_1 was incubated with 100 μM of [^{3}H] PLP for an indicated time and reduced with $NaBH_4$. The α and β subunits were separated by SDS-PAGE (12.5% gel). Fig. 23 shows that no remarkable differences were found in the rate of modification of the α and β subunits. When Ca^{2+}-ATPase was inactivated by 50%, 0.5 mole of PLP was found in each of the α and β subunit fractions per mole of CF_1.

Even when the time courses were measured with 40 or 70 or 140 μM of [^{3}H] PLP, no differences were detected between the rates of modification of the α and β subunits.

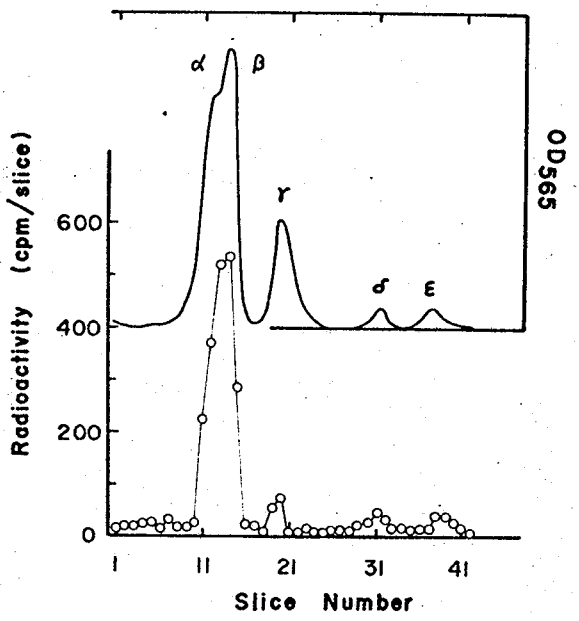


Fig. 22. Labeling of CF_1 subunits by [^{3}H] PLP. Isolated CF_1 was incubated with 120 μM [^{3}H] PLP for 60 min in the dark then modified. SDS-PAGE with 10% polyacrylamide was carried out with a CF_1 solution (40 μl) containing 40 μg protein.

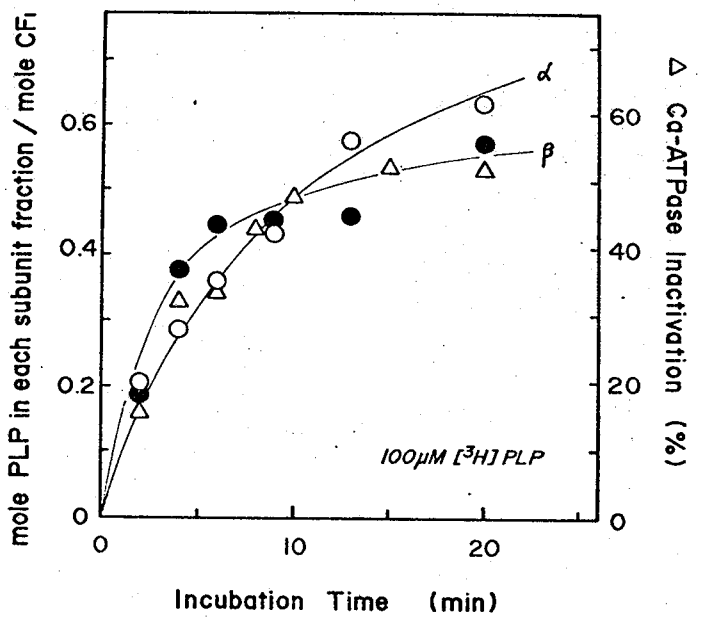


Fig. 23. Time courses of the increase of PLP in the α (○) and β (●) subunits and the inactivation of Ca^{2+}-ATPase (Δ). CF_1 was modified after incubation for an indicated time with [^{3}H] PLP (100 μM), then assayed for Ca^{2+}-ATPase and also applied to SDS-PAGE with 12.5% polyacrylamide (18 hours).

x) Circular Dichroism spectra of CF_1

Fig. 24 shows the CD spectra of CF_1 and PLP-modified CF_1 at pH 8.0.

The intensities of negative double minima at 208 and 222 nm, which reflect the α-helix conformation of the polypeptide backbone, and aromatic CD around 280 nm slightly increased after the PLP modification.

When ADP or ATP at a low concentration binds to CF_1, the intensity of negative CD signal at 260 nm increased and saturated at 100 μM ADP or ATP. Fig. 24 shows that the PLP modification had no effect on the binding of ATP to CF_1.

xi) Difference spectra of CF_1-PLP complex

Addition of PLP to a CF_1 solution (pH 8.0) resulted in characteristic spectral change which has been known to be typical of this kind of protein-ligand interaction (45). Positive absorption peaks were found at 435 and 330 nm, and a negative peak at 380 nm (Fig. 25). The shift of the absorption peak from 388 nm of PLP to 435 nm corresponds to formation of a Schiff base with a primary amine (45). Addition of $MgCl_2$ resulted in an increase in absorption of Schiff base. ATP in the presence of $MgCl_2$ lessened the absorption of Schiff base.

When Schiff base was reduced by $NaBH_4$, the spectrum changed to the absorption spectrum typical of covalently bonded PLP with a maximum at 325 nm which is characteristic of the formation of ε-amino-phosphopyridoxyl lysine (Fig. 26). A maximum at 278 nm is due to CF_1.

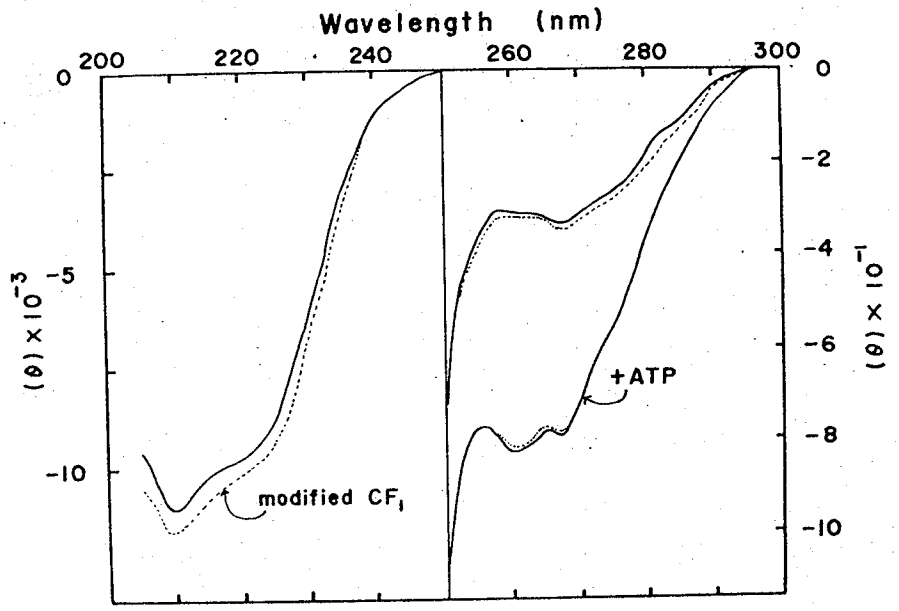


Fig. 24. CD spectra of CF_1 and PLP-modified CF_1. CF_1 was incubated with 100 μM PLP for 30 min then modified. This CF_1 sample bonded 1.6 mole PLP per mole CF_1 and retained 40% of the Ca^{2+}-ATPase activity. The concentration of CF_1 was 1.5 mg/ml for a region of 250-300 nm and 0.15 mg/ml for 205-250 nm. ATP and $MgCl_2$ were added at final concentration of 65 μM and 6.5 mM, respectively.

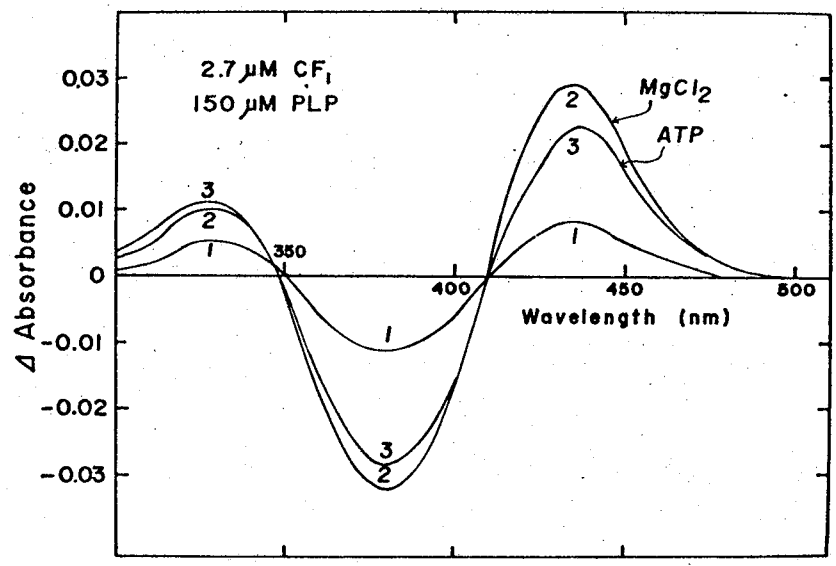


Fig. 25. Difference spectra of PLP-CF_1 complex. Spectroscopy was carried out after the incubation of 2.7 μM CF_1 with 150 μM PLP for 30 min at 25 °C (Spectrum 1). $MgCl_2$ (final concentration of 9.5 mM) was added and spectrum 2 was measured, then ATP (final concentration of 950 μM) was added and spectrum 3 was measured.

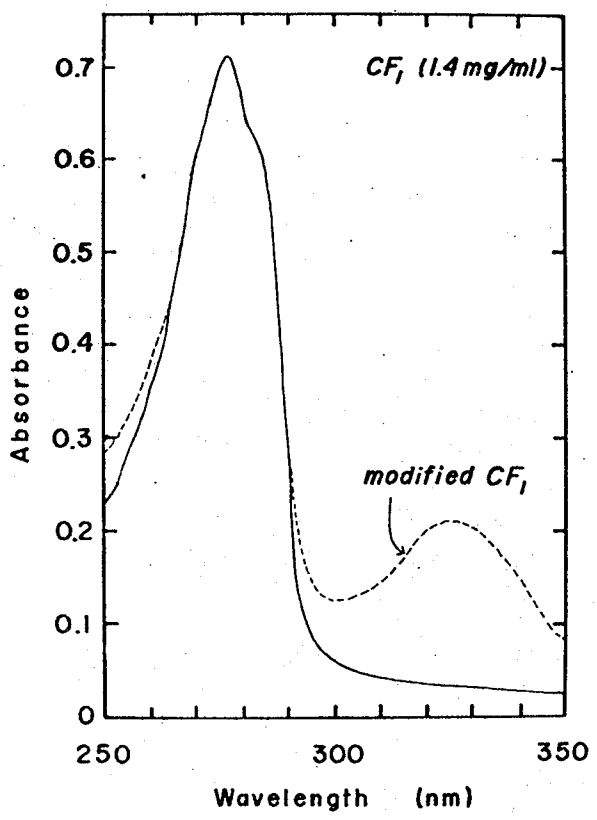


Fig. 26. Ultraviolet spectra of CF_1 and PLP-modified CF_1. CF_1 was modified with 0.3 mM PLP in the presence of 10 mM $MgCl_2$. The U.V. spectra were measured at a concentration of 1.4 mg CF_1/ml (pH 8.0).

IV DISCUSSION

IV-1 Effects of PLP Modification on the Activities of Membrane-bound and Isolated CF_1

i) PLP modification of chloroplasts in the dark causes energy transfer inhibition

The electron transport system stoichiometrically pumps protons into the inner space of the thylakoid membrane (42). Under steady state condition, the activity of electron transport is controlled by the proton gradient thus formed across the thylakoid membrane (ΔpH) (46) and corresponds to the rate of proton leakage through CF_1 and the thylakoid membrane (46). When ATP or ADP binds to membrane-bound CF_1, CF_1 undergoes conformation changes which reduce the rate of proton leakage through CF_1 (13) and increase the extent of ΔpH (13). Therefore, ADP or ATP inhibits (regulates) electron transport which pumps the proton to supplement the proton leakage through CF_1 (14). The remaining electron transport is called "basal electron transport" (14) due to the proton leakage through thylakoid membrane. The binding site for ADP or ATP (with apparent dissociation constant of the order of 10^{-6} M) on CF_1, related to the inhibition (regulation) of electron transport, is called "inhibition or regulatory site(s)" (14). The increment of electron transport due to phosphorylation

has been considered as electron transport which pumps protons to compensate the amounts translocated by CF_1 in the phosphorylation process, and is thus called "coupled electron transport" (14). There is thus a stoichiometry between ATP formation and the coupled electron transport. The site essential to ATP formation is the coupling site to which ADP binds with apparent dissociation constant (K_m) of around 2×10^{-5} M (14).

As shown in Fig. 2A, PLP modification of chloroplasts in the dark depressed the coupled electron transport in parallel with inactivation of phosphorylation. The ratio of phosphorylation to coupled electron transport is constant in agreement with the results obtained with phlorizin (47) and dicyclohexylcarbodiimide (48). Light-induced proton uptake and oxygen evolution of chloroplasts were almost unchanged after modification in the dark (Tables 1 and 2). This implies that the electron transport (and proton pump) machinery and thylakoid membrane itself were not damaged by the modification.

These results indicate that PLP modification in the dark causes energy transfer inhibition. The sites modified by PLP in chloroplasts would be located on the machinery which produces ATP from ADP and Pi in accordance with proton translocation (CF_1 and CF_o).

ii) PLP reversibly binds to membrane-bound CF_1

The ATPase activity of membrane-bound CF_1 was inhibited after PLP modification (Fig. 3) and PLP was actually found in

a CF_1 fraction isolated from chloroplasts modified with PLP (Table 6). Therefore, PLP modifies the membrane-bound CF_1 itself in the dark and consequently inhibits ATP hydrolysis or phosphorylation and coupled electron transport.

In contrast with the modification of membrane-bound CF_1 with N-ethylmaleimide (NEM) (27) which requires energization of the thylakoid membrane (by illumination or acid-base transition (29)), the inactivation of phosphorylation due to PLP modification occurs in the dark. This implies that PLP can readily reach the site(s) essential to phosphorylation without any energy-dependent changes (28) in CF_1 conformation. Furthermore, adenylates decreased the rate of phosphorylation inactivation (Fig. 4), suggesting that PLP modifies the active site or its vicinity of CF_1 for ATP synthesis.

The PLP modification consists of reduction of a Schiff base (49) which is formed between PLP and ε-NH_2 group of lysyl residue of the protein (CF_1). Addition of lysine to the incubation mixture before $NaBH_4$ reduction was able to restore phosphorylation (Table 4) and diminish the incorporation of PLP into CF_1 (Table 6). This implies that the formation of the Schiff base in the dark is reversible unless the base is reduced.

Other aldehydes showed no effect on the activities of chloroplasts (Table 5), which indicates that the specific binding and modification by PLP induce the observed

inactivation on chloroplasts.

iii) PLP reversibly binds to isolated CF_1

As shown in Fig. 24, PLP actually forms a Schiff base with isolated CF_1. When the Schiff base was reduced by $NaBH_4$, PLP was irreversibly linked to CF_1 and an absorption at 325 nm appeared (Fig. 25), then heat-activated Ca^{2+}-ATPase of CF_1 was inhibited (Fig. 14). The degree of Ca^{2+}-ATPase inactivation was saturated after the incubation for longer than 30 min, depending on the PLP concentration (Fig. 14). The Schiff base formation between CF_1 and PLP was reversible and thus seems to reach an equilibrium after 30 min.

Since the inhibition of heat-activated Ca^{2+}-ATPase of CF_1 by PLP modification is irrespective of whether CF_1 was activated before or after the modification, the specific functional site(s) modified by PLP would be exposed even before the heat activation.

2,3-butanedione and phenylglyoxal modified the arginyl residue(s) of membrane-bound (50,51) and isolated (52) CF_1 and inhibited phosphorylation and ATPase.

These results suggest that cationic amino acid (lysyl or arginyl) residue(s) would participate in the binding of anionic ligands (ADP, ATP and Pi) to CF_1.

iv) PLP modification of chloroplasts in the light causes uncoupling

When chloroplasts were modified with PLP in the light, the basal electron transport activity increased (Fig. 2B) which does not couple to the formation of ATP. This is phenomenologically identical to the results obtained with uncoupler, e.g., 2-amino-1-butanol and Gramicidin S (47). The light-induced proton uptake was decreased after PLP modification in the light (Table 2), implying the increase in proton leakage across the membrane. The phosphorylation was inhibited not only by PLP modification as the results in the dark but also by a loss of protons available for phosphorylation (by this increased leakage). These results indicate that PLP modification of chloroplasts in the light causes so-called uncoupling.

v) ATP-induced conformation change of membrane-bound CF_1 would protect chloroplasts from uncoupling due to PLP

Adenylates and PPi completely protect chloroplasts from the uncoupling due to PLP modification in the light (Table 3), suggesting that the uncoupling is also caused by the modification of CF_1 itself. Low concentrations of ATP (Fig. 5) prevented the uncoupling. In a similar concentration range, ATP, as a regulatory nucleotide, induces conformation change in the membrane-bound CF_1 (13), stimulates light-induced

proton uptake (13), inhibits electron transport (14) and protects from inhibition of phosphorylation by NEM (27). Therefore, it would be possible that a conformation change of CF_1 induced by binding of ATP to the regulatory site blocks binding of PLP to CF_1 and thus protects chloroplasts from the uncoupling.

vi) Uncoupling due to illumination in the presence of PLP would be caused by photosensitization

The amounts of PLP incorporated into CF_1 fraction was independent of the light during PLP modification (Table 6). This implies that the site(s) modified by PLP in the light would not differ from those in the dark. Addition of lysine was not able to depress the stimulation of basal electron transport and restore phosphorylation after the incubation in the light (Table 4). This would indicate that the reaction of PLP with chloroplasts in the light is irreversible even if the base is not reduced.

In addition to the formation of a Schiff base, it was reported that PLP irreversibly reacts with a nucleophilic (sulfhydryl or imidazole) group X and gives an X-azolidine ring (-X-CH(R)-NH-) as in Glutamate dehydrogenase (53) where R stands for the phosphopyridoxyl residue.

However, since addition of lysine was able to remove all PLP from chloroplasts (Table 6), the formation of an X-

azolidine ring can be excluded. PLP as a photosensitizer would give a secondary effect on membrane-bound CF_1, probably photooxidation of histidine (54).

vii) PLP-modified CF_1 could bind adenylates on its regulatory site

Adenylates inhibited electron transport to the basal level even after PLP modification in the dark which inhibited 50% of the phosphorylation activity (Fig. 8A), although the value of the binding constant of ATP and the Hill constant for the inhibition process by ATP decreased after the modification (Fi. 8B). Adenylates could also bind to isolated CF_1 after PLP modification which inhibited the Ca^{2+}-ATPase activity by 70% (Fig. 24). These results would support that the regulatory site (14) on CF_1 for ATP or ADP is not directly involved in the catalytic action of CF_1.

IV-2 Kinetic Analysis of PLP Mofication of Membrane-bound and Isolated CF_1

i) 1 : 1 Interaction between the active site on membrane-bound CF_1 and PLP

As shown in Figs. 6A and 7A, the process of inactivation of phosphorylation and Mg^{2+}-ATPase followed pseudo-first order kinetics. The reaction of PLP with CF_1 would thus be described by the following equation (55).

$$E + n\,I \xrightarrow{k_1} E\,I_n$$

where E, I and EI_n represent unoccupied active site, PLP and active site-PLP complex, respectively; n is mole of PLP bound to one active site (in other words, the reaction order) and k_1 is the second order rate constant for the reaction.

The rate of inactivation is given by

$$-d(E)/dt = k_1 \cdot (E) \cdot (I)^n$$

where the parentheses have ordinary meanings.

In the presence of excess (I) the differential equation may be integrated to

$$\ln (E)/(E)_o = - k_1 \cdot (I)^n \cdot t$$

where $(E)_o$ is the initial concentration of the active site. The plot of log $(E)/(E)_o$ (equal to logarithm of the remaining activity (%)) against time gives the slope of $2.303 \times k_1 (I)^n$. The pseudo-first order rate constant (k') can be substituted

for $k_1 (I)^n$ then, $\log k' = \log k_1 + n \log (I)$.

From the plot of log k' obtained from the data in Fig. 6A and Fig. 7A versus log (PLP), a slope of 1.0 was obtained for each process (Fig. 27). This suggests that the inactivation of phosphorylation and Mg^{2+}-ATPase by PLP is caused by 1 : 1 interaction between the active site and the modifier. The second order rate constant of the inhibition of phosphorylation was smaller than that of Mg^{2+}-ATPase (Figs. 5B and 6B), implying that Mg^{2+}-ATPase was more sensitive to PLP than photophosphorylation. Similar results were reported for the phenylglyoxal modification of chloroplasts (51). From the differences in sensitivity to the reagent, it was suggested (51) that the arginyl residue involved in the site of ATP synthesis might be different from that of ATP hydrolysis.

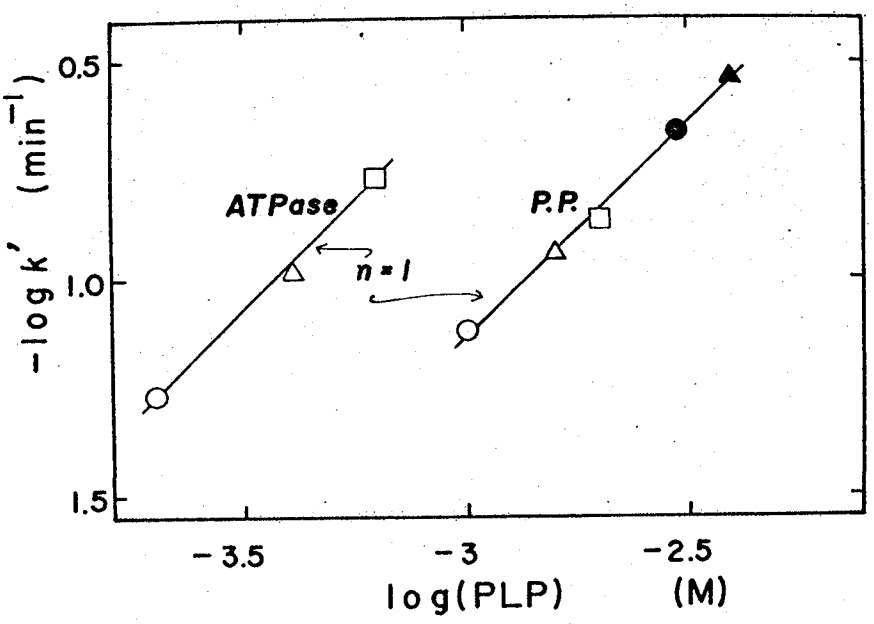


Fig. 27. Log-log plots of the rate constant (k') obtained from Fig. 5A and Fig. 6A versus PLP concentration. The slope n indicates the reaction order.

ii) One site with high affinity for PLP and many sites with lower affinities on the isolated CF_1

The formation of a Schiff base between CF_1 and PLP was reversible and reached an equilibrium after 30 min (Figs. 15 and 21).

When $NaBH_4$ was decomposed (56) by 5% TCA at either 5 sec or 5 min after the addition of a $NaBH_4$ solution to the incubation mixture, the equal amounts of PLP were found in both CF_1. This would show that the rate of irreversible bond formation (by an addition of $NaBH_4$ solution) between CF_1 and PLP is fast, compared with the rate needed to reach a new equilibrium after dilution, enough to assume that the amounts of the reduced Schiff base (PLP irreversibly bound to CF_1) indicate those of a Schiff base (PLP reversibly bound to CF_1) in an equilibrium with free CF_1 and free PLP.

The amounts of bonded PLP per CF_1 were measured in the presence or absence of 2 mM ATP and analyzed in Scatchard plots (57) (Fig. 28) under above assumption. The plots suggest that there are multiple groups of the binding sites on CF_1 differing in affinity to PLP and that total numbers of the sites are more than 10.

Assuming two independent groups of PLP binding sites on CF_1, which is the simplest case in various combinations of multiple groups, the binding constants and maximum numbers for those binding sites can be calculated by the following

equation (eq. 1) (58).

$$r = \frac{r_H K_H (PLP)}{1 + K_H (PLP)} + \frac{r_L K_L (PLP)}{1 + K_L (PLP)} \quad \text{eq. 1}$$

where r is the mole ratio of bound PLP to CF_1, (PLP) is concentration of free PLP, K is a binding constant (in M^{-1}, hereafter) of the ligand and subscripts H and L denote the higher and lower affinity sites, respectively.

The solid line in Fig. 28 was calculated with $r_H = 1$ at $K_H = 2 \times 10^4$ and $r_L = 10$ at $K_L = 650$.

ATP lessened the absorption of Schiff base between CF_1 and PLP (Fig. 25) and decreased the amounts of PLP bonded to CF_1 (Fig. 18). If ATP competes with PLP at the higher affinity site, r can be calculated by eq. 2.

$$r = \frac{r_H \left[K_H / \{ 1 + K_{ATP}(ATP) \} \right] (PLP)}{1 + \left[K_H / \{ 1 + K_{ATP}(ATP) \} \right] (PLP)} + \frac{r_L K_L (PLP)}{1 + K_L (PLP)} \quad \text{eq. 2}$$

The dashed line in Fig. 28 is calculated with $r_H = 1$ at $K_H / \{1 + K_{ATP}(ATP)\} = 2.5 \times 10^3$ and $r_L = 10$ at $K_L = 650$. Then binding constant of ATP is estimated to be $K_{ATP} = 3.5 \times 10^3$.

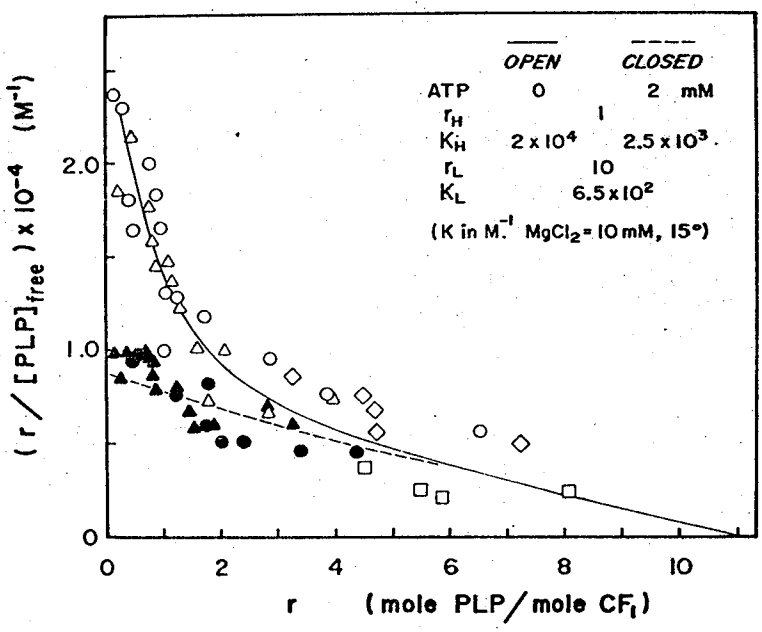


Fig. 28. Scatchard plots for the binding of PLP to CF_1. Different marks represent different experiments. The binding was determined in the presence (closed marks) or absence (open marks) of 2 mM ATP. The curves were calculated with the values listed in the figure. For notation, see DISCUSSION.

iii) ATP competes with PLP at the Ca^{2+}-ATPase active site which has high affinity to PLP

As shown in Fig. 18, in the absence of ATP the inactivation of the Ca^{2+}-ATPase increased with an increase of bonded PLP until the activity is halved at the PLP concentration of 60 μM. The remaining ATPase activity was plotted against the amounts of bonded PLP per mole CF_1 (Fig. 29A, circles). By extrapolation, about 1.5 mole of PLP per mole CF_1 would be required for complete inactivation. In the presence of ATP, ATPase is protected from inactivation but the amounts of bonded PLP do not decrease so largely (Fig. 18). The difference due to ATP in the remaining activity and in the amounts of bonded PLP are plotted in Fig. 29A (squares). The plots are extrapolated to give the ratio smaller than one for complete inactivation.

These results suggest that the specific modification of one site which relates to the inactivation of Ca^{2+}-ATPase would occur concomitant with the nonspecific modification of many sites which do not relate to the activity. The former is protected by ATP but the latter is not. If many sites were modified simultaneously with the specific active site(s), the former extrapolation would give an overestimated ratio and the latter an underestimated one.

If this one specific active site binds either PLP or ATP, neglecting other nonspecific sites, at an equilibrium,

$$(E) + (PLP) = (E\text{-}PLP) \qquad K'_{app} = \frac{(E\text{-}PLP)}{(E)(PLP)}$$

$$(E) + (ATP) = (E\text{-}ATP) \qquad K'_{ATP} = \frac{(E\text{-}ATP)}{(E)(ATP)}$$

where, (E), (PLP), (E-PLP), (ATP) and (E-ATP) are the concentration of free ATPase, free PLP, PLP-bound ATPase, free ATP and ATP-bound ATPase, respectively. K'_{app} and K'_{ATP} are apparent binding constant (in M^{-1}, hereafter) for PLP and ATP to the active site, respectively. Since E-PLP is inactive, the fractional remaining activity, F, is

$$F = \frac{(E) + (E\text{-}ATP)}{(E) + (E\text{-}PLP) + (E\text{-}ATP)} = \frac{1 + K'_{ATP}(ATP)}{1 + K'_{app}(PLP) + K'_{ATP}(ATP)}$$

then,

$$\frac{1}{F} = 1 + \frac{K'_{app}(PLP)}{1 + K'_{ATP}(ATP)} = 1 + K''_{app}(PLP)$$

therefore, plots of 1/F versus (PLP) should be linear at $(E) \ll (PLP)$. The data in Fig. 18 are replotted in Fig. 29B to find this relationship from which are estimated $K''_{app} = K'_{app} = 1.7 \times 10^4$ in the absence of ATP, and $K''_{app} = 1.5 \times 10^3$ in the presence of ATP then $K'_{ATP} = 5 \times 10^3$. These values from the activity data (K'_{app}, K'_{ATP}) are close to the corresponding K values from the binding data (K_H and K_{ATP}).

The value of the binding constant of ATP (K'_{ATP}) is in good agreement with the value obtained from Fig. 17 where 0.4 mM ATP showed half protection from Ca^{2+}-ATPase inactivation. This value is much higher than the value (a few μM) needed to protect chloroplasts from NEM inhibition and the dissociation constant of ATP binding to regulatory site, and is rather close to the K_m value (1-2 mM) for ATP in Ca^{2+}-ATPase reaction.

iv) Ca^{2+}-ATPase is completely inhibited by binding of one PLP per one CF_1

The results shown above suggest that the Ca^{2+}-ATPase activity is completely lost by PLP modification of only one site with the highest affinity to PLP among many sites with differing in affinity to PLP on one CF_1 molecule.

This is well supported by the results of two step modification (Fig. 19). At the first modification in the presence of ATP, PLP modified mostly non-specific sites and at the second modification in the absence of ATP, PLP modified only one site with a large loss of the Ca^{2+}-ATPase activity. The results of Fig. 19 also indicate that the first non-specific modification did not affect the second modification. This supports our first assumption that PLP modification of high and low affinity sites would occur independently each other.

It is thus concluded that the Ca^{2+}-ATPase is completely

inactivated by modification of single site with PLP.

Complete inactivation of ATPase by the single site modification was reported with NBD-Cl modification of CF_1 (25) or mitochondrial F_1 (59) or E. coli F_1 (60) and with 8-azido-ATP (photolabile ATP analogue) modification of bacterial F_1 (61).

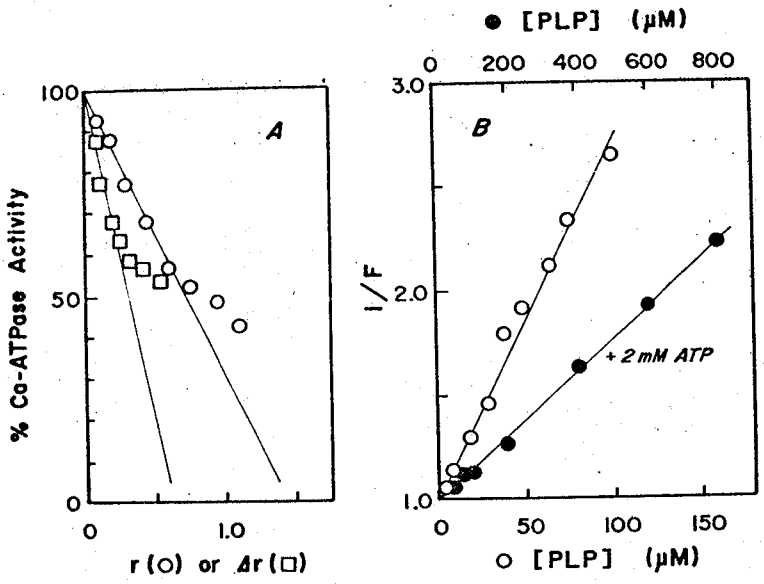


Fig. 29. Replots of the data in Fig. 18. (A) The remaining Ca^{2+}-ATPase activity against the mole ratio of bonded PLP to CF_1 (circles) and the differences due to 2 mM ATP in the ATPase activity against those in the ratio, Δr (squares). (B) Reciprocals of the fractional activity, F, of Ca^{2+}-ATPase after modification with varying concentrations of PLP in the presence (closed circles) or absence (open circles) of 2 mM ATP. For F, see DISCUSSION.

v) Divalent cation exposes the active site for Ca^{2+}-ATPase

Divalent cation (Mg^{2+}, Ca^{2+} and Mn^{2+}) is essential to the PLP modification of CF_1. As shown in Fig. 16, PLP modification of isolated CF_1 did not occur in the absence of divalent cation.

The amounts of bonded PLP to CF_1 were measured in the absence of divalent cation and analyzed in Scatchard plots (Fi. 30, closed marks). The plots show that PLP binding sites on CF_1 could not be grouped. The binding constant and the maximum number of binding sites may be obtained from the broken line to be 250 M^{-1} and 10 (this number is tentatively fixed to be identical to that of non-specific sites in the presence of $MgCl_2$), respectively. Since the binding constant of non-specific sites was lowered from 650 M^{-1} in the presence of Mg^{2+} to 250 M^{-1} in its absence, the binding constant of the specific site (K_H = 2 x 10^4 M^{-1} in the presence of Mg^{2+}) should be expected to be at least 7 x 10^3 M^{-1}. However, there is no plot indicating such high affinity. Therefore, the high affinity site, i.e., the active site is not exposed or does not reveal its high affinity to PLP in the absence of divalent cation.

Furthermore, formation of Schiff base itself, in general, does not require divalent cation (62) and is actually found in Fig. 25. Thus it would be more conceivable that divalent cation acts as an effector to change the conformation of CF_1

and expose the active site. Results suggesting such non-specific divalent cation dependence of enzyme conformation have been reported (63,64). It has been reported (63) that addition of divalent cations to a solution of CF_1 produced a significant increase in polarization of tyrosine fluorescence, suggesting that divalent cation alters the CF_1 structure in which, for example, the mobility of one or more tyrosyl residues of CF_1 is reduced.

With isolated CF_1 which binds three moles of nucleotides (ATP or ADP)/mole CF_1, the binding mode of nucleotide has been shown to change depending on Mg^{2+} (or Ca^{2+}) (65). In the presence of Mg^{2+}, the binding of the third nucleotide is positively cooperative to the second one, while in the absence of Mg^{2+}, it is slightly negatively cooperative to the second one. Although it has not been determined which one of three nucleotide binding sites in isolated CF_1 is (latently) catalytic, the change in the binding cooperativity would relate to the observed divalent cation dependent exposure-burial of the active site.

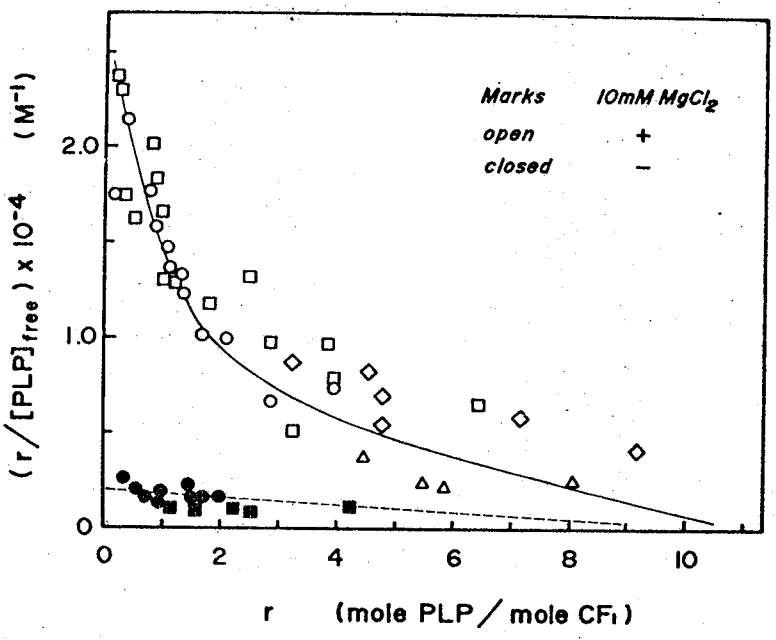


Fig. 30. Scatchard plots of PLP binding in the presence (open marks) or absence (closed marks) of 10 mM $MgCl_2$. The solid curve was copied from Fig. 28. The broken line was calculated with the value of r = 10 at K = 250 M^{-1}. For details, see DISCUSSION.

IV-3 Identification of PLP Modified Subunits of Membrane-bound and Isolated CF_1

i) The α, β and γ subunits in membrane-bound CF_1 were modified by PLP

When membrane-bound CF_1 was modified with [^{3}H] PLP, the α, β and γ subunits were equally labeled (Figs. 12 and 13) and phosphorylation was inhibited. For 50% inhibition, one mole of PLP was found in each of these three subunit fractions per mole CF_1. These results suggest the participation of one (or two) essential lysine per α and/or β and/or γ subunits. If the active site is located on the β subunit as suggested by many investigators (25,59-61,66), two moles of PLP may be required for complete inactivation of phosphorylation.

ii) The α and β subunits in isolated CF_1 were modified by PLP

When isolated CF_1 was modified with [^{3}H] PLP, the α and β subunits were equally labeled and heat-activated Ca^{2+}-ATPase was inhibited. Since Ca^{2+}-ATPase inactivation proceeded in parallel with the modification of both the α and β subunits (Fig. 22), the location of the active site has not yet been decided.

It has been reported that NBD-Cl modified one tyrosyl residue on the β subunit of CF_1 and inactivated Ca^{2+}-ATPase

(25). 8-azido-ATP specifically labeled one amino acid (unknown) on the β subunit of E. coli F_1 (61). When these results are also taken into account, present results would indicate that there is one active site on the one subunit (probably β), while there would be many non-catalytic sites on the other subunit which are modified at random but seemingly parallel to the active site modification.

iii) The γ subunit of CF_1 would be buried among other subunits by detaching from the membrane

The distribution of PLP among the subunits of CF_1 showed marked difference depending on whether CF_1 was modified on the membrane (Fig. 11) or in solution (Fig. 21). The absence of PLP in the γ subunit of CF_1 which was modified in a solubilized form would relate to the observation (20) that the anti-γ-antibody inhibited photophosphorylation but not Ca^{2+}-ATPase of isolated CF_1. The burial of the γ subunit among other subunits would make the anti-γ-antibody and PLP inaccessible to their sites of interaction.

The amounts of PLP required for complete inactivation of photophosphorylation were larger than those of ATPase of isolated CF_1. To the membrane-bound CF_1, at least two PLP molecules (probably on the two β subunits) seemed to be responsible for full inactivation, while one PLP was likely to be enough to inactivate solubilized Ca^{2+}-ATPase (Fig. 18).

These results also suggest that the detaching process of CF_1 from the membrane gives some effects on the subunit conformation and inter-subunit interaction of this multi-subunit enzyme.

IV-4 Tentative Scheme for Explanation of the Results of PLP Modification of CF_1

These results obtained by PLP modification require rather sophisticated consideration on the features of this multi-subunit enzyme. One simplest model is illustrated in Fig. 31.

The subunit conformation and inter-subunit interaction depend on the situation of CF_1.

By detachment from the membrane in the absence of Mg^{2+} (i.e., in a dilute EDTA solution), the γ subunit of CF_1 would be buried among other subunits. The PLP binding sites on the α and β subunits would also be hidden (unreactable with PLP). Upon addition of divalent cation, these binding sites are exposed. However, the two specific (active) sites on the β subunit pair would be so close to each other that binding of PLP to one site interferes binding of PLP or ATP to the other.

In membrane-bound CF_1, the γ subunit is exposed and maintains a distance between two active sites so as to for PLP modify both sites. The reversible conformation change of

the γ subunit by electrochemical potential gradient, proton translocation and/or exchange of H^+ to Mg^{2+} and vice versa, would change the inter-relationship between the α and β subunits, and thereby drive binding of ADP and Pi, condensation of these ligands and release of ATP etc. in cooperation with two pairs of α-β subunits. In isolated CF_1, since ATP hydrolysis is a downhill reaction, the driving force may not be neccesary and the movement of the γ subunit would not be required.

More complicated model involving cooperativity in subunit interaction and/or half of the site reactivity (67,68) would be possible and may be close to the real feature of the enzyme.

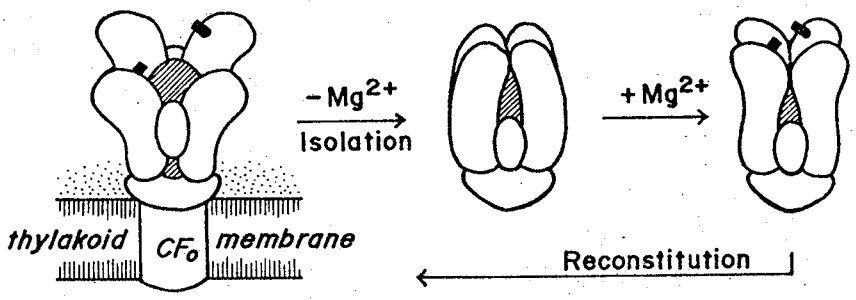


Fig. 31. A simple model illustrating the results of PLP modification. The active sites (block dots) with high affinity for PLP are located on one of the two large subunit pairs (probably 2β) and many non-specific PLP binding sites (not shown) on the other large subunit pair (probably 2α). The γ subunit to which small ε subunit(s) is attached is shadowed. The δ subunit forms a link to CF_o. For the explanation of the conformation change and the inter-subunit interaction, see DISCUSSION.

V SUMMARY

The effects of PLP modification on chloroplasts and isolated CF_1 were studied.

1) PLP modification of chloroplasts in the dark caused energy transfer inhibition. When the Schiff base formed between PLP and the lysyl residues of proteins on thylakoid membrane were reduced, PLP bonded covalently to the proteins present on the thylakoid membrane, but only inactivated the activity of CF_1. Furthermore, PLP modified a few of total 130 lysyl residues present in one CF_1 molecule. PLP modified the active site or its vicinity located at the surface of CF_1.

PLP modified the α, β and γ subunits of the membrane-bound CF_1. For 50% inhibition, 1 mole of PLP was found in each of these 3 subunit fractions per mole CF_1. The inactivation of phosphorylation was caused by 1 : 1 interaction between PLP and the active site of CF_1. Therefore, present results suggest the participation of one (or two) essential lysine per α and/or β and/or γ subunits in photophosphorylation.

2) PLP modification of chloroplasts in the light caused uncoupling which was protected by low concentrations (20 μM) of ATP or ADP. PLP gave a secondary effect on the membrane-bound CF_1 as a photosensitizer in the light, probably photo-oxidation of histidine.

3) In the presence of divalent cation such as Mg^{2+}, Mn^{2+} or Ca^{2+}, PLP modified isolated CF_1 which subsequently lost its heat-activated Ca^{2+}-ATPase activity. Isolated CF_1 seemed to possess at least one site with high affinity for PLP and many sites with lower affinities. PLP modified this high affinity site and caused complete inactivation of Ca^{2+}-ATPase. ATP competitively protected this specific PLP modification. PLP modified the α and β subunits of isolated CF_1 but negligibly the γ subunit. For 50% inhibition, 0.5 mole of PLP was found equally in these two subunit fractions per mole CF_1.

In the absence of divalent cation there was no site with high affinity for PLP and thus PLP did not cause the inactivation of Ca^{2+}-ATPase. The high affinity site for PLP, i.e., the active site, was not exposed or did not reveal its high affinity for PLP.

The distribution of PLP among the subunits of CF_1 markedly differed depending on whether CF_1 was modified on the membrane or in a solution. The amounts of PLP bonded to membrane-bound CF_1 in order to inactivate photophosphorylation completely were larger than those of ATPase of isolated CF_1 (1 PLP/CF_1). These results suggest that the detaching process of CF_1 from the membrane has some effect on the subunit conformation and the inter-subunit interaction of CF_1.

By detachment from the membrane in the absence of Mg^{2+}, the γ subunit of CF_1 would be buried among other subunits. The active site with high affinity for PLP on the β (or α) subunit would be hidden. The addition of divalent cation exposed this active site.

It was proposed that the subunit conformation and inter-subunit interaction of CF_1 participate in the process of photophosphorylation.

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