I. INTRODUCTION

Chemical modification of proteins is one of the important tools for the understanding of structural-functional relationship in the enzymatic reactions. Different functional groups in the proteins e.g., -COOH, -NH₂, -SH, have been modified by different chemical reagents and their role in enzymatic activities have been studied by several workers (Berg et al., 1974; Moroney et al., 1980; Oliver and Jagendorf, 1976; Vallejos, 1981; Weiss and McCarty, 1977). The energy dependent modification of amino group, by trinitrobenzenesulfonic acid (TNBS), of thylakoid membranes pretreated with methylacetiimidate in dark caused inhibition in chloroplast ATPase activity. One trinitrophenyl residue was found to be associated with one each on α- and β- and 2–3 trinitrophenyl residues were found on γ-subunits of CF₁. However, the inhibition and labelling with TNBS was protected by ADP or ATP plus P₁ (Oliver and Jagendorf, 1976). Similarly, modification of one lysyl residue of CF₁ by pyridoxal phosphate (PLP) completely inhibited its ATPase activity (Sugiyama and Mukohata, 1978, 1979). The inhibition of ATPase activity by PLP was protected by ADP or ATP. The essential lysine
molecule could be either on α-or β-subunit. PLP was found to inhibit photophosphorylation in spinach chloroplasts and modified the lysine residues in α-, β- and γ-subunits of CF$_1$.

Fluorescamine, a primary amine specific fluorescent label, first introduced by Udenfriend et al. in 1972, has been used to study energy dependent conformational changes in thylakoid membranes (Kraayenhof and Slater, 1974). Its effects on electron transport, proton translocation and phosphorylation activities in chloroplasts have been studied (Harnischfeger, 1979; Parkash and Singhal, 1984).

Here we describe the effect of chemical modification, by fluorescamine, of ε-NH$_2$ group of lysine residues of CF$_1$ in salt-washed thylakoid membranes on energy dependent release of [¹⁴C]-ADP and photophosphorylation.

II. MATERIALS AND METHODS

A. Plant Material

Fresh spinach (Spinacea oleracea) leaves obtained from local market were used for the isolation of chloroplasts.

B. Isolation of Type C Chloroplasts

Spinach leaves were washed thoroughly, first with tap water and then thrice with double distilled water, to
remove any impurity present on the surface of leaves. The washed leaves were illuminated with white light in cold room at 2-4°C for 30 minutes. The leaves were then deveined and chopped into small pieces and grinded in a prechilled Waring blender (Waring Company, New Hartford, Conn. USA) at maximum speed for 30-40 seconds in dark, in cold room. The grinding medium (also referred as isolation medium) consisted of 0.3M sucrose, 50mM NaCl, 1mM MgCl₂ and 10mM Tricine, pH 7.8. The resultant homogenate, a thick slurry, was squeezed through ten layers of cheese cloth and one layer of Miracloth (both the cheese cloth and Miracloth were thoroughly washed, first with tap water and then with double distilled water, before using them). The filtrate was centrifuged for 3 minutes at 300xg at 0°C in SS-34 rotor of RC-5 Superspeed Refrigerated Centrifuge (Sorvall, DuPont, USA) to remove cellular debris. The pellet was discarded and the supernatant was further centrifuged at 3000xg for 10 minutes in the same rotor at 0°C. The pellet, obtained after this step, consisted mainly of type C chloroplasts (Hall, 1972). It was washed once with isolation medium and then thrice with medium containing 50mM NaCl, 1mM MgCl₂ and 2mM Tricine, pH 7.8 and finally resuspended in the same medium at a chlorophyll concentration of 2 mg/ml (Strotmann et al., 1976).
C. Chemical Modification of Salt-Washed Thylakoid Membranes with Fluorescamine

(i) Modification in dark

The salt-washed thylakoid membranes were kept on an ice bucket in dark for 30 minutes before treating them with fluorescamine. After dark incubation, the thylakoid membranes were resuspended in Medium A (see footnote) to a chlorophyll concentration of 1 mg/ml in a final volume of 2 ml. To this resuspension 20 μl of fluorescamine (in acetone) was added with a Hamilton syringe and the reaction mixture was vortexed thoroughly on a vortex mixture for 15 seconds. These modified thylakoid membranes were used for prelabelling with [14C]-ADP.

(ii) Modification in light

The suspension of salt-washed thylakoid membranes in Medium A was illuminated with white light from a 250 watt projector lamp (Kodak Co., USA) for 1 minute. The light intensity at the reaction vessel was 290 Joules/m²/sec. Immediately after the illumination, 20μl of fluorescamine solution was added to the chloroplasts suspension. The contents were mixed thoroughly on a

Medium A consisted of 25mM Tricine, pH 8.00, 50mM NaCl, 1mM MgCl₂, 0.5mM methyl viologen and 2.5μM[14C]-ADP.
vortex mixture and transferred to ice-bucket. The modified thylakoid membranes were later used for pre-labelling with $[^{14}\text{C}]-\text{ADP}$.

D. Prelabelling of Thylakoid Membranes with $[^{14}\text{C}]-\text{ADP}$

The chloroplasts (control and fluorescamine treated) suspended in medium A with a chlorophyll concentration of 1 mg/ml in a final volume of 2 ml, were illuminated for one minute with white light from a 250 watt projector lamp (light intensity = 290 Joules/m$^2$/sec). The suspension was immediately centrifuged in RC-5 Sorvall Centrifuge at 20000xg for 5 minutes at $0^\circ\text{C}$ using SM-24 rotor. The pellet was washed thrice with 2 ml of ice-cold medium which consisted of 25mM Tricine, pH 8.0 and 50mM NaCl, so as to remove any unspecific labelling of thylakoid membrane surface by $[^{14}\text{C}]-\text{ADP}$. The pellet was finally suspended in the washing medium with a chlorophyll concentration of 1 mg/ml and was used later for determination of light-induced release of $[^{14}\text{C}]-\text{ADP}$.

E. Light-Induced Release of $[^{14}\text{C}]-\text{ADP}$ Bound to $\text{CF}_1$

The reaction mixture for light-induced release of $[^{14}\text{C}]-\text{ADP}$ in a final volume of 0.3 ml contained 25mM Tricine, pH 8.0, 50mM NaCl, 5mM MgCl$_2$, 0.5mM methyl viologen,
0.1mM ADP and prelabelled thylakoid membranes containing 100μg chlorophyll. The reaction mixture was taken in a 0.5ml semitransparent (~50% transparency) microfuge tube and then illuminated for 1 minute with white light (light intensity = 290 Joules/m²/sec). After illumination, the suspension was immediately centrifuged at maximum speed for 10 minutes at room temperature in a Beckmann Microfuge. The pellet was discarded and supernatant was used to determine the amount of [¹⁴C]-ADP released.

F. Determination of [¹⁴C]-ADP

[¹⁴C]-ADP was determined by using liquid scintillation method. The scintillation cocktail was prepared as follows: 5 gm PPO and 0.1 gm POPOP were dissolved in a final volume of 1 lt. of toluene, in a brown glass bottle with constant stirring for 1 hr on magnetic stirrer. The scintillation cocktail was kept in dark for 24 hrs before use.

100μl of supernatant containing [¹⁴C]-ADP was put in a glass scintillation vial and then 10 ml of scintillation cocktail was added. The vial was capped properly. The cap contained a proper gasket made of
cork and aluminium foil to prevent any leakage of the fluid. The outer surface of vial was cleaned with lens paper and the vial was then kept in dark for 2-3 hours before taking counts. The counting of \([^{14}C]\)-ADP was carried out in liquid scintillation counter (Beckmann, Model LS-7000, Microprocessor Controlled Liquid Scintillation Spectrometer, Beckman Instruments, USA).

G. Photophosphorylation

The non-cyclic photophosphorylation coupled to electron transport from water to methyl viologen was determined under the same experimental conditions as those used in determining light-induced release of \([^{14}C]\)-ADP. Fluorescamine modified thylakoid membranes were prepared as described earlier, with the exception that in place of \([^{14}C]\)-ADP either cold ADP or in some cases no ADP was present during chemical modification. In addition to dark and light modifications (See above) we have also carried out modification of thylakoid membranes with fluorescamine first in dark and then in light.

The reaction mixture in 0.3 ml consisted of 25mM Tricine, pH 8.0, 50mM NaCl, 5mM MgCl$_2$, 0.5mM methyl viologen, 0.1mM ADP, pH 8.0, $^{32}$P (carrier free, activity
4 x 10^7 cpm/ml, 1 mM P and thylakoid membranes equivalent to 100 µg chlorophyll. The samples were illuminated for 1 minute with white light (Intensity=290 Joules/m²/sec) and the reaction was stopped by adding 20 µl of 30% (w/v) TCA. The contents were then centrifuged at maximum speed in a Beckmann microfuge and the supernatant (hereafter referred as TCA extract) was used to determine [³²P]-ATP. The pellet was discarded carefully.

H. Determination of [³²P]-ATP

[³²P]-ATP was determined according to Avron (1960) with some minor changes. Isobutanol, benzene and water in a proportion of 1:1:0.2 were shaken thoroughly in a separatory funnel. The mixture was allowed to stand for some time for separation of phases. The two phases were separated and collected carefully for further use.

200 µl of TCA extract was taken in a screw cap vial (25 mm x 200 mm) and then 0.3 ml of water saturated with isobutanol-benzene (aqueous phase as prepared above) was added and the contents were mixed thoroughly on a vortex mixture. After this, 1.2 ml acetone and 160 µl of 5% (v/v) ammonium molybdate prepared in 4 N-HCl, were added. The contents were mixed well and then kept
for 2 minutes for completion of reaction. 7 ml of isobutanol-benzene saturated with water (as prepared above) was then added. The vial was stoppered and vortexed thoroughly for 30 seconds. The upper layer (organic phase, yellow in color) was aspirated off by a Pasteur pipette and discarded carefully. To the aqueous layer, 20μl of 0.02M KH₂PO₄ and 7 ml of isobutanol-benzene saturated with water were added. The vial was stoppered and vortexed thoroughly for 30 seconds. The upper layer was completely aspirated off by Pasteur pipette. The radioactivity contained in [³²P]-ATP (lower layer) was determined by using liquid scintillation method.

The scintillation cocktail was made up of 10 gm PPO, 0.25 gm POPOP and 112 gm naphthalene dissolved in a final volume of 1 lt. in 1,4-Dioxan. 0.5 ml of [³²P]-ATP and 10 ml of scintillation cocktail was taken in a glass vial and the counting was done in Beckmann LS-7000 (see above) liquid scintillation spectrometer.

I. Determination of Total Chlorophyll Concentration

Chlorophyll concentrations were determined according to Arnon (1949).
J. pH Adjustment

The pH adjustments were carried out by using pH meter (Radiometer A/S Copenhagen, Denmark, model PHM 26). The internal temperature compensation provided in pH meter was used to adjust pH of different solutions at different temperatures.

K. Preparation of Double Distilled Water

Double distilled water was prepared in all glass apparatus. The first distillation flask contained a very dilute solution of sulfuric acid whereas to the second flask some amount of alkaline KMnO₄ was added. The pH of water was checked routinely.

L. Disposal of Radioactive Waste

After the counting of radioactivity in different samples, the contents of the vials were poured into a big amber glass bottle with proper label, and kept in a safe place. Afterward it was disposed off safely by following usual procedures.

M. Sources of Chemicals and Other Materials

[8-¹⁴C]-ADP-ΝΗ₄⁺ salt (specific activity 56 mCi/mmol) was obtained from Amersham, England. ³²P₁(carrier free)
was purchased from Bhabha Atomic Research Centre, Bombay, India. Fluorescamine, Tricine, methyl viologen, DCCD, gramicidin D, ADP-Na₃, phloridzin and valinomycin were purchased from Sigma Chemical Co., USA. Ammonium molybdate was from Reanal Chemical Co., Budapest, Hungary and scintillation grade naphthalene was purchased from Koch-Light Laboratories, U.K. All other chemicals were of analytical reagent grade. Miracloth was obtained from Chicopee Mills, Milltown, N.J., USA. Shimadzu UV-240 double beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used for absorbance determination.

III. RESULTS

A. Light-Induced Release of $[^{14}\text{C}]$-ADP Bound to CF₁

A.1. Modification of thylakoid membranes with fluorescamine in dark

(i) In absence of ionophores and energy transfer inhibitors

The light-induced release of $[^{14}\text{C}]$-ADP from salt-washed thylakoid membranes increased on modification in dark with increasing concentrations of fluorescamine (See
Fig. 2.1.
The energy dependent release of $^{14}$C-ADP as a function of fluorescamine concentration. The salt-washed thylakoid membranes, incubated in dark for 30 minutes in medium A, were treated with increasing concentrations of fluorescamine. Prelabelling and energy dependent release of $^{14}$C-ADP were performed as described in MATERIALS AND METHODS. The final concentrations of gramicidin D (GmcD), valinomycin (Val), phloridzin (Phl) and DCCD, wherever used, were 0.4μM, 1.25μM, 2mM and 20μM respectively. Control rates for untreated chloroplasts and in absence of any inhibitor was 0.83 nanomoles $^{14}$C-ADP released/mg Chl.

Fig. 2.2.
Same as in Fig. 2.1. except that the salt-washed thylakoid membranes were illuminated for 1 minute before adding fluorescamine. Control rate for untreated chloroplasts was 0.69 nanomoles $^{14}$C-ADP released/mg Chl.
Fluorescamine, μ moles

% of Control Activity

Control
GmCD
Val.
Phl.
DCCD

Fluorescamine, μ moles

% of Control Activity

Control
GmCD
Val.
Phl.
DCCD

Fluorescamine, μ moles

% of Control Activity

Control
GmCD
Val.
Phl.
DCCD
Fig. 2.1). The maximum increase in light-induced release of $[^{14}\text{C}]$-ADP was about 80% at fluorescamine concentration of 4.00 µmoles. However, the increase in the activity was not linear with increasing concentration of fluorescamine.

(ii) In presence of ionophores
(a) Unmodified chloroplasts
The light-induced release of $[^{14}\text{C}]$-ADP decreased when ionophores like gramicidin D and valinomycin were added to the assay mixture. The decrease in the activity was 19.9% in the presence of 0.4µM gramicidin D and 29% in the presence of 1.25µM valinomycin (See Table 2.1).

(b) In fluorescamine modified chloroplasts
A similar increase in the energy dependent release of tightly bound $[^{14}\text{C}]$-ADP with increasing concentration of fluorescamine was observed in presence of ionophores as those observed in the absence of ionophores (See Fig. 2.1). The maximum increase in the activity was 62% in the presence of 0.4µM gramicidin D and 97% in presence of 1.25µM valinomycin when salt-washed thylakoid membranes were treated with 2.00 µ moles of fluorescamine. However, at higher concentration of fluorescamine there was no further enhancement in the activity (Fig. 2.1).
TABLE 2.1

EFFECTS OF IONOPHORES AND ENERGY TRANSFER INHIBITORS ON ENERGY DEPENDENT RELEASE OF \[^{14}C\]-ADP BOTH IN DARK ADAPTED AND PREILLUMINATED THYLAKOID MEMBRANES

<table>
<thead>
<tr>
<th>Sample adaptation</th>
<th>Control activity</th>
<th>Maximum change in [^{14}C]-ADP release in presence of</th>
<th>Energy Transfer Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ionophores</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4µM Gramicidin D</td>
<td>20µM DCCD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.25 µM Valinomycin</td>
<td>2mM Phloridzin</td>
</tr>
<tr>
<td>Dark</td>
<td>100</td>
<td>(-) 19.87</td>
<td>(-) 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-) 29</td>
<td>(+) 3</td>
</tr>
<tr>
<td>Light</td>
<td>100</td>
<td>(-) 3.5</td>
<td>(+) 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-) 15</td>
<td>(+) 24</td>
</tr>
</tbody>
</table>

(-) in parentheses indicates inhibition
(+ ) indicates enhancement in rates.

Control rates for untreated chloroplasts and in absence of any inhibitor was 0.83 nanomoles \[^{14}C\]-ADP released/mg Chl, for dark adapted chloroplasts and 0.69 nanomoles \[^{14}C\]-ADP released/mg Chl for illuminated chloroplasts.
(iii) In presence of energy transfer inhibitors

(a) Control chloroplasts

The light-induced release of $^{14}$C-ADP decreased by 5% when 20μM DCCD was added to the assay mixture (Table 2.1). However, in presence of 2mM phloridzin a slight increase, about 3%, in the energy dependent release of $^{14}$C-ADP was observed (Table 2.1).

(b) In fluorescamine treated chloroplasts

With increasing concentration of fluorescamine, an increase in the energy dependent release of $^{14}$C-ADP was observed when energy transfer inhibitors were present in the assay mixture (See Fig. 2.1). The maximum increase in the activity in presence of 20μM DCCD was 64% at a fluorescamine concentration of 4.00 μmoles. However, in presence of 2mM phloridzin in the assay mixture the maximum increase in the activity was about 35% at 4.00 μmole fluorescamine.

It may be noted that the modification of thylakoid membranes either in dark or in light (see later) with fluorescamine was done in absence of ionophores and energy transfer inhibitors. These modified thylakoid membranes were then used to study the energy dependent release of $^{14}$C-ADP in presence or absence of ionophores and energy transfer inhibitors.
A.2. Modification of thylakoid membranes with fluorescamine in light

(i) In absence of ionophores and energy transfer inhibitors

The energy dependent release of $^{14}$C-ADP increased when the salt-washed thylakoid membranes were treated in light with increasing concentrations of fluorescamine (See Fig. 2.2). The maximum increase in the activity was about 30% at a fluorescamine concentration of 1 μmole. However, at higher concentrations (> 1 μmole) of this modifier, no further increase in the activity was seen.

(ii) In presence of ionophores

(a) Control chloroplast (without any treatment)

The addition of 0.4μM gramicidin D to the assay mixture resulted in the decrease energy dependent release of $^{14}$C-ADP by 3.5% whereas in presence of 1.25μM valinomycin, the decrease in the activity was 15% (See Table 2.1).

(b) In fluorescamine modified thylakoid membranes

A similar increase in the light-induced release of $^{14}$C-ADP with increasing concentrations of fluorescamine was observed in presence of ionophores as was seen in
the absence of ionophores (See Fig. 2.2). The maximum increase in the activity was about 25% at a fluorescamine concentration of 4.00 μmoles in presence of 0.4 μM gramicidin D. In presence of 1.25 μM valinomycin in the assay mixture, the maximum increase in the activity was about 41% even at very low concentration (0.5 μmoles) of fluorescamine though at higher concentration (>0.5 μmoles) of the modifier the increase in the activity was less (See Fig. 2.2).

(iii) In presence of energy transfer inhibitors
(a) Control chloroplasts
In control chloroplasts, the addition of 20μM DCCD increased the energy dependent release of \([^{14}C]\)-ADP by about 14% whereas 2mM phloridzin increased the activity by 24% (See Table 2.1).

(b) In fluorescamine modified thylakoid membranes
In presence of energy transfer inhibitors a similar pattern of increase in the light-induced release of \([^{14}C]\)-ADP with increasing concentration of fluorescamine was observed as seen in the absence of energy transfer inhibitors (Fig. 2.2). The maximum increase in the activity in presence of 20μM DCCD was 62% at a fluorescamine concentration of 2.00 μmoles whereas the presence of 2mM phloridzin in the assay system caused
an increase of 31\% in the activity at fluorescamine concentration of 2.00 μmoles. However, at higher concentrations (> 2 μmoles) of the chemical modifier, the increase in the activity was less (Fig. 2.2).

B. Non-Cyclic Photophosphorylation

The non-cyclic Photophosphorylation coupled to electron transport from H₂O to methyl viologen was carried out under identical conditions of energy dependent release of [¹⁴C]-ADP. In addition to modification of salt-washed thylakoid membranes with fluorescamine in dark or in light, we have also carried out modification in dark+light and studied the effect of presence or absence of ADP during modification steps on non-cyclic photophosphorylation.

B.1. Modification of salt-washed thylakoid membranes with fluorescamine in dark

(i) In presence of ADP

An increase in the rate of non-cyclic photophosphorylation, coupled to electron transport from H₂O to methyl viologen, was observed when salt-washed thylakoid membranes were treated in dark with fluorescamine (See Fig. 2.3). The maximum increase in the activity was about 82\% at a fluorescamine concentration of 4.00 μmoles.
Fig. 2.3.
The non-cyclic photophosphorylation as a function of fluorescamine concentration. The treatment of salt-washed thylakoid membranes with fluorescamine was done in dark as described in Fig. 2.1. in presence or absence of cold ADP. For details see MATERIALS AND METHODS. Control activity for untreated chloroplasts was 113.5 μmoles ATP formed/mg Chl/hr.

Fig. 2.4.
Same as in Fig. 2.3. except that modification with fluorescamine was carried out in light. Control activity was 102.6 μmoles ATP formed/mg Chl/hr.
(ii) In absence of ADP
When the salt-washed thylakoid membranes were treated in dark with fluorescamine, in absence of ADP, a decrease in the rate of non-cyclic photophosphorylation was observed (See Fig. 2.3). A maximum inhibition of 20% in the activity occurred at 2.00 µmoles fluorescamine. At lower concentrations of fluorescamine there was no significant change in the activity.

B.2. Modification of salt-washed thylakoid membranes with fluorescamine in light

(i) In presence of ADP
A substantial reduction in the rate of non-cyclic photophosphorylation, coupled to electron transport form $H_2O$ to methyl viologen, was observed when salt-washed thylakoid membranes in their energized state (i.e. in light) were treated with fluorescamine in presence of ADP (See Fig. 2.4). The rate of decrease of photophosphorylation was more at lower concentrations of fluorescamine as compared to inhibition of phosphorylation activity at higher concentrations of fluorescamine. The decrease in activity was approximately 35% at 1.00 µmole fluorescamine whereas the maximum inhibition of 45% was observed at 4.00 µmoles fluorescamine.
(ii) In absence of ADP

However, when the salt-washed thylakoid membranes in their energized state (i.e. in light) were treated with fluorescamine, in absence of ADP, a very sharp decline in the rate of non-cyclic photophosphorylation was observed even at very low concentrations of fluorescamine (See Fig. 2.4). A maximum inhibition of 55% in the rate of photophosphorylation was observed at 0.5 μmoles fluorescamine and this inhibition in the activity was same even at 4.00 μmoles fluorescamine.

B.3. Chemical modification of salt-washed thylakoid membranes with fluorescamine, first in dark and then in light

(i) In presence of ADP

The rate of non-cyclic photophosphorylation, coupled to electron transport from water to methyl viologen, decreased when salt-washed thylakoid membranes were treated with increasing concentrations of fluorescamine, first in dark and then in light, in presence of ADP. The rate of decrease of activity was higher till 2.00 μmoles fluorescamine as compared to rate of decrease of activity at higher concentrations. The maximum inhibition of 41% was observed at 2.00 μmoles of fluorescamine (See Fig. 2.5).
Fig. 2.5.
Same as in Figs. 2.3. and 2.4. except that the salt-washed thylakoid membranes were treated with fluorescamine, first in dark and then in light, in presence or absence of ADP. Control activity was 104.3 μmoles ATP formed/mg Chl/hr.
Light + Dark + ADP

Light + Dark without ADP
(ii) In absence of ADP

A continuous decline in the rate of non-cyclic photophosphorylation was observed when thylakoid membranes were treated with fluorescamine, first in dark and then in light in absence of ADP (See Fig. 2.5). A maximum inhibition of 41% in the rate of photophosphorylation was observed at 4.00 μmoles fluorescamine.

IV DISCUSSION

It has been shown by Strotmann et al. (1973) that repeated washing of thylakoid membranes with medium having low salt concentration removes most of the extrinsic proteins except CF$_1$ which remains bound to thylakoid membranes. Fluorescamine is a specific covalent chemical modifier of primary amino group of amino acids, peptides and proteins (Udenfriend et al., 1972). In the salt-washed thylakoid membranes, fluorescamine basically modifies ε-NH$_2$ group of lysine residues of CF$_1$ under specified experimental conditions.

Our results as shown in Figs 2.1 and 2.3 indicate a close relationship between energy dependent release of ADP and photophosphorylation (Mills and Mitchell, 1984; Strotmann et al., 1979). These observations also suggest that ε-NH$_2$ group of lysine residues of CF$_1$ which are normally exposed in dark are involved in regulating both the energy dependent release of ADP and photophosphorylation. It seems that these ε-NH$_2$ group of lysine residues of CF$_1$ get buried
into the hydrophobic domain of CF₁ during energy dependent conformational rearrangement in CF₁ (Kraayenhof and Slater, 1974). When ε-NH₂ group of lysine residues of CF₁ in thylakoid membranes are first modified in dark with fluorescamine and then brought to high energy state by illumination, the new conformational state of CF₁ is expected to be quite different from the conformational state of CF₁ on unmodified illuminated thylakoid membranes. It can therefore be presumed that the regulation of CF₁ would also be different resulting in an increase in the rate of both the energy dependent release of ADP and photophosphorylation. Mills and Mitchell (1984) have proposed that protonation of ionizable groups on P-side of CF₁ and deprotonation of ionizable groups on N-side of CF₁ leads to activation of CF₁ whereas deprotonation of ionizable group on P-side of CF₁ and protonation of ionizable groups on N-side of CF₁ leads to inactivation of CF₁. Mills and Mitchell (1984) have further contented that modulation of these ionizable groups on P and N sides of CF₁ which leads to activation/deactivation of CF₁ results in an increase or decrease of fraction of CF₁ that are active respectively. It appears to us that the above mentioned (dark exposed) ε-NH₂ groups of lysine residues of CF₁ might be involved in protonation-deprotonation events in CF₀-CF₁ complex and modification of these
amino group by fluorescamine increases the $\phi$ as shown by increase in energy dependent release of ADP and concommittantly increase in the rate of non-cyclic photophosphorylation.

However, modification of salt-washed thylakoid membranes with fluorescamine during illumination and in presence of ADP caused a maximum increase in the rate of energy dependent release of ADP by 30\% (Fig. 2.2) whereas non-cyclic photophosphorylation activity was inhibited by 45\% (Fig. 2.4). This observation suggests that (1) $\varepsilon$-NH$_2$ group of lysine residues of CF$_1$ which get exposed during the high energy state and thereby become available for reaction with fluorescamine, are involved at the catalytic site of ATP synthesis. The modification of such $\varepsilon$-NH$_2$ group of lysine residues of CF$_1$ with fluorescamine would result in inactivation of the active site for ATP synthesis leading to inhibition of photophosphorylation. (2) the chemical modification, with fluorescamine, of $\varepsilon$-NH$_2$ group of lysine residues of CF$_1$ which get exposed during high energy state results in energy transfer inhibition. It has been shown with regard to certain energy transfer inhibitors and also with certain chemical modifiers that while they inhibit the rate of non-cyclic photophosphorylation (and ATPase activity)
at the same time they increase energy dependent release of ADP (or increase the number of binding sites for ADP in CF₁).

The sharp decline in the rate of non-cyclic photophosphorylation at low concentrations of fluorescamine was less when ADP was present during chemical modification (See Fig. 2.4). However, at higher concentrations of fluorescamine, protective effect of ADP was less. Substrates, in general, have been shown to protect the enzymes against chemical modification of active or regulatory site (in allosteric enzymes) by chemical modifiers and therefore protect them from inhibition in the enzyme activity. This is usually the case when substrate bind to either active or regulatory site or both of them and thus shield it from modifiers. These observations further support our suggestions that ε-NH₂ group of lysine residues of CF₁ which are exposed during the high energy state are involved at the catalytic site of ATP synthesis.

Our studies on the effect of fluorescamine treatment of salt-washed thylakoid membranes in light + dark or dark and light alone further suggest that (1) the ε-NH₂ group of lysine residue of CF₁ which get exposed during the energy dependent conformational changes in
CF$_1$ are involved at the catalytic site of ATP synthesis (2) the ε-NH$_2$ group of lysine residues of CF$_1$ exposed even in the dark, get buried during the energy dependent conformational rearrangement in CF$_1$ and then play a conformational role in regulating CF$_0$-CF$_1$ complex activities (see our discussion above regarding changes in $\phi$ e.g; compare dark and light modification).

Similarly the double modification of salt-washed thylakoid membranes with lower concentrations of fluorescamine in absence of ADP led to a slower decline in the rate of non-cyclic photophosphorylation (See Fig. 2.5) as compared to a very sharp decline in the rate of non-cyclic photophosphorylation observed when salt-washed thylakoid membrane were modified with very low concentration of fluorescamine in light alone in the absence of ADP (Fig. 2.4). The extent of maximum inhibition in the former case which was approximately 41% (Fig. 2.5) is much less as compared to 55% (Fig. 2.4) inhibition in the phosphorylation activity in the latter case. This observation and the discussion above suggest that double modification of thylakoid membranes by fluorescamine, first in dark and then in light, lead to not only energy transfer inhibition due to modification of ε-NH$_2$ group of lysine residues of CF$_1$ exposed during high energy
state but also causes a different conformational state and concomittantly different kind of regulation of CF$_0$-CF$_1$ complex due to modification of dark exposed ε-NH$_2$ group of lysine residues of CF$_1$. These changes in CF$_0$-CF$_1$ complex would cause alterations in δH$_+$ for activation (the fraction of active CF$_1$) resulting in changes in photophosphorylation activity of CF$_0$-CF$_1$ complex.

It is shown in Fig. 2.3 that in absence of ADP, modification of salt-washed thylakoid membranes in dark with increasing concentrations of fluorescamine caused 20% inhibition in the rate of non-cyclic photophosphorylation. This is in sharp contrast to 82% enhancement in the activity observed when ADP was added during the chemical modification (Fig. 2.3). We may speculate that modification of dark exposed ε-NH$_2$ group of lysine residues of CF$_1$ with fluorescamine, in presence of ADP leads to such a conformational state of CF$_1$ that when fluorescamine modified thylakoid membranes are brought to high energy state it results in the increase in $\phi$ (See Fig. 2.1). The increase in $\phi$ may be due to less requirement for δH$_+$ for activation (Mills and Mitchell, 1984). However, in absence of ADP during modification with fluorescamine, the new conformational
state could bring about decrease in $\phi$ arising due to increasing requirement for $\Delta \mu_{H^+}$ for activation. As we have mentioned previously that in general substrates protect the enzymes from chemical modification and subsequent inhibition of activity, by binding to the active site. However, there are other sites in the enzyme molecule which can be attacked by chemical modifiers. The modification of such sites might result in differential regulation of enzymatic activity.

A maximum inhibition of 20% and 29% in the rate of energy dependent release of ADP from dark adapted salt-washed thylakoid membranes were observed in presence of 0.4 $\mu$M gramicidin D and 1.25 $\mu$M valinomycin respectively (See Table 2.1). However, the inhibition in the activity was less when preilluminated thylakoid membranes were used (Table 2.1). This can be explained by the fact that collapse of $\Delta \psi$ by ionophores would lead to decrease in $\Delta \mu_{H^+}$ resulting in decrease in $\phi$. This is exactly reflected in the decrease in energy dependent release of ADP. The decrease in $\phi$ would also lead to decrease in the rate of non-cyclic photophosphorylation (See Chapter 6). However, the effect of preillumination causing a less decrease in the rate of energy dependent release of ADP by ionophores is not understood clearly.
Our studies on the effect of energy transfer inhibitors on dark adapted thylakoid membranes as well as on preilluminated thylakoid membranes show that dark adapted thylakoid membranes react with energy transfer inhibitor in a different way compared to preilluminated thylakoid membranes (See Table 2.1).

V. CONCLUSIONS

In conclusion we would like to suggest that it is difficult to assume that the putative protoneural network (Laszlo et al., 1984a, 1984b) would be affected by fluorescamine and cause changes in the energy dependent release of ADP and photophosphorylation. Since such a protoneural network is supposed to be located in the hydrophobic core of thylakoid bilayer where it would be difficult for fluorescamine to enter. Our results seem to be more compatible with the delocalized mechanism of energy transduction in thylakoid membranes (Mills and Mitchell, 1984). Our observations suggest that

(1) $\epsilon$-NH$_2$ group of lysine residues of CF$_1$ normally exposed in dark are involved in regulating energy dependent activation of CF$_1$ and photophosphorylation activity,
(2) $\epsilon$-NH$_2$ group pf lysine residues of CF$_1$ exposed during the energy dependent conformational rearrangement in
CF₁, are involved at the active site of ATP synthesis,

(3) Fluorescamine modification of ε-NH₂ group of lysine residues of CF₁ results in (a) changes in protonation-deprotonation profile of ionizable groups leading to changes in pKₐ values (b) significant conformational changes in CF₁. This is reflected in changes in Δμₗ⁺ as well as φ and consequently changes in energy dependent release of ADP and non-cyclic photophosphorylation.

VI. REFERENCES


Chapter 3

The Interaction of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazoole (NBD-Cl) with Thylakoid Membrane Bound CF₁

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I. INTRODUCTION

Deters et al. (1975) have shown that NBD-Cl reacts with one or two tyrosine residues on β-subunit of CF₁ resulting in the abolition of ATPase activity of CF₁. The inhibition of ATPase activity by NBD-Cl was completely reversed by DTT which removed the bound NBD-Cl. Spectral evidence suggested that a tyrosine residue on CF₁ was modified. Similar effects have also been observed with E. coli F₁ (Bragg and Hou, 1977). Cantley and Hammes (1975) observed that reaction of one mole of NBD-Cl per mole of spinach CF₁ resulted in complete inhibition of CF₁-ATPase activity, and it also prevented the binding of ADP and adenylyl imidodiphosphate to the heat activated CF₁.

The modification of Rhodospirillum rubrum chromatophores with NBD-Cl led to the complete inhibition of ATP synthesis and ATP hydrolysis in Rh. rubrum chromatophores (Khananshivili and Gromet-Elhanan, 1983). However, the site of membrane bound F₁ (RrF₁) responsible for inhibition of these activities by NBD-Cl is not known. In isolated F₁ from Rhodospirillum rubrum chromatophores (RrF₁) the binding of 1-2 moles of NBD-Cl per mole of RrF₁ caused complete inhibition of Ca⁺⁺-ATPase activity. However, NBD-Cl modification of β-
subunit which was isolated directly from *Rh. rubrum* chromatophores did not affect its reconstitution to β-less chromatophores. ATP synthesis as well as ATP hydrolysis were fully restored when NBD-Cl modified β-subunits were reconstituted with β-less chromatophores. Further treatment of this reconstituted system with NBD-Cl led to inhibition of both ATP synthesis and ATP hydrolysis. Based on these observations Khananshvili and Gromet-Elhanan (1983) proposed that modification of an additional tyrosine residue of membrane bound RrF₁ with NBD-Cl is responsible for inactivation of both ATP synthesis and ATP hydrolysis. But it is not known that whether this tyrosine residue is located on β-subunit or on some other subunit of RrF₁ (Gromet-Elhanan and Khananshivili, 1984).

It has been suggested that modification of CF₁ with NBD-Cl, which results in inactivation of ATPase activity is due to irreversible conformational changes in CF₁ rather than interaction of NBD-Cl with the catalytic site (Carlier et al., 1979; Holowka and Hammes, 1977). Based on several studies on modifications of tyrosine residues of F₁, it has been proposed that tyrosine residues in proton ATPases may be involved in (i) interactions with adenine at the site of nucleo-
tide binding, (ii) conformation of the enzyme and (iii) proton transfer reaction during ATP synthesis or hydrolysis (Ting and Wang, 1980; Mal'yan et al., 1985).

Here we present our studies on the effect of chemical modification of tyrosine residues of membrane bound CF₁ with NBD-Cl on energy dependent release of ADP as well as photophosphorylation.

II. MATERIALS AND METHODS

A. Isolation of Chloroplasts

The salt-washed thylakoid membranes were prepared and resuspended in the prelabelling medium as described earlier (See Chapter 2).

B. Modification of Salt-Washed Thylakoid Membranes with NBD-Cl

NBD-Cl solution was freshly prepared in methanol. 10 μl aliquot of NBD-Cl solutions of varying concentrations were added to chloroplasts suspensions in prelabelling medium in a final volume of 1 ml and chlorophyll concentration of 1 mg/ml. The final concentration of methanol did not exceed 1%. The samples were then incubated in dark at 0°C for four hours. After the dark
incubation the samples were illuminated with white light for 1 minute as described previously (See Chapter 2). The contents were centrifuged and the pellet was washed as described earlier in Chapter 2.

C. Light-Induced Release of $^{14}$C]-ADP Bound to CF$_1$

The light-induced release of $^{14}$C]-ADP was carried out in the same manner as described in Chapter 2.

D. Photophosphorylation

The rates of non-cyclic photophosphorylation both in control and in NBD-Cl thylakoid membranes were determined as described before (See Chapter 2). However, $^{14}$C]-ADP was replaced by cold ADP during chemical modification of salt-washed thylakoid membranes with NBD-Cl.

E. Source of Chemicals

NBD-Cl was purchased from Sigma Chemical Co., USA.

III. RESULTS

A. Energy Dependent Release of $^{14}$C]-ADP Bound to CF$_1$

(i) In absence of uncouplers, ionophores and energy transfer inhibitors

The modification of salt-washed thylakoid membranes with NBD-Cl did not affect significantly the rate of energy