Fig. 3.1.
The effect of chemical modification of salt-washed thylakoid membranes with increasing concentration of NBD-Cl, on energy dependent release of $[^{14}\text{C}]-\text{ADP}$. The salt-washed thylakoid membranes were incubated with NBD-Cl in dark at $0^\circ$ for 4 hours. Prelabelling and energy dependent release of $[^{14}\text{C}]-\text{ADP}$ were carried out as described in MATERIALS AND METHODS. The final concentrations of DCCD and phloridzin wherever used were $20\mu\text{M}$ and $2\text{mM}$ respectively. Control activity for untreated chloroplasts in absence of any inhibitor was $0.495$ nanomoles $[^{14}\text{C}]-\text{ADP}$ released/mg Chl.

Fig. 3.2.
Same as in Fig. 3.1. The concentrations of gramicidin D (GmCD) and valinomycin wherever used were $0.4\mu\text{M}$ and $1.25\mu\text{M}$ respectively. Control activity was $0.495$ nanomoles $[^{14}\text{C}]-\text{ADP}$ released/mg Chl.
% of Control Activity vs NBD-Cl, μM

- Control
- +DCCD
- +Phloridzin
- +GmCD
- +Valinomycin

NBD-Cl, μM
dependent release of $[^{14}C]$-ADP (Fig. 3.1). A maximum inhibition of $12\%$ in the energy dependent release of $[^{14}C]$-ADP was observed when the concentration of NBD-Cl was raised to 200 $\mu$M.

(ii) In presence of energy transfer inhibitors

(a) Control chloroplasts

The rate of energy dependent release of $[^{14}C]$-ADP from salt-washed thylakoid membranes increased by $10.5\%$ when 20 $\mu$M DCCD was present in the assay mixture (Table 3.1). However, in presence 2mM phloridzin $9\%$ decrease in the activity was observed (Table 3.1).

(b) In NBD-Cl modified chloroplasts

The presence of DCCD in the assay mixture did not affect significantly the changes in the rates of energy dependent release of $[^{14}C]$-ADP from salt-washed thylakoid membranes treated with NBD-Cl (Fig. 3.1). The maximum inhibition in the activity was $5\%$ at 200$\mu$M NBD-Cl. Similarly 2mM phloridzin did not affect the rate of change of energy dependent release of $[^{14}C]$-ADP from salt-washed thylakoid membranes treated with NBD-Cl (Fig. 3.1).

(iii) In presence of ionophores

(a) Control chloroplasts

The addition of either $0.4\mu$M gramicidin D or $1.25\mu$M valinomycin to the assay mixture did not affect the rate of
TABLE 3.1
EFFECT OF ENERGY TRANSFER INHIBITORS, IONOPHORES AND UNCOUPLERS ON ENERGY DEPENDENT RELEASE OF [$^{14}$C]-ADP FROM SALT-WASHED THYLAKOID MEMBRANES

<table>
<thead>
<tr>
<th>Control activity</th>
<th>Energy Transfer Inhibitors</th>
<th>Ionophores</th>
<th>Uncouplers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20μM DCCD</td>
<td>2mM Phloridzin</td>
<td>0.4μM Gramicidin D</td>
</tr>
<tr>
<td>100%</td>
<td>(+)10.5</td>
<td>(-)8.8</td>
<td>0</td>
</tr>
</tbody>
</table>

(-) in parentheses indicate inhibition
(+ ) in parentheses indicate enhancement

Control activity was 0.495 nanomoles [$^{14}$C]-ADP released/mg Chl.
energy dependent release of $[^{14}\text{C}]-\text{ADP}$ from salt-washed thylakoid membranes (Table 3.1).

(b) In NBD-Cl treated chloroplasts

The addition of ionophores to the assay mixture did not cause any significant alteration in the pattern of energy dependent release of $[^{14}\text{C}]-\text{ADP}$ from salt-washed thylakoid membranes treated with NBD-Cl (Fig. 3.2). In the presence of 6$\mu\text{M}$ gramicidin D the maximum inhibition of activity was 89% at 10$\mu\text{M}$ concentration of NBD-Cl whereas at higher concentrations of NBD-Cl the inhibition was less (Fig. 3.2). In presence of 1.25$\mu\text{M}$ valinomycin a maximum inhibition of 11% in the rate of energy dependent release of $[^{14}\text{C}]-\text{ADP}$ was observed at 200$\mu\text{M}$ NBD-Cl (Fig. 3.2).

(iv) In presence of uncouplers

(a) Control chloroplasts

A very small decrease ~3% in the rate of energy dependent release of $[^{14}\text{C}]-\text{ADP}$ was observed in presence of 10mM $\text{CH}_3\text{NH}_2$ or 10$\mu\text{M}$ nystatin (Table 3.1). However, in presence of 20$\mu\text{M}$ atebrin, increase in the activity was 10.7%.

(b) In NBD-Cl modified chloroplasts

The addition of either 10mM $\text{CH}_3\text{NH}_2$ or 20$\mu\text{M}$ atebrin to the assay mixture did not change the pattern of energy
dependent release of $[^{14}\text{C}]$-ADP observed in presence of NBD-Cl (Fig. 3.3). However, in presence of 10$\mu$M nystatin in the assay mixture, the rate of decline in the activity with NBD-Cl was more as compared to those without nystatin (Fig. 3.3). The maximum inhibition of activity was 23% at 200$\mu$M NBD-Cl.

B. Non-Cyclic Photophosphorylation

(i) In presence of ADP during chemical modification

The rate of non-cyclic photophosphorylation coupled to electron transport from water to methyl viologen, decreased when salt-washed thylakoid membranes were modified in dark with NBD-Cl in presence of ADP (Fig. 3.4). A maximum inhibition of 60% in the rate of photophosphorylation was observed at 400$\mu$M NBD-Cl.

(ii) In absence of ADP during chemical modification

In contrast to the inhibition of photophosphorylation (See Fig. 3.4), the chemical modification of salt-washed thylakoid membranes with NBD-Cl, in absence of ADP, resulted in increase in the rate of photophosphorylation. A maximum stimulation of 35% in the rate of photophosphorylation was observed at 400$\mu$M NBD-Cl (Fig. 3.4).
Fig. 3.3.

Same as in Fig. 3.1. The concentrations of CH$_3$NH$_2$, nystatin and atebrin wherever used were 10mM, 10μM and 20μM respectively. Control activity was 0.495 nanomoles [¹⁴C]-ADP released/mg Chl.

Fig. 3.4.

The non-cyclic photophosphorylation as a function of NBD-Cl concentration. For details see MATERIALS AND METHODS and Fig. 3.1. Control rate was 92.3 μmoles ATP formed/mg Chl/hr.
IV. DISCUSSION

NBD-Cl has been shown to react with phenolic hydroxyl group of tyrosine residues in proteins (Deters et al., 1975). In salt-washed thylakoid membranes, NBD-Cl would cause chemical modification of those tyrosine residues of CF$_1$ which are exposed in dark and thus available for modification. Our observations on the NBD-Cl modification of phenolic hydroxyl group of tyrosine residues of CF$_1$ in salt-washed thylakoid membranes show that while the rate of energy dependent release of ADP was not affected significantly, the rate of photophosphorylation declined sharply with increasing concentrations of NBD-Cl (compare Fig. 3.1 with Fig. 3.4). These observations suggest that phenolic hydroxyl group of tyrosine residues of CF$_1$ (normally exposed in dark) though involved in regulating the photophosphorylation activity, yet are not involved in the processes which cause energy dependent release of ADP. Therefore, we propose that modification of such tyrosine residues by NBD-Cl lead to a kind of energy transfer inhibition as observed with phloridzin (See Chapter 4). Thus, these tyrosine residues of CF$_1$ are involved at the catalytic site of the enzyme in ATP synthesis since their modification by NBD-Cl blocks only the terminal steps of ATP synthesis. Based on
our results we propose here a model (See Fig. 3.5 below) in which energy dependent release of ADP from the adenine nucleotide binding site activates the enzyme for ATP synthesis or hydrolysis. However, when the active site is modified by NBD-Cl, though the energy dependent release of ADP is not affected, the photophosphorylation activity is proportionately reduced.

**Fig. 3.5.** A diagrammatic representation of action of NBD-Cl on CF₁.
It has been proposed by Bicket-Sandkötter (1983) that the active site of \( \text{CF}_1 \) involved in ATP synthesis is physically the same as the site of binding of adenine nucleotide. The energy dependent release of adenine nucleotide from this site converts the inactive conformation into active conformation of \( \text{CF}_1 \). However, based on our result as described in this Chapter and also in Chapters 2 and 4 we would like to suggest that the active site of ATP synthesis and adenine nucleotide binding site on \( \text{CF}_1 \) are physically separate though they interact with each other. The energy dependent release of adenine nucleotide from adenine nucleotide binding site causes conformational changes in the ATPase molecule and this leads to conversion of inactive conformation of \( \text{CF}_1 \) to active conformation.

Our observations further suggest that phenolic hydroxyl group of tyrosine residues of \( \text{CF}_1 \) are present at the active site of ATP synthesis. This supports the earlier proposals of Ting and Wang (1980).

Our data (See Figs 3.1, 3.2 and 3.3 and Table 3.1) show that the energy dependent release of ADP both from control chloroplasts and from thylakoid
membranes treated with NBD-Cl is not affected significantly by ionophores, energy transfer inhibitors and uncouplers (except Nystatin). This shows that both unmodified and NBD-Cl modified thylakoid membranes interact with these perturbents in the same manner.

V. CONCLUSIONS
(1) Our observations indicate that phenolic hydroxyl group of tyrosine residues of CF$_1$ are involved at the active site of CF$_1$-ATPase.
(2) The active site of ATP synthesis and adenine nucleotide binding site on CF$_1$ are at different location on CF$_1$ however they interact with each other.

VI. REFERENCES


Chapter 4

The Interaction of Energy Transfer Inhibitors with CF$_0$ - CF$_1$ Complex

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