行政院國家科學委員會專題研究計畫 成果報告

單分子偵測技術探討 H(+)-ATP 合成胺催化反應動力學
研究成果報告(精簡版)

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計 畫 主 持 人：蘇志明

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行政院國家科學委員會補助專題研究計畫 成果報告

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計畫參與人員：

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Annual Report to the National Science Council

(95-2113-M-002-029)

Tzu-Min Su

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In the past few years, we have been working on the conformational structures and enzyme activity of the H^+-ATPase purified from the chloroplasts. Three general topics were addressed in this research. (A) The effects of the concentration gradients of the sodium and lithium ions on the activity of the H^+-ATPase. (B) The thermodynamics of the dissociation of the H^+-ATPase into its two major fragments CF_0 and CF_1. (C) The relative motion of the ε and b_1 subunits of the ATPsynthase during the hydrolysis and synthesis of the ATP molecules. In these studies, three different physical detection methods were employed. For the first one, the bioluminescence detection method through the luciferin/luciferase system was employed to measure the ATP initial synthesis rate. For the second experiment, the dynamic light scattering technique was employed to determine the concentrations of CF_0 and CF_1 under various experimental conditions, such as temperature and salt concentration. For the last experiment, the relative motions of the subunits were measured by the single-paired FRET technique.

We have made good progress on all these three subjects. Some observed and measured phenomena are new and also quite interesting. Since the biological system involved in this study is quite complicated, some loose ends of these studied are still needed to be tied up. The final formal papers have yet to be completed. We are currently working on it.

The major findings of the research are described as follows.

(A) The effects of the concentration gradients of the sodium and lithium ions on the activity of the H^+-ATPase.

(1) Sodium ion can be the driving force of H^+- ATP synthase

As shown in Fig. 1, with the condition of $[Na^+]_{in}$ /$[Na^+]_{out}$ =150 mM/ 8 mM at pH 8.0, the initial ATP synthesis rate was measured to be 3.5 s^{-1} per H^+-ATP synthase. To confirm that the ATP yield is not originated from a residual $\triangle$pH, 20 uM FCCP (proton ionophore) was added and the signal remains the same. A furthermore addition of sodium ionophore 30 uM, the signal of the ATP synthesis diminished to almost background level. Sodium ion can be the driving force to induce rotary torque and produce ATP in H^+-ATP synthase system. After that, we give the proteoliposome

the membrane potential of 30 mV by adding valinomycin 40 uM, and measure the initial rate rose to 14.7 s^{-1} . In order to confirm the signal is the Na^+ ion origin, we add the FCCP and valinomycin to the proteoliposome and find the initial rate decreases to 9.5 s^{-1}. It shows that Na^+ ion indeed produce the ATP synthesis (Vi= 9.5 s^{-1}, 30 mV) and the reduction of 5.2 s^{-1} after the addition of FCCP comes from the contribution of protons.

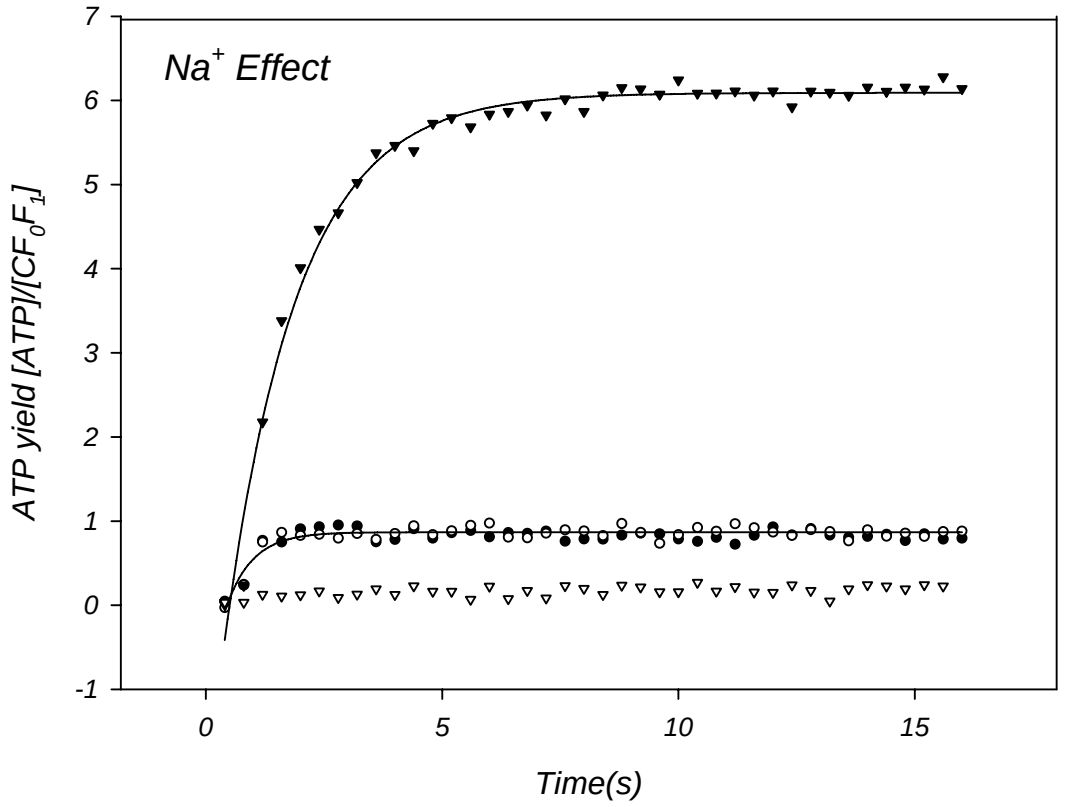


Fig 1: Effect of sodium ion on ATP synthase activity, $[Na^+]_i$ /$[Na^+]_o$ =150 mM /8mM ,pH_o is 8.0, and △ pH= 0 , ● represents $[Na^+]_i$ /$[Na^+]_o$ =150 mM/8 mM (V_i =3.5 s^{-1}), and ○ represents adding FCCP 20 uM,▼ represents with membrane potential 30 mV($[K^+]_o$/$[K^+]_i$= 130/50 mM; Valinomycin 10 uM; Vi=5.4 s^{-1})▽ represents adding sodium ionophore 30 uM.

(2) Lithium ion can not be the driving force of H^+- ATP synthase

Since the hydrogen, lithium and sodium cations are generally regarded to be similar in their ion conductivity properties, it is quite naturally to ask the effect of lithium ion on H^+-ATP synthase activity. As proteoliposomes under the condition of

$[Li^+]_i$ /$[Li^+]_o$= 150 mM/ 8 mM at pH 8.0, the initial rate was not detectable. It was apparent that lithium ion itself can not be the driving force of H^+-ATP synthase. However, as shown in Fig 2, the initial rate rises up to 13.2 s^{-1}/CF_0F_1 if a 140 mV membrane potential was introduced. In short summary, lithium ion itself can not be the driving force, however, in the presence of electrical potential 140 mV; the initial rate was accelerated to 13.2 s^{-1}/CF_0F_1. But this signal was completely quenched if 10 uM of FCCP (proton ionophore) was introduced. It appeared that the driving source is from proton. What happens in the process of lithium ion system?

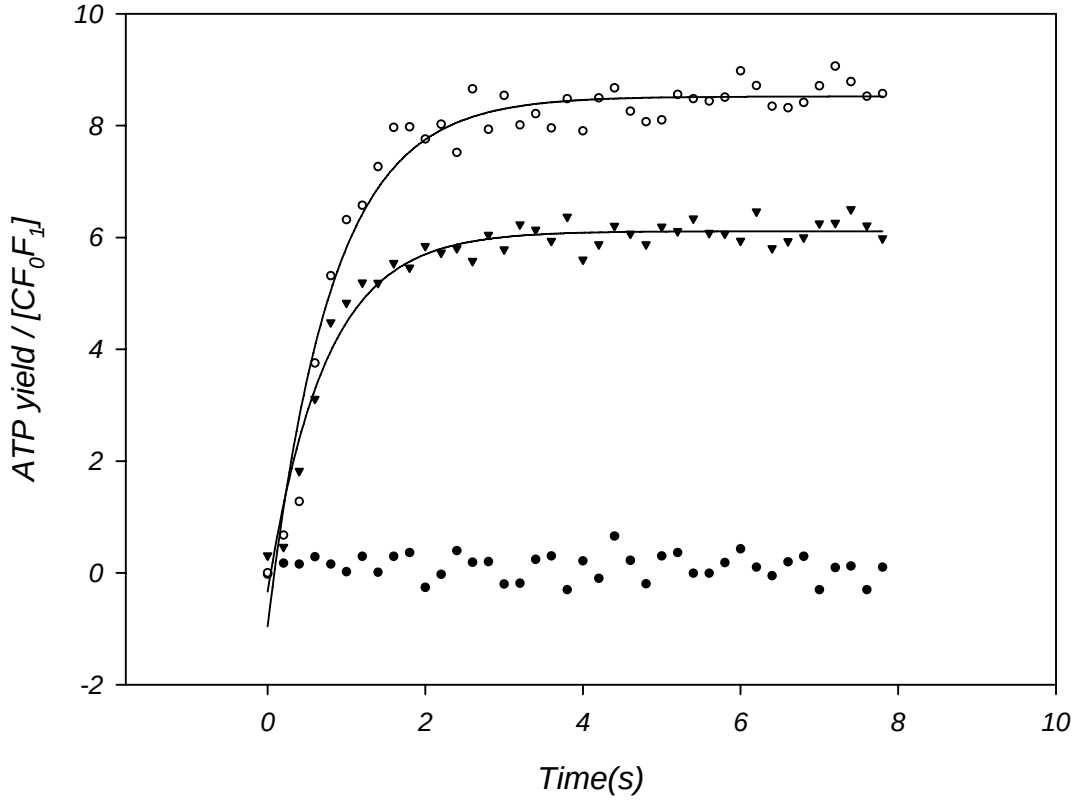


Fig 2: Effect of Lithium ion on ATP synthase activity with membrane potential 140 mV.○ represents $[Li^+]_{in}$ /$[Li^+]_{out}$ =100 mM /5 mM (Vi=13.2 s^{-1}) and Δφ =140 mV by adding Valinomycin 40 uM, and ▼ represents $[Li^+]_{in}$ /$[Li^+]_{out}$ =10 mM /5 mM and Δ φ =140 mV by adding Valinomycin 40 uM (Vi= 8.4 s^{-1}) ● represents $[Li^+]_{in}$ /$[Li^+]_{out}$ =100 mM /5 mM adding Valinomycin 40 uM and FCCP 10 uM.

(3) Lithium ion can produce proton by localized hydrolysis, i.e. local proton concentration could be enhanced by the presence of Li^+ ion

The concept that Li^+ in water could undergo localized hydrolysis with the nearby water molecules has long been suggested. As described in Fig. 3, a water molecule bound and polarized by Li^+ could donate its H^+ to a nearby proton acceptor and, in the present case that the proton acceptor is a water molecule, an OH^- ion was formed. The present initial rate measurements suggest that the lithium ion (150 mM) inside the ion channel of the ATPase could proceed the localized hydrolysis.

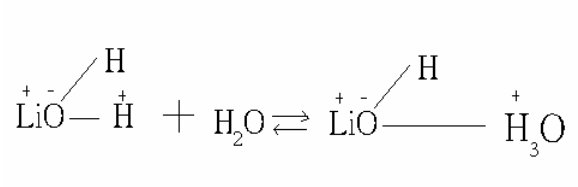


Fig. 3: Localized hydrolysis of lithium ion and produce proton

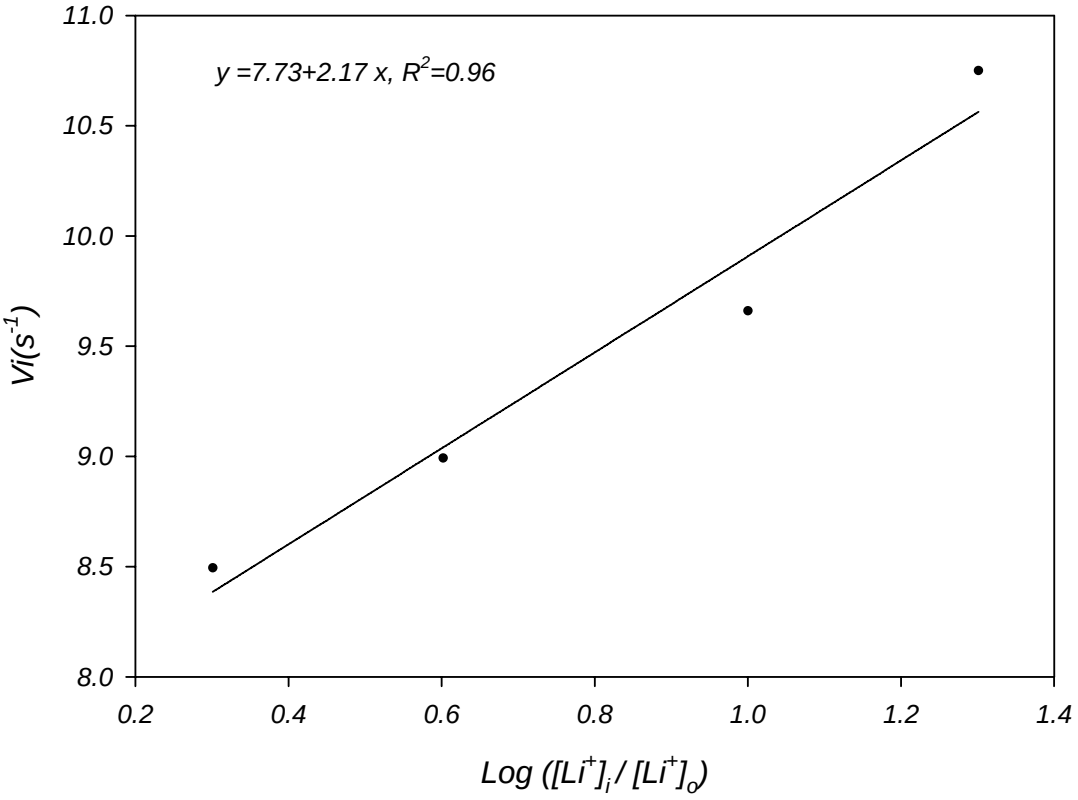


Fig 4: The relationship of pLi^+ and initial rate ($[Li^+]_i$ /$[Li^+]_o$ =10-100 mM /5 mM with $\Delta \varphi$ = 140 mV).

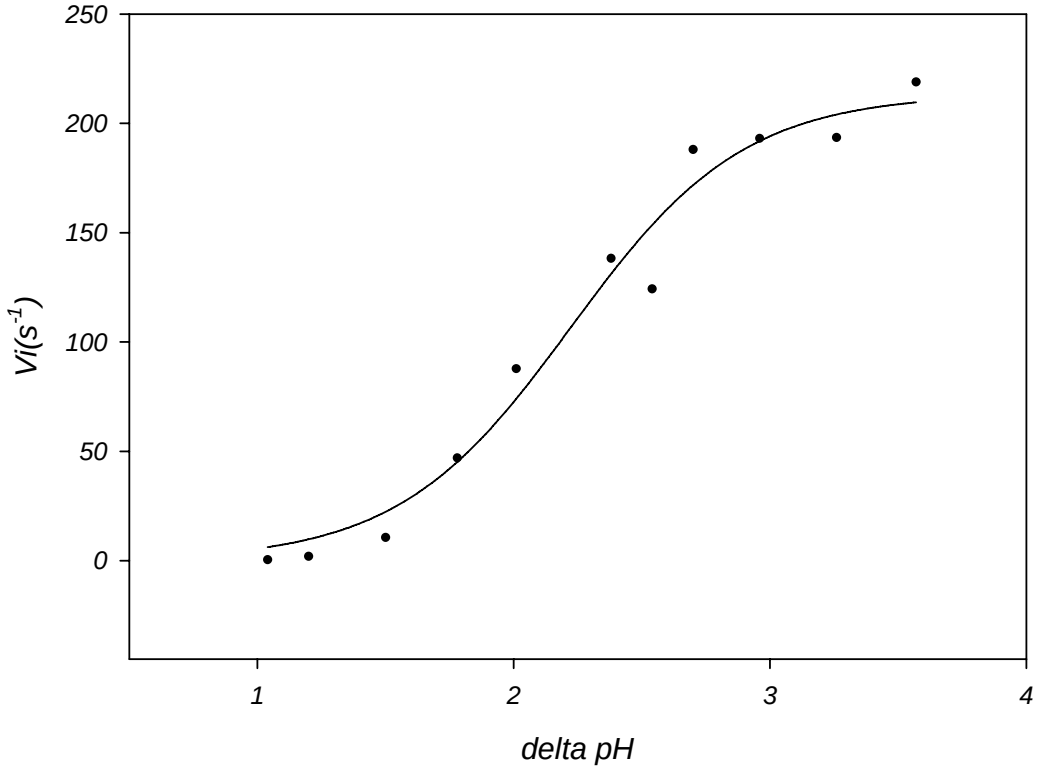


Fig 5: Initial Rate of ATP synthase energized by acid-base transition at $\Delta\varphi$ = 140 mV. pH_o = 8.0 and pH_i = 4.4-7.0 When pH_i =6.69 (ΔpH =1.31), initial rate (13.2 s^{-1}) is equal to the $[Li^+]_i$ /$[Li^+]_o$=100 mM /5 mM at ΔpH =0

($y = \frac{a}{(1+\exp(-\frac{(x-x_0)}{b})}$, a =426, b=0.337, x_0=2.22; R^2= 0.975)

Fig 5 shows that, at ΔpH=1.31 and a membrane potential 140 mV, the initial rate is equal to that under the condition of $[Li^+]_i$ /$[Li^+]_o$=100/ 5 mM. It is suggested that, when the concentration of lithium ion is 100 mM inside the proteoliposome, it's local proton concentration is equivalent to a medium with pH_i~ 6.7($[H^+]$ = 2*10^{-7} M).

If inside and outside lithium concentration are the same (no proton gradient), does it still produce ATP? Fig 6 compares the initial rate of $[Li^+]_i$/$[Li^+]_o$=75/5 mM and

$[Li^+]_i/[Li^+]_o$=75/75mM at ΔpH 4.4 ,and the ratio of initial rate is near 1. It means outer lithium ions not to affect the initial rate under our experimental condition or even no lithium ion gradient, the enzyme still produce ATP.

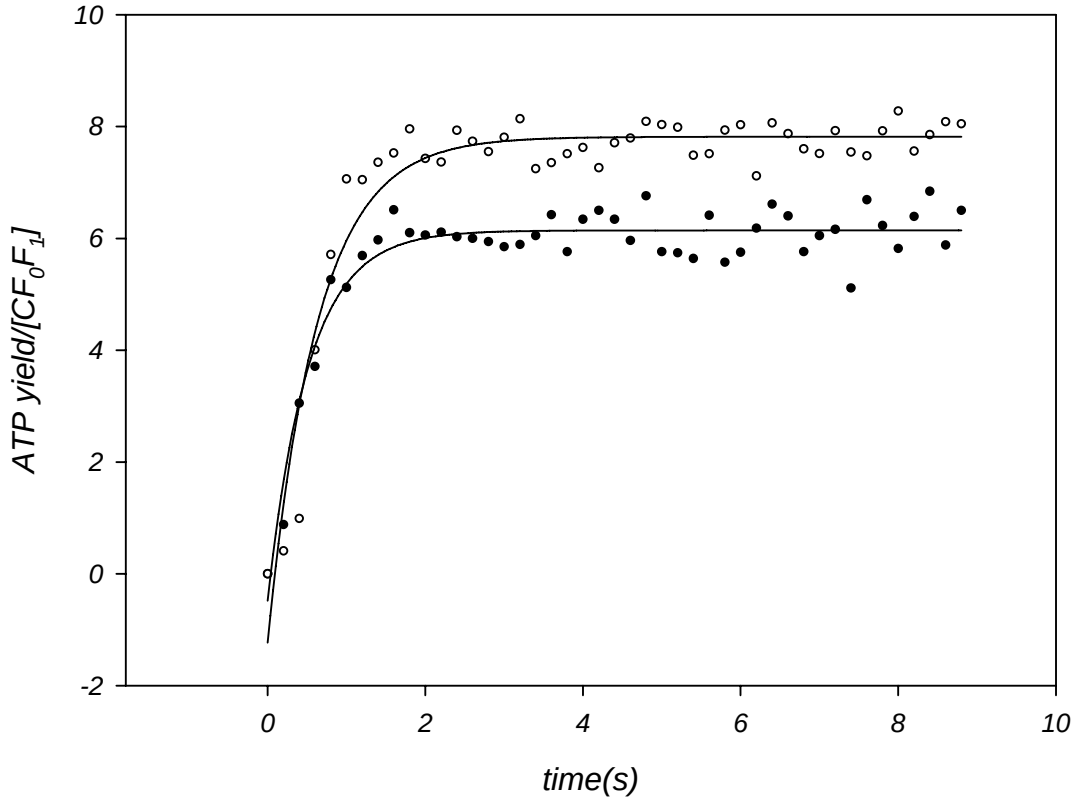


Fig 6: Comparison the initial Rate of $[Li^+]_i$ /$[Li^+]_o$ =75 mM /5 mM (○,V_i =12.8 s^{-1}) and $[Li^+]_i$ /$[Li^+]_o$ =75mM / 75 mM(●,V_i =12.4 s^{-1}) at Δ pH4.4 (pH_o=8.8, pH_i= 4.4) .

(4) Molecular orbital calculations on the enthalpies and free energies of Li^+ undergoing localized hydrolysis in the ion channel.

The enthalpy and free energy changes of the following reactions were calculated

under both the vacuum and ion channel environments

$$[CH_3COO^-Li^+ \cdot CH_3CO(NH_2)] \cdot (H_2O)_n \rightarrow$$
$$[CH_3COO^-H^+ \cdot CH_3CO(NH_2)](LiOH)(H_2O)_{n-1}$$

The number of the water molecules was varied from 1 to 5. The results with n = 5 were shown in the following two figures. It appears that the presence of the Li^+ could indeed stabilize the hydroxide species. In other words, the local H^+ concentration in the form of the CH_3COOH was greatly enhanced.

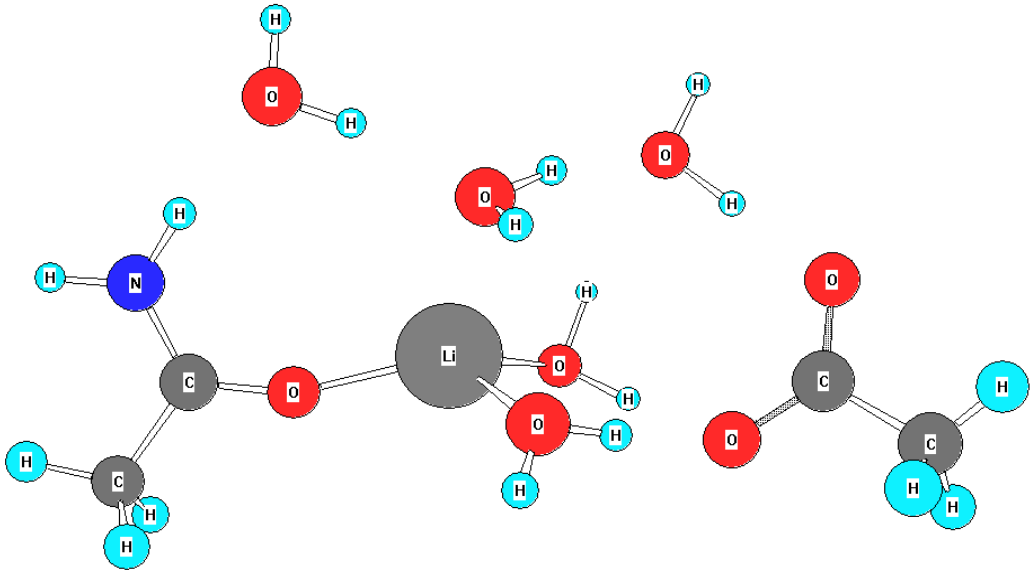


Fig 7: Molecular Orbital Simulation by running Gaussian for 5 molecules of water.H_2O does not dissociate to H^+ and OH^-.

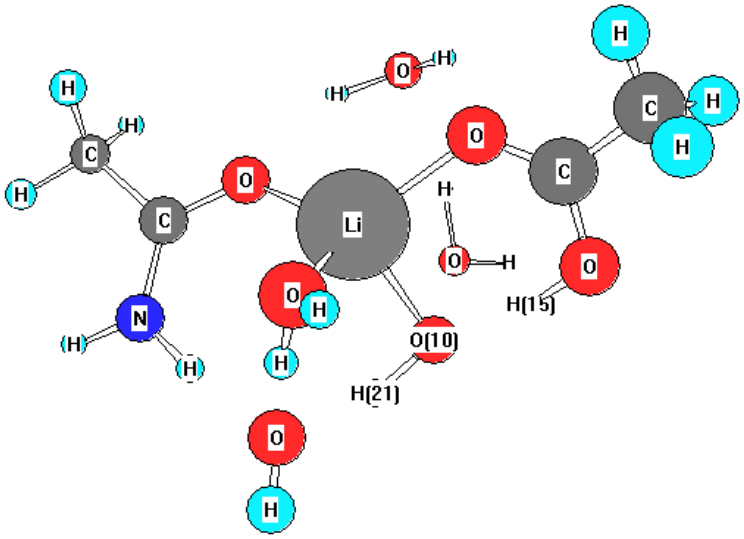


Fig 8: Molecular Orbital Simulation by running Gaussian for 5 molecules of water and dissociation of one. H_2O (O(10)) dissociates to H^+ at amino acid E and OH^- at Li^+.

These calculations show that the presence of the Li and also Na cations could help the dissociation of the water molecule into proton and the hydroxide ion. The local concentrations of the proton as well as the hydroxide ion are enhanced.

(B) The Thermodynamics of the Dissociation of the H^+-ATPase into CF_0 and CF_1 Fragments

(1) The dissociation of CF_0F_1 into CF_0 and CF_1 Fragments

The H^+-ATPase of the chloroplasts exists in either the oxidized or reduced forms. In the course of the separation of the ATPase subunts, it was found that the oxdized and reduced forms have different separation patterns. Fig. 9 shows the gel patterns of the CF_0 + ε –IV and CF_1- ε +IV fragments of the reduced ATP synthase after the sucrose

gradient centrifugation separation. Apparently, under the present separation condition, the ε subunit is attached to the CF_0 fragment, instead of the traditional CF_1 fragment. For further confirmation, the oxidized form was also prepared. Fig 10 shows that the ε subunit is attached to the CF_1 fragment for the oxidized H^+-ATPase. The interaction of the ε subunit with the rest of the ATPase is completely different in the oxidized and reduced forms.

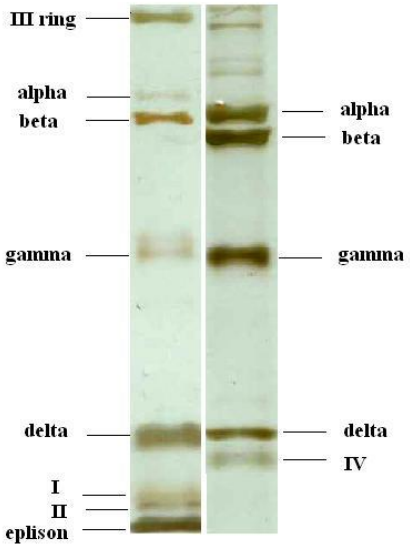


Fig 9: CF_0 + ε –IV and CF_1- ε +IV of reduced form ATP synthase (some contaminants of αβγ in CF_0+ε - IV gel)

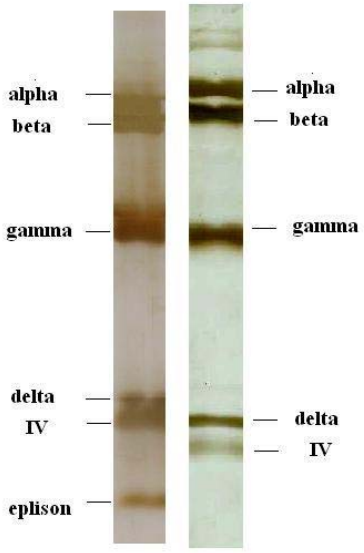


Fig 10: CF_1+IV (left, oxidized form) and CF_1-ε +IV (right, reduced form)of ATP synthase

(2) Thermodynamics of the dissociation of the ATPase in Salt Solutions

The determination of the dissociation constant and the reaction stoichiometry is an important step in the study of protein-protein interaction. It generally relies on measuring the equilibrium concentration of the complex and its components. The

range of K_d values observed in biological processes is extremely wide, and extends to at least 12 orders of magnitude from 10^{-4} to 10^{-16} M. A number of physicochemical methods are available when K_d is in the micromolar range above, including fluorescence quenching, equilibrium ultracentrifugation and microcalorimetry. The basic equilibrium relation is:

$$M_1+M_2 \leftrightarrow M_1M_2$$

$$Ka=\frac{[M_1M_2]}{[M_1][M_2]}=\frac{1}{K_d}$$

Where $[M_1]$ and $[M_2]$ are the interacting macromolecules. The strength of the interaction is described by the association constant K_a or the dissociation constant K_d, where $[M_1]$ and $[M_2]$ are the concentrations of the free reactants and $[M_1M_2]$ is the concentration of the complex. These constants are related to the Gibbs energy of association $\triangle G_a$ and dissociation $\triangle G_d$ and can be expressed in terms of the enthalpy, $\triangle H$ and entropy, $\triangle S$.

Fig 11 shows the size distribution of ATP synthase (oxidized form) in addition of 0.50 M NaSCN. Two peaks are located in the 13.6 nm and 3.5 nm, separately. Their diameters are smaller than 15 nm and 4.3 nm of the reduced form under the same condition.

—25℃

—25℃+ 0.5 M NaSCN

—28℃+ 0.5 M NaSCN

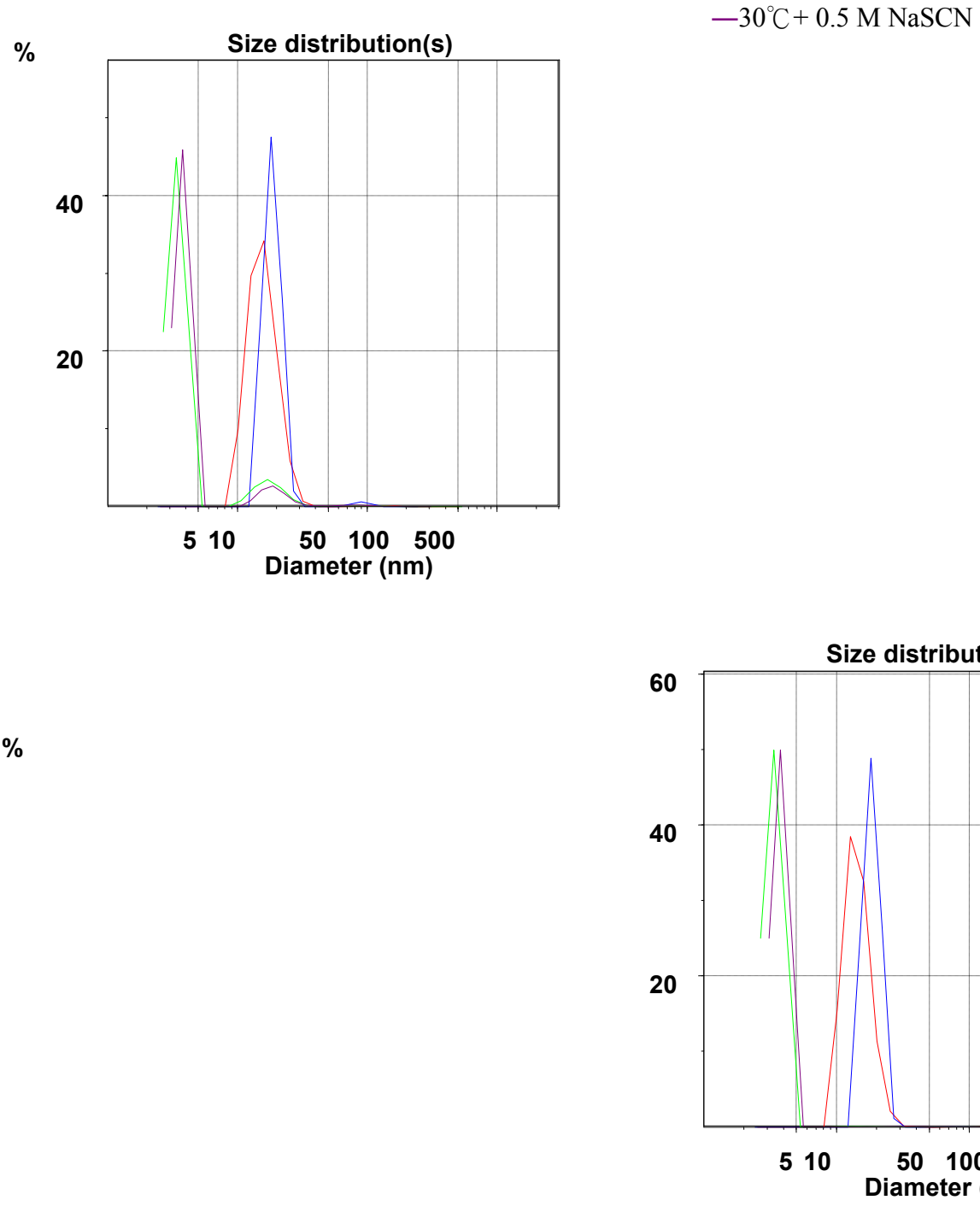


Fig 11: Size distribution of ATP synthase (oxidized form) in addition of 0.5 M NaSCN. The Volume anal
is up figure, and the Number analysis is down figure.
red:25℃
blue:25℃ +0.5 M NaSCN
green:28℃ +0.5 M NaSCN(3.4 nm, 17.8 nm)
purple:30℃ +0.5 M NaSCN (3.8 nm,19.2 nm)

From the van't Hoff plot as shown in Fig. 12, one could obtain the enthalpy and entropy changes of the dissociation of the oxidized and reduced forms, respectively. The final results are listed in Table 1. Apparently, the energetics between these two forms are a quite different.

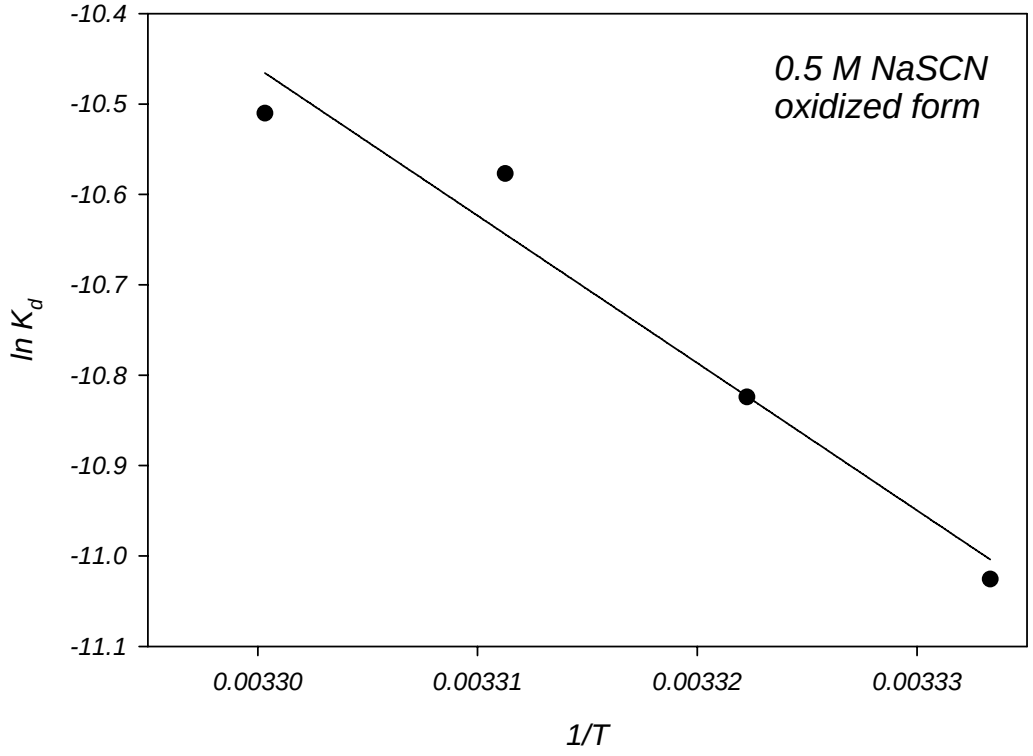


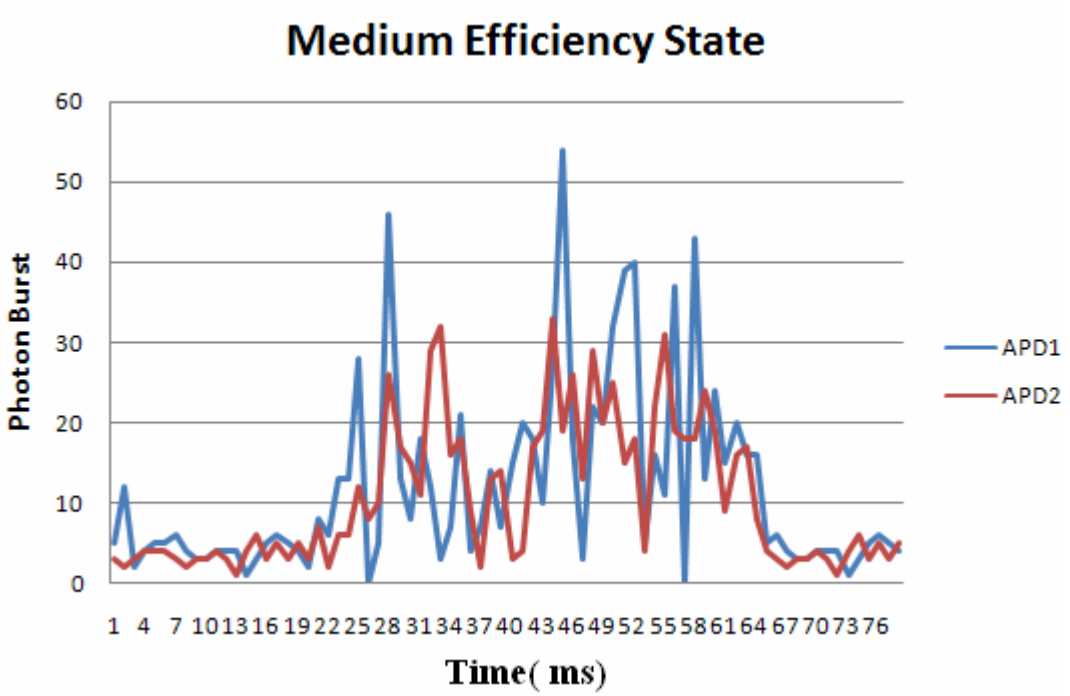
Fig 12: Calculation of enthalpy and entropy (ΔH and ΔS) by van't Hoff equation (y = 43.3 – 16308.3 x , R^2=0.958)

Table 1: Dissociation enthalpies and entropies of the reduced and oxidized forms of ATPase, respetively. (0.50 M NaSCN)

ATP synthase	ΔG (kj/mole)	ΔH (kj/mol)	ΔS (kj/mol·K)
Oxidized form	27.4(27℃)	135	0.36
	27.5 (28℃)		
	26.6(29℃)		
	26.5 (30℃)		
Reduced form	24.7(28℃)	40.7	0.05
	24.6 (30℃)		

(C) The relative motion of the ε and b_1 subunits of the ATPsynthase during the hydrolysis and synthesis of the ATP molecules.

There happens to be only one cysteine amino acid on each of the ε and b_1 subunits of the ATPase. Two dye molecues Cy5 and TMR were used as the FRET pair to label these two subunits. Under the experimental conditions that the microscope is set-up in confocal detection configuration and the sample is in the Brownian motion mode, the single-pair FRET signals were detected and analyzed. Some typical experimental measurements were shown in Figs. 13 -14. As shown in Fig. 15, three major steps were observed for the present system. Their corresponding distances between the two dye molecules were obtained. These parameters could be used for further analysis of the general rotational motion of the ATPase. Fig. 16 shows the generally-aceepted structure of the ATPase. The present experimental works could either support or even modify this general structure scheme. Related works are still in progress.



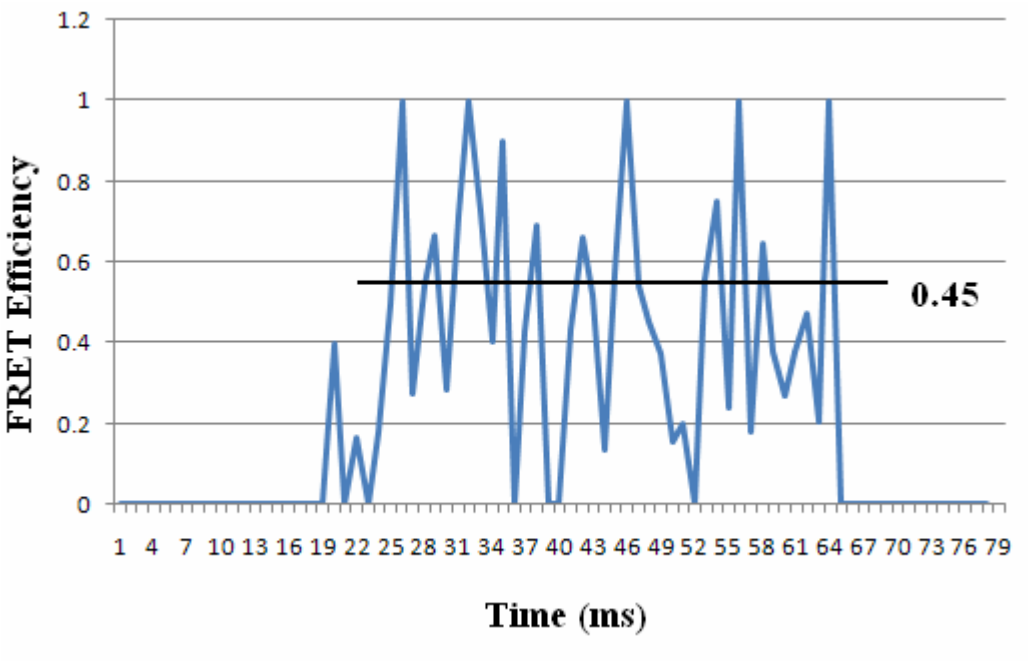
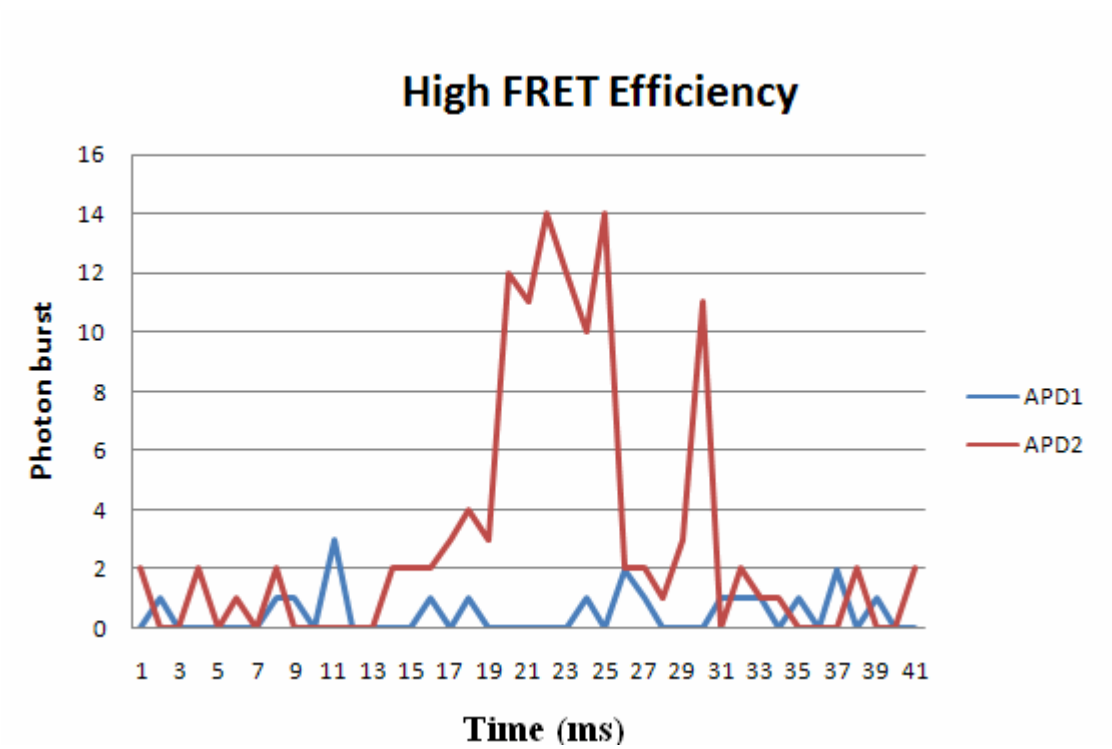


Fig 13: Medium efficiency of FRET. Inactive ATP synthase reconstituted in liposomes (buffer pH 8.0, no ADP, 4% glycerol,10-20 pM protein).FRET efficiency was 0.45



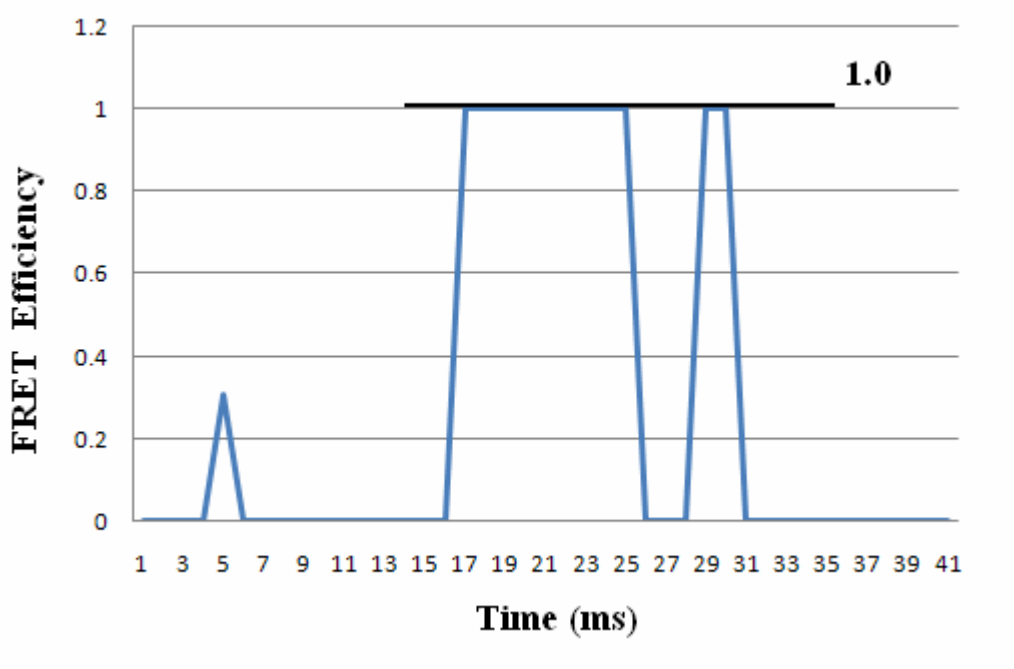


Fig 14: High efficiency of FRET. Inactive ATP synthase reconstituted in liposome (buffer pH 8.0, no ADP, 4% glycerol, 10-20 pM protein).FRET efficiency was 1.0.

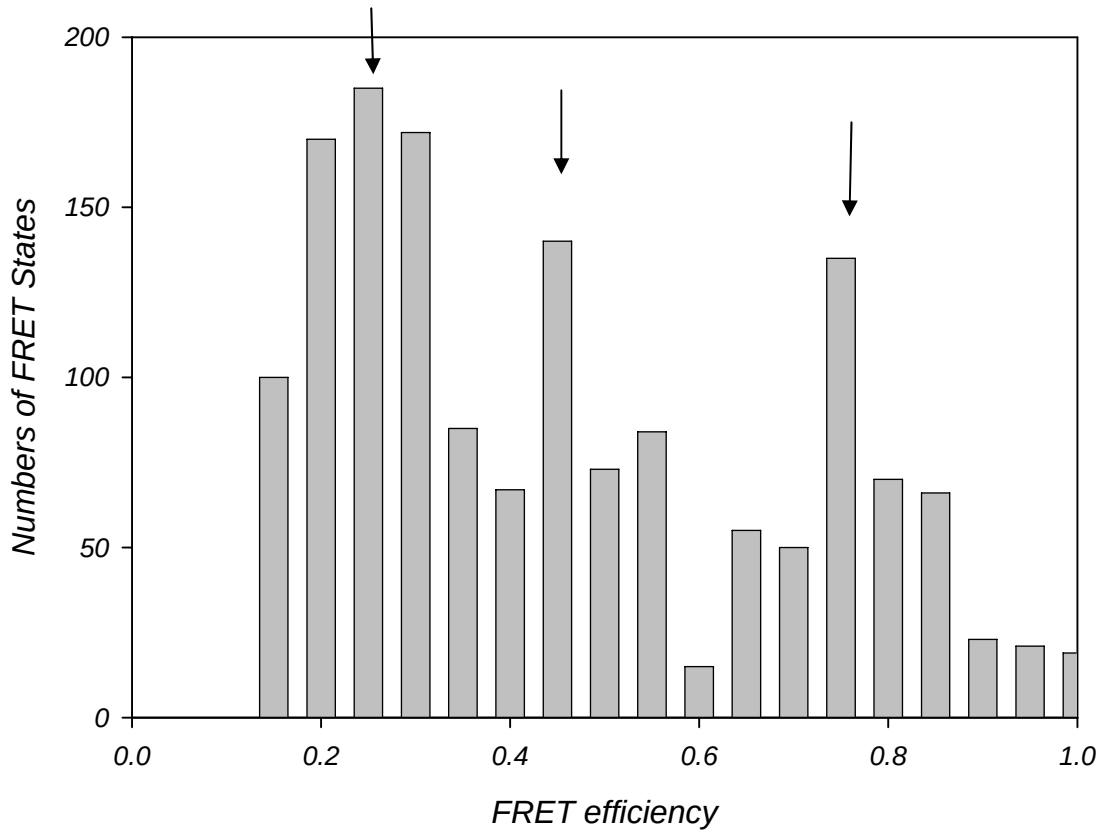


Fig 15: Histogram of the FRET efficiencies for the inactive CF_0F_1-liposome
There are apparently three Gaussian peaks at efficiency 0.25(7.6 nm),
0.45 (6.6 nm) and 0.75(5.3 nm), separately

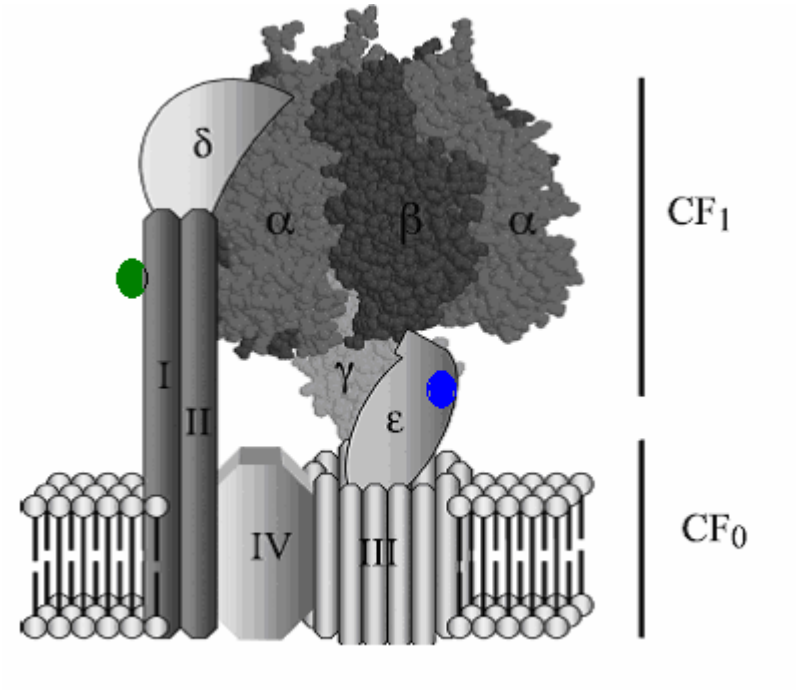


Fig16: Proposed subunit organization within the chloroplast ATP synthase complex