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KINETICS OF NUCLEOTIDE BINDING TO CHLOROPLAST COUPLING FACTOR (CF_1)

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Summary

Studies of the kinetics of association and dissociation of the formycin nucleotides FTP and FDP with CF_1 were carried out using the enhancement of formycin fluorescence. The protein used, derived from lettuce chloroplasts by chloroform induced release, contains only 4 types of subunit and has a molecular weight of 280 000.

In the presence of 1.25 mM $MgCl_2$, 1 mol of ATP or FTP is bound to the latent enzyme, with $K_d = 10^{-7}$ or $2 \cdot 10^{-7}$, respectively. The fluorescence emission (λ_{max} 340 nm) of FTP is enhanced 3-fold upon binding, and polarization of fluorescence is markedly increased. The fluorescence changes have been used to follow FTP binding, which behaves as a bimolecular process with $k_1 = 2.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$. FTP is displaced by ATP in a process apparently involving unimolecular dissociation of FTP with $k_{-1} = 3 \cdot 10^{-3}\ s^{-1}$. The ratio of rates is comparable to the equilibrium constant and no additional steps have been observed.

The protein has 3 sites for ADP binding. Rates of ADP binding are similar in magnitude to those for FTP. ADP and ATP sites are at least partly competitive with one another.

The kinetics of nucleotide binding are strikingly altered upon activation of the protein as an ATPase. The rate of FTP binding increases to at least $10^6\ M^{-1} \cdot s^{-1}$. This suggests that activation involves lowering of the kinetic barriers to substrate and product binding-dissociation and has implications for the mechanism of energy transduction in photophosphorylation.

Abbreviations used: CF_1, Chloroplast coupling factor; FMP, FDP, and FTP, formycin mono-, di- and triphosphate; Formycin, 7-amino-3(β-D-ribofuranosyl) pyrazolo-(4,3-d) pyrimidine; εADP, $1,N^6$-ethenoadenosine diphosphate; AMP-PNP, adenylyl imidodiphosphate; εAMP-PNP, $1,N^6$-ethanoadenylyl imidodiphosphate.

Introduction

The coupling factor isolated from chloroplasts (CF_1)[1] is believed to be the protein which catalyzes the formation of ATP in photophosphorylation [1]. This protein can be isolated from spinach or lettuce chloroplasts as a latent ATPase which can be activated by heat [2], trypsin [3], dithiothreitol [4] or other means. Various workers have studied the kinetics of the purified, activated ATPase and the association of nucleotides with the protein in both its latent and active forms [5—9]. However, many questions concerning the interaction of nucleotides with CF_1 remain open. In particular, it is unclear whether the 'tight' nucleotide binding sites ($K_d \approx \mu M$) in the isolated latent CF_1, the ATPase catalytic sites in the activated protein and the ATP forming sites in the protein in situ in chloroplast membranes can possibly be the same sites. It has been argued [7] that the rate of association of nucleotides (ϵADP and ϵAMP-PNP) with the tight sites of the latent enzyme is too slow for such sites to be involved in catalytic turnover. In addition, the dissociation constant for these sites [7—9] is 2 orders of magnitude lower than K_m for the ATPase [5—7].

Another question of great importance for the understanding of the role of CF_1 in photophosphorylation is the nature of the changes which occur in the protein upon its activation. Furthermore, the proposal that the coupling of ATP formation to earlier steps in photophosphorylation may involve, in part, conformational changes leading to changes in the properties of nucleotide binding sites [10,11] suggests that these sites might have interesting characteristics.

In the light of these questions, we have undertaken a study of the dynamics of nucleotide binding to CF_1, using the fluorescent nucleotides formycin di- and triphosphate (FDP and FTP). For technical reasons, we have used a protein prepared by chloroform-induced release of CF_1 [12] from lettuce chloroplasts. The preparation and properties of this protein, which has only four of the five subunits usually found in CF_1, will be described later (Shoshan, V. and Shavit, N., unpublished). The results presented here indicate that the association and dissociation of nucleotides with the 'tight' sites of latent CF_1 is a slow process. However, the association of nucleotides with the activated enzyme is much faster. This change in association kinetics may reflect on the mechanism of ATPase activation and may be relevant to the mechanism of photophosphorylation.

Materials and Methods

Protein preparation and properties

Chloroplasts were prepared from fresh market lettuce by the usual procedures [9]. The chloroplast pellet was washed 3 times with 50 mM NaCl and then CF_1 was released by shaking the suspension with half its volume of chloroform and purified by chromatography on DEAE-Sephadex A-50. The protein was stored as a suspension in 50% saturated ammonium sulfate at ph 7.1, containing 1 mM EDTA, 4 mM ATP, and 20 mM tricine. It was prepared for use free of ammonium sulfate and readily exchangeable nucleotides by Sephadex G-25 chromatography in 1 mM EDTA and 40 mM tricine at pH 8.0.

Disc-gel electrophoresis demonstrated that the protein was at least 98% pure, while SDS gel electrophoresis and other experiments showed that it only contained four types of subunit. The molecular weight was found to be 280 000 by sedimentation velocity (Shoshan, V. and Shavit, N., unpublished). Protein concentration was determined by the method of Lowry [13] with bovine serum albumin as standard, assuming the above molecular weight.

The ATP, ADP, and AMP content of the protein was examined by the methods of Kimmich et al. [14]. The enzyme after gel filtration contained 1 molADP/mol protein, less than 0.1 mol ATP/mol protein and no detectable AMP (Shoshan, V. and Shavit, N., unpublished). Ca^{2+}-ATPase activity of the protein was determined as described by Lien and Racker [15] after heat or dithiothreitol [4] activation. Nucleoside triphosphatase activities at low substrate concentration were also followed using the release of labelled phosphate. [γ-^{32}P]ATP or -FTP (0.01—0.25 mM) was incubated for 10 min at 37°C in 0.4 ml of a solution containing 9.4 μg of heat-activated CF_1 and $CaCl_2$ or $MgCl_2$ in quantities equimolar with the FTP or ATP, at pH 8.0 (40 mM tricine). For these experiments, as well as studies of nucleotide binding to activated protein, the CF_1 was passed once more through a Sephadex G-25 column as above after activation. $^{32}P_i$ released was determined by isobutanol-benzene extraction of the ammonium phosphomolybdate complex.

Nucleotides

The radioactive nucleotides [8-^{14}C]ATP and -ADP were obtained as ammonium salts from the Radiochemical Centre, Amersham. Formycin monophosphate [16] was the generous gift of Dr. S.J.D. Karlish. FTP was prepared by the simultaneous use of creatine kinase and myokinase. A mixture containing about 5 mM FMP, 10 μM FTP (i.e., a catalytic amount), 40 mM creatine phosphate, 25 mM tricine and 5 mM $MgCl_2$ was incubated at 37°C for 2 h with 100 units/ml of myokinase (Sigma, grade III) and 40 units/ml creatine kinase (Sigma, type I). The mixture was then heated at 100°C for 15 min and centrifuged at 500 × *g* for 10 min at 0—4°C. The supernatant was applied to a 0.5 × 7 cm Dowex-1 chloride column, which was then washed with 5 mM HCl. The FTP was eluted with 0.5 M HCl, with 1-ml fractions collected and examined spectrophotometrically (295 nm) for the presence of FTP [16]. The combined FTP-containing fractions were neutralized. FDP was prepared from FTP by the hexokinase reaction [17] and purified as described for FTP, except that 0.1 M HCl was used for its elution from Dowex-1. [γ-^{32}P]FTP was prepared from FDP and $^{32}P_i$ by photophosphorylation with lettuce chloroplasts [18].

The purity of the formycin nucleotides was checked by thin-layer chromatography on polyethyleneimine-impregnated cellulose using 0.5 M NaH_2PO_4, pH 3.5, as developer. The nucleotides were detected by their fluorescence. R_F values for FMP, FDP and FTP were 0.54, 0.30 and 0.145 respectively. The concentration of formycin nucleotides was determined spectrophotometrically on the basis of a molar extinction coefficient ϵ = 10 000 M^{-1} at 295 nm [16].

Binding measurements

The equilibrium binding of labelled nucleotides to the protein was determined by the forced dialysis method of Cantley and Hammes [19], employing

Amicon PM30 membranes, as previously described [9]. Enzyme concentrations ranged from 10^{-6} to $2 \cdot 10^{-6}$ M, and total [8-^{14}C]ATP and -ADP from 10^{-7} to $5 \cdot 10^{-6}$ M and from $5 \cdot 10^{-7}$ to $3 \cdot 10^{-5}$ M, respectively.

Fluorescence changes upon interaction of formycin nucleotides with the protein were measured using a Hitachi-Perkin Elmer MPF2A spectrofluorimeter. Excitation was at 296 nm and emission was measured at 380 nm, using slits giving an 8 nm bandwidth and the ratio mode of the instrument. All measurements were made at 22—23°C. To determine the equilibrium constant for binding of FTP to CF_1, 2.4 ml of a solution containing about $5 \cdot 10^{-8}$ M CF_1 in 50 mM tricine, 25 mM NaCl and 1.25 $MgCl_2$ at pH 8.0 was introduced into each of two matched 1-cm square fluorescence cells. Solutions of identical concentrations of FMP and FTP were prepared. The titration was carried out by adding FMP solution to one cell and FTP to the other. The fluorescence intensity for each cell was recorded when it stabilized (several minutes) and the difference between them taken as ΔF due to interaction of FTP with the protein. In general, 5-μl aliquots of solutions of 2×10^{-5} M, and then of 10^{-4} M nucleotide were added successively to give a concentration range of $4 \cdot 10^{-8}$ to 10^{-6} M.

The kinetics of fluorescence changes were measured using the same instrument, but with matched cylindrical microcells of 5 mm internal diameter. Enzyme solution as above (0.4 ml) was introduced into each of the two cells. FMP was added to one, and with the instrument recording, the same quantity of FDP or FTP introduced into the other on a thin Teflon rod, the cell contents rapidly mixed, and the cell compartment closed. Initial readings 3—5 s after addition could be obtained. The difference in fluorescence intensity between the cells was taken as ΔF.

For measurements of displacement of formycin nucleotides by non-fluorescent nucleotides, the displacing nucleotide was added to the cell containing CF_1 and FTP or FDP after its fluorescence had reached equilibrium. Measurements were recorded every few minutes, with the excitation shutter closed between readings.

The nonlinear least squares fits of data to theoretical equations were carried out using time-sharing programs on the CDC Cyber 73 computer. The numerical integration of the set of differential equations describing the displacement experiments employed a Runge-Kutta routine.

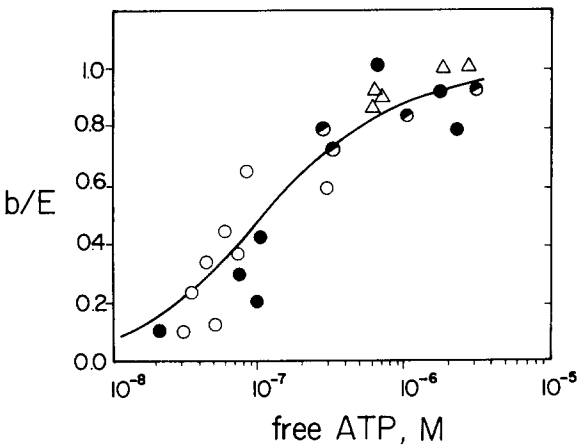
Polarization of fluorescence was determined using the fluorescence polarization accessory supplied by Perkin Elmer (manually controlled polarizers).

Results

We chose to investigate the nucleotide-binding properties of the 4 subunit chloroform released CF_1 rather than the usual EDTA-released 5-subunit CF_1 because of its much lower tendency to aggregate and cause light scattering. Unless otherwise indicated, CF_1 as used here will refer to this preparation.

Binding of nucleotides to CF_1

Although we feel that this protein is functionally similar to the usual 5-subunit CF_1 and is equally relevant to problems concerning the role of



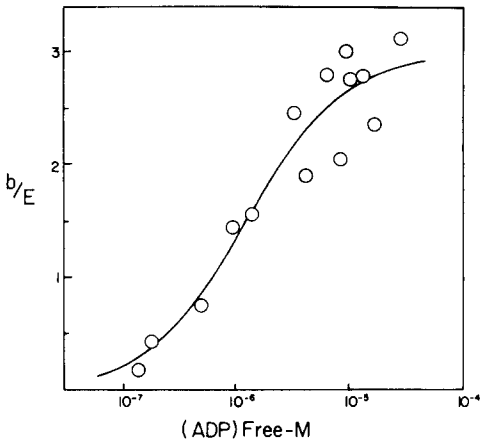


Fig. 1. Binding of ATP to chloroform-released 4-subunit CF_1. Mol ATP bound per mol protein (b/E) as a function of free ATP concentration was determined by forced dialysis at pH 8.0, 1.25 mM $MgCl_2$. Curve is theoretical line for one binding site with $K_d = 1.2 \cdot 10^{-7}$ M. Symbols represent data of four different experiments.

Fig. 2. Binding of ADP to CF_1. Mol ADP bound per mol protein (b/E) as a function of free ADP was determined as for Fig. 1. Curve is theoretical line for three independent binding sites with $K_d = 1.3 \cdot 10^{-6}$ M.

coupling factor, we can not infer its properties from previous studies of CF_1. For this reason, we have examined the binding of labelled ADP and ATP to this preparation in its latent form at pH 8.0 in the presence of 1.25 mM $MgCl_2$, by the method of forced dialysis.

Fig. 1 shows the results of measurements with [^{14}C]ATP, in the form of a titration curve. The protein appears to have a single, strong ATP-binding site with a dissociation constant of $1.2 \cdot 10^{-7}$ M under these conditions. Fig. 2 shows results of similar experiments with ADP. The latter data are best accomodated by the assumption that the protein has 3 equivalent non-interacting sites for the binding of ADP, with K_d about 10^{-6} M. However it should be emphasized that neither the exact number of sites nor their complete independence is established by the data, particularly since we do not know the fate of the ADP present in the isolated CF_1. Studies with latent EDTA-released 5-subunit CF_1 have indicated that it has two tight binding sites for either ADP or the ATP analog AMP-PNP [7,9].

The binding of ADP to CF_1 is inhibited by the presence of unlabelled ATP in a manner suggesting that ATP competes for one of the ADP sites, as shown in Table I.

When the formycin nucleotide FTP is added to CF_1, its fluorescence is strongly enhanced with no significant shifts. The fluorescence emission of FDP is also enhanced by CF_1, but that of FMP is completely unaffected at accessible protein concentrations (as expected for a compound of low affinity for CF_1) and the resulting spectrum is the sum of the free formycin spectrum and the weak but significant emission spectrum of the protein. Representative spectra are shown in Fig. 3. The enhancement relative to the sum of the component spectra is conveniently high (up to 50%) at 380 nm, and this emission wavelength was used in most of the work reported here.

TABLE I

INHIBITION BY ATP OF ADP BINDING TO CF_1

Latent CF_1 (final concentration 0.94 μM) was added to mixtures of [8-^{14}C]ADP and unlabelled ATP (in 1.25 mM $MgCl_2$/25 mM NaCl/50 mM tricine, pH 8.0) and binding determined by the forced dialysis technique.

^{14}ADP (μM)	ATP (μM)	ADP bound/mol protein
3.2	0	1.73
3.2	0.5	1.42
3.2	2.0	1.26
3.2	10	0.78
15.0	0	3.3
15.0	0.5	2.71
15.0	2.0	2.43
15.0	10	2.03

The observed fluorescence enhancement is dependent on the presence of Mg^{2+} or Ca^{2+} (Fig. 4), and in the absence of added divalent cations the spectra of CF_1 with FTP or FMP are identical. In all subsequent experiments, a final Mg^{2+} concentration of 1.25 mM, in the plateau region for binding, was used.

The fluorescence enhancement upon binding of FTP to CF_1 could be used to determine the dissociation constant for the complex. It can be shown that, so long as (a) the absorbance of the fluorescence cells is small, (b) FMP does not interact with the protein and (c) FTP reacts with sites of a uniform type with regard to spectral properties, the fluorescence enhancement will be propor-

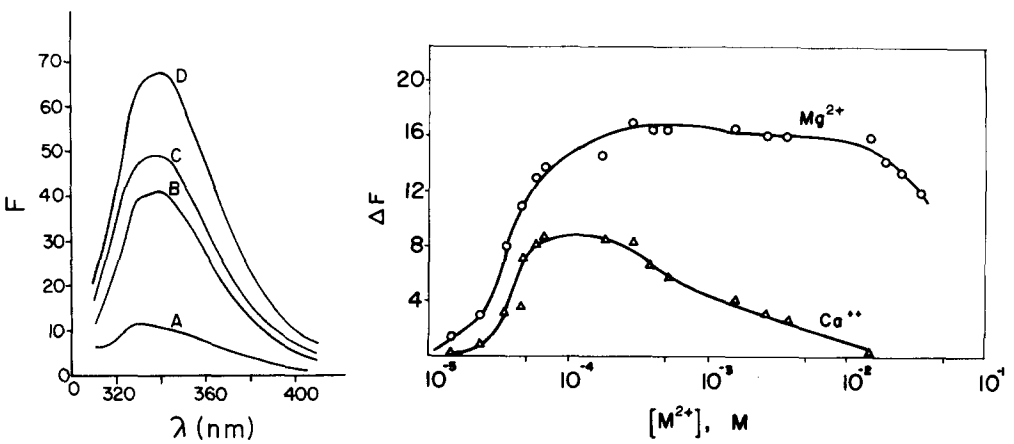


Fig. 3. Relative fluorescence emission spectra of formycin nucleotides, CF_1 and mixtures. A, chloroform-released 4 subunit CF_1 alone; B, FTP (or FMP); C, CF_1 + FMP; D, CF_1 + FTP. In all cases, CF_1 concentration was $5.5 \cdot 10^{-7}$ M, formycin nucleotide $2.2 \cdot 10^{-6}$ M, at pH 8.0 in the presence of 1.25 mM $MgCl_2$.

Fig. 4. Dependence of fluorescence of FTP + CF_1 on divalent cation concentration. The difference in fluorescence emission between a solution containing $9.2 \cdot 10^{-7}$ M CF_1 and $2 \cdot 10^{-6}$ M FTP and a solution containing CF_1 + FMP (ΔF, in arbitrary units) was measured at pH 8.0 in the presence of varying amounts of $MgCl_2$ or $CaCl_2$.

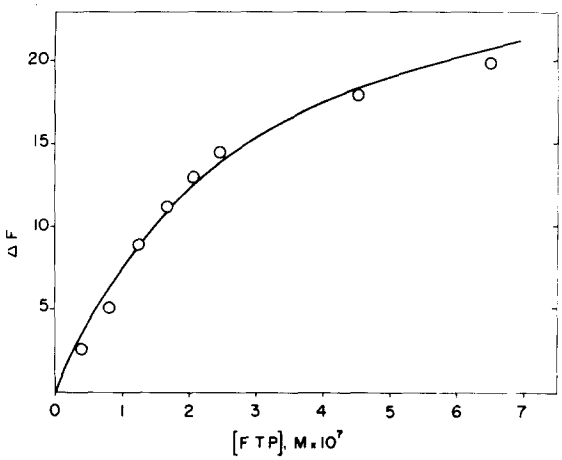


Fig. 5. Fluorescence titration of CF_1 with FTP. $1.29 \cdot 10^{-7}$ M CF_1 was titrated with FTP and FMP, respectively, as described in Materials and Methods, and the difference between the emission intensities of the two cells determined. Curve is theoretical for a single site with $K_d = 1.89 \cdot 10^{-7}$ M and $\Delta F = 28.4$ at saturation (arbitrary units).

tional to the concentration of protein-bound FTP; i.e.:

$$\Delta F = R \cdot [EL] \tag{1}$$

where ΔF is the difference in fluorescence emission intensity between cells containing FTP and FMP under identical conditions, [EL] is the concentration of protein-bound ligand and R is a fluorescence parameter dependent upon experimental conditions. A typical titration of CF_1 with FTP is shown in Fig. 5. Since it is not easy to make accurate measurements under conditions where total FTP is much larger than bound FTP, the analysis of data such as this requires the use of the quadratic equation:

$$\Delta F = \frac{R}{2}\{E_0 + L_0 + K_d) - [(E_0 + L_0 + K_d)^2 - 4\,E_0L_0]^{1/2}\} \tag{2}$$

where E_0 and L_0 are the total concentration of protein sites and FTP, respectively, and K_d is the dissociation constant for the complex. Eqn. 2 is the consequence of Eqn. 1 and the conditions for equilibrium when E_0, L_0 and [EL] are of comparable magnitude. The curve in Fig. 5 is the best fit of the data to Eqn. 2 (by non-linear least squares fit [20]). If one assumes that there are 2 equivalent FTP-binding sites per mol of enzyme rather than 1, a much poorer fit to the data is obtained. This result agrees with that for ATP, and it is safe to assume that this CF_1 preparation binds a single mol of FTP in a strong site. The dissociation constant for the complex is $(2.0 \pm 0.2) \cdot 10^{-7}$ M (average of 3 titrations).

On the basis of these results, it is possible to characterize the changes in the fluorescence properties of FTP on binding to CF_1. From the representative fluorescence data of Table II and the extent of binding predicted from the equilibrium constants, one can calculate that the fluorescence emission of FTP is enhanced about three-fold on binding. Such a calculation must, however,

TABLE II

FLUORESCENCE PROPERTIES OF CF_1-FORMYCIN NUCLEOTIDE MIXTURES

Solutions contained $1.0 \cdot 10^{-6}$ M CF_1 and $2.5 \cdot 10^{-6}$ M of the formycin nucleotide. Excitation 296 nm, emission 380 nm.

Sample	Concentration [a] (μM)			Fluorescence [b]	Polarization [c]
	[E]	[L]	[EL]		
1. FMP	—	2.5	—	26.0	0.090
2. CF_1	1.0	—	—	22.5	0.223
3. CF_1 + FMP	1.0	2.5	—	48.5	0.147
4. CF_1 + FTP	0.12	1.62	0.88	65.5	0.275
Calculated for 2.5 μM bound FTP [d]				74.3	0.422

[a] Calculated on the basis of a single site, $K_d = 2.0 \cdot 10^{-7}$ M for FTP and $K_d >> 10^{-5}$ M for FMP.
[b] Fluorescence intensity in arbitrary units.
[c] Polarization of fluorescence.
[d] Calculated using data of 1—4, calculated concentrations, and Weber's rule of additivity of fluorescence (ref. 21).

be based on the assumption that the fluorophors of the protein itself * are not affected at all by nucleotide binding. The increase in fluorescence polarization similarly implies that the polarization of fluorescence for FTP increases from 9% for the free nucleotide to 42% for the CF_1-bound nucleotide. The polarization observed with the CF_1 + FMP mixture, on the other hand, is rather close to the value expected for a mixture of non-interacting components.

Kinetics of binding

When FTP is added to a solution of CF_1, the fluorescence enhancement appears slowly (Fig. 6). If one assumes that one is observing a bimolecular process:

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL \tag{3}$$

where $k_{-1}/k_1 \doteq K_d$ and E, L represent the equilibrium concentrations of enzyme and FTP, respectively, then one expects [EL] to behave according to Eqn. 4:

$$\frac{d[EL]}{dt} = k_1\{(E_0 - [EL])(L_0 - [EL]) - K_d[EL]\} \tag{4}$$

Integration of Eqn. 4 with the boundary condition that [EL] = o at $t = 0$, and insertion of Eqn. 1, leads to Eqn. 5, which has 3 disposable parameters: R, K_d, and k_1.

$$\Delta F = \frac{2R\,E_0L_0(e^{-k_1q^{1/2}t} - 1)}{b + q^{1/2} - (b - q^{1/2})e^{-k_1q^{1/2}t}} \tag{5}$$

* The protein contains no tryptophan, and its only fluorophors are tyrosines and an unidentified non-protein fluorophor extractable into methanol/chloroform (Shoshan, V. and Shavit, N., unpublished results).

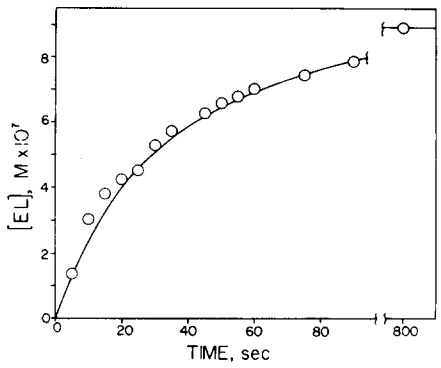


Fig. 6. Kinetics of association of FTP with CF_1. 1.27 μM FTP was added to 1.43 μM CF_1 at pH 8.0 in the presence of 1.25 mM $MgCl_2$. The fluorescence emission intensity was compared to that of a cell containing FMP and CF_1 at the same concentrations. Solid line is best fit of Eqn. 5 to data, with $k_1 = 1.92 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and $K_d = 2.0 \cdot 10^{-7}$ M. ΔF was converted to [EL] on ordinate scale by dividing by R.

where:

$$b = -(E_0 + L_0 + K_d),$$

$$q = (E_0 + L_0 + K_d)^2 - 4\,E_0L_0$$

A non-linear least squares fit can in principle be used to find the best values of all 3 parameters for a given experiment. In practice, of course, the uncertainty in the parameters derived in this way is very large. We chose instead to assume $K_d = 2.0 \cdot 10^{-7}$ M, as determined in the titration experiments above, and to fit only the remaining two parameters.

TABLE III

SUMMARY OF EXPERIMENTS ON THE KINETICS OF FTP BINDING TO CF_1

Experiments as shown in Figure 6 were carried out as described in Materials and Methods, and the best value of k_1 (see Eqn. 5) determined by least squares fit, assuming $K_d = 2.0 \cdot 10^{-7}$ M.

Initial concentration, μM		Calculated k_1 * ($M^{-1} \cdot s^{-1}, \cdot 10^4$)
E_0	L_0	
0.96	0.85	2.87 ± 0.17
1.43	1.27	1.92 ± 0.10
0.75	1.41	1.99 ± 0.18
0.76	1.96	3.78 ± 0.30
0.67	1.96	3.54 ± 0.33
0.62	2.00	2.00 ± 0.36
0.66	2.50	3.96 ± 0.40
0.66	2.50	3.75 ± 0.37
0.66	2.51	4.60 ± 0.50
0.75	2.77	1.90 ± 0.17
0.75	2.77	2.38 ± 0.19
0.75	2.77	3.18 ± 0.48
Weighted average **		2.4 ± 0.7

* Errors are "unbiased estimate of error" from least squares fit.

** Weighted in inverse proportion to square of errors.

Table III summarizes the results for a number of experiments. It should be noted that the calculated values of k_1 do not show a dependence on concentrations. The weighted average value for k_1 is $2.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$.

We have observed that the apparent kinetics of binding of FTP is independent of the order of addition of FTP and $MgCl_2$. When FTP is added to CF_1 in the absence of divalent cation, little or no fluorescence enhancement is observed. Upon addition of 1.25 mM $MgCl_2$ the fluorescence is enhanced with essentially the same time course as is seen for addition of FTP to CF_1 and $MgCl_2$.

The kinetics of binding of FDP to CF_1 also appear to be slow, with the half-time for development of the fluorescence change close to that for FTP at similar concentrations. However, since nucleotide diphosphates appear to bind to the protein at 2 or more sites, an analysis of the kinetics of FDP binding sould require unwarranted assumptions about the number and independence of binding sites and the lack of influence of binding at one site on the fluorescence of FDP at another site. For this reason, we have not attempted such an analysis.

The addition of a non-fluorescent nucleotide to a solution of CF_1 and FTP or FDP leads to a decrease in the fluorescence emission. A sufficient quantity of ATP can cause the fluorescence of a CF_1/FTP mixture to be identical to that of a CF_1/FMP mixture, i.e., reduce the enhancement to zero. This displacement of one nucleotide by another is also a slow process (Fig. 7). Since the complete displacement of FTP by high concentrations of ATP appeared to be a first-order process whose rate is nearly independent of ATP concentration, we considered that, at least for nucleoside triphosphates, the dissociation of one nucleotide from the enzyme is required for binding of another. One would then

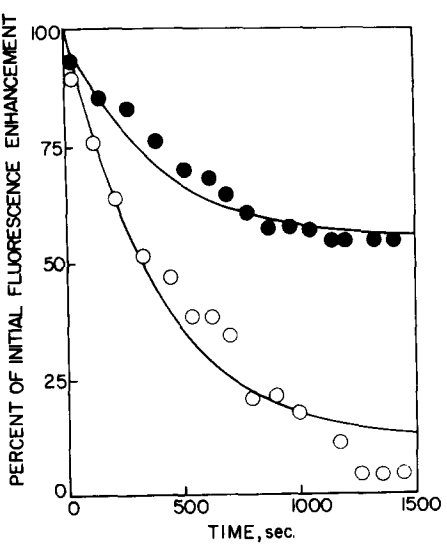


Fig. 7. The displacement of CF_1-bound FTP by ATP. A solution of 0.69 μM CF_1 and 1.4 μM FTP was allowed to reach equilibrium and its fluorescence compared with a CF_1-FMP solution. ATP was then added and the decrease in fluorescence recorded. ●, 0.74 μM ATP. ○, 7.2 μM ATP. Curves are theoretical for $k_1 = 2.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$, $k_{-1} = 3 \cdot 10^{-3}\ s^{-1}$, $k'_1 = 2.0 \cdot 10^4\ M^{-1} \cdot s^{-1}$, and $k'_{-1} = 1.2 \cdot 10^{-3}\ s^{-1}$.

expect Eqn. 6 to hold, where L is FTP and L' is ATP:

$$EL + L' \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + L + L' \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} EL' + L \qquad (6)$$

The set of differential equations completely describing the behavior of such a system, although simple, can not be integrated analytically and must be solved numerically. The theoretical curve for the change in fluorescence in a displacement experiment can be calculated by calculating the initial concentrations of EL, E and L (using a value of K_d), carrying out a numerical integration from this point with a given set of kinetic parameters to determine [EL] as a function of time, and inserting Eqn. 1 for the proportionality between ΔF and [EL]. The curves shown in Fig. 7 were derived in this way. All four kinetic parameters could not be determined simultaneously by fitting such data. We chose to assume that the values, $k_1 = 2.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$ (association rate constant for FTP) and $K'_d = k'_{-1}/k'_1 = 1.2 \cdot 10^{-7}$ M (dissociation equilibrium constant for ATP) as determined above were correct. For individual experiments using high ATP concentrations, it was then possible to obtain good fit of the curves and fairly precise values (±15%) for k_{-1} with relatively little dependence on k'_1. On the basis of several experiments, we conclude that $k_{-1} = (3 \pm 1) \cdot 10^{-3}\ s^{-1}$. The ratio of the rate constants k_{-1}/k_1 ($(1.3 \pm 0.6) \cdot 10^{-7}$ M) is thus within experimental error of the independently determined K_d for FTP. For reactions with relatively low ATP concentrations, on the other hand, k'_1 becomes partly rate-determining. Data from such reactions were used to estimate that the value of k'_1 is in the range $1—5 \cdot 10^4\ M^{-1} \cdot s^{-1}$, which is of the same order of magnitude as k_1. The parameters for the FTP-ATP-CF_1 system are summarized in Table IV.

Other nucleoside triphosphates and diphosphates, and even inorganic pyrophosphate in sufficient concentration, can cause the apparent displacement of FTP from CF_1. Some typical results are shown in Fig. 8. Although we have not attempted to analyze these processes, we have estimated from the extent of displacement of FTP by other nucleoside triphosphates that the dissociation constants for the complexes formed with CF_1 are in the ratio 0.4 : 1 : 2 : 5

TABLE IV

NUCLEOTIDE-BINDING PARAMETERS FOR CF_1

Summary of results for chloroform-released 4-subunit CF_1 at 22—23°C, pH 8.0, in presence of 1.25 mM $MgCl_2$.

Enzyme	Nucleotide	K_d (μM)	n	k_1 ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})
Latent	FTP	0.2 [a]	1	$2.4 \cdot 10^4$ [c]	$3 \cdot 10^{-3}$ [d]
	ATP	0.1 [b]	1	$1—5 \cdot 10^4$ [d]	$1—5 \cdot 10^{-3}$ [d]
	ADP	1.0 [b]	3		
Activated	FTP			$\geqslant 10^6$ [c]	
	ADP	1 [b]	3		

[a] Fluorescence titration.
[b] Forced dialysis.
[c] Association kinetics (12 experiments).
[d] Displacement kinetics (11 experiments).

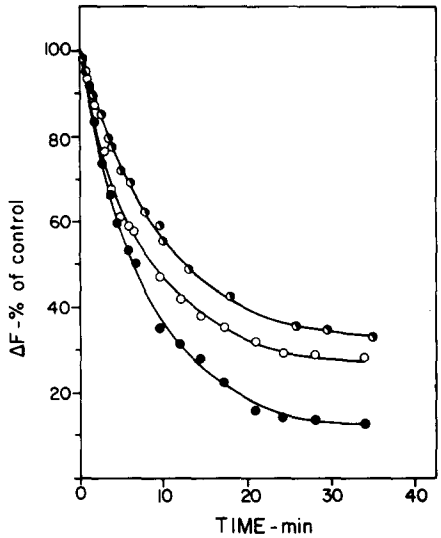


Fig. 8. The displacement of CF_1-bound FTP by ADP or PP_i. Experiments were carried out as for Fig. 7, with about 0.71 μM CF_1. ○, 1.46 μM FTP displaced by 1.4 μM ADP. ●, 1.46 μM FTP displaced by 7.3 μM ADP. ⊙, 1.96 μM FTP displaced by 1.40 mM PP_i.

for ATP, FTP, GTP and CTP respectively. This is quite similar to the specificity found for nucleoside diphosphate binding to EDTA-released 5-subunit CF_1 [9].

The displacement of FTP by high concentrations of ADP has about the same half-time as its displacement by ATP, suggesting either that ADP binding to additional sites on the protein before the dissociation of FTP does not occur, or that such binding does not affect the kinetics of FTP dissociation. On the other hand, the extent of displacement of FTP by a given concentration of ADP is greater than one would expect (e.g., see Fig. 8) for simple competition between ADP ($K_d \approx 10^{-6}$ M) and FTP ($K_d \approx 2 \cdot 10^{-7}$) for the same site.

Properties of activated CF_1

The CF_1 preparation we have studied is a latent ATPase which, when activated by heat or dithiothreitol treatment, has an ATPase activity of 20—40 μmol/mg protein · min, corresponding to a turnover of 100—200 s^{-1}. We did not have sufficient FTP to measure the FTPase activity of the activated protein at saturation, but when we followed the release of $^{32}P_i$ from [γ-^{32}P]ATP or FTP at low concentrations of nucleotide (up to 0.2 mM), we found that FTPase activity was less than 3% of ATPase activity. The presence of equimolar concentratins of FTP does, however, inhibit the hydrolysis of ATP by some 30—50% under these conditions. This nucleotide specificity of the ATPase activity is reminiscent of that observed with the EDTA-released 5-subunit CF_1 [22,23].

The association of [8-^{14}C]ADP with the activated protein, as determined by the forced dialysis method, is essentially unchanged from that observed for the latent enzyme, and the results (not shown) fit the same theoretical curve for 3 independent sites of $K_d \approx 1 \cdot 10^{-6}$ M. We have been unable to measure ATP

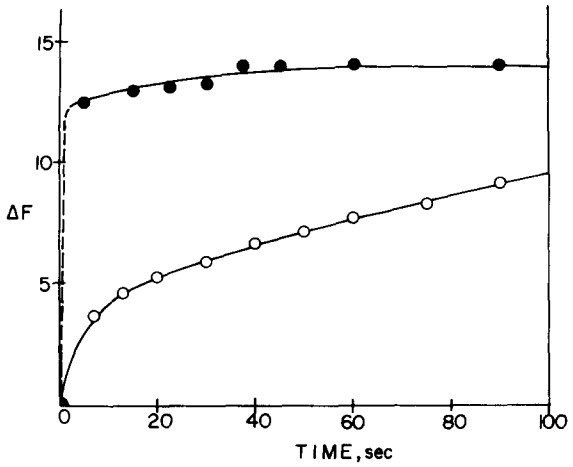


Fig. 9. Comparison of the kinetics of FTP binding to latent and activated CF_1. 1.96 μM FTP was added to 0.70 μM latent CF_1 (○) or to 0.48 μM dithiothreitol activitated enzyme (●) (15 μmol P_i released/mg protein · min) as described in Fig. 6. Fluorescence intensities normalized to same protein concentration on the assumption that near-saturation holds in both cases.

binding by this technique rapidly enough to avoid complications due to ATP hydrolysis.

The kinetics of nucleotide association is the property of the protein (other than the hydrolytic activity itself) which changes most strikingly on activation. The binding of FTP to latent and activated CF_1 is compared in Fig. 9. For the activated protein, the half time for the fluorescence increase is less than 1 s and no change in fluorescence is observed after 30 s. The final enhancements observed for the latent and activated protein are similar. These observations suggest that the protein retains the same number of FTP-binding sites upon activation, but that the kinetic properties of these sites have been altered. We estimate that the rate of association of FTP with the activated enzyme is equal to or greater than $10^6\ M^{-1} \cdot s^{-1}$. However, determination of this rate constant would require rapid kinetic techniques.

We have also observed that FDP binding becomes rapid upon activation of CF_1.

Discussion

We have shown above that the chloroform-released 4-subunit CF_1 has a single very strong site for FTP or ATP binding, which shows rather slow kinetics of association and dissociation. The parameters determined here for this site are summarized in Table IV.

On the basis of the observed fluorescence changes, the rotational freedom of the formycin base is severely restricted upon binding to this site. This suggests that both the base and the triphosphate moieties are involved in binding.

The protein has three apparently independent sites for ADP, and the kinetics of its interactions with nucleoside diphosphates appear to be similar to those of triphosphates. Several types of experiments indicate that binding of nucleotide diphosphates is at least partially competitive with the binding of triphosphates,

so it would seem that at least one ADP site is either coincident with the ATP site or allosterically inhibits binding to it.

The rates of nucleotide association and dissodiation observed for the strong sites of the latent enzyme are too slow for such sites to participate in the catalytic activity of the isolated protein, and are certainly too slow for them to be involved in ATP formation in photophosphorylation. However, activation of the enzyme is accompanied by a marked increase in the rate of binding to the strong site (by a factor of at least 40), and it is possible that this change is in fact the proximal cause of the activation phenomenon.

Many aspects of the interaction of nucleotides with this protein remain to be clarified. More definite experiments are required to characterize the ADP sites and the relation between ADP and ATP sites, particularly the possibility of either thermodynamic or kinetic interaction between them. It is possible that more than one of the ADP sites is potentially an ATP site, but that these sites have negative cooperativity for ATP binding. This question is particularly important in light of the fact that other workers have found 2 ATP sites per mol of latent EDTA-released CF_1 [7].

Perhaps the most crucial unanswered question is whether the strong sites whose kinetics have been studied here are in fact the active sites of the enzyme. There is no direct evidence for their identity, but in view of our results the only serious evidence against it is the observation that modification of a single tyrosine quite distant from two 'tight' sites [24] completely destroys the ATPase activity of activated EDTA-released CF_1. However, if CF_1 shows any allosteric properties or requires conformational changes for its activation and/or activity, it is entirely possible that modifications far from the active site might inhibit it.

Despite these questions, the results reported here have intriguing implications. The activation process observed has the property of converting 'tightly bound' nucleotides, that is, nucleotides exchanging slowly with the medium, to much more readily exchangeable nucleotides. Conversion of 'tightly bound' nucleotides to exchangeable nucleotides upon energization of thylakoid membranes has been observed directly [25] or indirectly in several laboratories [10,11], and implicated in energy coupling. The fact that the membrane-bound enzyme does not readily catalyze ATP hydrolysis might be the result of kinetic barriers to ATP binding at the catalytic site. The dropping of such kinetic barriers may be due to reorganization of the peptide chains of CF_1, in a process which may be controlled and reversible under the proper conditions in the thylakoid membrane but irreversible in the isolated protein.

One must be careful in drawing direct analogies between the isolated protein and the membrane-bound ATPase, but it is clear that the systems studied here can provide important insights into the role and function of CF_1. We are now pursuing further, more detailed studies, including interactions of formycin nucleotides with the activated enzyme.

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