Phosphate Binding to Isolated Chloroplast Coupling Factor (CF_1)

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A single binding site for phosphate was found on isolated chloroplast coupling factor in the absence of nucleotides. In our experiments the phosphate binding site showed a K_d of 170 μM. We did not observe any differences whether the ATPase activity of CF_1 had been activated or not.

If the enzyme was incubated with [γ-^{32}P]ATP the amount of ^{32}P bound per CF_1 depended on the pretreatment of the enzyme: In the presence of ADP no ATP or phosphate was bound to CF_1. After activation of ATPase activity one mol of ATP per mol CF_1 was rapidly bound and hydrolyzed while there was a slowly occurring binding of another phosphate without concomitant nucleotide binding.

We conclude that there are two different types of phosphate binding observed in our experiments: 1) Inorganic phosphate can be bound by one catalytic site per mol of CF_1. 2) The γ-phosphate of ATP is able to bind to an ATP binding domain of the enzyme if this domain can exchange substrates with the incubation medium. This ATP binding domain appears to differ from the site binding inorganic phosphate, because at least a portion of the coupling factor contains more than one labelled phosphate during our ATPase tests.

Introduction

The chloroplast coupling factor (CF_1) most probably contains three catalytic sites working cooperatively [1]. From photoaffinity labelling studies with 2-azido-ADP it may be concluded that these sites are identical in peptide structure [2]. The enzymatic reaction requires binding of both substrates, ADP and phosphate, in a precise orientation inside a catalytic domain to bring about the synthesis of ATP. Nucleotide binding to CF_1 (the isolated as well as the membrane bound protein) has been investigated in several papers (see ref. [3–5] for reviews). While it was shown that ADP can be bound to the catalytic sites at two different states [6] such results have not been reported for experiments on phosphate binding until now.

In this paper phosphate binding experiments with isolated CF_1 are shown. The number of phosphate binding sites per mol of CF_1 and their affinity to phosphate are estimated.

Abbreviations: DTE, dithioerythritol; PMS, N-methylphenazonium methosulfate; Chl, chlorophyll.
Enzyme: Chloroplast ATPase (EC 3.6.1.34).

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Materials and Methods

Labelled compounds were purchased from Amersham-Buchler, Braunschweig. γ-^{32}P labelled ATP was synthesized by illuminated spinach chloroplasts and purified by column chromatography as suggested by Magnusson *et al.* [7]. Enzymes and nucleotides were obtained from Sigma; purity of the nucleotides was controlled by chromatography on PEI-cellulose. All other chemicals were of reagent grade quality and purchased from Merck.

Filtration membranes, type XM-50, for experiments in a high pressure filtration cell were obtained from Amicon. Sephadex G-50 was bought from Pharmacia. 1 ml tuberculin syringes were bought from Henke-Sass Wolf, Tuttlingen, W.-Germany. These syringes were fitted with polyethylene discs with a pore size of 30 μm, obtained from Züricher Beuteltuchfabrik, Rüschlikon, Switzerland.

The endogenous nucleotide concentration was reduced to a value below 0.1 mol of adenine nucleotides per mol of CF_1 by pre-illumination of freshly prepared thylakoids, as detected by luciferase test. CF_1 was prepared from these thylakoids using the method of Strotmann *et al.* [8]. The CF_1 preparation was concentrated by forced dialysis and a final concentration of 1.2 mg protein per ml was adjusted. The protein concentration was measured by the method of Lowry *et al.* [9] using bovine serum albu-

min as a standard. In all calculations the molecular-weight of CF_1 was assumed to be 400,000 [10].

The binding of phosphate to CF_1 was measured by one of two procedures. The first one was membrane filtration employing Amicon XM-50 membranes. The second one was Penefsky's centrifuge column procedure [11]. But we varied Penefsky's technique with respect to sample application: We used a programmable centrifuge (Christ-Digifuge) with swinging bucket rotor. It reached a speed of 1,000 rpm ($170 \times g$) within 8 seconds. The columns were prepared as described by Penefsky [11]. Then the syringes were mounted by cut pipet tips. 50 µl samples were pipetted into these tips (when using columns of 1 ml Sephadex G-50). When the gels were centrifuged for the second time, these samples were applicated to the column material synchronously and completely separated immediately. The test tubes collecting the effluents from the columns contained 10 µl of 3 M $HClO_4$ in order to avoid any enzymic reaction after centrifugation.

Each CF_1 preparation was tested for its ATPase activity using the method of Franek and Strotmann [12]. Those preparations showing enhanced activity (more than 10 µmols of ATP hydrolysed per mg of protein and per hour) were discarded. ATPase activity of CF_1 preparations was activated by heat treatment using the method of Lien and Racker [13] or by detergent activation using octyl glucoside as described by Pick and Bassilian [14]. Only ATPase preparations with an activity of at least 400 µmols of ATP hydrolyzed per mg protein and per hour were used for further experiments.

For binding studies the standard experimental conditions were: 20 µl CF_1 suspension (containing 1.2 mg protein per ml; 2 mM tricin buffer, pH 8.0; 100 mM sucrose) were mixed with 25 µl incubation mixture (containing 50 mM tricine buffer, pH 8.0, and $MgCl_2$ or $CaCl_2$ as indicated) and another 25 µl phosphate buffer, pH 8.0, labelled with $^{32}P_i$ (the phosphate concentration is given in the figures). An aliquot of 50 µl was analyzed for phosphate binding to CF_1. – In experiments with activated ATPase the CF_1 suspension was prepared as described by Lien and Racker [13] or Pick and Bassilian [14], the protein concentration therefore was lower and it contained additional salts. In experiments with activated Mg-ATPase the incubation medium contained additional octylglucoside, giving a final concentration of 30 mM in the total volume of 70 µl test mixture.

Results

When starting phosphate binding studies we used thylakoid bound CF_1. But we failed in producing reliable results because we were not able to reduce unspecific binding of phosphate to the thylakoids. Similar problems arose when we measured phosphate binding to isolated thylakoids employing a high pressure filtration cell until we used Amicon XM-50 membranes. Nevertheless, though we rinsed the backs of the membranes after filtration, we still had numerous samples in each experiment showing a high degree of contamination with "unspecific bound" phosphate. Therefore we looked for a second, more reliable technique and found the centrifuge column technique published by Penefsky [11].

At first we had just the opposite problem with the "Penefsky-Columns": we found "too low" amounts of phosphate bound to CF_1 because we used too long incubation times on the columns. Before application of the samples, the columns had to be centrifuged to remove free buffer. Then the samples were applicated and immediately sucked into the column material. By this a first separation step of salts and proteins took place. Therefore the phosphate concentration in close vicinity to CF_1 was reduced and bound phosphate started to dissociate from the protein. – The effect of prolonged incubation of CF_1 on the centrifuge columns is shown in Fig. 1. A strong effect of incubation time can be seen especially with short periods of pre-incubation before starting the centrifuge. Therefore we put cut pipett tips on the tops of the centrifuge columns and the samples did not come in contact with the column medium until the centrifuge was started.

In the next set of experiments (Fig. 2) the binding of phosphate to isolated CF_1 was measured using varying phosphate concentrations. With 1 mM phosphate a maximal binding of 0.746 mols of phosphate bound per mol of CF_1 was found. In the double reciprocal plot a K_d of 170 µM phosphate was determined. This result was in good agreement with an earlier experiment using the filtration technique. We did not observe significant differences when the incubation media contained 5 mM $CaCl_2$ instead of 5 mM $MgCl_2$ (Table I). The amount of bound phosphate per mol of CF_1 was not changed if the ATPase was activated by heat or detergents (Table I). Because of the phosphate binding to trypsin or trypsin inhibitor we were not able to test trypsin activated ATPase.

When using centrifuge columns with detergent activated ATPase, we found that above a critical detergent concentration there was no longer a complete separation of proteins and phosphate, no matter which protein (CF_1, BSA, trypsin, GAP-Dh) was used. In these experiments we therefore had to employ the filtration technique. When measuring phosphate binding in the presence of added ATP we found different degrees of competition, depending on the kind of CF_1 pre-treatment. Competition appeared to be stronger with activated ATPase and became more pronounced with increasing incubation time in the presence of ATP.

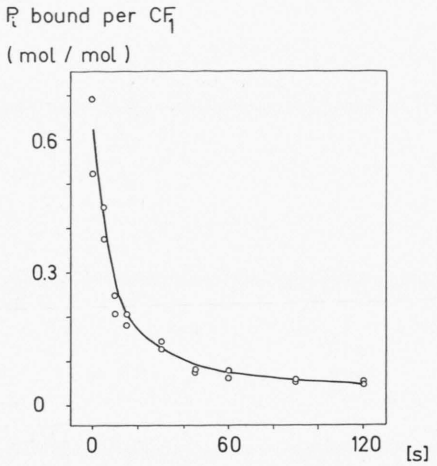

Fig. 1. Effect of prolonged incubation time inside the centrifuge columns. The complete incubation mixture was mixed and directly transferred on top of the column material. It took 7 to 9 seconds to put the columns into the centrifuge and to start it. In the figure the incubation time exceeding this value before starting the centrifuge is given. The incubation mixture contained in 70 µl: 24 µg CF_1; 2.5 µmol tricine buffer, pH 8.0; 0.25 µmol $MgCl_2$; 2 µmol sucrose; 35 µmol phosphate buffer, pH 8.0, labelled by $^{32}P_i$, 0.5 KBq/µmol. The results from two independent experiments are shown.

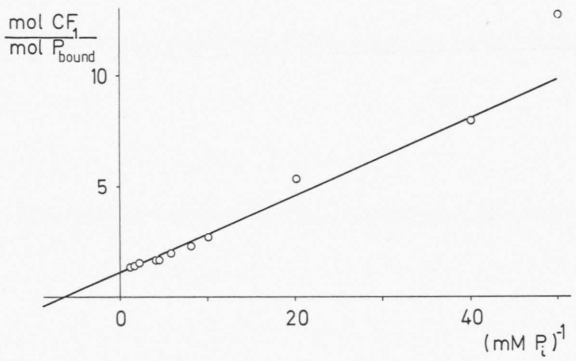

Fig. 2. Double-reciprocal plot of phosphate binding with P_i as the variable substrate. In this experiment CF_1 was mixed for 5 seconds with the incubation medium. Then an aliquot of 50 µl was transferred into the pipett tips on the top of a centrifuge column and incubated there for another 15 seconds before starting the centrifuge.

Table I. Effect of experimental conditions on phosphate binding. Results from the two techniques, membrane filtration and column centrifugation, are compared. CF_1 and the incubation medium containing labelled phosphate have been mixed for 5 seconds in Eppendorf vessels. Then the mixture was transfered to the membrane filtration apparatus or into the tips of the centrifuge columns. After another 5 seconds the N_2 valve was opened to start the membrane filtration or the centrifugation. The effects of the following experimental conditions on phosphate binding were tested:
1) Untreated CF_1 was incubated in the presence of 5 mM $MgCl_2$.
2) Heat activated ATPase was incubated in the presence of 5 mM $CaCl_2$.
3) CF_1 was incubated in the presence of 30 mM octylglucoside and 5 mM $MgCl_2$.
4) After activation of ATPase by incubation of CF_1 for 10 min in a solution containing 30 mM octylglucoside, CF_1 was incubated in the presence of 5 mM $CaCl_2$.

We were not able to use the centrifuge columns to separate CF_1 from a medium containing 30 mM octylglucoside, because we always found high amounts of labelled phosphate in the effluent. Therefore we suspect that such high concentrations of octylglucoside tend to form vesicles transporting some incubation medium within the protein fraction.

	Phosphate bound per CF_1 (mol/mol) measured by							
	Membrane filtration				Centrifuge column			
mM phosphate	0.025	0.125	0.50	1.00	0.025	0.125	0.50	1.00
Exp. conditions								
1	0.129	0.438	0.654	0.718	0.134	0.447	0.655	0.705
2	0.158	0.456	0.639	0.696	0.149	0.471	0.638	0.712
3	0.143	0.441	0.655	0.698	–	–	–	–
4	0.133	0.439	0.649	0.702	0.140	0.446	0.654	0.707

Fig. 3 shows that [γ-^{32}P]ATP is bound with a K_d of 2.3 μM. This value resembles quite well that one of [8-^{14}C]ATP binding to the high affinity nucleotide binding site of CF_1 (K_d = 7 μM [15] and K_d in the range of 3 μM [16]). Using the ammoniumheptamolybdate method we found that predominatly "inorganic" phosphate was bound to CF_1 though it had been incubated with labelled ATP. In a subsequent test we denaturated the CF_1 protein with 0.3 M $HClO_4$ after an incubation time of 15 seconds in a medium containing [8-^{14}C]ATP or [γ-^{32}P]ATP and analyzed the bound substrates using thin layer chromatography on PEI-cellulose plates. In agreement with experiments published by Bickel [17] we found 62% bound ADP and 38% bound ATP, but when comparing the results from different tests, we found some differences. We suspected that this might be due to difficulties we had with respect to standardization of the incubation time, especially in our earlier experiments. To test this we measured the amount of bound labelled phosphate and analyzed the percentage of bound ATP and P_i in identical samples (Fig. 4). By varying the incubation time of activated ATPase in the incubation medium we found that with increasing incubation time the amount of bound ATP did not vary while the amount of bound P_i increased.

In another experiment, we tried a competition between [γ-^{32}P]ATP and unlabelled ADP. As shown in Fig. 5 only a negligible amount of phosphate is

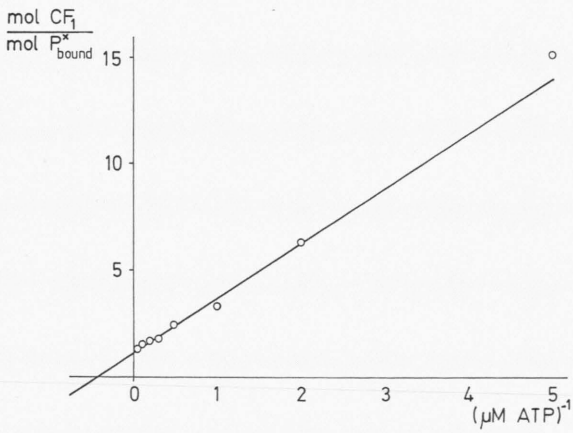

Fig. 3. Double-reciprocal plot of "phosphate" binding to heat activated ATPase with [γ-^{32}P]ATP as the variable substrate. The ATPase preparation was rapidly mixed with the incubation medium, containing the indicated ATP concentrations. Each sample was separated by column centrifugation within 5 to 6 seconds.

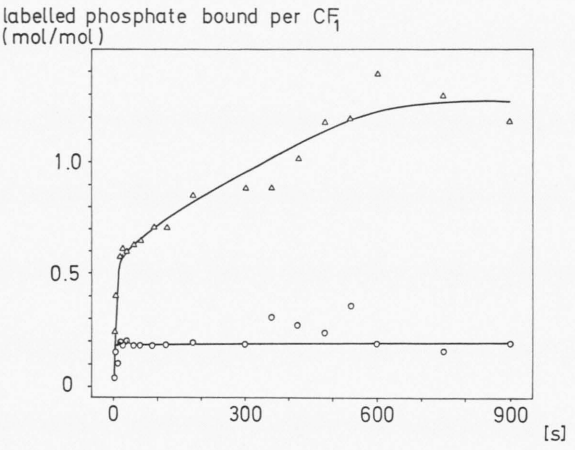

Fig. 4. Binding of [γ-^{32}P]ATP to heat activated CF_1-ATPase. Time course and identification of the bound phosphate species. Heat activated CF_1-ATPase was mixed with an incubation medium containing 50 μM labelled ATP and immediately transferred to the centrifuge columns. The effluent of the columns was denaturated by 0.3 M $HClO_4$. After centrifugation the percentage of bound ATP was determined by chromatography on PEI-cellulose. The time span that passed from the mixing step to the starting of the centrifuge is given in the figure. (△) Total amount of bound labelled ^{32}P; (○) bound [γ-^{32}P]ATP.

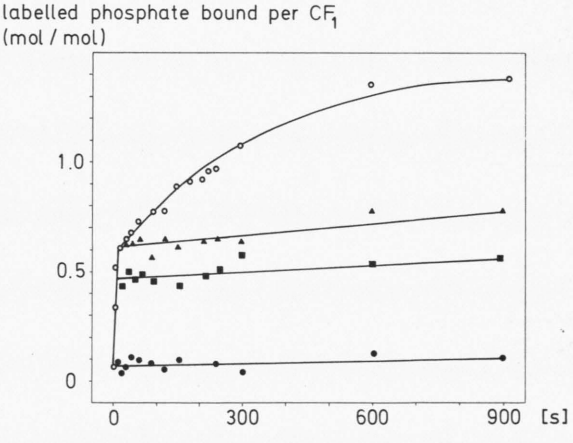

Fig. 5. Binding of [γ-^{32}P]ATP to heat activated ATPase. Effect of ADP on the binding kinetics. The experimental conditions were the same as described under Fig. 4 (○). But in three of the parallel tests ADP at a final concentration of 2 mM was added (filled symbols) at different instants:
a) together with the labelled ATP (●),
b) after 5 seconds of incubation (■), and
c) after 10 seconds of incubation (▲).

bound if 2 mM ADP were added in the presence of 50 μM ATP. But if ADP was added after a pre-incubation of 5 or 10 seconds, respectively, the bound labelled phosphate species was not displaced from the coupling factor any more.

Discussion

Until now very little is known on the binding of phosphate to chloroplast coupling factor. From the effects of phosphate on nucleotide exchange the existence of at least one phosphate binding site per CF_1 was assumed [18] and some phosphate was found to be bound to the enzyme during ATPase experiments [15, 19]. But there was a general agreement that binding of both substrates, ADP and P_i had to be studied.

We measured the binding of labelled phosphate using as well $^{32}P_i$ with untreated CF_1 as [γ-^{32}P]ATP with activated ATPase. We bargained for the fact that there are some differences with respect to substrate affinity but the number as well as the function of binding sites should be the same with the isolated and the membrane bound enzyme.

We found a single phosphate binding site on CF_1 in the absence of nucleotides (Fig. 1 and 2). The number of bound phosphates per CF_1 was not affected by activation of ATPase or using $CaCl_2$ instead of $MgCl_2$ (Table I). This means that the binding site is available on each CF_1 molecule independent of the state of catalytic activity. But we can not rule out that the binding kinetics is affected by such variations of the experimental conditions.

In a second set of experiments we measured the binding of labelled phosphate after incubation of CF_1 with [γ-^{32}P]ATP (Fig. 3) and found a biphasic binding of the label (Fig. 4). A rapid binding of ATP was succeeded by a phosphate binding. Both types of binding were suppressed by addition of ADP (Fig. 5), they did not occur with inactive or inhibited ATPase. Addition of unlabelled phosphate affected the slow ^{32}P-binding, not the rapid step. In agreement with results published by Bickel [17] we found that ADP + phosphate were predominantly bound during the rapid phase though ATP was added.

Our results suggest that ATP is hydrolyzed in its binding site very rapidly. Both, ADP and P_i, remain bound in this domain for a period of time. As indicated in Fig. 4 at least a portion of enzymes contains ATP (or ADP + P_i) and an additional phosphate. We think that inorganic phosphate from the incubation medium as well as the phosphate bound during the slow phase in our experiment were bound to orthophosphate binding sites of CF_1. Therefore we found a competition between medium phosphate and the hydrolyzed γ-phosphate of ATP.

The binding constants of 2.3 μM (ATP) and 170 μM (P_i) make it difficult to interpret the observation that we did not find a competition between ATP and P_i during ATP binding in the rapid step. The competition between ADP and labelled ATP (Fig. 5) indicates that ATP is bound by a nucleotide binding site and hydrolysis takes place by reversal of synthesis in a domain sequestered from the aqueous phase. During hydrolysis the γ-phosphate becomes bound to a phosphate binding site. When the enzyme switches to the succeeding state, this bound phosphate can be exchanged with medium phosphate or dissoziate from the enzyme. We think that the slow phosphate binding phase in our experiments indicates that with prolonged incubation an increasing number of ATPases contain labelled hydrolyzed γ-phosphate in their phosphate binding sites. In the competition experiment up to one mol of phosphate remained bound in the presence of ADP. But the slow kinetics of further phosphate binding was no longer detectable. This observation suggests that ATP does not bind to an orthophosphate binding site and that any turnover is stopped after binding of ADP.

[1] P. D. Boyer and W. E. Kohlbrenner, in: Energy Coupling in Photosynthesis (B. R. Selman and S. Selman-Reimer, eds.), pp. 231–240, Elsevier/North Holland, Amsterdam 1981.
[2] M. S. Abbott, J. J. Czarnecki, and B. R. Selman, J. Biol. Chem. **259,** 12271–12278 (1984).
[3] H. Strotmann and S. Bickel-Sandkötter, Ann. Rev. Plant Physiol. **35,** 97–120 (1984).
[4] R. E. McCarty and C. M. Nalin, Encycl. Plant Physiol. **19,** 576–583 (1986).
[5] H. Strotmann, Encycl. Plant Physiol. **19,** 584–594 (1986).
[6] I. Willms and A. Löhr, in: Proc. III. Europ. Bioen. Congr. (G. Schäfer, ed.), pp. 380–381, ICSU Short Reports, **Vol 3,** Cambridge University Press 1985.
[7] R. P. Magnusson, A. R. Portis, jr., and R. E. McCarty, Anal. Biochem. **72,** 653–657 (1976).
[8] H. Strotmann, H. Hesse, and K. Edelmann, Biochim. Biophys. Acta **314,** 202–210 (1973).
[9] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[10] J. V. Moroney, L. Lopresti, B. F. McEwen, R. E. McCarty, and G. G. Hammes, FEBS Lett. **158,** 58–62 (1983).
[11] H. S. Penefsky, J. Biol. Chem. **252,** 2891–2899 (1977).
[12] U. Franek and H. Strotmann, FEBS Lett. **126,** 5–8 (1981).
[13] S. Lien and E. Racker, Methods Enzymol. **23,** 547–555 (1971).
[14] U. Pick and S. Bassilian, Biochemistry **21,** 6144–6152 (1982).
[15] R. P. Magnusson and R. E. McCarty, J. Biol. Chem. **251,** 7417–7422 (1976).
[16] H. Strotmann, S. Bickel-Sandkötter, K. Edelmann, F. Eckstein, E. Schlimme, K. S. Boos, and J. Lüstorff, Biochim. Biophys. Acta **545,** 122–130 (1979).
[17] S. Bickel-Sandkötter, Biochim. Biophys. Acta **723,** 71–77 (1983).
[18] V. Shoshan and H. Strotmann, J. Biol. Chem. **255,** 996–999 (1980).
[19] D. Wu and P. D. Boyer, Biochemistry **25,** 3390–3396 (1986).