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SPECIFICITY OF NUCLEOTIDE BINDING SITES IN ISOLATED CHLOROPLAST COUPLING FACTOR (CF_1)

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Summary

The binding of various nucleotides to chloroplast coupling factor CF_1 was studied by two dialysis techniques. It was found that the number of nucleoside diphosphate sites and their specificities for the base moiety is dependent on the magnesium concentration. In the presence of 50 μM added $MgCl_2$, the protein has a single strong site/mol protein with K_d = 0.5 μM for ADP and high specificity (K_d > 20 μM for ϵADP, GDP, CDP). In the presence of 5 mM $MgCl_2$, the protein has two independent tight ADP sites (K_d = 0.4 μM) of low specificity ($K_d \approx$ 0.8, 2, and 2 μM, respectively for ϵADP, GDP, and CDP). These results are compared with the specificity of the partial reactions for photophosphorylation.

Introduction

The chloroplast coupling factor protein, CF_1[1], is generally believed to contain the sites responsible for the final steps in photophosphorylation (e.g. ATP formation) [1], as well as the partial reactions of photophosphorylation in chloroplasts such as light-dependent or light-triggered ATPase [2], ATP $\leftrightarrow$ P_i exchange [3], and the formation of ATP in the 'acid-base' reaction [4]. The isolated, homogeneous protein itself is a latent ATPase which can be activated in a number of ways. Although the treatments leading to Ca^{2+}-ATPase activity in purified CF_1 destroy its ability to reconstitute photophosphorylation in CF_1-depleted chloroplasts [5], Mg^{2+}-ATPase activity can be induced in the protein without destroying this ability [6].

Despite the fact that the same protein is apparently responsible for a number

Abbreviations used: CF_1, chloroplast coupling factor; ϵADP, 1-N^6-ethenoadenosine diphosphate; ϵCDP, 3,N^4-ethenocytidine diphosphate, TLC, thin-layer chromatography.

of varied reactions, the nucleotide specificity of the reactions differs widely [7,8]. Chloroplasts can phosphorylate a number of nucleoside diphosphates (NDPs), such as GDP, IDP, ϵADP and ϵCDP, with maximal rates over 50% that for ADP and apparent Michaelis constants only 2—5 times higher [2,7]. At the other extreme, chloroplast catalyzed ATP $\leftrightarrow$ P_i exchange is very specific, with other nucleoside triphosphates having activities 1% that of ATP at best [3,7,8]. We have suggested [7] that one possible explanation for these results is that the nature of the nucleotide binding sites of CF_1 varies with changes in the protein conformation induced by its environment, including changes in the state of energization of the chloroplast membrane.

With these observations in mind, we have attempted to examine the nucleotide binding specificity of isolated CF_1. To our surprise, the binding specificity of non-activated CF_1 from lettuce chloroplasts varies with the concentration of Mg^{2+} ions. At Mg^{2+} concentrations similar to those generally used in studies of photophosphorylation and the partial reactions (5 mM), the protein has two strong ADP-binding sites of low specificity, while in low Mg^{2+} concentrations, CF_1 has a single, strong site highly specific for ADP.

Materials and Methods

Chloroplast suspensions were prepared from fresh market lettuce by standard methods [9]. CF_1 was isolated by washing the chloroplast membranes (500—600 μg/ml) with aqueous EDTA, and purified by the method of Lien and Racker [10] with minor modifications. The protein showed a single band on disc gel electrophoresis [11,12], and five subunits of appropriate molecular weight [13] upon SDS polyacrylamide gel electrophoresis. Upon activation with heat, the protein showed Ca^{2+}-ATPase activity (method of Lien and Racker [10]) of 27 μmol P_i released/mg protein/min. A single CF_1 preparation was used to obtain all the data reported here.

For binding measurements, portions of the CF_1 preparation stored as a suspension at 4°C in 2 M ammonium sulfate and 4 mM ATP at pH 7.1 [10] were chromatographed on a 1 X 16 cm Sephadex G-50 column equilibrated and eluted with 40 mM tricine/NaOH buffer containing 2 mM EDTA at pH 8.0. The protein fractions contained about 1—2 mg/ml protein, and were free of ammonium sulfate as judged by the Nessler test. Since the storage medium contained 4 mM ATP [10], the protein was examined for ATP content after the Sephadex G-50 column by using the luciferase assay [14]. It was found to contain 1 mol ADP/mol protein and <0.1 mol ATP/mol protein. The standard solution for binding measurements contained 0.01 M tricine/NaOH buffer, pH 8.0/0.01 M NaCl/1—$3 \cdot 10^{-6}$ M CF_1. Protein concentrations were determined by the method of Lowry [15], using the correction factor of 1.15 suggested by Farron [16] and an assumed mol wt. of 325 000 [16].

Equilibrium dialysis experiments were carried out in lucite cells with a volume of 0.4 ml per half-cell, using asymmetric cellulose acetate ultrafiltration membranes (types 5D00 and 3B00) prepared by Dr. Melvin S. Weintraub of the Research and Development Authority of Ben Gurion University (Final Report NEG-ES-73-6). These membranes, with a very thin semipermeable layer on a more porous support, had much higher diffusion rates for nucleotides than

commercial cellulose acetate membranes, and complete equilibration was obtained in 16 h. The semipermeable layer always faced the side of the cell containing the protein. At equilibration, duplicate 25-μl samples were taken from each side of the cell for scintillation counting, and 25- and 50-μl samples taken for protein determinations.

Forced dialysis experiments were carried out by the method of Cantley and Hammes [17] using an 8-chambered device made by the MRA Corporation (Boston, Mass.) on the basis of the design of Paulus [18]. Amicon PM 30 membranes were employed. Solutions were prepared in test tubes and incubated for up to 2 h before introduction into the apparatus (volume introduced 0.35 ml) and dialysis under 40 lb/inch^2 nitrogen pressure.

The radioactive nucleotides [2-^{3}H]AMP, [2-^{3}H]ADP, [8-^{14}C]ADP, [8-^{14}C]-ATP, [U-^{14}C]GDP, and [U-^{14}C]CDP were obtained as ammonium salts from the Radiochemical Centre, Amersham. The specific activities of solutions diluted with unlabelled nucleotides were determined for each experiment by scintillation counting of aliquots under the same conditions used for the analysis of binding results and parallel spectrophotometric determinations of concentration. In all cases, specific activities of $5-10 \cdot 10^7$ cpm/μmol were used. [2-^{3}H]-ϵADP was synthesized by reacting [2-^{3}H]ADP with chloroacetaldehyde by the method of Secrist et al. [19]. The very small sample could not be recrystallized, and excess solvent and reagents were removed by evaporation several times, after addition of distilled water. The purity of the product was ascertained in several ways. The conversion of adenosine to ϵ-adenosine proceeded to completion according to the spectroscopic criterion of Secrist et al. [19]. A sample of the product was chromatographed on a thin layer of cellulose (Merck aluminum-backed cellulose F 254), eluting with isobutyric acid/concentrated NH_3/water (39 : 1 : 60 v/v). Standards of AMP, ADP, ϵAMP, and ϵADP were run in parallel. Very little radioactivity was detected in the region where the ADP standard ran. The radioactive ϵADP, diluted with unlabelled ϵADP, was also chromatographed on a thin layer of polyethyleneimine-impregnated cellulose on a plastic backing (Merck) [20]. After development, the sheet was cut into 1-cm strips and the strips counted in scintillation vials. About 4% of the radioactivity was contained in ϵAMP.

The polyethyleneimine-impregnated cellulose TLC method was also used to check the radiochemical purity of other nucleotides used, as well as their stability to hydrolysis under the experimental conditions.

Results and Discussion

The binding of ADP and other nucleotides to purified CF_1 in solutions of low magnesium concentration (50 μM total added $MgCl_2$) was measured by equilibrium dialysis, with 16—20 h allowed for equilibration. Under these conditions (pH 8.0, 0.01 M tricine/NaOH buffer/0.01 M NaCl/$1-3 \cdot 10^6$ M CF_1 at 20—21°C) the hydrolysis of nucleoside diphosphates to the monophosphates proceeds to the extent of about 5—20% (as determined by ion-exchange TLC [20]). This hydrolysis was corrected for in the analysis of the data by assuming that the monophosphates have very low affinity for CF_1, an assumption justified by direct measurements of AMP binding.

Because of the uncertainty of the above-mentioned corrections for hydrolysis, the association of ADP and CF_1 was also measured by the forced analysis technique. Protein and ADP were incubated together in buffer for two hours before forcing the dialysis (largely for experimental convenience). Such a preincubation leads to insignificant hydrolysis (even at higher Mg^{2+} concentrations), but can be shown to be sufficient for complete equilibration of the association process. When identical mixtures were incubated over times from 10 to 120 min before forcing the dialysis, the measured b/E (mol ligand bound per mol enzyme) was independent of time within the limits of experimental accuracy.

The association of ADP with CF_1 in the presence of $5 \cdot 10^{-5}$ M $MgCl_2$ was measured over a free nucleotide concentration range of $4 \cdot 10^{-8}$—10^{-5} M, using both experimental methods. The results of these measurements are shown in Fig. 1, in the form of a titration curve (b/E as a function of free nucleotide concentration). Data from the forced dialysis technique is clearly more accurate, for the reasons mentioned above, and was given more consideration. From the titration curve, as well as from a Scatchard plot of the same data (insert in Fig. 1), it appears that ADP is bound to CF_1 in a 1 : 1 molar ratio under these conditions, with a dissociation constant of about $5 \cdot 10^{-7}$ M. Additional binding sites for ADP may exist at this low magnesium concentration, but their dissociation constants must be above 10^{-5} M and cannot be determined by the methods used here.

The binding of other nucleotides to CF_1 in the presence of $5 \cdot 10^{-5}$ M added $MgCl_2$ is very much weaker than that of ADP, as can be seen from the data in Fig. 1. The dissociation constants estimated on the basis of this data are summarized in Table I. The specificity of nucleotide binding is unusually high, with

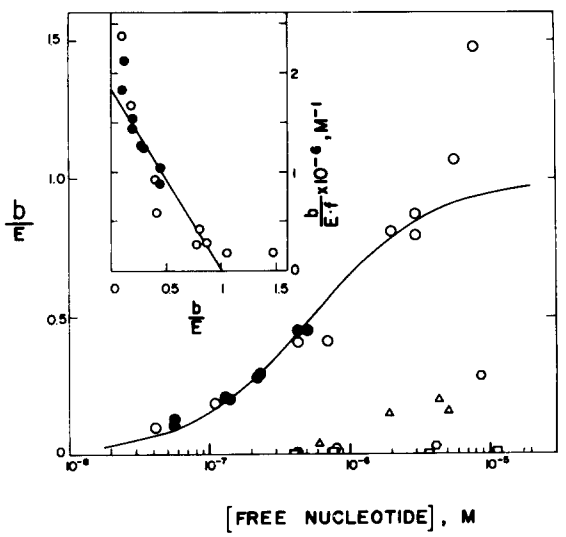


Fig. 1. Binding of nucleotides to CF_1 in the presence of $5 \cdot 10^{-5}$ M added $MgCl_2$ (pH 8.0; 10 mM tricine/10 mM NaCl). Open symbols from equilibrium dialysis experiments, filled symbols from forced dialysis (see text). Circles, ADP; triangles, ϵADP; squares, CDP; hexagons, GDP. Inset is Scatchard-type plot of ADP-binding data. Solid line in both main plot and inset represents binding to a single site with $K_d = 5.4 \cdot 10^{-7}$ M.

TABLE I

DISSOCIATION CONSTANTS FOR INTERACTION OF NUCLEOTIDES WITH CF_1

Constants are estimated from the data of Fig. 1—3, based on equilibrium dialysis and forced dialysis (pH 8.0, 10 mM tricine, 10 mM NaCl).

Nucleotide	Dissociation constant (M)	
	$5 \cdot 10^{-5}$ M $MgCl_2$ (1 site)	$5 \cdot 10^{-3}$ M $MgCl_2$ (2 sites)
ADP	$(5 \pm 1) \cdot 10^{-7}$	$(4 \pm 1) \cdot 10^{-7}$
ϵADP	approx. $1—2 \cdot 10^{-5}$	approx. $8 \cdot 10^{-7}$
GDP	$\geqslant 2 \cdot 10^{-5}$	approx. $2 \cdot 10^{-6}$
CDP	$>10^{-4}$	approx. $2 \cdot 10^{-6}$
AMP	$>10^{-4}$	approx. $1—3 \cdot 10^{-5}$

even ϵADP apparently binding 50 times more weakly than ADP itself.

Since ϵADP is a good analog of ADP in many enzymic reactions [21], including chloroplast photophosphorylation [7], we considered the possibility that the poor binding of ϵADP to CF_1 observed at low magnesium concentration might be an artifact due to inhibition of the protein by impurities in our preparation of [2-^{3}H]ϵADP. In order to rule out this possibility, we carried out a competition experiment in which approximately equal amounts of ADP and ϵADP were incubated with CF_1 simultaneously in the same equilibrium dialysis cells, and the results compared with experiments in which each nucleotide was present separately (Table II). As can be seen, the results are in accord with competition by the two nucleotides for the same site (although they do not prove that the same sites are involved) and show that the results of Fig. 1 are due to a difference of some 30-fold in the affinity of the protein for the two nucleotides and not to any 'poisoning' of the protein by impurities in the ϵADP.

The above results imply that at very low added $MgCl_2$ concentrations, CF_1 contains a single site of high affinity for ADP which is highly specific for the adenine moiety, and perhaps derives most of its affinity from interactions between the base and the protein. It should be pointed out that because the analysis mixtures contained about 0.5 mM EDTA (residual from Sephadex

TABLE II

BINDING COMPETITION BETWEEN ADP AND ϵADP

Equilibrium dialysis experiment, conditions as in Fig. 1.

Experiment	E_{total} (μM)	Ligand (μM)		b/E	b/E, expected *
		Bound	Free		
1. [^{14}C]ADP	3.86	3.12	2.01	0.807	0.801
2. [^{3}H]ϵADP	2.88	0.575	4.38	0.200	0.226
3. [^{14}C]ADP	3.59	2.79	3.25	0.774	0.829
+ [^{3}H]ϵADP		0.199	5.15	0.055	0.044

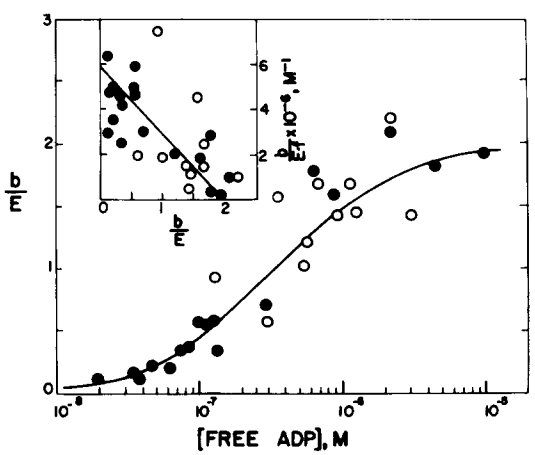
* Expected moles ligand bound/total enzyme concentration assuming competition for the same site, with K_d for ADP = $5 \cdot 10^{-7}$ M and for ϵADP = $1.5 \cdot 10^{-5}$ M, and the given free ligand concentrations.

G-50 desalting of the protein, see Materials and Methods), the free Mg^{2+} concentration can be calculated as being less than $3 \cdot 10^{-8}$ M [22]. The nucleotides in solution are thus almost entirely present in their free form, as opposed to Mg complexes, although one can not rule out the possibility that the protein's binding site has a sufficiently high affinity for Mg^{2+} [23] to bind significant amounts of Mg^{2+} under these conditions.

We must also consider the possibility that the observed binding of labeled nucleotides involves exchange with the mol of tightly bound ADP isolated with the protein under our conditions. While the experiments cannot rule this out, the time course of binding does not seem to fit the slow exchange rate of the tightly bound nucleotide, and we have not detected the expected systematic deviations from the titration curve at low added nucleotide concentrations which such an exchange would show. What is important to note is that, whether or not the binding of labelled nucleotides involves exchange with 'tightly bound ADP', it involves a site which is highly specific for the adenosine moiety.

The association of nucleotides with CF_1 in the presence of $5 \cdot 10^{-3}$ M added $MgCl_2$ has also been examined by the same methods. From Fig. 2 it can be seen that under these 'high magnesium' conditions, the protein appears to have more than one strong binding site for ADP. While we cannot rule out the possibility that there are three such sites, the data appear to be fit best by a theoretical line for two identical, independent sites with a dissociation constant of about $4 \cdot 10^{-7}$ M. A Hill plot of the data (not shown) has a slope of one, confirming a lack of interaction between the sites.

Fig. 3 presents the data for the binding of other nucleotides to CF_1 in the



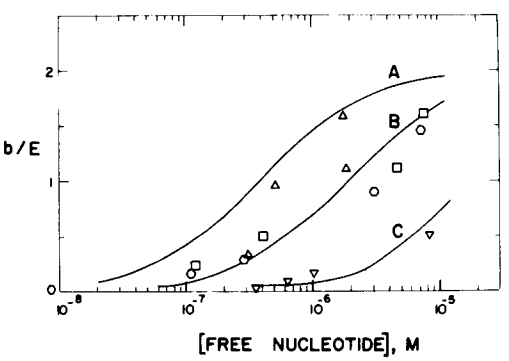


Fig. 2. Binding of ADP to CF_1 in the presence of $5 \cdot 10^{-3}$ M added $MgCl_2$ (pH 8.0, 10 mM tricine, 10 mM NaCl). Open circles from equilibrium dialysis experiments, filled circles from forced dialysis (see text). Inset is Scatchard-type plot of same data. Solid line in each plot represents binding to two identical non-interacting sites with $K_d = 3.5 \cdot 10^{-7}$ M.

Fig. 3. Binding of nucleotides other than ADP to CF_1 in the presence of $5 \cdot 10^{-3}$ M $MgCl_2$ (by equilibrium dialysis, as in Fig. 2). Triangles, ϵADP; squares, CDP; hexagons, GDP; inverted triangles, AMP. A: theoretical curve for ADP binding as in Fig. 2. B: theoretical curve for binding to two identical sites, $K_d = 2 \cdot 10^{-6}$ M. C: curve for 2 sites, $K_d = 2 \cdot 10^{-5}$.

presence of $5 \cdot 10^{-3}$ M added $MgCl_2$, and includes for comparison the 'theoretical line' for ADP binding (A). In sharp contrast with the results with a much lower $MgCl_2$ concentration, the various nucleoside diphosphates tested are quite similar in affinity to ADP under these conditions (e.g., ADP and CDP differ by only 5-fold). The approximate dissociation constants are summarized in Table I. The tight nucleotide sites of CF_1 are thus not specific for the base moiety of the nucleotide in the presence of $5 \cdot 10^{-3}$ M $MgCl_2$.

Cantley and Hammes have previously determined that CF_1 (in its latent ATPase form) has two independent binding sites for ADP in the presence of 2 mM Mg^{2+}, with K_d of about $1.8 \cdot 10^{-6}$ M [24]. They also found that these sites bind ϵADP with a similar affinity. Girault et al. made similar observations on ADP-binding [25] in the presence of 20 mM Mg^{2+}. It has been reported by others [27] that the pattern of binding sites for ADP and ATP is affected by Mg^{2+} concentrations.

The tight nucleotide sites observed here in the presence of 5 mM Mg^{2+} have the same sort of specificity observed in chloroplast photophosphorylation [2,7]: while ADP is apparently the 'best' ligand, other nucleoside diphosphates are only slightly poorer. The presence of a single, tight nucleotide site of quite different specificity under a diffferent set of conditions (very low Mg^{2+} concentration) is intriguing (and reminiscent of the highly base-specific partial reactions, e.g. ATP $\leftrightarrow$ P_i exchange [3,7,8]). One trivial explanation could be that the sites in question are totally unrelated. The non-specific sites at high Mg^{2+} concentration may derive much of their nucleotide affinity from a pyrophosphate-divalent cation-protein bridge and thus be 'tight' sites only in the presence of sufficient cation concentrations. The specific site observed at $5 \cdot 10^{-5}$ M added $MgCl_2$ might be only coincidentally of similar ADP affinity and be blocked in the presence of higher magnesium concentrations.

An interesting alternative explanation is that the sites involved are the same sites, showing different properties because of local or general changes in CF_1 as a result of magnesium binding. The 'single' ADP site observed at low Mg^{2+} might reflect strong negative cooperativity between two potential sites under these conditions. While extreme changes in the divalent cation concentration are not relevant to the function of CF_1 in chloroplast membranes, if this explanation is correct it does reflect the capacity of the protein to undergo changes in the properties of its nucleotide binding sites in response to its environment. Such changes were suggested as a possible explanation for the varied specificity of the reactions presumably catalysed by CF_1 [7].

In the accompanying paper, we present data on the kinetics of nucleotide interactions with CF_1 (albeit a preparation of a different sort). These data suggest that the tight binding sites of CF_1 may be the catalytic sites. In addition, the data demonstrate another way in which the properties of the binding sites of this complex enzyme may be altered. Such effects are of general interest in light of the suggestion that conformational changes in coupling proteins may be intimately involved in energy transduction (e.g. ref. 26).

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References

1 Avron, M. (1963) Biochim. Biophys. Acta 77, 699—702
2 Bennun, A. and Avron, M. (1965) Biochim. Biophys. Acta 109, 117—127
3 Carmeli, C. and Avron, M. (1967) Eur. J. Biochem. 2, 318—321
4 Jagendorf, A.T. and Uribe, E. (1967) in Energy Conversion by the Photosynthetic Apparatus (Brookhaven Symposium No. 19) pp. 215—241, Brookhaven National Laboratory, Upton, N.Y.
5 Farron, F. and Racker, E. (1970) Biochemistry 9, 3829—3836
6 Nelson, N., Nelson, H. and Racker, E. (1972) J. Biol. Chem. 247, 6506—6510
7 Shahak, Y., Chipman, D.M. and Shavit, N. (1974) in Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., ed.), pp. 859—866, Elsevier, Amsterdam
8 Shahak, Y., Chipman, D.M. and Shavit, N. (1973) FEBS Lett. 33, 293—296
9 Avron, M. (1961) Anal. Biochem. 2, 535—543
10 Lien, S. and Racker, E. (1971) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 23, pp. 547—555, Academic Press, New York
11 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
12 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321—349
13 Nelson, N., Deters, D.W., Nelson, H. and Racker, E. (1973) J. Biol. Chem. 248, 2049—2055
14 Kimmich, G.A., Randles, J. and Brand, J.S. (1975) Anal. Biochem. 69, 187—206
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Farron, F. (1970) Biochemistry 9, 3823—3828
17 Cantley, Jr., L.C. and Hammes, G.G. (1973) Biochemistry 12, 4900—4904
18 Paulus, H. (1969) Anal. Biochem. 32, 91—100
19 Secrist, III, J.A., Barrio, J.R., Leonard, N.J. and Weber, G. (1972) Biochemistry 11, 3499—3506
20 Randerath, K. and Randerath, E. (1967) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.D., eds.), Vol. 12A, pp. 323—347, Academic Press, New York
21 Secrist, III, J.A., Barrio, J.R. and Leonard, N.J. (1972) Science, 175, 646—647
22 Chaberek, S. and Martell, A.E. (1959) Organic Sequestering Agents, p. 513, John Wiley, New York
23 Hochman, Y., Lanir, A. and Carmeli, C. (1976) FEBS Lett. 61, 255—259
24 Cantley, Jr., L.C. and Hammes, G.G. (1975) Biochemistry 14, 2968—2975
25 Girault, G., Galmiche, J.M., Michel-Villaz, M. and Thiery, J. (1973) Eur. J. Biochem. 38, 473—478
26 Boyer, P.D. (1974) in Dynamics of Energy Transducing Membranes (Ernster, L., Estabrook, R.W. and Slater, E.C., eds.), pp. 289—301, Elsevier, Amsterdam
27 Higashida, M. and Mukohata, Y. (1976) J. Biochem. (Tokyo) 80, 1177—1179