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Binding of Modified Adenine Nucleotides to Isolated Coupling Factor from Chloroplasts as Measured by Polarization of Fluorescence

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1. Fluorescent nucleotides (1,N^6-ethenoadenosine diphosphate and triphosphate, εADP and εATP) replace the natural nucleotides rather efficiently (65–85%) in several chloroplast reactions (ADP inhibition of electron transport, ATP inhibition of electron transport, ATP stimulation of proton uptake, *etc.*).

2. εAMP does not bind to coupling factor protein.

3. The latent isolated coupling factor protein, which reconstitutes phosphorylation well when added to depleted chloroplasts, binds (in less than 1 min) both εADP and εATP at two divalent cation-sensitive sites each of similar binding affinity (dissociation constant $K_d \approx 1-3$ μM) although the εADP and εATP sites are not the same. 0.1 M NaCl increases K_d by 3–5-fold.

4. Polarization of fluorescence of nucleotides and coupling factor suggest that cation-induced changes of protein conformation exist.

5. Binding of coupling factor to nucleotides is maximal at pH 8 where phosphorylation in chloroplasts is also maximal, but changes (with isolated coupling factor) at other pH values are not directly related to the enzyme action.

6. Interaction of inorganic phosphate (P_i) with coupling factor in the presence of nucleotide occurs as monitored by a sizable increase in K_d for εADP-coupling factor and εATP-coupling factor binding sites, and by decrease in polarization of fluorescence of coupling factor; this suggests negative interaction of adenine nucleotides and P_i on coupling factor.

7. Interaction of coupling factor with εADP and ATP on the one hand, and with εATP and ADP on the other, occurs as indicated by sizable increases in the K_d for εADP-coupling factor and ATP-coupling factor, respectively; this suggests s negative interaction of ADP and ATP on coupling factor.

8. The kinetics of release of εADP and εATP from coupling factor when excess ADP or ATP were added in competition were relatively slow compared to the rate at which photophosphorylation proceeds *in vivo*; this suggests that coupling factor may have to be bound to the hydrophobic HF_0 protein and/or the thylakoid membrane for fully functional adenine nucleotide binding to be expressed. Already bound εATP was more difficult to remove, even with Dowex 2X, from coupling factor than already bound εADP suggesting a more 'internal' binding of the former.

To date, the gap has been rather large between what is known of nucleotide, ionic and conformational effects observed in functioning chloroplasts and what is specifically known about the binding and interaction of these factors with the ATP synthetase (coupling factor) which plays a central role in the process of energy transduction. (For a review of photophosphorylation and the current knowledge of coupling factor, see Jagendorf [1].) Therefore, to extend the present limited knowledge of the molecular mechanism of photophosphorylation in chloroplasts, the binding of adenine nucleotides to coupling factor was systematically investigated by measuring the fluorescence polarization of εAMP, εADP and εATP (1,N^6-

Abbreviations. εAMP, 1,N^6-ethenoadenosine monophosphate; εADP, 1,N^6-ethenoadenosine diphosphate; εATP, 1,N^6-ethenoadenosine triphosphate; K_d, dissociation constant; K_1, smallest K_d in a two-binding site system; K_2, largest K_d in a two-binding site system; p, polarization of fluorescence; tricine, *N*-tris(hydroxymethyl)methylglycine.

ethenoadenosine monophosphate, diphosphate and triphosphate) which are the fluorescent analogs of AMP, ADP and ATP. Some spectroscopic properties of these fluorophores and their biological activities in other systems [2–7] and chloroplasts [8,9] have been described. The intact chloroplast, although fully functional, is so complex that it is often difficult to ascertain what is specifically occurring at the molecular level. Therefore, we have undertaken the present study using a relatively simpler, better defined system, *i.e.* coupling factor isolated from chloroplasts, in which the data can be more readily interpreted.

Recently, the fluorescent compounds εADP and $1,N^6$-ethenoadenylylimidodiphosphate were used in conjunction with the non-fluorescent nucleotides to partially characterize binding sites on soluble chloroplast coupling factor [10]; in this study, the ATPase function of coupling factor was investigated, and a model was proposed in which nucleotide binding sites on heat-activated coupling factor act as allosteric conformational 'switches' for the ATP hydrolysis activity of solubilized coupling factor. In the present work, which is an extension of preliminary data we have published [11], attention has been focussed on the unmodified (latent) coupling factor protein as it may relate to the forward process of phosphorylation and, therefore, only limited comparison is possible with the studies of Cantley and Hammes [10]. A comparative study, using circular dichroism and chemical isolation, of coupling factor-ligand complexes is also available [12].

It has been demonstrated that εADP acts nearly as well as ADP substrate for photophosphorylation [8, 9]. εATP substitutes poorly for ATP in the ATPase reaction using heat-activated coupling factor [9], but it functions like ATP in stabilizing coupling factor against heat inactivation [9]. In this study, the above result for εADP phosphorylation was confirmed. In addition, for the first time, εADP and εATP were shown to substitute well for ADP and ATP in several other coupling-factor-mediated chloroplast reactions (ADP inhibition of electron transport, ATP inhibition of electron transport, ATP stimulation of light-induced proton uptake), thus, demonstrating that the fluorescent-modified nucleotides are good functional analogs, and data obtained using these compounds (presented below, see Results and Discussion) can be interpreted with confidence. Binding of εADP and εATP to coupling factor, as measured by polarization of nucleotide fluorescence, is presented here under various conditions: time of incubation, pH of the medium, different concentrations of fluorescent and non-fluorescent nucleotides, and of divalent cations, presence of phosphate (P_i), *etc.* We also present data on the conformational status of coupling factor, through measurements on the polarization of tyrosine fluorescence, in the presence of cations, ADP and P_i.

MATERIALS AND METHODS

Chloroplast Preparation

Class II chloroplasts were isolated from lettuce (*Lactuca sativa*), unless otherwise indicated, by homogenizing in a buffer containing 50 mM phosphate or tricine-NaOH (pH 7.8), 10 mM NaCl plus 10 mM KCl, and 400 mM sucrose, and filtering through several layers of cheese-cloth and a 10 μm mesh cloth; the filtrate was centrifuged at $1500 \times g$ for 10 min. The chloroplast pellet was washed in hypotonic medium (homogenizing buffer, minus sucrose) to rupture the outer chloroplast membrane envelope, and then resuspended in a small volume of homogenizing medium. Chlorophyll concentration was determined by the method of MacKinney [13] with a Cary 14 spectrophotometer.

Chloroplast Activity

Ferricyanide reduction (Hill reaction) was measured by following the decrease in absorbance at 420 nm on a Cary 14 spectrophotometer when the chloroplast suspension was simultaneously illuminated with saturating (700 W/m^2) red actinic light (white light passed through Corning C.S. 2-64 and heat filters); the absorption coefficient used for calculation of ferricyanide reduced was 1000 cm^{-1} M^{-1} [14].

Photophosphorylation and light-induced proton flux were measured as described by Dilley [15] by monitoring the rate of alkalinization of the medium using a recording pH meter; illumination was with saturating (600 W/m^2) white light passed through a heat filter. Details of reaction mixture are given in the Table legends.

Preparation of Coupling Factor Protein

Coupling factor protein was isolated from market lettuce (*Lactuca sativa* var. Romaine) leaves by a procedure based on the method of Strotmann *et al.* [16] in which coupling factor is released from the thylakoids by resuspending chloroplast pellets (previously washed several times with 10 mM $Na_4P_2O_7$, pH 7.5) in 2 mM tricine-NaOH and 300 mM sucrose at pH 8.0. After addition of 2 mM EDTA and 4 mM ATP to stabilize coupling factor [17], solid $(NH_4)_2SO_4$ was slowly added, with stirring, to the supernatant to give a final concentration of 2 M (roughly 50% saturation at 4 °C) in order to concentrate coupling factor. After storage at 4 °C overnight, the precipitate was separated from sucrose and collected by centrifugation (10000 $\times g$ for 10 min). The pellet was dissolved in a small volume (several ml) of 40 mM tricine-NaOH (pH 8), 4 mM ATP, 2 mM EDTA and 2 M $(NH_4)_2SO_4$.

For use in fluorescence experiments, aliquots of stored coupling factor were centrifuged for 5 min at

about $10000 \times g$, dissolved in a small volume (about 1 ml) of 30 – 40 mM tricine-NaOH (pH 8), centrifuged for 2 min at about $10000 \times g$ to remove any undissolved material, and then desalted and stripped of bound nucleotides on a Sephadex G-50 column (1×50 cm) at about 12 – 15 °C since unprotected coupling factor in solution is cold (0 – 10 °C) labile [17,18]. Protein concentration was determined by the Lowry method [19]. A molecular weight of 325000 for coupling factor [20] was used in determining the molarity.

Our preparation was tested for purity by comparing the fluorescence spectra of soluble coupling factor with a solution of tyrosine in the same buffer (25 mM tricine-NaOH, pH 8.1). Both samples, when excited at 270 nm, had emission maxima at 305 nm (not shown). Lien and Racker [17] have shown a direct correlation of the ratio of fluorescence at 300 and 350 nm to the purity of coupling factor preparations; all fractions with a 300:350 nm intensity ratio exceeding 1.85 were homogenous by polyacrylamide gel electrophoresis. In our preparations, a ratio of about 2 for fluorescence at 300 and 350 nm was obtained when coupling factor in a solution containing 25 mM tricine-NaOH (pH 8) was excited with 270 – 275 nm light. Thus, our samples were pure by the above criterion.

The activity of isolated coupling factor was checked by its ability to rebind to thylakoid membranes depleted of coupling factor and restore photophosphorylation. (See Shoshan and Shavit [21] for the method used here as well as for a study on factors affecting the reconstitution of photophosphorylation in chloroplast membranes.) Our coupling factor preparations restored phosphorylation with an efficiency of 90% (sample reconstituted at 0.11 mg chlorophyll/ml and at least 65% (at 0.18 mg chlorophyll/ml) compared to control (not shown).

Chemicals

The fluorescent nucleotides, εAMP, εADP, εATP, were purchased from P & L Biochemicals (Milwaukee, Wis.); tricine, AMP, ADP, ATP, KH_2AsO_4, *N*-methylphenazonium methylsulfate and dithioerythritol were from Sigma (St Louis, Mo.). Purity of fluorescent nucleotides was assured by thin-layer chromatography.

Polarization of Fluorescence

Fluorescence polarization measurements on fluorescent nucleotides were made on an instrument similar to that described by Weber [22] (D. M. Jameson *et al.*, unpublished) but with photon counting. In this instrument, parallel and perpendicular components of polarized fluorescence are monitored simultaneously by two photomultipliers at right angles to the exciting beam. This apparatus also allows convenient subtraction of background scattering at low signal-to-noise values. A 450-W xenon arc, cooled by air draft, was the light source. The 310-nm exciting light was selected by a Bausch and Lomb grating monochromator (1-mm slit width; 1.7 nm/mm disperison) and passed through a Corning glass C.S. 7-54 filter. The fluorescence emission was filtered through a Corning glass C.S. 0-52 filter, which also blocks the exciting light from detection by the photomultiplier tube.

For measurements of polarization of protein fluorescence, the same photon-counting instrument was used; the exciting wavelength was 280 nm and the fluorescence emission was filtered through a Corning C.S. 0-54 filter. Initial base levels of polarization of protein fluorescence varied somewhat with the particular preparation and condition, and so the data from different experiments were normalized to the polarization with no addition for easier comparison. All experiments were made at 20 °C.

Values of $I_\parallel$ and $I_\perp$, where $I_\parallel$ is the intensity of the emission light polarized parallel to the exciting beam (which was polarized in a plane perpendicular to the axis of observation) and $I_\perp$ is the intensity of the emission light polarized perpendicular to the exciting beam, were obtained by repeated counting over 20 – 30-s intervals. Unless otherwise indicated, the polarization values reported here are the average of 2 – 3 such determinations. The polarization of fluorescence (p), defined as $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, is reported.

Fluorescence Intensity

Fluorescence intensity of the modified adenine nucleotides was measured on the photon-counting instrument described above under the same conditions and settings. (See below for use of fluorescence intensity in calculations of binding parameters.) Fluorescence emission of proteins was measured on a Hitachi Perkin-Elmer MPF-2A spectrofluorometer; excitation was at 270 – 275 nm.

Calculation of Binding Parameters

Data for the binding of the fluorescent-modified nucleotides to coupling factor was calculated and plotted according to the equation derived by Klotz *et al.* [24], namely, $S/DX = 1/n + K/nD(1-X)$, where n is the number of adsorbing sites, $K(= K_d)$ is the equilibrium constant of dissociation of the complex; S and D are the total protein and ligand concentrations, respectively, and X is the fraction of ligand bound. The reciprocal of the intercept of a plot of S/DX *versus* $1/D(1-X)$, *i.e.* the [protein]/[bound ligand] *versus* 1/[free ligand], gives n, and K/n is the slope. The polarization data were analyzed for binding

according to this method in the range of nucleotide concentration from about 2–35 μM with coupling factor at concentrations of 4–8 μM (further details given in figure legends or Results and Discussion). The essential features of the binding plot are as follows. The ordinate is unitless since the protein and ligand concentrations cancel out and X is a fraction; the abscissa is expressed in μM^{-1}. In this paper, K_1 will refer to the region of smaller slope (smaller K_d or stronger binding) and K_2 will refer to the region of steeper slope (greater K_d or weaker binding). If the two sites are of intrinsically the same affinity, nonlinearity observed in the binding plot is probably due to electrostatic interactions between the two negatively charged ligands as first one, and then the second nucleotide binds [24]. In this case the 'true' K_d will in the first approximation be some average of K_1 and K_2; this will generally not have much effect on the results reported here since for both εADP and εATP the values of K_1 and K_2 fall within a very narrow range [1–3 μM].

The fraction of ligand bound, X, was calculated according to the relation $X = R\ (p/p_b) - R + 1$. R is the fluorescence intensity of the ligand-protein system relative to that of the ligand alone at the same concentration and pH; R was computed using the sum of the parallel and perpendicular components of fluorescence intensity when the sample was excited with light polarized in a plane perpendicular to the axis of observation [4]. The observed polarization at a given ligand concentration is p and the value for complete binding of ligand, p_b, was determined by extrapolation of the raw binding data (polarization of fluorophor *versus* added concentration of ligand, at low concentrations of εADP or εATP); $p_b = 0.20$ for εADP and 0.25 for εATP. Confidence can be placed in these values since the K_d for binding of εADP, using a forced dialysis technique [10], was very close to those obtained here. (For literature on the use of the polarization method in binding studies, see [4,25,26].)

RESULTS AND DISCUSSION

Chloroplast Reactions

We have confirmed earlier work [8,9] showing that in chloroplasts supporting either cyclic (*N*-methylphenazonium methosulfate) or non-cyclic (H_2O to methyl viologen) electron transport, rates of εADP phosphorylation are nearly as high (about 70%) as for ADP (not shown). In addition, we measured the activity of εAMP, εADP, and εATP in several other chloroplast reactions.

Adenine nucleotides exert a regulatory effect on the rate of electron transport and proton transport mediated by the interaction of ADP or ATP with coupling factor [27–30]. In coupled chloroplasts, electron transport is inhibited under non-phosphorylating conditions (*e.g.*, by addition of ADP or ATP only, without P_i) and this inhibition is partially reversed under phosphorylating conditions (ADP, P_i, Mg^{2+} added). Lines 1–2 in Table 1 show that 100 μM ADP inhibits the rate of ferricyanide reduction by 22% in lettuce chloroplasts under nonphosphorylating conditions. If the ADP concentration is increased to 500 μM, no further inhibition is produced (line 3); this confirms data on the saturation of this effect [28]. Inhibition of electron transport was obtained also by εADP (about 13%, lines 1 and 6, Table 1), but the effect was 60% as compared to that of ADP (line 2).

If a 1 mM KH_2PO_4 (P_i) is present to allow photophosphorylation to proceed, a partial recovery (about 40%) of the rate of electron transport was observed with 500 μM ADP (line 4, Table 1); εADP substituted for ADP in this reaction, and gave a recovery rate of 70% (line 8, Table 1) compared to the unmodified nucleotide.

Addition of 100 μM ATP results in an inhibition of about 25% of the rate of ferricyanide reduction (line 5, Table 1). This decrease is also produced by εATP with activity of about 75% (line 9, Table 1) compared to unmodified ATP (line 5).

It has been shown [29,30] that 10 μM ATP stimulates the extent of the proton gradient by as much as 2–3-fold in spinach chloroplasts. This is due to a reduction in the rate of proton efflux at the level of coupling factor. We observed that the extent of stimulation was in the range of 1.5–2.0-fold in lettuce chloroplasts (Table 2). Approximately 0.11 μmol H^+/mg chlorophyll increased to about 0.18 μmol H^+/mg chlorophyll when 10 μM ATP was added, giving a 64% stimulation (line 4, Table 2); an increase from about 0.11–0.17 μmol H^+/mg chlorophyll was observed when 10 μM εATP was added, giving a stimulation of 56% (line 8, Table 2). Thus, the fluorescent-modified ATP substituted well for ATP with about 85% of the activity of the unmodified nucleotide.

Arsenate (As) plus ADP (catalyzing arsenylation) substitute for phosphate (P_i) plus ADP (catalyzing phosphorylation) in restoring the ADP-inhibited rate of ferricyanide reduction [27,31]. Mukohata and Yagi [31] demonstrated that AMP + ATP + As partially restored the inhibition of ferricyanide reduction by ATP, ATP + As, or ATP + AMP whereas AMP + As produced little or no inhibition of electron transport with nucleotide concentration up to 100 μM. This reaction facilitated by the combined presence of ATP + AMP + As was termed 'quasi-arsenylation'; phosphate could not substitute for arsenate in this reaction. We estimated the functional efficiency of εAMP compared to AMP by investigating this unusual reaction [31]. Using pea chloroplasts, we observed substantial restoration of the ATP-in-

Table 1. *Effects of adenine nucleotides on ferricyanide reduction*
Lettuce chloroplasts equivalent to 45 μg chlorophyll/2 ml were suspended in a medium containing 15 mM tricine-NaOH (pH 8.3), 100 mM sucrose, 6 mM $MgCl_2$ and 0.6 mM $K_3Fe(CN)_6$. At this concentration of ferricyanide it accepts electrons mostly from photosystem I. The decrease in absorbance at 420 nm was measured during exposure of chloroplasts to saturating (700 W/m^2) red light using a Corning C.S. 2-64 glass filter; values for ferricyanide reduction correspond to initial rates. Shown here is representative data of three experiments. The range of error indicated in the data represents the approximate minimum resolution of the measurement system

Addition	Ferricyanide reduced	Inhibition
	μmol × mg chlorophyll^{-1} × h^{-1}	%
1. No ADP	300 ± 8	–
2. 100 μM ADP	233	22.4
3. 500 μM ADP	235	21.7
4. 500 μM ADP + 1 mM KH_2PO_4	257	14.3
5. 100 μM ATP	224	25.5
6. 100 μM εADP	261	13.0
7. 500 μM εADP	263	12.4
8. 500 μM εADP + 1 mM KH_2PO_4	275	8.7
9. 100 μM εATP	242	19.2

Table 2. *Effects of adenosine triphosphate and its fluorescent analog on proton uptake*
Lettuce chloroplasts equivalent to 130 μg chlorophyll/3 ml were suspended in a medium containing 1.5 mM tricine-NaOH (pH 8), 5 mM $MgCl_2$, 25 mM NaCl, 25 mM KCl and 25 μM *N*-methylphenazonium methylsulfate, then illuminated with white light passed through a heat filter (Corning number 7440) and a circulating water bath surrounding the reaction chamber. ATP or εATP concentration was 10 μM. Values for proton uptake are ± 0.006 μmol, indicating the approximate minimum resolution of the measurement system

	Proton uptake		Stimulation
	− ATP	+ ATP	
	μmol/mg chlorophyll		%
	0.087	0.151	73
	0.120	0.191	59
	0.129	0.206	60
Average	0.112	0.183	64

	Proton uptake		Stimulation
	− εATP	+ εATP	
	μmol/mg chlorophyll		%
	0.085	0.130	53
	0.096	0.153	59
	0.140	0.220	57
Average	0.107	0.168	56

Table 3. *Restoration of ATP-inhibited electron transport by quasi-arsenylation*
Pea (dwarf, progress number 9) chloroplasts equivalent to 46 μg chlorophyll/2 ml were suspended in a medium containing 15 mM tricine-NaOH (pH 8.3), 100 mM sucrose, 6 mM $MgCl_2$, 0.6 mM $K_3Fe(CN)_6$ and 1 mM KH_2AsO_4 (As). Ferricyanide reduction was measured as in Table 1. See text for details on restoration

A	Addition	Ferricyanide reduced
		μmol × mg chlorophyll^{-1} × h^{-1}
	50 μM AMP	288 ± 7[a]
	50 μM εAMP	276
	75 μM ATP	210
	75 μM ATP + 50 μM AMP	261
	75 μM ATP + 50 μM εAMP	221
B	Addition	Restoration of ferricyanide reduction
		%
	ATP + AMP	65
	ATP + εAMP	20

[a] Electron transport rates are the average of three measurements.

hibited electron transport rate by AMP; εAMP substituted for AMP (Table 3). In the presence of 1 mM KH_2AsO_4 (As), the rates of ferricyanide reduction were 288 and 210 μmol × mg chlorophyll^{-1} × h^{-1} when 50 μM AMP and 75 μM ATP were added, respectively. However, addition of 75 μM ATP and 50 μM AMP together yielded a value of 261 μmol ferricyanide × mg chlorophyll^{-1} × h^{-1}. The restoration by AMP was calculated as follows:

$$\% \text{ restoration} = \frac{\% \text{ Inhibition (+ ATP)} - \% \text{ inhibition (+ ATP + AMP);}}{\% \text{ inhibition (+ ATP)}}$$

in calculating % inhibition, control rates were the values with AMP(εAMP) + As. Such a calculation demonstrated a 65% restoration by AMP, whereas εAMP was 30% as efficient as AMP.

Binding of Nucleotides to Isolated Coupling Factor

Since εAMP substituted less well (about 30%, Table 3) for AMP than εADP and εATP for ADP and ATP, respectively (about 65–85%, Tables 1 and 2), and since we found that εAMP, under a variety of conditions, shows little or no binding to isolated coupling factor (not shown) and, since also a transphosphorylation reaction involving AMP [32, 33] is a slow side reaction [34], εAMP was not studied further in the present work.

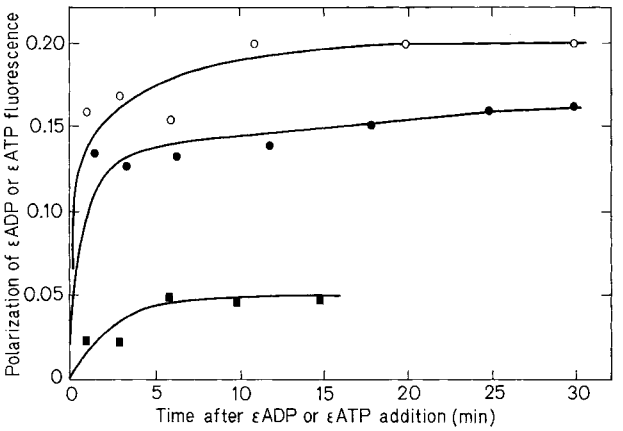

Fig. 1. *Time course of the binding of εADP (4.9 μM, filled circles) and εATP (4.2 μM, open circles) to 7 μM coupling factor.* The lower curve (squares) is the time course of binding of 4.9 μM εADP to coupling factor (from the same stock preparation as the upper curves) which had first been titrated in steps from pH 8 to 7 with 1 M HCl and then from pH 7 to 8 with 1 M NaOH before adding εADP. Medium contained 40 mM tricine-NaOH (pH 8) and 5 mM $MgCl_2$. Shown is representative data from three experiments. Values for the time given here are $\approx \pm 1/2$ min due to inherent time limitation in making a polarization measurement

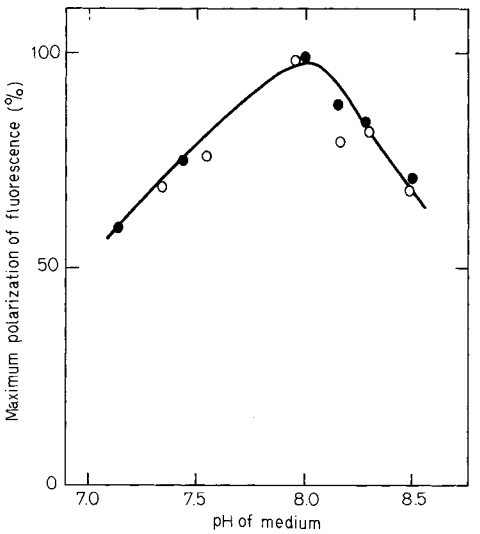

Fig. 2. *pH dependence of the binding of εADP (4.9 μM, filled circles) and εATP (4.2 μM, open circles) to coupling factor (two experiments with 4.5 and 6.5 μM coupling factor were normalized together).* Maximum polarization values were 0.16 and 0.20 for εADP-coupling factor and εATP-coupling factor, respectively. Changes in pH were made by titrating with 1 M HCl and 1 M NaOH. Medium contained 40 mM tricine-NaOH (pH 8) and 5 mM $MgCl_2$

Time Course of εADP and εATP Binding to Coupling Factor

Polarization values of nucleotide fluorescence above the zero level were observed when εADP was added to a solution of coupling factor isolated from lettuce chloroplasts. Fig. 1 shows the time course of binding of εADP (filled circles) and εATP (open circles) to coupling factor. In both cases binding was rapid, at least 80% complete within one minute. By comparing this time course with the time interval between successive additions of εADP and εATP in titrations made to ascertain binding constants (see below), one sees that essentially the equilibrium values are being determined. These kinetics are in contrast to the very slow (1−2 h time course) binding of ^{14}C-labelled ADP to coupling factor [32]; however, in the latter case, only very tightly bound enzyme-ADP complexes (able to pass through Sephadex column intact) which were insensitive to the presence of divalent cations were studied. (Such very tightly bound nucleotides may be present in our coupling factor preparation, but our study was of nucleotide-enzyme complexes of higher K_d, closer to the range for the K_m of nucleotides in chloroplast phosphorylation [1].) We note that reactions involving such tightly bound species may not be in the main path of chloroplast phosphorylation [34], but this remains to be determined with certainty. In another study with isolated coupling factor [10], 0.5−2 h incubation was also allowed; however, in this case 0.1 M NaCl was included in the reaction medium, which we have shown (see below) suppresses binding of εADP to coupling factor.

Effect on pH on εADP and εATP Binding to Coupling Factor

When a solution of coupling factor was subjected to pH titration from pH 8 to 7 and back to pH 8 before εADP binding was measured (Fig. 1, filled squares), the kinetics were similar (within error of measurement), but the maximal level of polarization of εADP-coupling factor attained was considerably less (one third of control) indicating some irreversible effects. Fig. 2 shows the pH dependence of binding of εADP and εATP to coupling factor in the pH range 7 to 8.5; maximal binding under these conditions was at pH 8. The changes in polarization during pH titration were not reversible (not shown).

Further support for an irreversible effect on coupling factor was found with measurements of the effect of pH changes on the intensity of fluorescence of the tyrosine residues of coupling factor protein. During titration with 1 M HCl from pH 8 to 7, a change (20% increase, not shown) in intensity of protein fluorescence at 310 nm was observed indicating an (as yet unspecified) alteration in the structural state of coupling factor; titration with 1 M NaOH from pH 7 back to

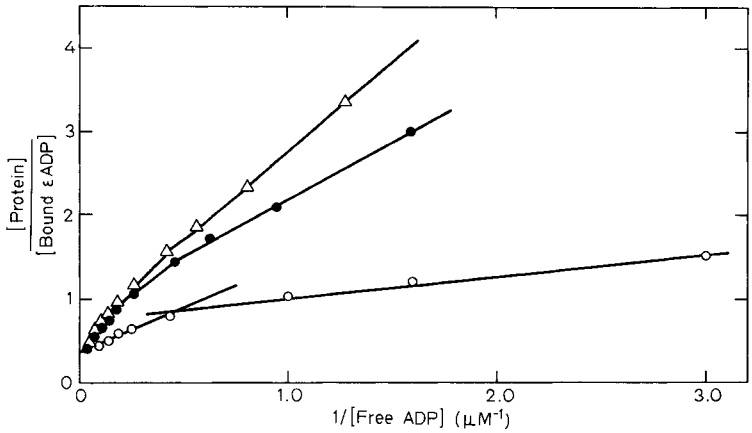

Fig. 3. *Klotz binding plot for εADP-coupling factor as a function of ionic content.* Medium contained 4.1 μM coupling factor and 30 mM tricine-NaOH, pH 8 (△), or buffer plus the following additions: 5 mM $MgCl_2$ (○), or, 5 mM $MgCl_2$ + 100 mM NaCl (●). The wavelength of excitation was 310 nm, and fluorescence emission was filtered through a Corning C.S. 0-52 glass filter placed before the photomultiplier. See Materials and Methods for further experimental details. Calculated values for dissociation constants (K_d) are: K_1 = 0.5 μM, K_2 = 2 μM (5 mM $MgCl_2$, open circles); K_1 = 2.8 μM, K_2 = 6 μM (5 mM $MgCl_2$ + 100 mM NaCl, filled circles); K_1 = 4 μM, K_2 = 6 μM (30 mM tricine-NaOH only, triangles). K_1 and K_2 refer to regions to the right and left of the break in binding curves, respectively

8 demonstrated that this effect also was not reversible (allowing time intervals of several minutes between additions of acid or base). A small irreversible change in the clarity of the coupling factor solution was also noted upon visual inspection representing nonspecific aggregation, denaturation or separation of coupling factor complex into subunits. (Lien *et al.* [35] also observed such changes with a cold inactivation treatment at pH 6.5; irreversible inactivation of isolated coupling factor could be prevented by including glycerol plus ATP in the medium, but we could not test the effects of these factors since ATP at the high concentrations necessary completely prevents observation of binding of the fluorescent nucleotides to coupling factor.) Isolated unprotected coupling factor is quite sensitive to changes in the pH of the environment as indicated both by binding of fluorescent nucleotides to coupling factor and by protein fluorescence; the observed changes are apparently not related to enzyme mechanism. It is concluded that any H^+-coupling factor interactions affecting adenine nucleotide binding and/or coupling factor conformation which may be important for phosphorylation require the thylakoid membrane for expression. (For reference to data and models involving the interaction of membrane-bound coupling factor with protons see [1, 8, 28, 30, 36–39].)

Effect of [$MgCl_2$] and High Ionic Strength on εADP Binding to Coupling Factor

Fig. 3 shows a plot of [protein]/[bound εADP] *versus* 1/[free εADP]. Coupling factor shows two binding sites for εADP of fairly high affinity with 5 mM $MgCl_2$, one (K_1 = 0.5 μM) being apparently somewhat tighter than the other (K_2 = 2 μM) (open circles). However, the observed heterogeneity may be a reflection of the negative interaction upon binding to two sites of essentially the same affinity rather than an indication of sites of intrinsically different K_d values.

As the $MgCl_2$ concentration is reduced from 5 mM to 0.5 mM and/or the ionic strength is increased by about an order of magnitude (100 mM NaCl added, filled circles) the dissociation constants are correspondingly increased (K_1 = 2.8 μM; K_2 = 6 μM). With buffer only (triangles), the K_d values are 4 μM (K_1) and 6 μM (K_2) and the 'degree of separation' between the sites is decreased (K_2/K_1 is about 4 for 5 mM $MgCl_2$, but 1.5 for no $MgCl_2$). Thus, it appears that Mg^{2+} acts not only in facilitating stronger εADP binding, but affects the heterogeneity or negative interaction between the two sites as well. High ionic strength (0.1 – 0.5 M) media are known to enhance the lability of coupling factor (as monitored by ATPase activity) in the cold, and also to a lesser extent at room temperature [40]. A related, but not neccessarily identical,change in coupling factor producing a conformation less favorable for εADP binding may be responsible for the effect of 0.1 M NaCl observed in Fig. 3.

Effects of Mg^{2+}, Ca^{2+} and P_i on εADP and εATP Binding to Coupling Factor

The binding of εADP to coupling factor in the presence of $MgCl_2$, $CaCl_2$ and $MgCl_2$ + P_i is shown in Fig. 4. The binding affinity with 5 mM $MgCl_2$ (K_1 = 1.5 μM; K_2 = 2.5 μM, open circles) is slightly greater than with 5 mM $CaCl_2$ (K_1 = 2.5 μM; K_2

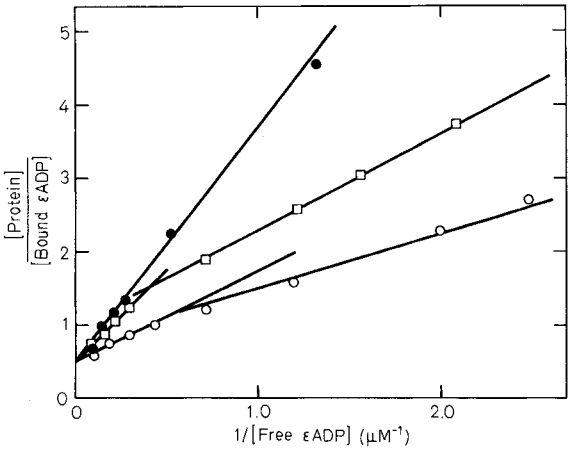

Fig. 4. *Klotz binding plot for εADP-coupling factor.* Medium containing 5.7 μM coupling factor and 25 mM tricine-NaOH (pH 8) plus the following additions affected the K_d as indicated: 5 mM $MgCl_2$ (open circles, K_1 = 1.5 μM, K_2 = 2.5 μM), 5 mM $MgCl_2$ + 0.5 mM KH_2PO_4 (filled circles, $K_1 = K_2$ = 7 μM), and 5 mM $CaCl_2$ (open squares, K_1 = 2.5 μM, K_2 = 5 μM)

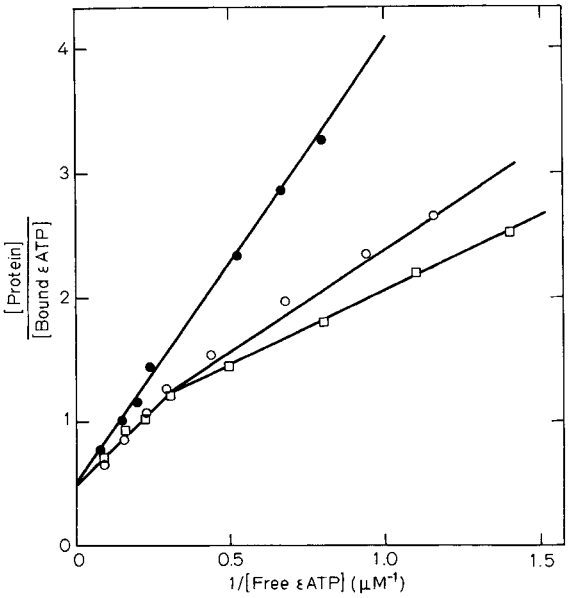

Fig. 5. *Klotz binding plot for εATP-coupling factor.* Medium containing 5.7 μM coupling factor and 25 mM tricine-NaOH (pH 8), plus the following additions affected the K_d as indicated: 5 mM $MgCl_2$ (open circles, K_1 = 2.2 μM, K_2 = 4 μM), 5 mM $MgCl_2$ + 0.5 mM KH_2PO_4 (filled circles, $K_1 = K_2$ = 7 μM), and 5 mM $CaCl_2$ (open squares, K_1 = 1.8 μM, K_2 = 4 μM)

= 5 μM, open squares). Qualitatively, the same difference was observed in different experiments. However, in view of the variance in quantitative values of K_1 and K_2 (corresponding to some variability in preparations and the limitations and experimental errors inherent in the fluorescence measurements) we do not attach much significance to the differences observed between the effects of $CaCl_2$ and $MgCl_2$.

If phosphate (0.5 mM KH_2PO_4) is present with 5 mM $MgCl_2$ (Fig. 4, filled circles), the dissociation constants (K_1, K_2) of both the magnesium-mediated binding sites are significantly increased to a value of 7 μM. Using data of Fig. 2 from our preliminary publication, see [11], we observed a similar trend: K_1 = 1 μM, and K_2 = 2 μM for 5 mM $MgCl_2$ and $K_1 = K_2$ = 6 μM for 5 mM $MgCl_2$ + 0.5 mM KH_2PO_4. Thus, the divalent cation Mg^{2+} (or Ca^{2+}) facilitates εADP binding to coupling factor at 2 sites with only small difference, whereas P_i exhibits a substantial negative interaction with both εADP sites.

Fig. 5 shows the effects of 5 mM $MgCl_2$ (open circles), 5 mM $CaCl_2$ (open squares), and 5 mM $MgCl_2$ plus P_i (0.5 mM KH_2PO_4, filled circles) on the binding of εATP to coupling factor. If $MgCl_2$ or $CaCl_2$ is present in the medium there are two binding sites on coupling factor for εATP ($K_1 \approx$ 2 μM, $K_2 \approx$ 4 μM). As in the case of εADP, phosphate substantially increases the K_d for both Mg-εATP binding sites ($K_1 = K_2$ = 7 μM). Both the divalent cations Mg^{2+} and Ca^{2+} facilitate εATP binding to coupling factor, and P_i exhibits a substantial negative interaction with both sites.

Komatsu and Murakami [41] showed that Mg ADP complex, rather than free ADP, is the active form of the substrate in photophosphorylation. The effect of Mg^{2+} observed here in the binding of εADP to coupling factor is an effect not directly involving concentration of Mg · ADP since some binding of εADP to coupling factor is observed even in the absence of added $MgCl_2$, and also 0.5 mM $MgCl_2$ is already about 100 times the concentration needed for a 1:1 complex of Mg · ADP, and yet increasing [$MgCl_2$] to 5 mM has a further effect in binding. The possible involvement of ion-induced conformational changes is considered in the following section.

Ion-Induced and Nucleotide-Induced Changes in Conformation of Coupling Factor

Since it is probable that the substrates or cofactors of phosphorylation might induce functional changes in the conformation of coupling factor [42,43], the polarization of fluorescence of the tyrosine residues in coupling factor was measured to monitor possible alterations in the physical state of the protein which could be responsible for the effects of ions on εADP binding to coupling factor. In contrast to tryptophan, the emission spectrum of tyrosine is relatively insensitive [44] to changes in its environment (*e.g.* polarity), and therefore spectral changes could not be utilized.

The addition of divalent cations to a solution of coupling factor produced a significant (about 20%) increase in polarization of tyrosine fluorescence (Fig. 6). $MgCl_2$ is somewhat more efficient than $CaCl_2$ in producing this effect in the 2.5–20 mM

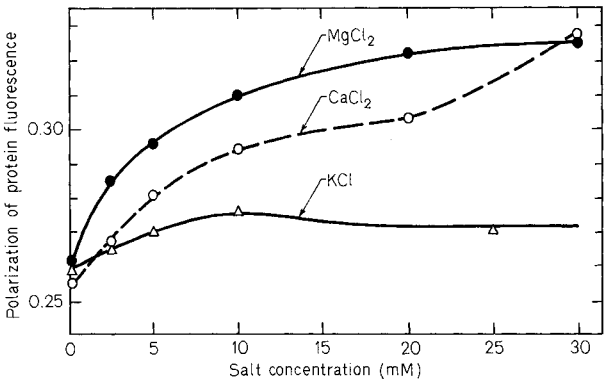

Fig. 6. *The effect of various salts on the polarization of coupling factor (tyrosine) fluorescence.* Medium contained 25 mM tricine-NaOH (pH 8.2) and 3.6 μM coupling factor. See Materials and Methods for details of measurement

range, although 30 mM $CaCl_2$ response is the same as that for 30 mM $MgCl_2$. The concentration for half-maximal effect is about 5 mM $MgCl_2$ under these conditions. With KCl, the resulting changes were much smaller (< 4%) in the same concentration range (Fig. 6); results with KH_2PO_4 were similar to the KCl data (not shown).

Addition of 2.5–20 mM $MgCl_2$ had little or no effect on both ultraviolet absorption (280 nm) by coupling factor and the intensity of protein fluorescence (not shown). This indicates that the observed salt-induced changes in polarization of coupling factor fluorescence are not due merely to a decrease in the lifetime of tyrosine fluorescence, which could also account for the observed changes. For example, a shorter lifetime would result in fluorescence being emitted before molecular motions causing a decrease in polarization. Rather, it is probable that these changes are due to an alteration in protein structure in which, for example, the mobility of one or more tyrosine residues of coupling factor is reduced.

The magnesium-induced increase in polarization of fluorescence is reversible as shown by the addition of 5.0–7.5 mM EDTA (Table 4). This indicates that the effect of the salt is not an artifact produced by some irreversible destructive effect (*cf. e.g.* effect of pH shift).

Since we have shown that $MgCl_2$ (in the millimolar range) does reversibly change the physical state of coupling factor as detected by polarization of protein (tyrosine) fluorescence (Table 4, Fig. 6), the $MgCl_2$ effect on the binding of εADP may be partially due to a change in conformation of coupling factor.

With both Mg^{2+} and Ca^{2+}, the binding of εADP and εATP to sites on coupling factor is similar with K_d values of the order of 1–3 μM (Fig. 4 and 5). Thus, the known specificity of the photophosphorylation reaction for Mg^{2+} [1] may not be due strictly to an effect on adenine nucleotide binding *per se*,

Table 4. *Effects of EDTA and $MgCl_2$ on polarization (p) of coupling factor fluorescence*
Medium contained 25 mM tricine-NaOH (pH 8) and 0.2 μM coupling factor. Additions were made sequentially in the same sample, in the order listed. Instrumental fluctuations introduce error in *p* values of about 0.003. Results are average of two experiments. See Materials and Methods for further details of measurement

Additions	*p*	Change from control
None	0.260	–
2.5 mM $MgCl_2$	0.274	0.014
5.0 mM EDTA	0.263	0.003
5.0 mM $MgCl_2$	0.276	0.016
7.5 mM EDTA	0.262	0.002

Table 5. *Effect of ADP on the polarization (p) of coupling factor fluorescence*
Medium contained 25 mM tricine-NaOH (pH 8.2) and 5.4 μM coupling factor. In each experiment (A, B, C) the indicated additions were made sequentially in the same sample; note that the number in parentheses refers to the total concentration in the sample at that point in the addition sequence. Instrumental fluctuations introduce error in *p* values of about 0.004

	Additions	*p*	Change from control
A	1. None	0.260	–
	2. $MgCl_2$ (5 mM)	0.290	0.030
	3. $MgCl_2$ (15 mM)	0.312	0.052
	4. ADP (20 μM)	0.341	0.081
B	5. $CaCl_2$ (5 mM)	0.283	0.023
	6. $CaCl_2$ (15 mM)	0.313	0.053
	7. ADP (20 μM)	0.327	0.067
C	8. KH_2PO_4 (15 mM)	0.274	0.014
	9. $MgCl_2$ (15 mM)	0.290	0.030
	10. ADP (20 μM)	0.302	0.042

but rather to cation-dependent differences in coupling factor conformation as $MgCl_2$ was more efficient than $CaCl_2$ in producing changes in (tyrosine) fluorescence of coupling factor (Fig. 6).

Table 5 shows that 20 μM ADP causes an increase in polarization of coupling factor (tyrosine) fluorescence above the saturated $MgCl_2$ (Table 5, lines 1–4) and $CaCl_2$ (lines 1, 5–7) enhanced level (about 31%) to about 34% and 33%, respectively.

If 15 mM KH_2PO_4 is present, the polarization increase produced by either 15 mM $MgCl_2$ or 15 mM $MgCl_2$ + 20 μM ADP addition is smaller (29% and 30%, respectively; see lines 8–9, Table 5). Actual enhancement in polarization of fluorescence is 0.03 (line 9) instead of 0.05 (line 3) with 15 mM $MgCl_2$, and is 0.04 (line 10) instead of 0.08 (line 4) with 15 mM $MgCl_2$ + 20 μM ADP; these differences are significant.

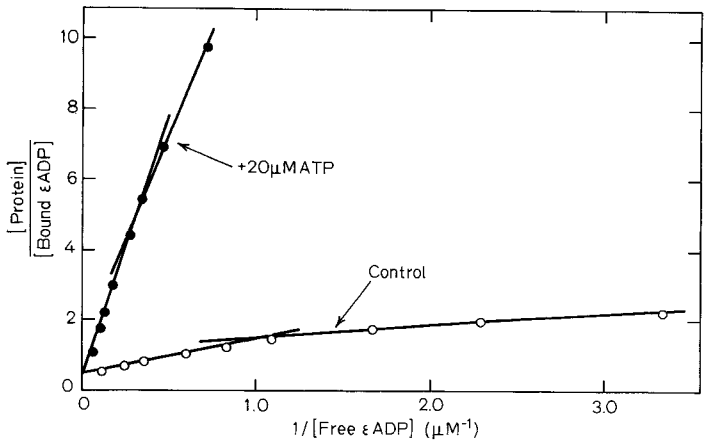

Fig. 7. *Effect of 20 μM ATP on Klotz binding plot for εADP-coupling factor.* Medium contained 8.5 μM coupling factor, 30 mM tricine-NaOH (pH 8) and 5 mM $MgCl_2$. Calculated values for K_d are: $K_1 = 0.7$ μM, $K_2 = 2$ μM (-ATP, open circles) and $K_1 = 25$ μM, $K_2 = 30$ μM (filled circles, 20 μM ATP added first and 10 min allowed before εADP additions)

Thus, ADP as well as divalent cations exert some control on the conformation of coupling factor. The negative interaction of P_i and ADP presented above in binding curves (Fig. 4) is also evident in the effects on tyrosine fluorescence polarization indicating conformation changes of coupling factor, which are probably a physical expression of the ADP + P_i interaction. This type of negative interaction in general, perhaps involving phosphate, was proposed in a model for phosphorylation by Weber [45, 46]. More specifically, interaction between ADP and P_i was proposed by Boyer [47], which, he suggests, might be due to close binding and thereby result in an effective increase in the local concentration of ADP and P_i. This is then supposed to contribute to an increase in the ATP/ADP ratio from 10^{-8} to about 1.0 catalyzing ATP formation with essentially no energy input; release of ATP, in his view, requires energy. Although our data, on the surface, is not inconsistent with such a concept, the existence of a mutual interaction between P_i and ADP does not prove that ATP can be formed as Boyer suggests. There are two problems: firstly, it is not clear that an increase by P_i of the K_d for ADP binding by only an order of magnitude would result in a dramatic increase in the ATP/ADP ratio, when the [P_i] is already in excess of ADP, which is in the same range, 2–30 μM, as the known K_m (20–50 μM in spinach chloroplasts) for ADP in phosphorylation; and secondly, P_i also increases the K_d for ATP.

As an alternative way of explaining how ATP might be formed (before release) with little or no energy input, Boyer [47] states that there is a preferential tight binding of ATP at the catalytic site. Our data does not support this concept, since the K_d values for both εADP-coupling factor and εATP-coupling factor binding sites are similar. (It may be that in chloroplasts, as in submitochondrial particles [48], energy input linked to a conformational change, in the complete system, is required to promote P_i and ADP binding to a mode competent for phosphorylation; a possibility that should be tested.)

Effect of ADP and ATP on εADP Binding to Coupling Factor

At relatively low concentration (10 μM), ATP has sizable effect on εADP binding as indicated by a decrease (about 60% at 10 μM εADP) in polarization of fluorescence (not shown). The presence of 10 μM ATP reduces the binding affinity of εADP to coupling factor by about an order of magnitude for both sites: $K_1 = 0.5$ μM, $K_2 = 1.5$ μM (-ATP); $K_1 = 2.6$ μM, $K_2 = 11$ μM (+ ATP) (not shown). Increasing the ATP concentration to 20 μM affects (Fig. 7) εADP binding further ($K_1 = 25$ μM, $K_2 = 30$ μM). Thus, ATP exhibits a strong negative interaction with both εADP sites on coupling factor.

The effect of 10 μM ADP on the polarization of εADP-coupling factor was also investigated. With a solution of coupling factor that produced a typical polarization (binding) curve for εADP, 10 μM ADP decreased the level of εADP polarization to a value close to zero (less than 10% of control) at [εADP] in the range of 2–35 μM (not shown). Thus, the negative interaction of unmodified ADP with εADP binding is apparently stronger than the interaction of ATP (at the same concentration) with εADP.

Since it is known that both ADP and ATP form complexes with Mg^{2+}, one might suggest that the effect of either or both of these nucleotides on the binding curves for εADP (or εATP, see below) is due to a reduction in the available Mg^{2+} necessary for maximal binding. However, using association constants for formation of Mg-ATP ($K_a = 1 \times 10^4$) and Mg-ADP ($K_a = 1 \times 10^{3.1}$) and the concentrations of

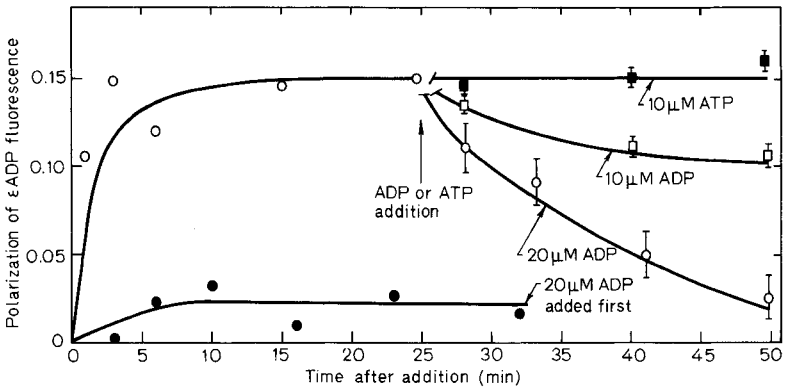

Fig. 8. *Time course of the effect on the polarization of εADP fluorescence of addition of an excess of ADP or ATP to a solution containing 2 μM εADP bound to coupling factor.* Experiments with square symbols (7.3 μM coupling factor present) were normalized at the level of maximum polarization data obtained in another set of experiments indicated by circular symbols (6.9 μM coupling factor present). ADP or ATP was added at the point indicated, at the concentrations shown in the figure. (Vertical error bars indicate the approximate uncertainty in polarization introduced by the effect (< 2%) of added ADP or ATP on the background scattering intensity subtracted in the calculation of fluorescence polarization.) In the experiment in which 20 μM ADP was added first (filled circles), a 15-min interval was allowed for equilibration before adding 2 μM εADP. Medium contained 5 mM $MgCl_2$ and 30 mM tricine-NaOH (pH 8)

ADP or ATP (10 μM) and $MgCl_2$ (5 mM) used, one can calculate that ATP and ADP would reduce the concentration of free Mg^{2+} by only about 0.5 mM and 50 μM, respectively. Decreasing [$MgCl_2$] by nearly ten times the calculated value of Mg-ATP could not account for the dramatic changes induced in εADP binding by 10 μM nucleotide (Mg-ADP would have even less effect).

In an experiment in which the order of addition of nucleotide was reversed, an excess of ADP or ATP was added to a solution of 2 μM εADP after it had achieved maximal binding to coupling factor, and the time course of the effect on εADP fluorescence polarization was followed. There was no change in polarization of εADP fluorescence even up to 30 min after the addition of ATP (Fig. 8). On the other hand, addition of 10 μM or 20 μM ADP caused a substantial decrease in polarization, proportional to the ADP concentration. After about 30 min, with a 10-fold excess of ADP (20 μM), the polarization was reduced to nearly zero (about 0.025). When 20 μM ADP was added first and allowed to equilibrate with coupling factor before adding εADP, the polarization of fluorescence of εADP-coupling factor equilibrated within 30 min to the same value (about 0.025) as when ADP was added after εADP; this trend was also observed with 10 μM ADP (not shown). Thus, in comparing the effects of unmodified ADP and ATP, these results are consistent with the degree of effect these nucleotides have on the εADP binding curves discussed above (Fig. 7), *i.e.* ADP has a stronger apparent interaction with εADP than ATP does. It was observed that ADP equilibrates directly with the εADP binding sites, but ATP interacts poorly with the εADP sites (Fig. 8).

Since the polarization of εADP fluorescence is reduced in the presence of 10 μM ATP (see above), there is an apparent contradiction with the lack of effect of 10 μM ATP on εADP fluorescence (Fig. 8) even when present in the coupling factor solution for 25 min, longer than the incubation period of Fig. 7. However, there is a difference in the order of adding ATP and εADP in the two cases. When ATP is added first, it can bind to coupling factor, presumably at a non-εADP, regulatory or allosteric site, such that the K_d for εADP binding is increased (Fig. 7); this proposed effect may be mediated by an ATP-induced change in coupling factor conformation. On the other hand, if added first, εADP can bind to coupling factor so as to prevent the ATP-dependent decrease in polarization of εADP fluorescence, possibly mediated indirectly by an ADP-induced change in the ATP regulatory site *via* a change in coupling factor conformation. Support for an ADP-induced change in coupling factor conformation was indicated by a change in the polarization of protein fluorescence (Table 5). Moreover, the suggestion of an ADP-induced effect on the ATP binding is consistent with the decrease in the K_d for binding of εATP to coupling factor which we observed under the same conditions (*i.e.* when ADP was added first; presented below).

Effect of ADP and ATP on εATP Binding Coupling Factor

The effects of ADP, the nucleotide substrate for photophosphorylation, on the binding of εATP to coupling factor were also examined in analogy to the results for εADP in the section above. At relatively low concentration (10 μM), ADP significantly affects εATP binding as indicated by a decrease (about 60% at 10 μM εATP) in polarization of fluorescence (not shown). The presence of 10 μM ADP reduces the binding affinity of εATP by about an order of

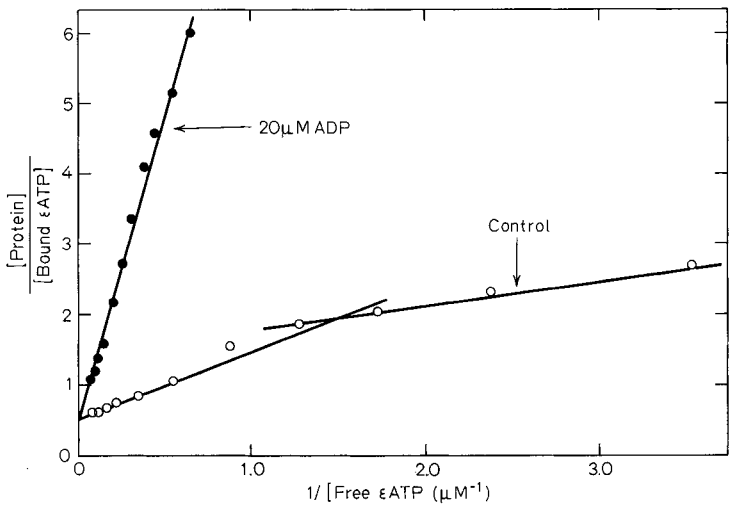

Fig. 9. *Effect of 20 μM ADP on the Klotz binding plot for εATP-coupling factor.* Medium contained 8.5 μM coupling factor, 30 mM tricine-NaOH (pH 8) and 5 mM $MgCl_2$. Calculated values for K_d are: $K_1 = 0.75$ μM, $K_2 = 2$ μM (-ADP, open circles) and $K_1 = K_2 = 18$ μM (filled circles, 20 μM ADP added first and 10 min allowed before εATP additions)

magnitude: $K_1 = 0.5$ μM, $K_2 = 4$ μM (− ADP) and $K_1 = 2.5$ μM, $K_2 = 16$ μM (+ 10 μM ADP) (not shown). By increasing the ADP concentration to 20 μM, εATP binding at the tighter site is further reduced (Fig. 9) with an apparent tendency for the dissociation constants for the two sites to become closer to each other (in terms of binding affinity), *i.e.* $K_1 = 18$ μM. Thus, ADP exhibits a strong negative interaction with both εATP sites on coupling factor.

The effect of 10 μM ATP on the polarization of εATP-coupling factor was also investigated. In a solution of coupling factor that yields a typical polarization (binding) curve for εATP, 10 μM ATP decreases the level of εATP polarization to a value close to zero (less than 5% of control) at concentrations of εATP in the range of 2−35 μM (not shown). Thus, the negative interaction of unmodified ATP with εATP binding is apparently stronger than the interaction of ADP (at the same concentration) with εATP.

In an experiment in which the order of addition of nucleotide was reversed, an excess of unmodified ADP or ATP was added to a solution of 2 μM εATP after it had achieved maximal binding to coupling factor and the time course of the effect on εATP fluorescence polarization was followed (Fig. 10). A slow small decrease in polarization of εATP fluorescence was observed when a 10 μM or 20 μM ATP was added. In this case 10 μM ADP also caused a small decline in polarization, which, within experimental error, was not very different from the effect of 10 μM ATP. In all cases, about 30 min after addition of unmodified nucleotide, there was only about a 15% decrease from the initial maximal level. When 20 μM ATP was added first and allowed to equilibrate with coupling factor before adding εATP, even after 30 min the polarization of fluorescence of εATP-coupling factor did not reach the value attained when ATP was added after εATP; this trend was also observed with 10 μM ATP (not shown).

Dowex-2 is a cross-linked polystyrene ion exchange resin which has a very high affinity for negative ions, including nucleotide anions [49]. If εATP bound to coupling factor is readily accessible to the medium, Dowex-2 would be expected to complex with the anion and prevent binding, thus causing, without delay, a decrease in polarization of εATP fluorescence. When about 1 mg of Dowex-2 was added to the εATP-coupling factor sample just described (Fig. 10) polarization decreased after a few minutes, but only to about half the initial maximal level. Based on the exchange capacity of this resin (about 3 mequiv. per g), the binding capacity of 1 mg of Dowex-2 is about 1000-fold in excess of the amount of εATP in a 2 μM solution of 2 ml volume; we expect this to assure a rapid removal of εATP bound at relatively exterior regions of the coupling factor complex. Since a rather slow incomplete removal was observed (Fig. 10), a significant portion of the εATP must have been bound (to coupling factor) rather tightly and/or at a relatively unexposed site. Thus, while ATP substitutes well for εATP and when added first suppresses the binding of εATP, some rearrangement prevents the efficient release from coupling factor of εATP already bound by ATP added second. If the εATP site(s) on coupling factor is involved in the process of phosphorylation, our data is not inconsistent with an energy-requiring step needed to effectively release ATP from the coupling enzyme during net phosphorylation; though Boyer's proposal [47] includes such a step, the present data does not of course prove that it, or any other part of this model, is valid.

The relatively slow time course for 'unbinding' or release of εATP in Fig. 10 suggests that a small part of the effect ADP has on the calculated K_d

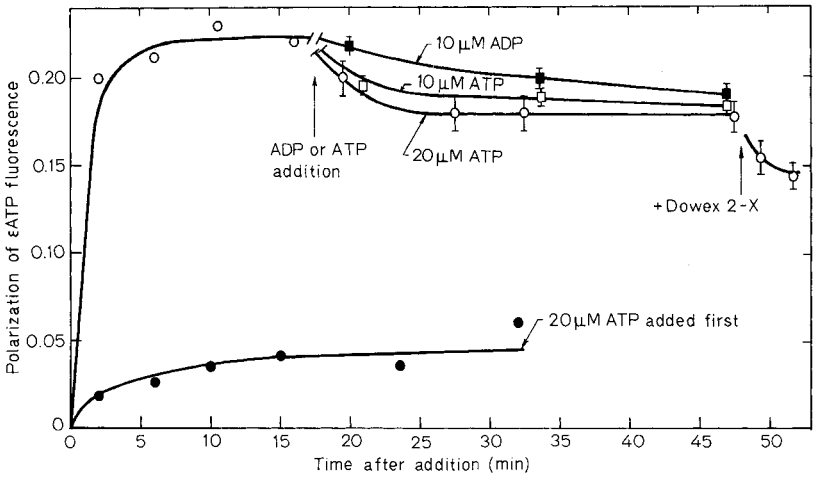

Fig. 10. *Time course of the effect on the polarization of εATP fluorescence of addition of an excess of ADP or ATP to a solution containing 2 μM εATP bound to coupling factor.* Experiments with square symbols (6.8 μM coupling factor present) were normalized, at the level of maximum polarization, to data obtained in another set of experiments indicated by circular symbols (6.9 μM coupling factor present). ADP or ATP was added at the point indicated, at the concentrations shown in the figure. (See the legend of Fig. 8 for explanation of error bars.) In the experiment in which 20 μM ATP was added first (filled circles), a 15-min interval was allowed for equilibration before adding 2 μM εATP. Medium contained 5 mM $MgCl_2$ and 30 mM tricine-NaOH (pH 8). Approximately 1 mg Dowex-2X was added to εATP-coupling factor solution as indicated in the figure; after mixing, measurement of fluorescence polarization was not made until after a 2-min interval to allow Dowex particles to settle. Before use, the Dowex-2X was washed according to the procedure outlined by Brewer *et al.* [49]

values for εATP binding sites (Fig. 9), and to a lesser extent the effect of ATP on the K_d values for εADP binding sites (Fig. 7), may be due to kinetic factors (*e.g.* equilibrium not entirely achieved during binding experiment). However, the binding of ADP and ATP to sites on coupling factor shows a sizable negative interaction even though the 'true' K_d values may be slightly different, the latter being difficult to ascertain (*e.g.* due to problems with coupling factor stability or enzymatic conversion of nucleotides with experiments involving hours rather than minutes).

The binding of εADP or εATP to coupling factor with 5 mM $MgCl_2$ was fast (Fig. 1), but with the limitations of measurements we could only say that almost maximal (80 %) binding is attained within about a minute. On the other hand, the release of εADP or εATP from coupling factor, when excess ADP or ATP is added in competition, is relatively slow (at least minutes, Fig. 8 and 10) compared to the rate at which chloroplast phosphorylation proceeds (several milliseconds after initiation by light or acid-base treatment). This suggests that coupling factor may have to be bound to the hydrophobic protein and/or thylakoid membrane for fully functional adenine nucleotide binding to be expressed.

Do ADP and ATP Bind to Coupling Factor at the Same Sites?

Since εADP and εATP bind to coupling factor with similar kinetics (Fig. 1) and K_d values (Fig. 4, 5, 7, 9), if ADP and ATP are interchangeable at the same sites, then they should exhibit similar competition for both εADP and εATP sites. However, at equivalent concentrations, ADP reduces the binding of εADP to coupling factor more strongly than ATP (see above) and similarly, ATP reduces the binding of εATP to coupling factor more strongly than ADP (section above), and also ADP binds well at the εADP site in causing the release of bound εADP from coupling factor whereas ATP does not (Fig. 8). Thus, the binding sites for ADP and ATP on coupling factor are not the same; in any case, these nucleotides do not exchange with coupling factor interchangeably. Any model for phosphorylation in chloroplasts which is contingent upon equivalent sites for ADP and ATP on coupling factor when not energized would, therefore, be questionable.

On the basis of the characteristics of the binding of ADP and ATP with coupling factor obtained in this investigation, it is premature to delineate any model for the mechanism of energy transduction in chloroplasts. The data of this paper does, however, lay the background for future studies on the interaction of adenine nucleotides with the 'latent' coupling enzyme; moreover, instructive correlations with existing data and proposed models were made at several points.

The reader is referred to a recent paper by Lee *et al.* [50] on the binding of nucleotides to purified coupling factor-latent ATPase from *Myobacterium phlei* for comparison.

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REFERENCES

1. Jagendorf, A. T. (1975) in *Bioenergetics of Photosynthesis*, (Govindjee, ed.) pp. 413–492, Academic Press, New York.
2. Secrist III, J. A., Barrio, J. R. & Leonard, N. J. (1972) *Science (Wash. D.C.)*, *175*, 646–647.
3. Secrist III, J. A., Barrio, J. R., Leonard, N. J. & Weber, G. (1972) *Biochemistry*, *11*, 3499–3506.
4. Chien, Y. & Weber, G. (1973) *Biochem. Biophys. Res. Commun.* *50*, 538–543.
5. Barrio, J. R., Secrist III, J. A., Chien, Y., Taylor, P. J., Robinson, J. L. & Leonard, N. J. (1973) *FEBS Lett. 29*, 215–218.
6. Hilborn, D. A. & Hammes, G. G. (1973) *Biochemistry*, *12*, 983–990.
7. Tondre, C. & Hammes, G. G. (1973) *Biochim. Biophys. Acta*, *314*, 245–249.
8. Girault, G. & Galmiche, J. M. (1976) *Biochem. Biophys. Res. Commun. 68*, 724–729.
9. Shahak, Y., Chipman, D. M. & Shavit, N. (1973) *FEBS Lett.* *33*, 293–296.
10. Cantley, L. C. & Hammes, G. G. (1975) *Biochemistry*, *14*, 2968–2975.
11. VanderMeulen, D. L. & Govindjee (1975) *FEBS Lett. 57*, 272–275.
12. Girault, G., Galmiche, J.-M., Michel-Villaz, M. & Thiery, J. (1973) *Eur. J. Biochem. 38*, 473–478.
13. MacKinney, G. (1941) *J. Biol. Chem. 140*, 315–322.
14. Trebst, A. (1972) *Methods Enzymol. 24B*, 146–165.
15. Dilley, R. (1972) *Methods Enzymol. 24B*, 68–74.
16. Strotmann, H., Hesse, H. & Edelman, K. (1973) *Biochim. Biophys. Acta*, *314*, 202–210.
17. Lien, S. & Racker, E. (1971) *Methods Enzymol. 24A*, 547–555.
18. Inada, Y., Yamazaki, S., Miyake, S., Hirose, S., Okada, M. & Mihama, H. (1975) *Biochem. Biophys. Res. Commun. 67*, 1275–1280.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
20. Farron, F. (1970) *Biochemistry*, *9*, 3823–3828.
21. Shoshan, V. & Shavit, N. (1973) *Eur. J. Biochem. 37*, 355–360.
22. Weber, G. (1955) *J. Opt. Soc. Am. 46*, 962–970.
23. Reference deleted.
24. Klotz, I. M., Walker, F. M. & Pivan, R. B. (1946) *J. Am. Chem. Soc. 68*, 1486–1490.
25. Laurence, D. J. R. (1952) *Biochem. J. 51*, 168–180.
26. Velick, S. F. (1958) *J. Biol. Chem. 233*, 1455–1467.
27. Avron, M., Krogmann, D. W. & Jagendorf, A. T. (1958) *Biochim. Biophys. Acta*, *30*, 144–153.
28. Telfer, A. & Evans, M. C. W. (1972) *Biochim. Biophys. Acta*, *256*, 625–637.
29. Mukohata, Y., Yagi, T., Sugiyama, Y., Matsuno, A. & Hagashida, M. (1975) *Bioenergetics*, *7*, 91–102.
30. McCarty, R. E., Fuhrman, J. S. & Tsuchiya, Y. (1971) *Proc. Natl Acad. Sci. U.S.A. 68*, 2522–2526.
31. Mukohata, Y. & Yagi, T. (1975) *Bioenergetics*, *7*, 111–120.
32. Roy, H. & Moudrianakis, E. N. (1971) *Proc. Natl Acad. Sci. U.S.A. 68*, 464–468.
33. Roy, H. & Moudrianakis, E. N. (1971) *Proc. Natl Acad. Sci. U.S.A. 68*, 2720–2724.
34. Smith, D. J., Stokes, B. O. & Boyer, P. D. (1976) *J. Biol. Chem. 251*, 4165–4171.
35. Lien, S., Berzborn, R. J. & Racker, E. (1972) *J. Biol. Chem. 247*, 3520–3524.
36. Jagendorf, A. T. & Neumann, J. (1965) *J. Biol. Chem. 240*, 3210–3220.
37. Mitchell, P. (1974) *FEBS Lett. 43*, 189–194.
38. Boyer, P. D. (1975) *FEBS Lett. 58*, 1–6.
39. Schmid, R., Shavit, N. & Junge, W. (1976) *Biochim. Biophys. Acta*, *430*, 145–153.
40. McCarty, R. E. & Racker, E. (1966) *Brookhaven Symp. Biol. 19*, 202–214.
41. Komatsu, M. & Murakami, S. (1976) *Biochim. Biophys. Acta*, *423*, 103–110.
42. Boyer, P. D., Stokes, B. O., Wolcott, R. G. & Degani, C. (1975) *Fed. Proc. 34*, 1711–1717.
43. Jagendorf, A. T. (1975) *Fed. Proc. 34*, 1718–1721.
44. Konev, S. V. (1967) *Fluorescence and Phosphorescence of Proteins and Nucleic Acids*, Plenum Press, N.Y.
45. Weber, G. (1972) *Proc. Natl Acad. Sci. U.S.A. 69*, 3000–3003.
46. Weber, G. (1974) *Ann. N.Y. Acad. Sci. 227*, 486–496.
47. Boyer, P. D. (1974) in *Dynamics of Energy Transducing Membranes* (Ernster, L., Estabrook, R. W. & Slater, E. C., eds), pp. 289–301, Elsevier, Amsterdam.
48. Kayalar, C., Rosing, J. & Boyer, P. D. (1976) *Biochem. Biophys. Res. Commun. 72*, 1153–1159.
49. Brewer, J. M., Pesce, A. J. & Ashworth, R. B. (1974) *Experimental Techniques in Biochemistry*, pp. 5–6, 75–76, Prentice-Hall, Englewood Cliffs, N.J.
50. Lee, Soon-Ho, Kalra, U. K., Ritz, C. J. & Brodie, A. F. (1977) *J. Biol. Chem. 252*, 1084–1091.

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