Biochimica et Biophysica Acta, 589 (1980) 10—20

BBA 47779

AMP PHOTOPHOSPHORYLATION AND BINDING BY CHLOROPLASTS

V. VAMBUTAS [a], W. BRONSTEIN [a] and W. BERTSCH [b]

[a] *Department of Chemistry, Queens College of the City University of New York, Flushing, NY 11367 and* [b] *The John Stauffer Photosynthesis Laboratory, Biology Department, Pomona College, Claremont, CA 91711 (U.S.A.)*

(Received July 19th, 1979)

Key words: Photophosphorylation; Coupling factor; Nucleotide binding; AMP phosphorylation; (Chloroplast)

Summary

Stoichiometric amounts of chloroplast thylakoids photophosphorylate free AMP to tightly bound ADP. Free ADP is a poor competitor for this AMP photoreaction, which saturates below 16 μM AMP. The inhibitor, diadenosine pentaphosphate, abolishes AMP photophosphorylation, and inhibits dark ADP binding. Taken together, these data imply that this photoreaction involves the high affinity nucleotide binding site(s) of chloroplast coupling factor CF_1, and that little mixing with free nucleotides occurs.

Introduction

Several observations indicate that chloroplasts can photophosphorylate AMP to ADP. Roy and Moudrianakis [1] isolated CF_1-bound [^{3}H]ADP after incubation of substrate amounts of energized thylakoids with [^{3}H]AMP and P_i. Similarly [^{32}P]ADP has been found after short illumination (5—100 ms) of substrate amounts of thylakoids with either $^{32}P_i$ [2] or with $^{32}P_i$ + ADP [3,4]. Furthermore, AMP + P_i inhibits electron flow with catalytic amounts of chloroplasts to the same extent as ADP alone [5].

The chemical pathway of AMP photophosphorylation remains unclear, and a number of questions might be asked about it. (1) Does the ADP synthesized from AMP first appear in the medium and then bind to CF_1, or does its synthesis and binding take place directly on CF_1? (2) Which nucleotide binding site(s) of CF_1 are involved? (3) Is the phosphate donor for AMP photophosphorylation free ATP, P_i itself or CF_1-bound ATP? (4) What is the functional role of AMP photophosphorylation? The present experiments partially answer the first three questions. Photophosphorylation of AMP results in thylakoid-

Abbrebiations: $(Ado)_2P_5$, diadenosine pentaphosphate Ado(5′)P_5(5′)Ado; CF_1, coupling factor I; Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

bound ADP, apparently without prior passage of the ADP through the medium. At least one of the tight nucleotide binding sites of CF_1 is involved. The phosphate donor for AMP photophosphorylation is uncertain, and present data do not differentiate between P_i and ATP as possible donors.

Methods and Materials

Spinach chloroplasts were isolated [5], washed first with grinding medium [5], then with 60—80 ml of 2 mM Tricine buffer (pH 7.8) containing 50 mM NaCl. Before both washes, chloroplasts were gently dispursed using a glass homogenizer. After the second wash, chloroplasts were resuspended to about 2 mg chlorophyll/ml for storage at 2° C in a medium containing 0.2 M sucrose, 5 mM Hepes, pH 7.6, 4 mM $MgCl_2$, 0.05% fatty acid-free bovine serum albumin. Chlorophyll content was determined [6], and photophosphorylation measured [5], as described earlier. [^{3}H]ADP binding studies were essentially performed as described by Strotmann and Bickel-Sandkotter [7]. Reaction mixtures contained hexokinase + glucose as a trap for free ATP. After 10 or 15 s illumination with white light [5], reaction mixtures in glass centrifuge tubes were quickly sedimented by centrifugation in the dark for 2 min at 15 000 × *g* and 3° C to remove unbound nucleotides from the thylakoids. The sedimented thylakoids were washed twice with 5-ml portions of cold 50 mM NaCl containing 25 mM Tricine buffer, pH 7.8. Each time the pellet was gently homogenized. The final pellet was rinsed (without suspension) with 0.5 ml of water to minimize the amounts of salts, and then suspended in water to about 0.5—0.8 mg chlorophyll/ml. To release the bound nucleotides, 0.50 ml of labeled thylakoids + 0.50 ml of 1 mM EDTA, pH 7.6, were heated in a boiling water bath for 5 min. EDTA was added to minimize adenylate kinase activity [8]. After the denatured thylakoids were sedimented by centrifugation, the supernatant containing the nucleotides was saved and the pellet was washed once with 0.5 ml containing 0.8 mM ATP, 0.8 mM ADP and 0.4 mM AMP. A 0.1 ml aliquot of the combined supernatants was counted to determine the total amount of bound nucleotides/mg chlorophyll. The remainder was lyophilized and the residues dissolved in 0.05 ml of water. Nucleotides were separated on a polyethylene imine-impregnated cellulose plate with a solvent mixture containing 2 M HCOOH and 0.5 M LiCl, eluted with 0.5 N HCl overnight at 2° C, then concentration and counts were determined as described earlier [5]. Hexokinase was freed of $(NH_4)_2SO_4$ [9]. The activity of $(NH_4)_2SO_4$-free hexokinase [10] and the protein concentration were determined spectrophotometrically [11].

The extinction coefficient of $(Ado)_2P_5$ was assumed to be $30.8 \cdot 10^3$ at 259 nm (two adenosine molecules/one $(Ado)_2P_5$ molecule); sodium salts of AMP, ADP, ATP and hexokinase (e.g. 400 U/mg) were purchased from Sigma; [^{3}H]-AMP and [^{3}H]ADP from New England Nuclear; $(Ado)_2P_5$ from PL Biochemicals; precoated plastic sheets, CEL 300 polyethylene imine-impregnated cellulose, from Brinkmann Instruments; freshly harvested spinach from local markets.

Results

Fig. 1. shows that light increases AMP binding to thylakoids. The light-induced portion of AMP binding saturates below 16 μM AMP. In contrast, the

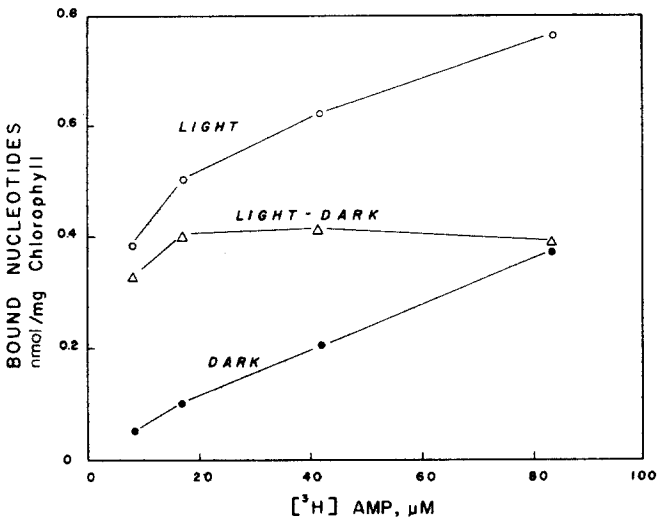

Fig. 1. Light-dependent binding of [^{3}H]AMP to thylakoids. Reaction mixtures contained: 13 mM Tricine buffer, pH 7.5, 4 mM NaCl, 1.7 mM $MgCl_2$, $7.29 \cdot 10^6$ cpm of [^{3}H]AMP at each AMP concentration, 0.8 mM P_i, 1.5 units of $(NH_4)_2SO_4$-free hexokinase, 3 mM glucose, 0.05 mM *N*-methyl phenazonium methosulfate and 0.84 mg of chlorophyll in a final volume of 1.2 ml. The reactions were illuminated at 22°C for 15 s, and immediately centrifuged in the dark to pellet the thylakoids. Thylakoid-bound nucleotides were measured as described in Methods and Materials.

dark binding of AMP saturates above 80 μM AMP. Nucleotide binding was measured in presence of 0.7 mM P_i and hexokinase plus glucose to regenerate free ADP from any free ATP which might have been generated during incubation. Table I shows that both the dark and the light-induced AMP binding are relatively independent of pH from pH 7.2 to 8, with slight inhibition at pH 8.5. This broad pH profile is similar to that for ATP binding [12] and for ADP

TABLE I

pH OPTIMUM FOR [^{3}H]AMP BINDING TO THYLAKOIDS

Reaction conditions were as described in Fig. 1. Hepes buffer was used at pH 7.2; Tricine buffer at all other pH values. [^{3}H]AMP was adjusted to $2.9 \cdot 10^5$ cpm/nmol. The final volume of 1.5 ml contained 0.75 mg of spinach chloroplasts and 1.5 units of hexokinase. The reactions were exposed to light for 10 s. Thylakoid-bound nucleotides were measured as described in Materials and Methods.

Conditions (pH)	Addition of [^{3}H]AMP (nmol/1.5 ml)	Bound nucleotides (nmol/mg chlorophyll)	
		Total	Light—dark
Light (7.2)	20	0.370	0.240
Dark (7.2)	20	0.154	
Light (7.5)	20	0.387	0.267
Dark (7.5)	20	0.120	
Light (8.0)	20	0.387	0.257
Dark (8.0)	20	0.130	
Light (8.5)	20	0.285	0.175
Dark (8.5)	20	0.110	

TABLE II

EFFECT OF HEXOKINASE ON [^{3}H]AMP AND [^{3}H]ADP BINDING TO THYLAKOIDS

Each reaction mixture contained 26 mM Tricine buffer, pH 7.8, 6 mM NaCl, 2.6 mM $MgCl_2$, 0.7 mM P_i, 0.05 mM *N*-methyl phenazonium methosulfate, 0.304 mg chlorophyll and other components as indicated in a final volume of 1.5 ml. [^{3}H]AMP was $1.58 \cdot 10^5$ cpm/nmol, and [^{3}H]ADP was $1.56 \cdot 10^5$ cpm/nmol. Illumination was 10 s. Washed thylakoids were suspended in 1 ml H_2O. An aliquot (0.2 ml) of each was supplemented with 0.6 ml H_2O, 0.1 ml of 35% $HClO_4$ and 0.1 ml of 10 mM ADP. The precipitate was removed by centrifugation and the supernatant was counted. The boiling procedure described in Materials and Methods gave almost identical results.

	Additions				Bound nucleotides (nmol/mg chlorophyll)	
	Hexokinase (units/1.5 ml)	Glucose (μmol/1.5 ml)	[^{3}H]AMP (nmol/1.5 ml)	[^{3}H]ADP (nmol/1.5 ml)	Total	Light–dark
Light			28.8		0.82	0.64
Dark			28.8		0.18	
Light	8.9	10	28.8		0.47	0.29
Dark	8.9	10	28.8		0.19	
Light				28.7	1.65	1.19
Dark				28.7	0.46	
Light	8.9	10		28.7	1.89	1.04
Dark	8.9	10		28.7	0.85	

photophosphorylation at a low ADP concentration: 10 μM [12]. Photophosphorylation at higher ADP concentrations is strongly dependent on pH over this pH range [12].

McCarty observed that hexokinase inhibited AMP binding [13]. Table II shows that hexokinase inhibits light-induced AMP binding by about 50% with-

TABLE III

EFFECT OF HEXOKINASE ON [^{3}H]AMP BINDING TO THYLAKOIDS

Each reaction mixture contained 26 mM Tricine buffer, pH 7.8, 6 mM NaCl, 2.6 mM $MgCl_2$, 0.7 mM P_i, 0.05 mM *N*-methyl phenazonium methosulfate, 19.2 μM AMP, 0.45 mg chlorophyll, and other components as indicated, in a final volume of 1.5 ml. [^{3}H]AMP was $2.5 \cdot 10^5$ cpm/nmol. Since sucrose contained small amounts of glucose, about 19 nmol glucose was also present in each 1.5 ml reaction. After a 10 s illumination, chloroplasts were washed three times and [^{3}H]AMP binding measured.

	Additions		Bound nucleotides (nmol/mg chlorophyll)	
	Glucose-free hexokinase (units/1.5 ml)	Glucose (μmol/1.5 ml)	Total	Light–dark
Light	3.2	10	0.25	0.17
Dark	3.2	10	0.076	
Light	12.9	10	0.25	0.20
Dark	12.9	10	0.048	
Light	25.8	10	0.38	0.31
Dark	25.8	10	0.066	
Light	25.8	0	0.37	0.31
Dark	25.8	0	0.061	

out affecting dark AMP binding. Hexokinase had only a slight effect on light-induced ADP binding, but increased dark ADP binding by nearly a factor of two.

Table III shows that hexokinase addition up to 25.8 units did not abolish AMP binding, and in fact enhanced it. This enhancement was not due to contaminating adenylate kinase, since no adenylate kinase activity could be detected spectrophotometrically over a 4 min interval in 8 units of hexokinase (sensitivity about 0.2 nmol NADPH/min). Hexokinase also enhanced AMP-dependent $^{32}P_i$ photoincorporation into organic phosphates, as shown in Table IV. No AMP-dependent $^{32}P_i$ uptake was observed in absence of hexokinase (Expt. 2), or in presence of 21.8 units of hexokinase without glucose (Expt. 1). Clearly these chloroplast preparations formed little or no free ADP from added AMP and $^{32}P_i$ in a 10 s illumination period. Increasing amounts of hexokinase increasingly stimulated AMP-dependent $^{32}P_i$ photoincorporation. Hexokinase also stimulated $^{32}P_i$ incorporation into organic phosphates in absence of added AMP, but to a lesser extent (Expt. 1). Stimulation by hexokinase of AMP-dependent $^{32}P_i$ photoincorporation into organic phosphates suggests that ADP (bound or free) is turning over, and that AMP is causing enrichment of the ADP pool. Large amounts of hexokinase failed to abolish the AMP contribution to this ADP pool.

TABLE IV

EFFECT OF HEXOKINASE ON AMP-DEPENDENT $^{32}P_i$ UPTAKE

Reaction conditions were as described in Table III. In Expt. 1, $^{32}P_i$ was $1.3 \cdot 10^6$ cpm/μmol, chlorophyll was 0.3 mg/ml. Thylakoids washed four times (first wash with grinding medium). In Expt. 2, $^{32}P_i$ was $6.3 \cdot 10^5$ cpm/μmol, chlorophyll was 0.4 mg/ml. Thylakoids washed twice, as in Materials and Methods. Chloroplasts for these experiments were resuspended in the washing medium, which excludes sucrose, Hepes, $MgCl_2$ and bovine serum albumin. Time of illumination was 10 s. The reactions were inactivated by addition of 0.1 ml of 35% $HClO_4$ and 0.5 μmol ADP. $^{32}P_i$ was quantitatively precipitated with 1.15 ml molybdate reagent [5]. After centrifugation, 1 μmol P_i was added to each tube. The resultant precipitate was removed by centrifugation and 0.5 ml aliquots of clear supernatant were counted in a scintillation counter.

	Additions			Bound nucleotides ($^{32}P_i$ incorporated into organic phosphates) (nmol/mg chlorophyll per 10 s)		
	Glucose-free hexokinase (units/1.5 ml)	Glucose (μmol/1.5 ml)	AMP (nmol/1.5 ml)	Light	Dark	Light—dark
Expt. 1 (four washes)	2.4	10	28.8	9.9	5.5	4.5
	7.3	10	28.8	12.4	5.4	7.0
	12.1	10	28.8	13.9	5.2	8.7
	16.9	10	28.8	15.0	5.1	9.9
	21.8	10	28.8	16.0	5.2	10.8
	21.8	0	28.8	6.3	6.4	—0.1
	21.8	10	0	11.6	5.4	6.2
	2.4	10	0	11.9	5.7	6.2
Expt. 2 (two washes)	0	10	28.8	14.9	15.7	—0.8
	2.2	10	28.8	21.5	14.8	6.7
	6.7	10	28.8	27.1	14.9	12.2
	13.2	10	28.8	28.9	14.9	14.0
	26.3	10	28.8	32.3	14.8	17.5
	26.3	0	28.8	16.0	15.7	0.3
	0	10	0	15.5	15.4	0.1

With Swiss chard chloroplasts (200 μg/ml, 2.3 units hexokinase/ml) AMP-dependent $^{32}P_i$ uptake was 2—4 times faster than with spinach chloroplasts, although $^{33}P_i$ uptake in absence of AMP was 5—10 times slower. This observation may suggest that the bound nucleotide content of Swiss chard thylakoids is small compared to spinach thylakoids. The pH optima, on the other hand, were similar for both reactions in both systems: pH 7.1—7.5.

P_i slightly increased AMP binding, and more ADP was bound than AMP. The small P_i requirement might indicate that the thylakoids were not entirely free of bound P_i.

Table V shows that at equimolar concentrations, unlabeled AMP inhibits a larger portion of light-induced [^{3}H]ADP binding than unlabeled ADP inhibits of the light-induced [^{3}H]AMP binding. In various experiments equimolar unlabeled ADP reduced [^{3}H]AMP binding from 5 to 30%, while in most cases unlabeled AMP inhibited [^{3}H]ADP binding from 40 to 75%. Unlabeled ADP appeared to have an even smaller effect on [^{3}H]AMP binding with 9 units of hexokinase than with 1.5 units. In the majority of experiments, AMP binding was about half of the ADP binding. We observed, however, on two occasions, large amounts of ADP binding (above 1 nmol/mg chlorophyll), and only 0.2—0.3 nmol/mg chlorophyll of AMP binding. In one such experiment, unlabeled AMP inhibited [^{3}H]ADP binding by only 20%. 50—70% inhibition by AMP was more typical. The light-induced ADP binding saturates at 20—30 μM [9,12,14], while light-induced AMP binding saturates below 16 μM (Fig. 1). Similarly, the apparent K_m for release of ATP-induced inhibition of electron transport is 6 μM AMP + arsenate [14].

Since AMP itself binds only loosely to CF_1, or to thylakoids [8,15], we tested the possibility that [^{3}H]AMP binds in light as the photophosphorylated derivative, [^{3}H]ADP. Table VI shows this is the case. After chromatographic separation of bound nucleotides, nearly all the [^{3}H]AMP bound due to light was recorded as [^{3}H]ADP. The inhibitor, $(Ado)_2P_5$ ($2.8 \cdot 10^{-6}$ M) caused 92%

TABLE V

EFFECT OF AMP ON [^{3}H]ADP BINDING

Reaction conditions were as described in Fig. 1, but at pH 8.0, [^{3}H]AMP was $4.17 \cdot 10^5$ cpm/nmol; [^{3}H]ADP was $4.69 \cdot 10^5$ cpm/nmol; hexokinase, 1.6 units; spinach chloroplasts, 0.75 mg and the final volume 1.5 ml. The reactions were exposed to light for 10 s. Thylakoid-bound nucleotides were measured as described in Materials and Methods.

	Additions (nmol/1.5 ml)				Bound nucleotides (nmol/mg chlorophyll)		Percentage inhibition by unlabeled ADP or AMP
	[^{3}H]AMP	ADP	[^{3}H]ADP	AMP	Total	Light—dark	
Light	0	0	25	0	0.551	0.27	
Dark	0	0	25	0	0.281		
Light	0	0	25	25	0.404	0.074	69
Dark	0	0	25	25	0.330		
Light	25	0	0	0	0.394	0.19	
Dark	25	0	0	0	0.203		
Light	25	25	0	0	0.314	0.14	26
Dark	25	25	0	0	0.175		

inhibition of AMP phosphorylation and binding in absence of added ADP and 81% if ADP was added (Table VI). Lower concentrations of $(Ado)_2P_5$ ($1.1 \cdot 10^{-6}$ M) inhibited AMP phosphorylation 64% when no ADP was present. Muscle adenylate kinase on the other hand was inhibited 94% by $3 \cdot 10^{-7}$ M $(Ado)_2P_5$ which contained 0.2 mM ADP.

In contrast to AMP, Table VII shows that $(Ado)_2P_5$ did not inhibit light-induced binding of [^{3}H]ADP, but reduced dark [^{3}H]ADP binding from 25 to 50%. The inhibition of photoinduced [^{3}H]ADP binding by unlabeled AMP was completely released by $(Ado)_2P_5$. The near absence of bound [^{3}H]ATP may have been due to either postillumination exchange between free [^{3}H]ADP and bound [^{3}H]ATP [12,16], or to ATPase action [12].

TABLE VI

INHIBITION OF [^{3}H]AMP BINDING TO THYLAKOIDS BY $(Ado)_2P_5$

Reaction conditions were as described in Fig. 1, except at pH 7.8. Also 0.50 mg of spinach chloroplasts were added to the reaction mixture prior to the addition of AMP or ADP so as to allow $(Ado)_2P_5$ to interact with the thylakoids. After 2 or 3 min the remainder of the components were added as shown: $2.78 \cdot 10^5$ cpm/nmol of [^{3}H]AMP, ADP, 3.0 units of hexokinase and *N*-methyl phenazonium methosulfate to a final volume of 1.5 ml. After 10 s exposure to light, thylakoid-bound nucleotides were measured as described in Materials and Methods.

Expt. No.	Additions (nmol/1.5 ml)			Bound nucleotides (nmol/mg chlorophyll)		Amount of [^{3}H]AMP bound as ATP, ADP or AMP (nmol/mg chlorophyll)		Light—dark
	[^{3}H]AMP	ADP	$(Ado)_2P_5$	Total	Light—dark			
1. Light	25	0	0	0.68	0.49	ATP	0.014	0.002
						ADP	0.506	0.444
						AMP	0.023	—0.006
Dark	25	0	0	0.19		ATP	0.012	
						ADP	0.062	
						AMP	0.029	
2. Light	25	0	4.25	0.17	0.04	ATP	0.007	0.002
						ADP	0.033	0.012
						AMP	0.011	—0.001
Dark	25	0	4.25	0.13		ATP	0.005	
						ADP	0.021	
						AMP	0.012	
3. Light	25	25	0	0.54	0.36	ATP	0.013	0.009
						ADP	0.391	0.333
						AMP	0.023	0.000
Dark	25	25	0	0.18		ATP	0.004	
						ADP	0.058	
						AMP	0.023	
4. Light	25	25	4.25	0.20	0.07	ATP	0.009	0.006
						ADP	0.051	0.032
						AMP	0.026	0.004
Dark	25	25	4.25	0.13		ATP	0.003	
						ADP	0.019	
						AMP	0.022	

TABLE VII

EFFECT OF $(Ado)_2P_5$ ON [^{3}H]ADP BINDING TO THYLAKOIDS

The experiments were performed as described in Table IV. The amount of chloroplasts per 1.5 ml was 0.50 mg; [^{3}H]ADP, $3.17 \cdot 10^5$ cpm/nmol and hexokinase, 3 units.

Expt. No.	Additions (nmol/1.5 ml)			Bound nucleotides (nmol/mg chlorophyll)		Amount of [^{3}H]ADP bound as ATP, ADP or AMP (nmol/mg chlorophyll)		Light—dark
	[^{3}H]ADP	AMP	$(Ado)_2P_5$	Total	Light—dark			
1. Light	25	0	0	1.06	0.66	ATP	0.019	—0.005
						ADP	0.844	0.538
						AMP	—	—
Dark	25	0	0	0.40		ATP	0.024	
						ADP	0.306	
						AMP	0.026	
2. Light	25	0	4.25	0.98	0.70	ATP	0.035	0.018
						ADP	0.983	0.757
						AMP	0.053	0.002
Dark	25	0	4.25	0.28		ATP	0.017	
						ADP	0.226	
						AMP	0.051	
3. Light	25	25	0	0.59	0.23	ATP	0.011	0.015
						ADP	0.444	0.213
						AMP	0.052	0.017
Dark	25	25	0	0.36		ATP	0.026	
						ADP	0.231	
						AMP	0.035	
4. Light	25	25	4.25	1.09	0.75	ATP	0.027	—
						ADP	1.029	0.862
						AMP	0.065	0.023
Dark	25	25	4.25	0.34		ATP	—	
						ADP	0.167	
						AMP	0.042	

Discussion

Competition between AMP and ADP for the thylakoid nucleotide binding sites indicates that the [^{3}H]ADP photoproduced from [^{3}H]AMP and P_i does not mix with free ADP in the medium (Tables V and VI). Hexokinase was included in all the reaction mixtures to exclude any free ATP. Hexokinase up to 25.8 units did not abolish AMP photophosphorylation and binding. The extremely low concentrations of AMP which saturate AMP photophosphorylation (Fig. 1), and the inhibition by ADP of [^{3}H]AMP photophosphorylation and binding (Table V), suggest that this photoreaction occurs on one or both of the two high-affinity ADP (ATP) binding sites of chloroplast coupling factor, CF_1. This suggestion is supported by the earlier observation that the AMP + P_i-induced inhibition of electron transport is abolished by the antibody to CF_1 [5]. Furthermore, $(Ado)_2P_5$ increases light-dependent binding of [^{3}H]ADP both in presence and absence of added AMP. Thus this effect of $(Ado)_2P_5$ cannot be explained in terms of isotope dilution of [^{3}H]ADP by unlabeled AMP (mediated by adenylate kinase).

Taken together, these various observations seem to eliminate soluble chloroplast adenylate kinase as a possible catalyst of this AMP reaction. In order for adenylate kinase to mediate [^{3}H]AMP photophosphorylation and binding, the enzyme would have to be directly associated with membrane-bound CF_1, mediating phosphate transfers only between nucleotides bound to CF_1. Otherwise, we must invoke an intramembrane pool of nucleotides [17] which is not accessible to hexokinase. The concept of close association between CF_1 and adenylate kinase could explain our observation that the added hexokinase did not trap the hypothetical ATP intermediate.

The simplest interpretation of our data might be that AMP can be a substrate for CF_1 in spite of the fact that AMP itself does not measurably bind to the coupling factor [15]. Nevertheless, at least one of the two tight nucleotide binding sites of the energized CF_1 may have a greater affinity for AMP phosphorylation than for ADP binding (Table V). Since thylakoids bind more ADP than AMP (Table V), perhaps only one of the energized high-affinity sites can mediate AMP phosphorylation. The role of high-affinity nucleotide binding sites in photosynthetic phosphorylation remains unclear.

One possible hypothesis is that CF_1 directly catalyses photophosphorylation of free AMP to tightly bound ADP, utilizing a different mechanism (and different nucleotide binding sites) from those which mediate ADP photophosphorylation at high ADP concentrations [18]. This interpretation is consistent with two other observations: (1) relative lack of pH dependence in AMP photophosphorylation and binding (Table I) as compared to ADP photophosphorylation at saturating ADP concentrations [12], and (2) inhibition of AMP photophosphorylation by $(Ado)_2P_5$ concentrations which stimulate ADP photophosphorylation (Table VI and unpublished results).

McCarty [13] found that large amounts of hexokinase prevented ADP and ATP accumulation in the medium from added AMP. We found that hexokinase did indeed inhibit AMP phosphorylation and binding (Table II). However, even 25.8 units hexokinase/1.5 ml reaction did not inhibit all AMP phosphorylation and binding (Table III). Furthermore, 21.8 units hexokinase did not abolish AMP enhancement of $^{32}P_i$ incorporation into organic phosphates (Table IV). The difference between McCarty's results and ours might be due to the different pH ranges at which the reactions were performed: pH 8.3 in McCarty's work as versus pH 7.5—7.8 in our experiments. The rate of AMP phosphorylation and binding diminished above pH 8 (Table I). We have noted a similar pH effect on AMP-dependent $^{32}P_i$ exchange with ATP, above pH 8. In addition, the pH optimum of AMP-dependent $^{32}P_i$ incorporation into organic phosphate is between pH 7.1 and 7.5.

It is interesting that hexokinase stimulates three reactions studied here: (1) AMP photophosphorylation and binding; (2) AMP-dependent $^{32}P_i$ photoincorporation into organic phosphates, and (3) dark ADP binding. These three observations might suggest that hexokinase, in addition to removing medium ATP, may also pull tightly bound ATP from CF_1. This would produce empty sites on CF_1, which then could bind ADP in the dark. These hypothetical empty sites might also photophosphorylate and bind AMP.

The $^{32}P_i$ exchange into bound nucleotides, described by Harris and Slater [8] and AMP-dependent photoincorporation of $^{32}P_i$ into organic phosphates

appear to be related. The pH optima of both reactions are between 7.1 and 7.5, and both reactions are enhanced by hexokinase. These two reactions are probably also related to the rapid $^{32}P_i$ photoincorporation into thylakoid-bound ADP [2—4]. Dark absorption or binding of both AMP and ADP is considerable (Fig. 1, Tables V—VII; Ref. 12). AMP does not inhibit dark ADP binding (Table VII).

The source of phosphate for AMP photophosphorylation is unknown. It is unlikely that free ATP donates phosphate for the reaction because an ATP trap does not inhibit, and because little mixing occurs with free nucleotides (Table V). The inhibition of $(Ado)_2P_5$ suggests that AMP photophosphorylation may involve transphosphorylation. Such transphosphorylation could be mediated either by CF_1 itself or by adenylate kinase specifically associated with CF_1. $(Ado)_2P_5$ was previously considered a specific inhibitor of adenylate kinase [19]. Inhibition of adenylate kinase would be an explanation for the $(Ado)_2P_5$ inhibition of AMP phosphorylation, except for observations which eliminate conventional adenylate kinase as a mediator.

Three observations indicate that $(Ado)_2P_5$ interacts with CF_1: (1) $(Ado)_2P_5$ inhibits dark binding of ADP (Table VII); (2) $(Ado)_2P_5$ stimulates photophosphorylation of ADP to ATP (unpublished observations), and (3) $(Ado)_2P_5$ enhances light-induced H^+ uptake in chloroplasts (unpublished observations). The differential inhibition by $(Ado)_2P_5$ of phosphorylation and binding of AMP, as versus ADP, could reflect an $(Ado)_2P_5$ affinity for CF_1 which is intermediate between the affinities of AMP and ADP for CF_1.

The overall rate of AMP photophosphorylation to free ADP is very slow, but has nevertheless been observed with catalytic amounts of chloroplasts [5]. This reaction, though, might be a composite of several steps, where one of them is limiting. If AMP phosphorylation yields only tightly bound ADP (as our data indicates) then the steady-state rate of this phosphorylation, after the first round, might be limited by a very slow release of the bound ADP [6]. That the intrinsic rate of AMP phosphorylation might be fast is indicated by rapid light and AMP-induced ATP-$^{32}P_i$ exchange [5], as well as by occasional observation of more rapid $^{32}P_i$ labeling of bound ADP than of ATP [2,4].

Acknowledgements

This work was supported by Grant PCM77-07259 from the National Science Foundation and by CUNY Faculty Research Award. The authors wish to thank Dr. J. Krakow for sharing his expertise on nucleotide analogues.

References

1 Roy, H. and Moudrianakis, E.N. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2720—2724
2 Boyer, P.D., Stokes, B.O., Wolcott, R.G. and Degani, C. (1975) Fed. Proc. 34, 1711
3 Harris, D.A. and Crofts, R.A. (1978) Biochim. Biophys. Acta 502, 87—102
4 Beyeler, W. and Bachofen, R. (1978) Eur. J. Biochem. 88, 61—67
5 Vambutas, V. and Bertsch, W. (1976) Biochem. Biophys. Res. Commun. 73, 686—693
6 Arnon, I.D. (1949) Plant Physiol. 24, 1—15
7 Strotmann, H. and Bickel-Sandkotter, S. (1977) Biochim. Biophys. Acta 460, 126—135
8 Harris, D.A. and Slater, E.C. (1975) Biochim. Biophys. Acta 387, 335—348
9 Vambutas, V. and Bertsch, W. (1974) Biochim. Biophys. Acta 376, 169—179

10 McCarty, R.E., Fuhrman, S.J. and Tsuchiya, Y. (1971) Proc. Nat. Acad. Sci. U.S. 68, 2522—2526
11 Warburg, O. and Chirstian, W. (1941) Biochem. Z. 310, 384
12 Magnusson, R.P. and McCarty, R.E. (1976) J. Biol. Chem. 251, 7417—7422
13 McCarty, R.E. (1978) FEBS Lett. 95, 299—302
14 Mukohata, Y. and Yagi, T. (1975) Bioenergetics 7, 111—120
15 Roy, H. and Moudrianakis, E.N. (1971) Proc. Natl. Acad. Sci. U.S. 68, 464—468
16 Shavit, N., Lien, S. and San Pietro, A. (1977) FEBS Lett. 73, 55—58
17 Pflugshaupt, C. and Bachofen, R. (1975) Bioenergetics 7, 49—60
18 Rosing, J., Smith, D.J., Kayalar, C. and Boyer, P.D. (1976) Biochem. Biophys. Res. Commun. 72, 1—8
19 Lienhard, G.E. and Secemski, I.I. (1973) J. Biol. Chem. 248, 1121—1123