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THIOPHOSPHATE ANALOGS OF ADP AND ATP AS SUBSTRATES IN PARTIAL REACTIONS OF ENERGY CONVERSION IN CHLOROPLASTS

H. STROTMANN [a], S. BICKEL-SANDKÖTTER [a], K. EDELMANN [a], F. ECKSTEIN [b], E. SCHLIMME [c], K.S. BOOS [c] and J. LÜSTORFF [d]

[a] *Botanisches Institut, Abteilung für Biochemie der Pflanzen, Tierärztliche Hochschule Hannover,* [b] *Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Göttingen,* [c] *Fachgebiet Organische Chemie im Fachbereich Naturwissenschaften, Gesamthochschule Paderborn, and* [d] *Institut für Klinische Biochemie und Physiologische Chemie, Medizinische Hochschule Hannover, Hannover (F.R.G.)*

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Summary

Thiophosphate analogs of ADP and ATP have been employed in partial reactions of photosynthetic energy conversion in chloroplasts. Substitution of oxygen by sulfur at the α-phosphate yields a pair of diastereomers (ADPαS, ATPαS, A and B forms). Two diastereomeric compounds are also obtained by replacement of oxygen by sulfur in the β-phosphate group of ATP (ATPβS, A and B form) (Eckstein, F. and Goody, R.S. (1976) Biochemistry 15, 1685—1691).

The A form of ADPαS is phosphorylated by chloroplasts with a K_m comparable to that of ADP but with a lower V. The B form of ADPαS as well as ADPβS is not a substrate in photophosphorylation and only weakly competes with ADP.

The A forms of ADPαS and ATPαS strongly compete with ADP for the tight nucleotide binding site of CF_1 in the light-induced exchange reaction, whereas the B forms display a much smaller competitive effect. The efficiencies of ADPβS and the A isomer of ATPβS are intermediate, and the B form of ATPβS is a weaker competitor.

The A forms of ATPαS and ATPβS are hydrolyzed by light-triggered ATPase, whereas the B forms are not. The efficiency of the A isomer of ATPαS is comparable to that of normal ATP, and the A form of ATPβS is cleaved at a lower rate. In trypsin-activated Ca^{2+}-dependent ATPase the A form of ATPαS is the only thiophosphate analog to be hydrolyzed.

Abbreviations: ADPαS, adenosine 5′-(*O*-1-thiodiphosphate); ATPαS, adenosine 5′-(*O*-1-thiotriphosphate); ADPβS, adenosine 5′-(*O*-2-thiodiphosphate); ATPβS, adenosine 5′-(*O*-2-thiotriphosphate); Plasmocorinth B, 3-(5-chloro-2-hydroxyphenylazo)-4,5-dihydroxy-2,7-naphthalenedisulfonic acid; Tricine, tris-(hydroxymethyl)-methylglycine; CF_1, chloroplast coupling factor.

The results indicate a stereospecific interaction of ADP and ATP at the catalytic sites as well as the tight nucleotide binding site of coupling ATPase of chloroplasts.

Introduction

Several ADP and ATP analogs have been used in order to investigate the nucleotide specificities of photophosphorylation and the partial reactions related to this process [1—5]. As a phosphate chain-modified analog, α,β-methylene ADP (AOPCP) has been recently employed [6]. It was found to be phosphorylated by chloroplasts, although at rather low rates. The corresponding nucleoside triphosphate (AOPCPOP) was not hydrolyzed by light-triggered ATPase and did not undergo γ-phosphate exchange [6].

In the present study a new class of phosphate-modified adenine nucleotide analogs, the thiophosphate derivates, have been investigated in photophosphorylation, ATPase reactions and binding to the tight nucleotide binding site of chloroplast coupling factor. In these nucleotide analogs one oxygen atom not involved in a C-O-P or P-O-P bond, is replaced by sulfur either in the α- or β-phosphate group. Such replacement can result in the formation of an additional chiral center. Since this new chiral center is linked to an optically active ribose, pairs of diastereomers of ADPαS, ATPαS and ATPβS exist. They have been arbitrarily named A and B [7]. They can be discriminated by their different reactivities in several kinase reactions [7]. The diastereomers of ADPαS can also be separated by high pressure liquid chromatography [8].

Both isomers of ADPαS and also ADPβS were found to be inactive in oxidative phosphorylation [9,10]. The A form of ADPαS, however, was a good substrate in photophosphorylation [5]. Our previous studies have been extended in the present paper. The results obtained with these nucleotide analogs allow conclusions to be drawn about the functional role of the phosphate chain in nucleotide binding to chloroplast ATP synthetase as well as the stereospecific aspects of this interaction with respect to the phosphate moiety.

Experimental

Chloroplasts were isolated from spinach leaves [11]. Photophosphorylation was carried out as described [12] with either $K_3[Fe(CN)_6]$ (1 mM) or methylviologen (0.5 mM) as electron acceptors. The samples were illuminated for 1 min with white light ($1.2 \cdot 10^6$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}). If ferricyanide was used as electron acceptor, the rate of electron transport was calculated from end point measurements in aliquots of the supernatants [12].

Thin layer chromatography of phosphorylation products was performed as described [13]. ^{32}P activity was detected by scanning with a thin layer scanner (Berthold).

Light-induced incorporation of [^{14}C]ADP by chloroplasts has been described in a previous paper [11].

The medium for measurement of light-triggered ATPase contained 25 mM Tricine buffer, pH 8.0, 5 mM $MgCl_2$, 5 mM dithiothreitol and 50 μM *N*-methyl-

phenazonium methosulfate. After addition of chloroplasts (80 μg/ml) the light was switched on for 3 min. Immediately after illumination nucleoside triphosphate was added. The final volume was 1 ml and the temperature 20°C. After 15 s, 1, 2, 5 and 10 min, 0.2-ml samples were taken and deproteinized by 3% trichloroacetic acid. P_i was determined colorimetrically in aliquots of the supernatants [15]. From the time courses of P_i release the initial rates of nucleoside triphosphate hydrolysis were computed.

For measurement of Ca^{2+}-dependent ATPase, chloroplasts were activated by trypsin treatment according to ref. 16 as modified in ref. 17. The ATPase assay was run at 36°C as described previously [17]. Time courses of P_i release were obtained as described for the light-triggered system.

The chemical synthesis of ATPαS and ATPβS as well as the enzymatic preparation of the diastereomers of ATPαS, ADPαS and ATPβS has been published in detail elsewhere [7,13].

Results

1. Photophosphorylation of thiophosphate analogs of ADP

The abilities of the two isomers of ADPαS and of ADPβS to act as phosphoryl acceptors in photophosphorylation are illustrated in Table I. Only ADPαS, A form, was found to be phosphorylated whereas the B form and ADPβS were not substrates in the process. It could be demonstrated by thin layer chromatography that ATPαS was the product when the A form of ADPαS was phosphorylated by chloroplasts (Fig. 1). A phosphoryl transfer caused by nucleoside diphosphate kinase and ATP formed from small amounts of ADP can be excluded since the chloroplast preparations used (washed 3 times in hypotonic medium) did not contain any detectable activities of this enzyme.

In Fig. 2 the photophosphorylations of ADP and ADPαS, A form, are compared as functions of substrate concentrations. The Lineweaver-Burk plots revealed that the apparent K_m values were identical (17 μM under the condi-

TABLE I

PHOTOPHOSPHORYLATION OF THE THIOPHOSPHATE ANALOGS OF ADP

The assay medium contained 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM ^{32}Pi, 1 mM $K_3Fe(CN)_6$ chloroplasts equivalent to 29.6 μg chlorophyll/ml and the indicated concentrations of nucleoside diphosphates. The total volume was 2.0 ml, the illumination time 1 min.

Nucleotide	Conc. (μM)	μmol Fecy red. / mg chl/h	μmol $^{32}P_i$ inc. / mg chl/h
—		250.4	
ADP	50	242.4	83.8
	500	484.9	227.5
ADPαS, A form	50	219.1	85.7
	500	362.2	160.3
ADPαS, B form	50	128.5	2.7
	500	153.0	12.0
ADPβS	50	127.9	0.0
	500	151.9	2.0

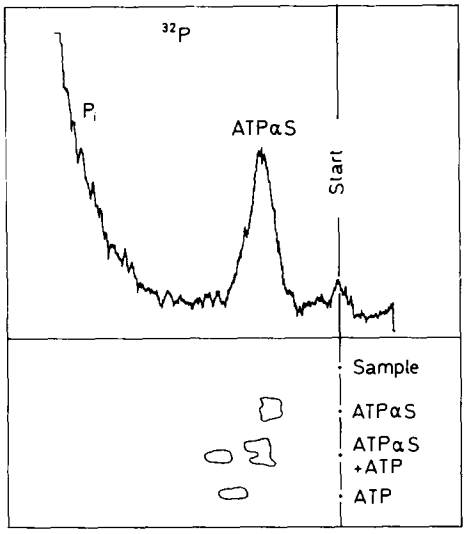

Fig. 1. Thin layer chromatography of the phosphorylation product of ADPαS, A form. Lower part: ultraviolet spots of authentic compounds, upper part: ^{32}P scanning diagram of the sample. The sample was taken from the experiment shown in Table I (ADPαS, A form, concentration 500 μM).

tions employed). However, with the thiophosphate analog a lower V was obtained. In order to investigate whether the two inactive analogs were able to bind to the catalytic ADP site, the effects of ADPαS, B form, and ADPβS on ADP phosphorylation were measured. As demonstrated in Table II, both analogs only slightly inhibited photophosphorylation of ADP, even at 2.5 fold excess. ADPβS showed a competitive inhibition with a K_i of 180 μM.

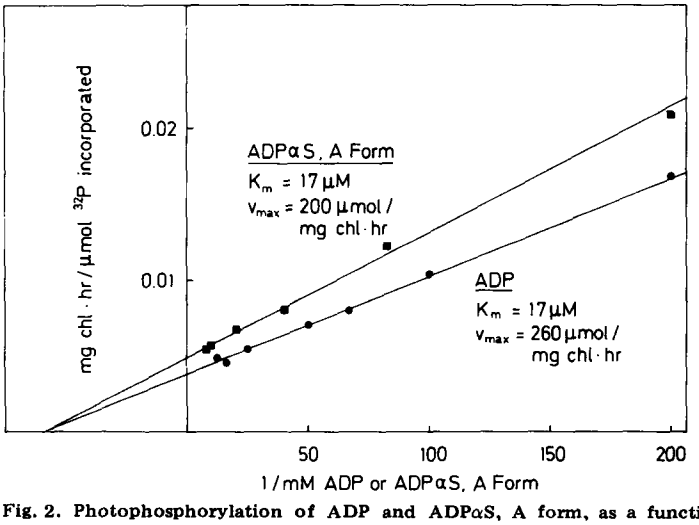

Fig. 2. Photophosphorylation of ADP and ADPαS, A form, as a function of substrate concentration (Lineweaver-Burk plot). The incubation conditions were the same as in Table I, except that 0.5 mM methylviologen instead of ferricyanide was present and a hexokinase trap was employed (10 mM glucose and 13 units/ml salt-free hexokinase from Sigma). The total volume was 0.5 ml, the chlorophyll content 25.0 μg/ml, the illumination time 1 min.

TABLE II

EFFECTS OF ADPαS, B FORM, AND OF ADPβS ON PHOTOPHOSPHORYLATION OF ADP

The experimental conditions were the same as described in Fig. 2, except that 20 μM ADP and the indicated concentrations of the thiophosphate analogs were present in the incubation medium.

Nucleotide analog	Conc. (μM)	μmol $^{32}P_i$ incorp. / mg chl/h
—		106.9
ADPαS, B form	2.5	103.6
	5	101.9
	10	108.3
	25	105.6
	50	93.0
ADPβS	2.5	108.3
	5	107.3
	10	110.0
	25	95.3
	50	91.1

2. Interaction of thiophosphate analogs of ADP and ATP with the tight nucleotide binding site of CF_1

Chloroplast coupling factor (CF_1) is known to contain tightly bound ADP which is exchanged for free ADP or ATP only on energization of the thylakoid membranes [11,18—20]. Exchange can be followed by the light-induced

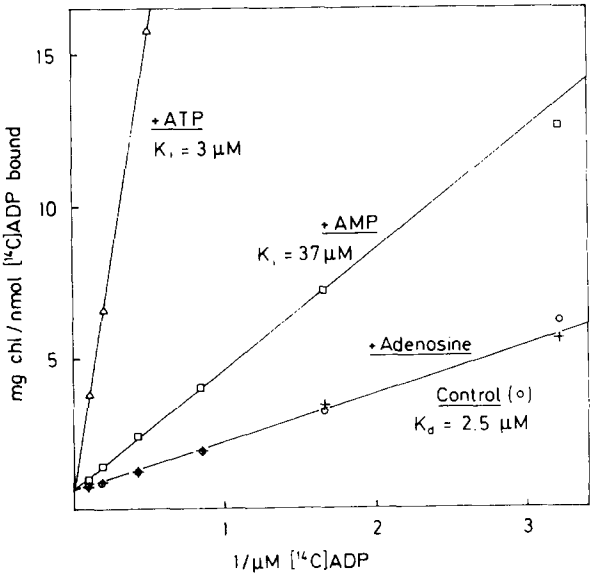

Fig. 3. Effects of ATP, AMP and adenosine on light-induced incorporation of [^{14}C]ADP into thylakoid membranes. The unlabeled compounds were added at a concentration of 50 μM. [^{14}C]ADP was varied between 0.25 and 10 μM. The medium contained 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$ and 0.5 mM methylviologen. The chlorophyll concentration was 0.634 mg/ml, the illumination time 1 min.

TABLE III

THIOPHOSPHATE ANALOGS OF ADP AND ATP AS COMPETITIVE INHIBITORS OF LIGHT-INDUCED INCORPORATION OF [^{14}C]ADP INTO THYLAKOID MEMBRANES

Apparent K_i values were determined as described in Fig. 3.

Nucleotide	Apparent K_i (μM)
ADPαS, A form	2
ADPαS, B form	17
ADPβS	8
ATPαS, A form	2
ATPαS, B form	12
ATPβS, A form	7
ATPβS, B form	12

incorporation of [^{14}C]ADP [11]. Apparent dissociation constants (K_d) for ADP of between 2 and 3 μM have been reported from our laboratory [5,11]. In accordance with the results obtained by Magnusson and McCarty [20], similar apparent K_d values were found with ATP. In a competition experiment it could be demonstrated that both ADP and ATP interact with the same binding site (Fig. 3). A much weaker competition was found with AMP and adenosine had no effect on [^{14}C]ADP binding. It should be emphasized that the calculated apparent K_i values as well as the apparent K_d for [^{14}C]ADP binding are not actual equilibrium constants but depend on the chlorophyll concentration in the assay. Nevertheless they give a relative measure for the affinity of a nucleotide to the tight binding site of CF_1.

Table III summarizes the relative affinities of the thiophosphate analogs to this particular site as calculated from their competitive effect on light-induced [^{14}C]ADP binding. The A forms of ADPαS as well as ATPαS showed affinities comparable with those of the parental compounds ADP and ATP respectively. On the other hand, the B forms exhibited much higher apparent K_i values. The affinity of ADPβS to this binding site was found to be intermediate and corresponded to that of the A fom of ATPβS. The B form of ATPβS had a

TABLE IV

THIOPHOSPHATE ANALOGS OF ATP AS SUBSTRATES IN LIGHT-TRIGGERED AND IN TRYPSIN-ACTIVATED Ca^{2+}-DEPENDENT ATPase

The nucleoside triphosphates were present at a concentration of 1 mM. The given values indicate initial rates. In both ATPase system the ATP concentration is below saturation.

Nuclotide	μmol P_i released · mg^{-1} chl · h^{-1}	
	Light-triggered ATPase	Ca^{2+}-ATPase
ATP	84.4	20.0
ATPαS, A form	82.4	29.8
ATPαS, B form	2.4	3.3
ATPβS, A form	38.6	0.8
ATPβS, B form	2.0	1.0

lower affinity than the A isomer and inhibited [^{14}C]ADP incorporation with an apparent K_i comparable to that of ATPαS, B form.

3. Hydrolysis of thiophosphate analogs of ATP by chloroplasts

The efficiencies of the diastereomers of ATPαS and ATPβS in light-triggered ATPase [21—23] are shown in Table IV. The A form of ATPαS was found to be a substrate in this process, whereas virtually no hydrolysis was observed in the case of the B isomer. The A form of ATPβS was also cleaved, but the rate was only 50% of the control. The B isomer was inactive.

A Ca^{2+}-dependent ATPase is induced by controlled trypsin digestion or heat treatment of chloroplasts or isolated CF_1 [16,24]. With trypsin-induced Ca^{2+}-ATPase a different specificity for the thiophosphate analogs of ATP was obtained. The A isomer of ATPαS was an even better substrate than ATP, whereas the other three analogs showed virtually no hydrolysis (Table IV).

Discussion

The absolute configurations of the diastereomers of ADPαS, ATPαS and ATPβS have as yet not been determined and it is therefore not known which of the isomers exhibits the *S*- and which the *R*-configuration. Metal complexes of the diastereomeric pairs have different geometries as outlined previously [7]. Thus, assuming that the metal ion only complexes with the oxygen of the phosphate chain and not with sulfur, and it also interacts with the adenine ring one can construct models of the diastereomeric pairs. They show that in the *S*-configuration of ADPαS the β-phosphate is adjacent to C-3′ of the ribose whereas in the *R*-configuration this position is taken by α-phosphate [7]. It has been suggested that similar structures are formed when the nucleotide analog interacts with charged groups of a protein, the role of the metal ion being fulfilled by cationic groups at the binding site [7].

The results described here on the activity of thiophosphate analogs of ADP and ATP in photophosphorylation and related processes show a dependence on the position of the sulfur (in α- or β-phosphate) as well as on the configuration of the diastereomers.

Since only the A isomer of ADPαS is a substrate in photophosphorylation and the B isomer is neither a substrate nor a competitive inhibitor in this process one has to conclude that the structure of the B isomer or its Mg^{2+} complex [25] is such that it can not be accomodated in the active site. The same conclusions are to be drawn for the diastereomers of ATPαS in the ATPase systems.

Binding of nucleotides to the tight site of CF_1 depends on the length of the phosphate chain of the nucleotide. Adenosine does not interact and AMP displays a much weaker affinity than ADP and ATP. Since Mg^{2+} is not required in this process [19,26] a direct interaction between the negatively charged phosphate chain and positive groups of CF_1 is likely. Both isomers of ADPαS and ATPαS bind to this particular site, but with different affinities, the A isomer being preferred.

It is reasonable to assume that substrate analogs in a given enzymatic reaction have to adopt a structure similar to that of the natural substrate at the

binding site. ADP and ATP as well as their Mg^{2+} complexes can assume conformations which correspond to the structures of both the A and B forms of ADPαS and ATPαS, respectively. Since only the A isomers of the thiophosphate analogs are active in photophosphorylation as well as in ATP hydrolysis, and are preferred in binding to the tight site of CF_1, the natural substrates ADP or ATP must be present in a conformation which is equivalent to the structure of only the A isomer of ADPαS and ATPαS at these sites.

Oxygen substitution by sulfur on α-phosphate yields adenine nucleotide analogs which are as active as the parent compounds in all the reactions investigated, provided that they exist in the A form. In contrast, oxygen substitution by sulfur on β-phosphate results in compounds which exhibit reduced affinities to the binding sites, independent of the configuration. This is particularly evident in phosphorylation where ADPβS is not a substrate. This is due to its weak ability to interact with the catalytic ADP binding site, as demonstrated by the poor competitive effect of ADPβS on ADP phosphorylation. If Mg^{2+}-ADP were the substrate in photophosphorylation, the low affinity of ADPβS might reflect an instability of its Mg^{2+} complex. However, this possibility can be excluded since similar complex constants were obtained for ADP (log K = 3.9) and ADPβS (log K = 3.7) at pH 8.0 using a dye competition method with Plasmocorinth B. Probably the catalytic ADP binding site of CF_1 exhibits a more stringent requirement for the unmodified β-phosphate group.

The particular significance of the β-phosphate in substrate binding can also be demonstrated in the trypsin-activated Ca^{2+}-ATPase neither of the two diastereomers of ATPβS being cleaved in this reaction. In the Mg^{2+}-dependent light-triggered ATPase the A form of ATPβS was hydrolyzed, but with a lower rate as compared to the natural substrate. Assuming that the ATP binding sites in both ATPase systems are the same, a considerable alteration of its conformation by the trypsin treatment has to be concluded from this result.

β-Phosphate specificity is much less pronounced in ADP and ATP binding by the tight site of CF_1, as demonstrated by the relatively high affinity of ADPβS as well as both diastereomers of ATPβS. These results suggest that the tight site is not identical with the catalytic ADP or ATP binding site in the process of photophosphorylation. The same conclusion has been drawn from kinetic experiments [11,27] and comparative studies of base-modified adenine nucleotide analogs [5].

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