PHOTOPHOSPHORYLATION OF BASE-MODIFIED NUCLEOTIDE ANALOGS BY SPINACH CHLOROPLASTS

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Received 15 July 1979

1. Introduction

Nucleotide analogs have proved to be of exceptional utility in the investigation of the interaction of nucleotides with energy transducing systems [1–5]. Here, our interest has been directed toward the action of a series of modified nucleoside 5′-diphosphates in photophosphorylation.

The ADP molecule exhibits two recognition centers with respect to its interaction with the catalytic site of the chloroplast ATP synthetase (CF_1): in the diphosphate chain; and in the base moiety [4,5]. The reaction of the diphosphate residue is highly specific from the stereochemical point of view as was shown by the chiral thiophosphate analogs of ADP [5]. On the other hand, the specificity for the base is less stringent. It is well known that other purine nucleotides, e.g., GDP, IDP [6] or the fluorescent ADP analog, 1,N^6-etheno ADP [7], can replace ADP in photophosphorylation. Nevertheless, the structure and properties of the heterocycle largely influence the affinity of a nucleotide to the catalytic site of the ATP synthetase and, furthermore, affect the catalytic process itself.

In order to obtain a more complete mapping of the nucleotide binding site, the ability of the chloroplast ATPase to phosphorylate a series of base-modified nucleoside diphosphate analogs was determined. The results suggest that the C6–N1–C2 region of the purine ring is particularly important in the binding of the base to the ATPase. The formation of hydrogen bonds between the heterocycle and the enzyme seems to establish a conformation of the catalytic center that facilitates the phosphoryl transfer to the β-phosphate group of the nucleoside diphosphate.

2. Experimental

Chloroplasts from spinach leaves were prepared as in [8] and photophosphorylation measured as in [9]. The reaction medium contained in 0.5 ml: 12.5 μmol tricine buffer (pH 8.0), 25 μmol NaCl, 2.5 μmol $MgCl_2$, 2.5 μmol ^{32}P-labeled orthophosphate, 0.25 μmol methylviologen, 5 μmol D-glucose, 14 units hexokinase (Sigma-Chemie, München, sulfate-free) and the nucleoside diphosphates at varying concentrations. The chlorophyll content was ~12.5 μg. The hexokinase trap was sufficient to recycle the nucleoside diphosphates even at low turnover rates with the generated nucleoside triphosphates. At 10 μM, the triphosphates of the analogs exhibited activities with hexokinase 2–12% of that obtained with ATP, except the N^6-methylated derivatives whose activities were ~80% and triazolo ITP with only 0.2%.

Samples were usually illuminated for 1 min with white light (1.2×10^6 ergs .cm^{-2}.s^{-1}) although in some experiments red light (Filter RG 630, Schott, 5.8×10^5 ergs .cm^{-2}.s^{-1}) was used. K_m values were calculated from Lineweaver-Burk plots. If an analog was not a substrate for phosphorylation, the competitive effect of the analog on ADP phosphorylation was determined. In those experiments, the ADP concentration was varied at two fixed concentrations of the analog and compared with the control curve. A

phosphoryl transfer caused by nucleoside diphosphate kinase can be excluded since the chloroplast preparations used did not contain any detectable activities of this enzyme [5]. Chemical syntheses of the nucleoside 5′-diphosphates (table 1) were performed as follows: 1-amino-IDP and 1-amino-GDP were prepared in the μM range from IDP and GDP, respectively, by reaction with hydroxylamine-*O*-sulfonic acid in 1 M NaOH at room temperature for 48 h as described for the appropriate nucleosides [10]. The N1-aminated nucleoside diphosphates were separated from the reaction mixture by DEAE-cellulose column chromatography (3 × 50 cm) using a linear triethylammonium hydrogen carbonate gradient (0–0.4 M, 3 litre). 1-Oxido-ADP was prepared from ADP by *N*-oxidation with monoperphthalic acid [11]. Isolation of the product from the reaction mixture was achieved by DEAE-cellulose chromatography (1 × 12 cm) using a linear NaCl gradient (0–0.3 M, 1 litre) followed by desalting over a Sephadex G-10 column (2 × 15 cm). 8-Bromo ADP was generated from ADP by bromination with Br_2 as in [12,13]. Triazolo-inosine was synthesized from guanosine via N1-amination followed by cyclisation in formamide at 180°C for 45 min [14]. 2-Amino-adenosine and 2-amino-nebularine were prepared as in [15]. N^6-Methyl- and N^6-dimethyl-adenosine were purchased from Sigma-Chemie, München. All the mentioned nucleosides were phosphorylated chemically to the appropriate 5′-monophosphates using phosphorous oxychloride ($POCl_3$) in either acetonitrile [16] or trimethylphosphate [17], respectively. In the case of triazolo-inosine, 2-amino-adenosine and 2-amino-nebularine, the 5′-diphosphates were generated in a one-step reaction using $POCl_3$ in trimethylphosphate with an average yield of 10%. The N^6-methylated compounds were prepared according to [16] followed by phosphorylating the imidazolides of the obtained monophosphates with pyrophosphate to the appropriate 5′-triphosphates as in [18]. For the preparation of the corresponding nucleoside diphosphates, N^6-methyl- as well as N^6-dimethyl-ATP were treated with yeast hexokinase (EC 2.7.1.1) as in [3]. The nucleoside diphosphates were separated from the reaction medium by DEAE-cellulose column chromatography exactly as above. The phosphorylation at the 5′-position was controlled by periodate oxidation of the 2′,3′*cis*-diol group in each of the

Table 1
Some analytical data of the employed synthesized nucleoside 5′-diphosphates

NuDP[a]		λ_{max} (nm)	ϵ ($cm^2 \cdot \mu mol^{-1}$)		R_F-values	
					I[c]	II[d]
N^6-Methyl-ADP	2	266	15.9	(aq.pH 7)	0.60	0.72
N^6-Dimethyl-ADP	3	275	18.3	(aq.pH 7)	0.61	0.81
2-Amino-ADP	4	280 255	10.0 9.0	(aq.pH 6)	0.55	0.36
1-Oxido-ADP	6	259 232	9.3 40.6	(aq.pH 7)	0.61	0.43
8-Bromo-ADP	7	265	15.1	(aq.pH 7)	0.40	0.66
2-Amino-NeDP[b]	8	305	6.3	(aq.pH 7)	0.53	0.62
1-Amino-IDP	10	251	9.9	(aq.pH 11)	0.67	0.47
1-Amino-GDP	12	254	13.6	(aq.pH 11)	0.54	0.42
Triazolo-IDP	13	285	14.3	(aq.pH 11)	0.32	0.33

[a] NuDP, nucleoside 5′-diphosphate
[b] NeDP, nebularine 5′-diphosphate
[c] System I: polygram CEL 300 PEI/UV thin-layer plates (Macherey and Nagel, Düren) with 0.75 M KH_2PO_4 (pH 4.1) as mobile phase. Reference standard: ADP $R_F = 0.51$
[d] System II: cellulose F 1440 LS 254 thin-layer plates (Schleicher and Schüll, Dassel) with ethanol/1 M ammonium acetate (7:3, v/v) as mobile phase. Thin-layer chromatography was carried out with the above nucleotides after digestion with alkaline phosphatase. Reference standard: adenosine $R_F = 0.57$

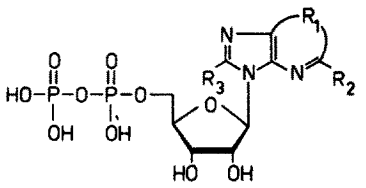
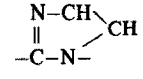


Table 2
Photophosphorylation of base modified nucleoside 5′-diphosphates

NuDP		R_1	R_2	R_3	K_m (μM)	(s^a) (μM)	V_{max} (%)
ADP	1		–H	–H	14	(4)	100
N^6-Methyl-ADP	2		–H	–H	51	(1)	100
N^6Dimethyl-ADP	3		–H	–H	66	(10)	60
2-Amino-ADP	4		$-NH_2$	–H	80	(17)	100
1,N^6-Etheno-ADP	5		–H	–H	54	(6)	50
1-Oxido-ADP	6		–H	–H	70[b]	(5)	90
8-Bromo-ADP	7		–H	–Br	> 775[c]	(80)	< 30
2-Amino-NeDP	8	–CH=N–	$-NH_2$	–H	170	(40)	90
IDP	9		–H	–H	150	(30)	100
1-Amino-IDP	10		–H	–H	210[d]	(40)	< 20
GDP	11		$-NH_2$	–H	115	(5)	100
1-Amino-GDP	12		$-NH_2$	–H	135	(35)	100
Triazolo-IDP	13		–NH–CH=N–	–H	not phosphorylated[e]		

[a] standard deviation;
[b] illumination with red light;
[c] a very poor substrate only, not a competitive inhibitor of ADP phosphorylation;
[d] illumination with red as well as white light leads to identical results, not a competitive inhibitor of ADP phosphorylation;
[e] competitive inhibitor with K_i = 127 (s = 42) μM calculated from the double-reciprocal plots according to: $K_i = K_m \cdot [I]/(K_m' - K_m)$

described nucleotides. The diphosphate moiety was identified by ^{31}P NMR spectroscopy (Bruker WP 60 DS, 60 MHz) and showed two groups of signals. The resonances centered at $\delta = -6$ (d, $J = 20$ Hz) for P2 and -11 (d, $J = 20$ Hz) ppm for P1 upfield to 85% H_3PO_4 as the external standard. 1,N^6-etheno-ADP was a generous gift from Professor N. Shavit, Beersheva. All other nucleoside diphosphates and enzymes employed in this study were commercially available from Boehringer, Mannheim.

3. Results and discussion

The app. K_m and rel. V_{max} values of the nucleoside diphosphate analogs in photophosphorylation are shown in table 2. All kinetic parameters were determined under comparable experimental conditions using methylviologen as the electron acceptor in non-cyclic electron transport. The V_{max} for the individual compounds was always related to the V_{max} for ADP which was measured as a control in every experiment. The K_m values for the single compounds are quite reproducible, but they strongly depend on the efficiency of electron transport. The Briggs-Haldane formulation of the phosphorylation process with respect to the substrate ADP ($\simeq$ S):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

reveals that K_m is equal to $(k_{-1} + k_2)/k_1$. It is reasonable to assume that k_2 is affected by the amount of energy input (i.e., the magnitude of the protonmotive force) generated by photosynthetic electron transport. Thus, when the rate of electron transport is accelerated, k_2 increases and, therefore, an increase in K_m would be expected. This is in fact observed when the rate of electron transport is increased by using a phenazine methosulfate (PMS)-catalyzed cyclic system instead of a non-cyclic system. The K_m values for some selected nucleotides using PMS as the cofactor for cyclic electron transport are presented in table 3. Compared to the K_m values measured in the methylviologen system, the K_m values for all the compounds increase in the PMS system. However, the ratio of the K_m values of the analogs to the K_m for ADP agree well in both systems. Therefore, the app. K_m values measured under identical conditions can be taken as approximate relative measures for the affinities of the substrate analogs, although they are different from the K_d of the substrate–enzyme complex.

Table 3
Apparent K_m values for some nucleoside diphosphates obtained in a PMS-catalyzed cyclic system

NuDP	K_m	K_m (NuDP)/K_m (ADP)	
		PMS system[a]	Methylviologen system[b]
ADP	40	1	1
1,N^6-Etheno-ADP	170	4	4
2-Amino-ADP	256	6	6
2-Amino-NeDP	416	10	12
GDP	400	10	8

[a] Instead of methylviologen 50 μM PMS was present in the incubation medium
[b] Values calculated from the data given in table 2

The nucleotide analogs used in this study can be divided into two classes, adenine-type nucleotides (compounds 1–7 in table 2) and nucleotides derived from the hypoxanthine base (compounds 9–13).

A rough comparison of the results indicates that adenine-type nucleoside diphosphates generally exhibit a lower K_m (from 14 μM in the case of ADP itself to ~80 μM) than the lactam type nucleotides derived from IDP (115–210 μM). The only exception is 8-bromo-ADP (7) which is a very poor substrate for phosphorylation. 8-Bromo-ADP exists in a sterically preferred *syn* conformation [19]. Binding of 8-bromo-ADP might occur in *syn* or *anti*. However, in both cases a *gauche,gauche* orientation of the C4′–C5′ exocyclic group is hindered thus leading to a distortion of the phosphate chain conformation. Its low affinity, therefore, suggests that recognition and binding of ADP to the catalytic center requires an *anti gauche,gauche*-structured nucleotide. Similar results were obtained with 8-azido-ADP [20] which probably exhibits the same conformation as 8-bromo-ADP.

The specificity of nucleotide binding for the adenine moiety is probably due to the amidino region which includes N1,C6 and the exocyclic C6-amino group. Alteration of the basal structure of this region as in the lactam type bases and in 2-amino-NeDP

(compounds 8–13) reduces the affinity by one order of magnitude. When the basal structure is maintained but modified by methylation or cyclisation (compounds 2,3,5), the effect on the binding properties is less pronounced. In adenine-type and lactam-type bases, the structure as well as the charge distribution in that region of the molecule is fundamentally different. Thus, from the chemistry of the base it is unlikely that the same portion of the protein is able to contact both regions of base. The geometry of the binding site is probably so that both types of nucleotides can be accomodated but in a different manner. In the above reaction scheme, V_{max} is given by $E_t.k_2$. k_2 is, of course, a complex constant which includes all the reaction constants involved in the formation of ATP on the enzyme as well as ATP release. V_{max} is, therefore, a measure of the sum of all consecutive reaction steps when the enzyme is saturated with the substrates.

With the adenine-type nucleotides 2,4, and the lactam-type nucleotides 9, 11, 12, the same V_{max} is obtained as with the natural substrate, ADP, but with the other derivatives, a lower V_{max} is measured. In adenine-type derivatives, the structure of the exocyclic C6-substituent appears to be responsible for the high V_{max}. If the exocyclic 6-amino N contains at least one hydrogen atom, phosphorylation proceeds with the unrestricted maximum velocity. However, if both hydrogens of the amino group are replaced by methyl groups or disappear by cyclisation, the corresponding derivatives 3 and 5 are phosphorylated with only ~50% of the maximum rate.

These results suggest that the phosphorylation process is accelerated by the formation of a hydrogen bond between the enzyme and the substrate via the C6 amino group. It is quite possible that such an interaction leads to a conformational change of the catalytic center which promotes the phosphoryl transfer to β-phosphate of the nucleotide even when the site of reaction is not in close vicinity to the base binding site.

In lactam-type nucleotides a similar hydrogen bridging can take place either via the N1 hydrogen or, if present, the amino substituent at C2. If the latter group is not present and N1 is blocked (compound 10), V_{max} is extremely reduced. In the presence of a C2 amino group, a fully active derivative (12) is obtained; however, if both groups are blocked by cyclisation (13), this compound is virtually not phosphorylated although recognized and bound by the catalytic binding site ($K_i \sim 127\ \mu M$).

In summary, the results suggest that the base moiety of the nucleoside diphosphate exhibits an important role in substrate binding as well as in triggering the phosphorylation process. The interaction of adenine- and lactam-type nucleotides is probably different and determined by the structure and charge distribution of the C6–N1–C2 region of the heterocycle, including the exocyclic groups. In addition, this region probably forms hydrogen bonds with the enzyme counterpart. Hydrogen bonding appears not to be essential for the stability of the substrate–enzyme complex but largely affects the velocity of the catalytic process.

Acknowledgements

Professor K. H. Scheit, MPI für Biophysikalische Chemie, Göttingen, is gratefully acknowledged for kindly supplying us with 2-amino-adenosine and 2-amino-nebularine. This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds des Verbandes der Chemischen Industrie.

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