PROPERTIES OF RIBOSE MODIFIED ADP ANALOGUES IN PHOTOPHOSPHORYLATION OF SPINACH CHLOROPLASTS

Karl-Siegfried BOOS, Joachim LÜSTORFF and Eckhard SCHLIMME

Institut für Klinische Biochemie und Physiologische Chemie, der Medizinischen Hochschule Hannover, D-3000 Hannover, and *Laboratorium für Biologische Chemie der Universität – Gesamthochschule, D-4790 Paderborn GFR*

and

Harald HESSE and Heinrich STROTMANN

Botanisches Institut der Tierärztlichen Hochschule Abteilung für Biochemie der Pflanzen, D-3000 Hannover GFR

Received 20 September 1976

1. Introduction

Our interest is directed to investigations into structural requirements of membrane integrated enzyme systems for adenine nucleotides as substrates [1–3]. In this publication we report on the binding and photophosphorylation properties of ribose modified ADP analogues. The experiments were carried out using the ribose ring opened ADP, rroADP (fig.1), 2′-dADP and 3′-dADP (fig.2).

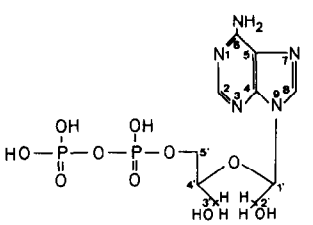

Fig.1. Formula of rroADP (heterocycle drawn in syn-position).

Abbreviations: NTP, nucleoside triphosphate; rroAT(D)P, 2,2′[1-(9-adenyl)-1′-(tri-)-diphosphoryl-oxymethyl]-dihydroxydiethylether; 2′-dAT(D)P, 9-(β-D-2′-deoxyribofuranosyl)-adenine di-, triphosphate; 3′-dAT(D)P, 9-(β-D-3′-deoxyribofuranosyl)-adenine di-, triphosphate; AOPCP, α,β-methylene adenosine 5′-diphosphate; AOPOPCP, β,γ-methylene adenosine 5′-triphosphate; R_t, retention time

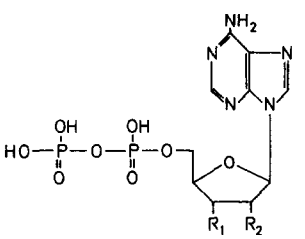

Fig.2. Formula of 2′-dADP, R_1 = OH, R_2 = H; and 3′-dADP, R_1 = H, R_2 = OH; (heterocycle drawn in syn-position).

Though rroADP is specifically bound to the photosynthetic system, it is not phosphorylated, whereas 2′-dADP as well as 3′-dADP can act as substrates in the photophosphorylation step. The experimental results obtained with these substrate analogues will be discussed on the basis of their structural features in comparison with the phosphate chain modified ADP analogue AOPCP [4].

2. Methods and materials

Chloroplasts were prepared from 30 g freshly harvested spinach leaves, using 250 ml of a medium contained 0.3 M sucrose and 10 mM sodium pyrophosphate pH 7.8. After centrifugation (5 min, 1000 × *g*) the chloroplasts were suspended in 10 mM sodium

North-Holland Publishing Company – Amsterdam

pyrophosphate, pH 7.8, and thus broken. Chlorophyll was determinated according to Arnon [5].

Pre-loading of the chloroplasts with the nucleotides and release experiments were done using the method of Strotmann et al. [6]. Photophosphorylation was essentially carried out as described previously [7].

The ribose-ring-opened AT(D)P analogues were prepared by cleavage of the C(2-)–C(3′) bound by periodate oxidation and subsequent borohydride reduction according to the method described previously [2,8]. The compounds were characterized by ultraviolet spectroscopy (λ_{max} = 256 nm; ϵ = 14 800 M^{-1} cm^{-1}), and thin-layer chromatography on polyethyleneimine-impregnated cellulose plates (PEI-cellulose F 1440 PEI/LS 254, Schleicher and Schüll, Dassel) in 0.75 M KH_2PO_4 [9] pH 4.1. The following R_F values were obtained: AMP = 0.52, ADP = 0.42, ATP = 0.20 rroADP = 0.55, rroATP = 0.31.

Anion-exchange liquid chromatography of the adenine nucleotides was performed as published [10]. Contamination by unmodified adenine nucleotides was less than 1.5%. All rroAT(D)P preparations show identical properties compared to an authentic product characterized by elementary analysis and proton magnetic resonance spectroscopy [11]. The antibiotic 9-(β-D-3′-deoxyribofuranosyl) adenine (Cordycepin; Sigma Chemie, München) was chemically phosphorylated to the triphosphate according to [12,13]. For preparation of the corresponding nucleotide diphosphates, 2′dATP (Boehringer, Mannheim) and 3′dATP were treated with yeast hexokinase (EC 2.7.1.1). The incubation assay contained: 4 μl yeast hexokinase (2 mg/ml; 140 U/mg), 80 μl 0.1 M glucose, 400 μl 0.1 M TRA pH 7.6, 10 μl 0.25 M $MgCl_2$ and 8 μmol of nucleotide; incubation time was 20 h at 24°C. After digestion the samples were applied to a DEAE-cellulose column (Whatman DEAE 52 cellulose; 3 × 80 cm) and eluted by a linear gradient of triethylammoniumbicarbonate (0–0.3 M; 3 litre) at pH 7.3. The sodium salts of 2′-dADP and 3′-dADP were obtained by passing through a cation-exchange column (Dowex 50 WX 4, Na^+ sign).

Purity control of the diphosphates by cation-exchange chromatography [10] revealed no detectable amount of triphosphates. The photophosphorylated ^{32}P-labelled nucleoside triphosphates were characterized by the same method. Following R_t values (min) were obtained: ATP = 5.0, 3′-dATP = 5.8, 2′-dATP = 6.6. Conditions: column: 300 × 5 mm; Nucleosil^R 5 SA, silica gel spherical TP, particle size 5 μm (Machery & Nagel, Düren Germany); mobile phase: 0.3 M $LiNO_3$, 0.02 M KH_2PO_4 pH 1.5; flux rate: 21 ml/h, 71 atm, 24°C; detection system: Zeiss PMQ IV, 258 nm.

3. Results

As shown in table 1 3′-dADP and 2′-dADP act as phosphoryl acceptor, whereas rroADP is not photophosphorylated by spinach chloroplasts. The ^{32}P-labelled photophosphorylated products 2′-dATP and 3′-dATP were characterized as described in Materials and methods. Separation of rroADP and rroATP was achieved by anion-exchange liquid chromatography [10]. Figure 3 shows a typical elution diagram of a standard mixture containing AMP, ADP, ATP, rroADP and rroATP. Analysis of phosphorylation assays performed with rroADP did not result in any detectable amount of [^{32}P]rroATP (cf. table 1).

On the basis of this experiment one might expect that rroADP is not bound to the active site of CF_1 and therefore not phosphorylated. In order to clarify this, CF_1 of spinach chloroplasts was preloaded with ^{14}C-labelled ADP [6], and light dependent exchange of the CF_1 bound labelled nucleotides by free rroADP was measured (fig.4). Exchange was well above background release of CF_1 bound [^{14}C]adenine nucleotides. The initial rate was about half of the usual rate found for the unmodified compound. In a similar experiment spinach chloroplasts were preloaded with

Table 1
Photophosphorylation with ADP and ribose modified adenine nucleoside diphosphates as substrates

Substrate	μmol NTP/mg chlorophyll × h
ADP	174.7
2′-dADP	70.8
3′-dADP	166.8
rroADP	0.0

Isolation and pretreatment of the chloroplasts and photophosphorylation measurements as described under Methods and materials. The chlorophyll content during illumination (1 min white light and saturated conditions) was 26.5 μg/ml. Concentration of the nucleoside diphosphates was 5 μmol/ml.

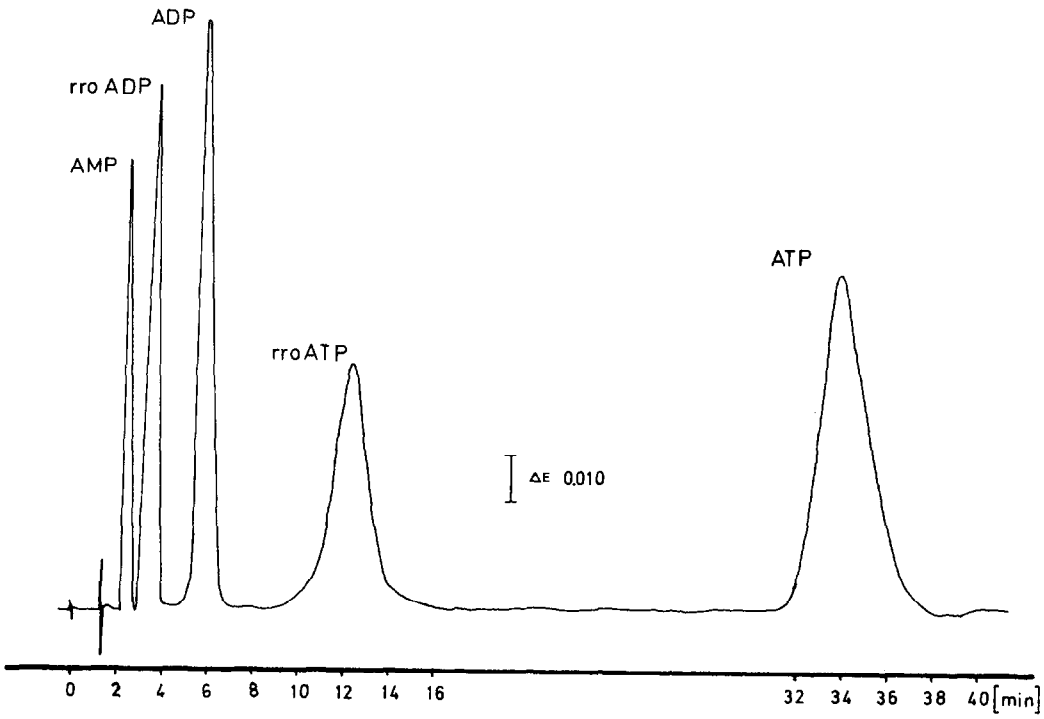
ADP
rro ADP
AMP
ATP
rroATP
ΔE 0.010
0 2 4 6 8 10 12 14 16
32 34 36 38 40[min]

Fig.3

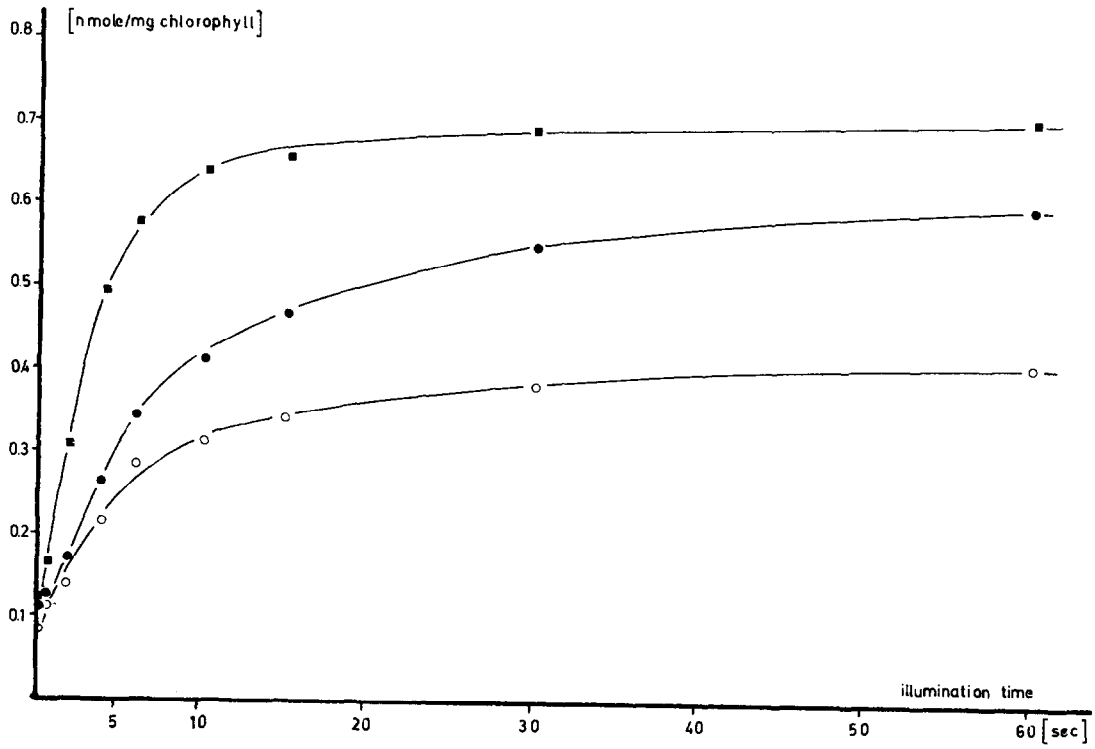
[nmole/mg chlorophyll]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
illumination time
5 10 20 30 40 50 60 [sec]

Fig.4

[^{14}C]rroADP (specific activity; 1.08 μCi/μmol) up to 0.91 nmol [^{14}C]rroADP/mg chlorophyll. As to be expected replacement with unlabelled ADP as well rroADP was achieved.

These findings strongly suggest that binding of rroADP occurs in the same mode and at the same binding site of CF_1 as ADP. Photophosphorylation of this analogue, however, is not obtained (cf. table 1).

4. Discussion

Comparison of the structures of the nucleotide analogues with the natural substrate may elucidate the possible mode of protein–nucleotide interactions and may provide some insight into the phosphorylating step.

Based on NMR studies [14,15] adenine nucleotides can be considered in terms of three structural features: (1) pseudorotation (puckering) of the ribose ring with a rapid equilibrium between *N*-type [C(3′)-endo, C(2′)-exo] and *S*-type [C(2′)-endo, C(3′)-exo] conformers, (2) exocyclic group C(4′)–C(5′) orientation with three possible rotamers (gauche-gauche, gauche-trans, trans-gauche) and (3) the syn–anti equilibrium of the heterocycle/ribose ring orientation.

Chemical shift measurements [16,17] of 3′-deoxyribonucleotides revealed an equally populated rotamer distribution of the exocyclic group, which only slightly affects the conformational state of the sugar moiety and the torsion around the C(1′)–N(9) glycosidic linkage. On the other hand, substitution of a hydroxyl group by a hydrogen atom in the 2′-position causes an increase of the *S*-type sugar population. The marked preference of the *S*-conformer results in the predominance of the syn-conformation [16,17]. Furthermore, the removal of the 2′-hydroxyl group, which can form an intramolecular hydrogen bond with an anti-positioned N(3) of the heterocycle [14] favours the syn-conformation. From the proton magnetic resonance spectrum of rroATP [11] it is concluded that a fixed conformation around the glycosidic linkage and a preferred exocyclic group orientation cannot be retained. Summarizing, the structure of rroADP [2] is more flexible than that of the unmodified adenine nucleotide, which exists predominantly in the anti, gauche-gauche conformation [14,15].

This preferred nucleoside diphosphate conformation allows metals, i.e. Mg^{2+}, to be simultaneously bound to the heterocycle nitrogen N(7) and α- and β-phosphate group. The fourth remaining site of the quadrivalently chelated metal may be bound to the enzyme [18,19]. On the other hand, as shown for the active complex of pyruvate kinase [20], a direct metal–ATP coordination is not strictly necessary. Water ligands or functional groups of the protein might intervene between the nucleotide and the divalent metal ion. In this case, a transition from a 'folded' binary chelate complex to an 'extended' ternary second sphere complex takes place.

On the basis of these structural features we will now discuss the different properties of the ADP analogues in phosphorylation as summarized in table 2. It is well known that the overall process of photophosphorylation by spinach chloroplasts requires Mg^{2+}, probably Mg–ADP is the actual substrate in photophosphorylation [22]. On the other hand, ADP binding and exchange is largely independent of Mg^{2+} [23]. This is in accordance with our findings, because each of the three adenine nucleotide analogues is capable of binding to the same site as the natural substrate. Even rroADP is bound, though it is unable to form a stabilized Mg^{2+} complex involving the α- and β-phosphate group and N(7) of the heterocyclic base. Concerning the pseudorotation model of ATP-synthesis [24–26] the metal ion should be coordi-

Fig.3. Anion-exchange liquid chromatography. Column: 300 × 5 mm; Nucleosil^R 5 SB, silica gel spherical TP, particle size 5 μm (Machery & Nagel, Düren Germany); mobile phase: 0.1 N KNO_3, 0.02 M KH_2PO_4, pH 2.6; flux rate: 21 ml/h, 67 atm, 24°C; detection system: Zeiss PMQIV, 258 nm.

Fig.4. Light-dependent release of CF_1 bound [^{14}C]adenine nucleotides from [^{14}C]ADP pre-labelled chloroplasts (1.07 nmol [^{14}C]ADP/mg chlorophyll) in the absence (o——o) and presence (■——■) of ADP and rroADP (●——●), respectively. Incubation medium: 50 mM Tricin pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM methylviologen, 100 μM ADP; 100 μM rroADP; 105.0 μg chlorophyll; total volume: 300 μl; experimental procedure see Methods and materials.

Table 2
Properties of phosphoryl acceptors

	Syn–anti equilibrium[a]	Metal–complexation (involving N(7))	Photophos-phorylation	Oxidative phos-phorylation[b]
ADP	anti	yes (+++)	yes (+++)	yes (+++)
3′-dADP	anti	yes (+++)	yes (+++)	yes (+++)
2′-dADP	syn	complicated (+)[c]	lowered (+)	---[d]
rroADP	distored	no	no	---[d]
AOPCP	anti	facilitated (++++)	slightly[e]	slightly[e]

[a] Preferred orientation of the heterocycle with respect to the ribose-ring
[b] Rat liver mitochondria
[c] Due to the fact that N(3) is involved in metal-complexation with the syn-conformer [21]
[d] 2′-dADP and rro ADP are not transported across the inner mitochondrial membrane
[e] Due to the strongly altered phosphate chain

nated directly or via water molecules to the α- and β-phosphoryl group in the active complex [27].

This stereo-specific Mg^{2+}–ADP complex seems to be necessary for a proper positioning of the nucleotide after binding to the active site. This metal induced substrate fixation allows an apical (in-line) nucleophilic attack of the nucleotidyl moiety on the enzyme- or metal-bound tetrahedrogenal inorganic phosphate and formation of a trigonal bipyrimidal transition state. Since in the case of rroADP this fixation cannot be restored, photophosphorylation by spinach chloroplasts does not occur. 3′-dADP, on the other hand, seems to be in the proper preorientation to coordinate with the metal due to the fact that only the equilibrium of the rotamer distribution of the exocyclic group is affected compared to ADP. As we could demonstrate previously [28,3] 3′-dADP is also transported and phosphorylated by rat liver mitochondria.

The lower photophosphorylation rate of 2′-dADP compared to ADP could be related to the preferred syn-orientation of the hetero-cyclic base, thus complicating the coordination.

Recent studies with α,β-methylene adenosine 5′-diphosphate (AOPCP) revealed only a very low activity in photophosphorylation. Moreover, AOPCP is if any a very pure phosphate acceptor for oxidative phosphorylation by rat liver digitonin particles [29]. As shown for the corresponding triphosphate (AOPOPCP) the lack of reactivity is not due to the inability of the analogue to bind divalent metal ions. The affinity constant of AOPOPCP for Mg^{2+} is even 2.5 times larger than for ATP [30]. The structural characteristics of the P–C–P grouping are quite different from the P–O–P or P–N–P linkages. The more acute P–C–P bond (117°; P–O–P 130°) angle and the longer P–C bond (1.79 Å; P–O 1.61 Å) distances [31] complicates the equatorial nucleophilic attack of AOPCP, thus resulting in a very low photophosphorylation rate.

According to the pseudorotational concept [24,25], ATP-synthesis is linked to energy dependent conformational changes at the catalytic site. Concerning the conformational transitions of the coupling factor protonation and deprotonation may be involved [6,24,25,32,33]. Additionally, metal complexation may affect the stereo-chemical parameters (in-line mechanism) of the nucleotidyl transfer by lowering the energy requirements for the pseudorotation process.

Acknowledgements

We are indebted to Professor W. Lamprecht for generous support. K.-S. B. gratefully acknowledges the personal grant of the Graduierten-Förderung. The authors thank the government of Nordrhein-Westfalen and the Deutsche Forschungsgemeinschaft for financial support.

References

[1] Schlimme, E., Lamprecht, W., Eckstein, F. and Goody, R. S. (1973) Eur. J. Biochem. 40, 485–491.
[2] Boos, K. S., Schlimme, E., Bojanovski, D. and Lamprecht, W. (1975) Eur. J. Biochem. 60, 451–458.
[3] Boos, K. S. and Schlimme, E. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 290–291.
[4] Horak, A. and Zalik, S. (1976) Biochim. Biophys. Acta 430, 135–144.
[5] Arnon, D. J. (1949) Plant Physiol. 24, 1.
[6] Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194–198.
[7] Strotmann, H. (1970) Ber. dtsch. Bot. Ges. 83, 443.
[8] Cramer, F., v.d. Haar, F. and Schlimme, E. (1968) FEBS Lett. 2, 136–139.
[9] Goody, R. S. and Eckstein, F. (1971) J. Amer. Chem. Soc. 93, 6252–6267.
[10] Stahl, K. W., Schlimme, E. and Bojanovski, D. (1973) J. Chromatogr. 83, 395–404.
[11] v.d. Haar, F., Schlimme, E., Gomez-Guillen, M. and Cramer, F. (1971) Eur. J. Biochem. 24, 296–302.
[12] Yoshikawa, M., Kato, T. and Takenishi, T. (1967) Tetrahedron Lett. 50, 5065–5068.
[13] Hoard, D. E. and Ott, D. G. (1965) J. Amer. Chem. Soc. 87, 1785–1788.
[14] Tso, P. O. P. in: Basic Principles in Nucleic Acid Chemistry (1974) Vol. 1, pp. 453–517, Academic Press New York.
[15] Sundaralingam, M. (1975) Ann. N.Y. Acad. Sci. 255, 3–43.
[16] Davies, D. B. and Danyluk, S. S. (1974) Biochemistry 13, 4417–4434.
[17] Davies, D. B. and Danyluk, S. S. (1975) Biochemistry 14, 543–554.
[18] Sundaralingam, M. (1969) Biopolymers 7, 821.
[19] Cohn, M. and Hughes, T. R. (1960) J. Biol. Chem. 235, 3250–3253.
[20] Sloan, D. L. and Mildvan, A. S. (1976) J. Biol. Chem. 251, 2412–2420.
[21] Freist, W., v.d. Haar, F., Sprinzl, M. and Cramer, F. (1976) Eur. J. Biochem. 64, 389–393.
[22] Komatsu, M. and Murakami, S. (1976) Biochim. Biophys. Acta 423, 103–110.
[23] Bickel-Sandkötter, S. and Strotmann, H. (1976) FEBS Lett. 65, 102–106.
[24] Korman, E. F. and Mc Lick, J. (1970) Proc. Natl. Acad. Sci. USA 67, 1130–1136.
[25] Young, J. H., Korman, E. F. and Mc Lick, J. (1974) Bioorg. Chem. 3, 1–15.
[26] Benkovic, S. J. and Schray, K. Y. (1973) Enzymes 8, 201–238.
[27] Amsler, P. E. and Sigel, H. (1976) Eur. J. Biochem. 63, 569–581.
[28] Boos, K. S., Lüstorff, J. and Schlimme, E. (1976) Abstr. Symp. Biochem. Membrane Transport, p. 401, Zürich.
[29] Duée, E. D. and Vignais, P. V. (1969) J. Biol. Chem. 244, 3920–3931.
[30] Yount, R. G., Babock, D., Ballantyne, Wm. and Ojala, D. (1971) Biochemistry 10, 2484–2489.
[31] Yount, R. G. (1975) Adv. Enzymol. 43, 1–56.
[32] Slater, E. C. (1975) in: The Structural Basis of Membrane Function (Hatefi, Y., Djavadi-Ohaniance, eds) JUB symposium series No. 72, pp. 161–168, Elsevier, Amsterdam.
[33] Rosing, J., Kayalar, C. and Boyer, P. D. (1975) in: The Structural Basis of Membrane Function (Hatefi, Y., Djavadi-Ohaniance, eds) JUB symposium series No. 72, pp. 189–204.