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PHOTOPHOSPHORYLATION STUDIES WITH FLUORESCENT ADENINE NUCLEOTIDE ANALOGS

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1. Introduction

The synthesis of fluorescent analogs of nucleotides with enzymic activity was recently reported [1]. The fluorescence properties of 1,N^6-ethenoadenosine di- and triphosphate (εADP and εATP) promise to provide useful tools for the study of the interaction of adenine nucleotides with the energy conservation system of chloroplast membranes and the transport of nucleotides across these membranes, if these can replace nucleotides in the various chloroplast reactions.

This communication deals with the activity of εADP as a substrate for photophosphorylation and of εATP as substrate for several hydrolytic and exchange reactions related to photophosphorylation. Significant differences in the capability of the ε-adenine analogs to replace adenine nucleotides in the various reactions tested suggest that more than one type of site, with different specificity for the nucleotide phosphates, exists.

2. Methods and materials

Chloroplasts were isolated from fresh market lettuce leaves by standard procedures. The coupling factor protein (CF_1) was isolated from lettuce chloroplasts and purified following Lien and Racker [2].

Abbreviations:
PMS – phenazine methosulfate;
CF_1 – chloroplast coupling factor;
NTP/e_2 or ATP/e_2 – εATP or ATP formed per $2e^-$ transferred;
DTT – dithiothreitol.

Yeast hexokinase (Type III) and the sodium salts of ADP, ATP and GTP were purchased from Sigma Chemical Co.

εADP and εATP were prepared by the method of Secrist et al. [1], and had satisfactory melting points. By the spectral criteria suggested by Secrist et al. [1] the nucleotides were at least 98% ethenoadenine derivatives. Thin-layer chromatography (on Merck microcrystalline cellulose sheets with fluorescent indicator using isobutyric acid–NH_4OH–H_2O (60:1:39 v/v/v) showed that the final products obtained (εATP and εADP) contained only traces of their corresponding hydrolysis products. [γ-^{32}P]ATP was prepared from [^{32}P]phosphate and ADP [3]. [γ-^{32}P]εATP was similarly prepared from εADP and was shown to be chemically identical to εATP prepared as above, by co-chromatography on the above TLC system. Yeast hexokinase transferred the label from either [^{32}P]nucleotide to glucose [3]. The [γ-^{32}P]nucleotides were diluted with cold nucleotide triphosphates for ATPase activity assays. ATP formation and ferricyanide reduction were assayed as described [3,4].

3. Results

εADP can replace ADP as the substrate for photophosphorylation in chloroplasts. Substrate amounts of εADP were phosphorylated in this reaction, and it was demonstrated that the phosphorylated product obtained is in fact [γ-^{32}P]εATP by co-chromatography with cold εATP and by [^{32}P]phosphate transfer to glucose with yeast hexokinase (EC 2.7.1.1) [5]. As shown in table 1 the apparent Michaelis constants for

Table 1
Photophosphorylation with ADP and εADP substrates.

Reaction	Substrate	K_m (appar) (mM)	V_{max} (appar) (μmoles ATP/mg CHL/hr)	NTP/e_2^*
Ferricyanide	ADP	0.06	313	0.95
	εADP	0.11	162	0.65
Phenazine methosulfate	ADP	0.04	1193	–
	εADP	0.10	662	–

Reaction mixtures contained the following components in μmoles in a total volume of 3 ml at pH 7.8: Tris-HCl, 90; NaCl, 150; $MgCl_2$, 10; P_i, 10 (containing 4×10^6 cpm ^{32}P); chloroplasts containing about 50 μg chlorophyll and $K_3Fe(CN)_6$, 1.0 or PMS, 0.1 as indicated. In the reaction with PMS, 60 μmoles of glucose and hexokinase, 2 mg were used. Light intensity was 160 000 lux. Samples were illuminated for 1 min at 22°C.
*NTP/e_2 was calculated at optimal εADP concentrations.

ADP and εADP are similar in the two photophosphorylation assays tested. In each case the maximal velocity observed with εADP was about 50% of that attained with ADP. The NTP/e_2 ratio with εADP was somewhat lower than ATP/e_2 suggesting less efficient coupling. In contrast, εATP was not a good substitute for ATP in the partial reactions of photophosphorylation. In the light triggered chloroplasts ATPase or the CF_1-Ca^{2+} ATPase reactions, maximal rates with εATP were about 10–15% of those ATP (table 2). Even more striking was the low activity observed with εATP in the ATP ↔ P_i exchange reaction (less than 1%). εATP was found to be a competitive inhibitor of ATP in this reaction with K_i of the same order of magnitude as K_m for ATP, and εADP inhibited the light triggered ATPase as ADP.

Since these reactions were performed with either purified, heat-activated CF_1 or with modified chloroplasts, it might be argued that they are not directly comparable to a normal chloroplast preparation. We therefore examined the activity of εADP in a photophosphorylation reaction in a medium containing DTT, conditions comparable to those of the light-triggered ATPase and ATP ↔ P_i exchange reactions.

Table 2
εATP as substrate in partial reactions of photophosphorylation.

Reaction	Substrate	Optimal concentration range (mM)	Rate*	Relative activity (%)
A) CF_1-ATPase	ATP	4 – 10	17	
	εATP	4 – 10	2	12
B) Light-triggered ATPase	ATP	2 – 4	105	
	εATP	2 – 5	14	13
C) Light-dependent ATPase	ATP	0.4 – 0.9	23	
	εATP	0.4 – 0.9	6.5	28
D) ATP ↔ P_i	ATP	1 – 3	19	
	εATP	1 – 3	0.03	0.2

A) Desalted CF_1 was heat activated in the presence of 33 mM ATP and 5 mM DTT at 60°C for 4 min. ATPase activity was determined using ^{32}P-labelled substrates as described, [6] except that a constant Ca^{2+}/[NTP] ratio of 1 was maintained. Rates are given in μmoles P_i released per mg protein per minute. B and C) ATPase activities were determined using ^{32}P-labelled substrates as described [10] except that in the case of light-dependent ATPase assay a Ca^{2+}/[NTP] ratio of 5 was maintained. Rates are given in μmoles P_i released per mg chlorophyll per hour. D) ATP ↔ P_i exchange activity was determined as described [11] except that $MgCl_2$ and P_i were 10 and 5 mM, respectively.
* Rates are given in μmoles P_i exchanged per mg chlorophyll per hour.

Table 3
Protection of CF_1 during heat activation.

Additions to heat activation mixture	ATPase (μmoles P_i/mg protein/min)	(% of control)
None	0	0
ATP	14.5	100
GTP	0	0
ϵATP	8.5	59
PP_i	0	0

Heat activation of CF_1 was as described in table 2. The concentration of nucleotides or PP_i during heat activation was 33 mM. ATPase activity was then determined using [γ-^{32}P]ATP [10]. ATPase activity obtained after heat activation in the presence of ATP is referred to as 100%.

The relative rates of phosphorylation of ϵADP (70% of ADP) was similar to that obtained with normal chloroplast preparations. Although ϵATP was a relatively poor substrate for ATPase activities, the competitive nature of the inhibition of the ATP $\leftrightarrow$ P_i exchange by ϵATP suggested that it could bind to the protein(s) involved in these reactions. Therefore we tested the ability of ϵATP to replace ATP in protecting the latent CF_1-ATPase during heat activation of the protein. As shown in table 3, ϵATP was 60% as effective as ATP, while heating CF_1 with GTP or pyrophosphate was much less effective [6].

4. Discussion

It has been assumed that the ATPase and ATP $\leftrightarrow$ P_i exchange reactions represent reversal of one or more of the last steps in ATP formation during photophosphorylation [7]. This assumption implies that these reactions occur at a catalytic site identical with that involved in photophosphorylation. One might therefore expect a similar specificity towards the adenine nucleotide substrates in all these reactions. The results described above, contrary to this expectation, show large differences among the specificities of these reactions. ϵADP is nearly as good (50–70%) a substrate as ADP for photophosphorylation, while ϵATP is a very poor substrate (0.2% of ATP rate) for the ATP $\leftrightarrow$ P_i exchange reaction. In none of the ATPase reactions was the relative effectiveness of ϵATP comparable to that of ϵADP in photophosphorylation even with similar chloroplast preparations. However, ϵATP was a competitive inhibitor of the ATP $\leftrightarrow$ P_i exchange, and could protect CF_1 from heat inactivation. Published data involving comparison of other nucleotides also suggests that the ATP $\leftrightarrow$ P_i exchange reaction is highly specific [7], while photophosphorylation has a much wider specificity for nucleotides [8, 9].

One explanation for these results is the existence of at least two types of catalytic sites involved in the reactions studied, and that those sites with high nucleotide specificity may not be directly related to the process of ATP formation. It is of interest that other workers have found evidence for multiple nucleotide sites in chloroplast and mitochondrial isolated coupling factors, although the relevance of these observations to the problem raised here is uncertain. Roy and Moudrianakis, in proposing a mechanism for the formation, from ADP, of ATP tightly bound to the isolated CF_1 protein, have suggested that the protein has two different ADP binding sites [12]. It has recently been reported that the solubilized mitochondrial ATPase has two nucleotide binding sites with different base specificities [13]. Investigation of the binding of nucleotides to the isolated CF_1 protein and the membrane-bound enzyme, as well as fluorescence studies with ϵ-adenine analogues, should provide further information regarding the interactions between these nucleotides and the membrane-bound enzymes, and should help clarify the relevance of these interactions to the energy conservation mechanism in chloroplasts.

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References

[1] J.A. Secrist III, J.R. Barrio, N.J. Leonard and G. Weber, Biochemistry 11 (1972) 3499.
[2] S. Lien and F. Racker, in: Methods in enzymology, Vol.

24B, eds. S.P. Colowick and N.O. Kaplan (Academic Press, 1972) p. 547.
[3] M. Avron, Anal. Biochem. 2 (1961) 535.
[4] M. Avron and N. Shavit, Anal. Biochem. 6 (1963) 549.
[5] M.O. Kamen, in: Methods in enzymology, Vol. 6, eds. S.P. Colowick and N.O. Kaplan (Academic Press, 1963) p. 313.
[6] F. Farron and E. Racker, Biochemistry 9 (1970) 3829.
[7] C. Carmeli and M. Avron, European J. Biochem. 2 (1967) 318.
[8] M. Avron, Biochim. Biophys. Acta 40 (1960) 257.
[9] A. Bennun and M. Avron, Biochim. Biophys. Acta 109 (1965) 117.
[10] C. Carmeli and M. Avron, in: Methods in enzymology, Vol. 24B, eds. S.P. Colowick and N.O. Kaplan (Academic Press, 1972) p. 92.
[11] N. Shavit, in: Methods in enzymology, Vol. 24B, eds. S.P. Colowick and N.O. Kaplan (Academic Press, 1972) p. 318.
[12] H. Roy and E.N. Moudrianakis, Proc. Natl. Acad. Sci. U.S. 68 (1971) 464.
[13] D.A. Hilborn and G.G. Hammes, Biochemistry 12 (1973) 983.