Biochimica et Biophysica Acta, 766 (1984) 570–575
Elsevier

BBA 41567

OXIDATION-LINKED FORMATION OF INORGANIC PYROPHOSPHATE IN MAIZE SHOOT MITOCHONDRIA

STANISŁAW KOWALCZYK and PIOTR MASŁOWSKI

Department of Biochemistry, Institute of Biology, Mikołaj Kopernik University, Pl 87-100 Toruń (Poland)

(Received November 30th, 1983)
(Revised manuscript received May 3rd, 1984)

Key words: Pyrophosphate synthesis; Oxidative phosphorylation; Pyrophosphataze inhibitor; Uncoupler; (Maize seedling)

The coupled mitochondria of maize seedlings are the site of electron-transport-dependent synthesis of inorganic pyrophosphate. The inorganic-pyrophosphate synthesis depends on the presence of Mg^{2+} and exogenous phosphate; it is inhibited by electron transport inhibitor, uncoupler and by inorganic pyrophosphatase inhibitors (methylene diphosphonate, NaF, Ca^{2+}).

Introduction

It is generally accepted that in chromatophores from the photosynthetic bacterium, *Rhodospirillum rubrum*, takes place formation of PP_i in parallel with, and independently of, ATP [1–4]. The PP_i synthesis as a phosphorylating energy acceptor is coupled with the photosynthetic cyclic electron flow. More recently, a special system for energy-dependent synthesis of PP_i was discovered and characterized [5,6]. The findings of these authors show that the formation of PP_i is catalyzed by a membrane-bound inorganic pyrophosphatase. This enzyme, isolated from chromatophores and incorporated into phospholipid vesicles, can function alone as H^+-pump [7–9].

So far, an unanswered question of great significance is whether *R. rubrum* is a unique organism that can couple the electron flow to the synthesis of PP_i. In fact, as far back as in the 1950s, the evidence has been presented for the synthesis of PP_i in *Acetobacter suboxidans*, *Escherichia coli* and yeast [10,11], and lately in mitochondria of *Endomyces magnusii* [12] and rat liver [13]. The experiments reported in this communication examine more closely the formation of PP_i in plant mitochondria.

Materials and Methods

Isolation of mitochondria

Mitochondria were isolated from 3-day-old etiolated shoots essentially by the method of Day and Hanson [14]. Approx. 50 g of tissue was ground in an ice-cold mortar lined with two layers of fine nylon net with 100 ml of 0.4 M sucrose/50 mM Tes/5 mM EGTA/0.1% bovine serum albumin, adjusted to pH 7.8 with KOH. The homogenate was squeezed through three layers of cheesecloth and centrifuged. The resultant mitochondrial pellet was resuspended in 0.5–1 ml of 0.25 M sucrose/10 mM Tes-KOH (pH 7.2)/0.1% bovine serum albumin. All steps were carried out at 0–4°C.

Determination of ADP / O and acceptor control ratio

Oxygen consumption was measured with a Clark

Abbreviations: Dimethyl POPOP, 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PPO, 2,5-diphenyloxazole; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

oxygen electrode in 1.4 or 4.3 ml of standard reaction medium which consisted of 0.25 M sucrose/10 mM Tes/5 mM $MgCl_2$/5 mM KH_2PO_4/0.1% bovine serum albumin, adjusted to pH 7.2 with KOH. Mitochondrial protein was approx. 0.7–3.8 mg per vessel. Reactions were run at 25 °C and started by the sequential addition of mitochondria, 1 mM NADH or 10 mM succinate and ADP. The final incubation volume was 1.5 or 4.5 ml. The oxygen electrode was calibrated according to Misra and Fridovich [15], ADP/O and acceptor control ratios were determined graphically [16].

Pyrophosphate determination

Pyrophosphate was assayed by the enzymatic method according to Drake et al. [17]. The standard assay medium contained 0.1 M triethanolamine-HCl (pH 6.5)/0.2 M glucose/10 mM $MgCl_2$/0.8 mM NADP/0.25 mM adenylyl sulfate/2 units of hexokinase/2 units of glucose-6-phosphate dehydrogenase. After addition of the assay samples (600 µl), 1 unit of ATP sulfurylase (30 µl) is introduced into the reaction mixture to initiate PP_i analysis. The incubation volume was 1.25 ml. In the control, ATP sulfurylase was omitted.

Protein determination

Protein was determined by the method of Lowry et al. [18] with bovine serum albumin (fraction V) as a standard.

Separation of ^{32}P-labelled compounds

The separation of ^{32}P-labelled PP_i and nucleotides (AMP, ADP, ATP) was carried out by high-voltage paper electrophoresis. After neutralization and centrifugation of the reaction mixtures, 10 µl samples were taken and separated on Whatman 3MM filter paper in 50 mM citrate buffer (pH 4.1) at the potential gradient of approx. 40 V/cm. At the origin, 5 µl of a solution containing 20 mM PP_i, P_i, AMP, ADP and ATP were additionally applied. The electrophoresis was carried out below 6 °C. After electrophoresis was completed, the location of compounds was determined according to Hanes and Isherwood [19]. The stained spots containing PP_i and ATP were cut out, placed in scintillation vials containing 10 ml of a toluene-based scintillator (0.3 g/l dimethyl POPOP and 5 g/l PPO) and counted in scintillation counter.

Electron microscopy

For electron microscopy the organelles were fixed in suspension according to Pomeroy [20].

Chemicals

Adenylyl sulfate, antimycin A, dimethyl POPOP, EGTA, methylene diphosphonate, oligomycin, PPO, Tes and the auxiliary enzymes were purchased from Sigma; 2,4-dinitrophenol, NADH, NADP, all nucleotides and all chemicals for electron microscopy from Serva. Bovine serum albumin and succinate were obtained from BDH and sucrose from Merck. $KH_2{}^{32}PO_4$ was from the Institute of Nuclear Research (Świerk). All other chemicals were of analytical reagent grade and obtained from commercial sources.

Results and Discussion

Characteristics of isolated mitochondria

The quality of the mitochondrial preparation was evaluated by the measurement of certain characteristic parameters. High acceptor control ratios were obtained with either NADH or succinate (results not shown) as the respiratory substrate. Also the observed ADP/O ratios approached the expected value of 2, suggesting the normal operation of the coupling site that comprise oxidative phosphorylation. Electron micrographs indicate that mitochondria are the dominating organelles in the prepared fraction (not shown).

Essentially, the mitochondria obtained by this method were considered to be functionally intact, relatively undamaged and suitable for the attempted study.

Oxidation-linked PP_i formation

Fig. 1 shows the oxygen consumption record and the corresponding changes in the PP_i level. It is of interest that the PP_i level increases markedly 12–13 nmol/mg protein per min in the respiration state IV and in the presence of Mg^{2+} only. The rapid rise of PP_i is only in a small extent accompanied by the increase in oxygen consumption initiated by the addition of Mg^{2+}. It is worth noting that the rate of PP_i formation drops sud-

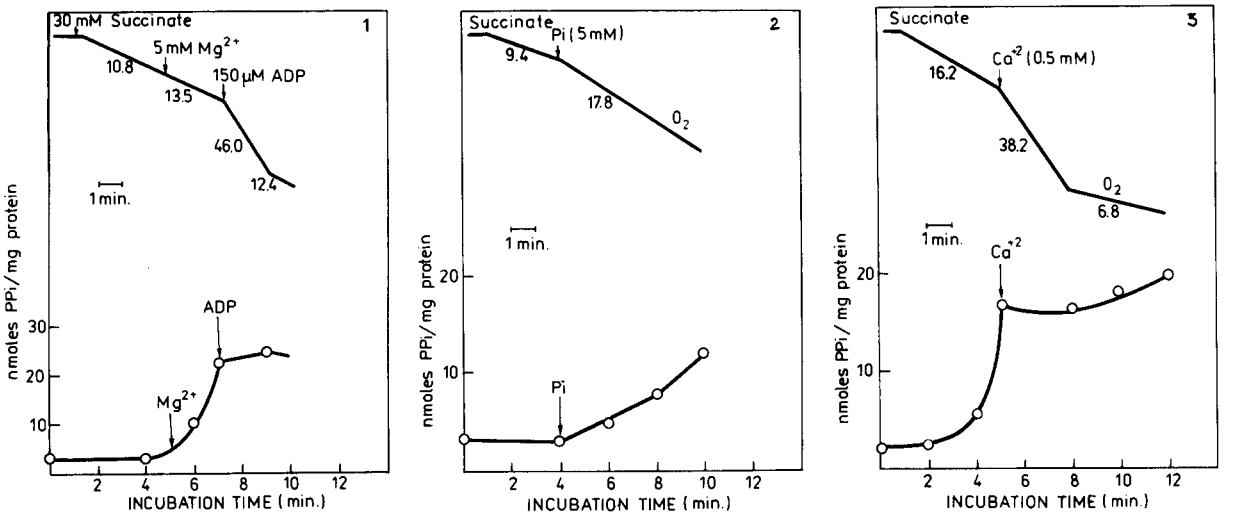

Fig. 1. Relationship between the PP_i formation and oxygen consumption rate in the respiration states IV and III. The reaction medium was of identical composition as standard medium, except that Mg^{2+} was omitted. To 4.3 ml of the incubation mixture, 0.2 ml of a suspension of freshly isolated mitochondria was added; the mixture was incubated at 25 °C in an oxygraphe vessel, and changes in oxygen consumption rate were recorded. At indicated time intervals (see arrows), succinate, $MgCl_2$ and ADP (5–10 μl) were successively added. From a sample incubated in parallel, at definite time intervals, 0.6 ml samples were taken up and added to 50 μl of 45% (v/v) $HClO_4$. Immediately after protein denaturation, the samples were neutralized with 50 μl of 45% (w/v) KOH. $KClO_r$ and denaturated proteins were removed by centrifugation, and PP_i was determined by the enzymatic method as described in the Materials and Methods section.

Fig. 2. Effect of orthophosphate on PP_i accumulation and oxygen consumption rate. The composition of reaction medium was the same as standard mixture (see Materials and Methods), except that orthophosphate was omitted. The assay was performed as in Fig. 1. At the appropriate time, orthophosphate was added.

Fig. 3. Relationship between Ca^{2+} transport and changes in PP_i formation rate. The assay conditions were as in Fig. 2, except that the standard reaction medium contained 10 mM (potassium) phosphate buffer (pH 7.2). At appropriate time intervals, $CaCl_2$ (0.5 mM, final concentration) was added.

denly after subsequent addition of ADP (Fig. 1). As previously observed, state-IV respiration rates in corn mitochondria depend on internal phosphate and only a small respiratory increment due to its active transport was found [21]. In the present experiments, the oxygen consumption rate increases after addition of phosphate from 9.4 to 17.8 nmol/mg protein per min (Fig. 2). This al-

TABLE I

EFFECT OF SOME COMPOUNDS ON PP_i FORMATION

To 0.5 ml of standard medium additionally containing appropriate concentrations of 2,4-dinitrophenol, antimycin A, NaF, methylene diphosphonate or oligomycin, 0.1 ml of the mitochondrial suspension was added. After 5 min of incubation at 25 °C, the reaction was interrupted by $HClO_4$ and PP_i determined enzymatically.

Addition (concn.)	nmol PP_i synthesized in 5 min	Percentage of control
Control (no addition)	64	100
Dinitrophenol (0.1 mM)	5	8
Antimycin A (10 μM)	21	33
NaF (1 mM)	3	5
Methylene diphosphonate (1 mM)	5	8
Oligomycin (0.1 mg/ml)	62	97

most two-fold accelerated respiration is accompanied by an evident increase in PP_i level, though slower than in the previous experiments. The invariably low PP_i level prior to phosphate addition suggests that the observed rise of PP_i takes place via de novo synthesis from orthophosphate. This finding also suggests that the respiratory increase upon addition of phosphate is largely due to the formation of PP_i, not to the process of transporting of phosphate or substrate only.

In view of the facts that the formation of PP_i is inhibited by ADP and that the increase of oxygen consumption which accompanies PP_i formation is negligible (Fig. 1), it seemed interesting to investigate more closely the mechanism of PP_i synthesis. As a result of recent studies [12,13], it seems appropriate to offer the suggestion that PP_i synthesis is associated with the functioning of the mitochondrial electron-transport chain. On the other hand, the accumulation of PP_i was not found during oxidation of substrates other than fatty acids, indicating that the PP_i formation is a result of the fatty-acids activation reactions [22]. In the light of the fact that freshly isolated plant mitochondria contain a high level of free fatty acids [23], this suggestion must be taken into consideration.

To test whether or not the synthesis of PP_i is oxidation-linked, several experiments concerning the effect of 2,4-dinitrophenol and antimycin A on the PP_i formation were carried out. As shown in Table I, 2,4-dinitrophenol (uncoupler) inhibits PP_i formation by nearly 92%, while antimycin A (an electron transport inhibitor) by about 67%. These results suggest that the synthesis of PP_i is associated with the functioning of the electron-transport chain.

Years ago, Boyer [24] pointed out that the slow rate of pyrophosphate synthesis may be the result of the substitution of a phosphate for ADP at the enzymatic site identical to that utilized for ATP synthesis. Inhibition of PP_i formation by ADP can support this assumption, but it also suggests that the pathways of PP_i formation are ATP-independent; the rise of PP_i level is not the result of ATP hydrolysis to AMP and PP_i. On the other hand, the data on the light-dependent synthesis of PP_i in *R. rubrum* chromatophores and animal mitochondria bring forward that this process involves membrane inorganic pyrophosphatase [3,4,13]. In order to examine the possibility of the H^+-ATPase participation in the synthesis of PP_i, the effect of some inhibitors on formation of this compound was studied. A previous comparative analysis of the effect of various inhibitors on mitochondrial ATPase and pyrophosphatase has shown that NaF and methylene diphosphonate preferentially inhibited pyrophosphatase, whereas oligomycin inhibited only ATPase activity (results not shown). As shown in Table I, the inhibition of PP_i formation was effective even in the case of NaF and methylene diphosphonate, in whose presence the

TABLE II

EFFECT OF METHYLENE DIPHOSPHONATE AND OLIGOMYCIN ON LABELLING PP_i AND ATP BY [^{32}P]PHOSPHATE

The incubation mixture used in the experiments comprised orthophosphate concentrated 5-times lower than previously, and contained additionally $K_2H^{32}PO_4$ (approx. $1.5 \cdot 10^6$ cpm per 0.2 ml). To 0.2 ml samples, in the presence of an appropriate inhibitor, 50 µl of mitochondrial suspension was added. After 5 min incubation at 25 °C, during which the labelling of PP_i and ATP increased linearly, the reaction was stopped with 20 µl 45% (v/v) $HClO_4$. The separation and counting of labelled products was performed as described in the Materials and Methods section. The percentage of inhibition of the labelled product formation is given in parentheses.

Addition (concn.)	[^{32}P]Phosphate incorporation (cpm) into:			
	PP_i		ATP	
	−ADP	+ADP	−ADP	+ADP
Control (no addition)	5550	750	820	92400
Methylene diphosphonate (1 mM)	650	150	640	74500
	(12)	(20)	(78)	(81)
Oligomycin (0.1 mg/ml)	5900	800	450	52700
	(106)	(107)	(55)	(57)

increase in the PP_i level was only 5–8% as compared with the control. The exertion of oligomycin caused no essential changes in PP_i formation. The above-mentioned evidence has been supported by experiments concerning the effect of methylene diphosphonate and oligomycin on labelling of PP_i and ATP by [^{32}P]P_i in the presence or absence of ADP. Table II indicates that methylene diphosphonate inhibits ADP phosphorylation by 19% and PP_i synthesis by about 88%, whereas oligomycin inhibits labelling of ATP by 43% and stimulates the PP_i synthesis by about 6%. These results may indicate that the PP_i synthesis proceeds independently of the ATP synthesis and is not catalyzed by H^+-ATPase.

With regard to the energy-dependent accumulation of high numbers of sparingly soluble calcium and magnesium pyrophosphate granules occurring in *Tetrachymena piriformis* [25], a possible role of Ca^{2+} in regulating the level of mitochondrial PP_i can also be taken into consideration. Lately, evidence has been presented for PP_i-stimulated uptake of Ca^{2+} in *Phytophthora infestans* [26], and for Ca^{2+}-dependent elevation of PP_i as a result of butyrate oxidation [27].

The relationship between the PP_i level changes and Ca^{2+} transport in corn-shoot mitochondria is shown in Fig. 3. Calcium ions increase the oxygen consumption rate from 16.2 to 38.2 nmol/mg protein per min, whereas they completely stop the increase in PP_i level, being rapid in the first minutes. These findings indicate that energy-dependent Ca^{2+} transport probably competes with PP_i formation and does not confirm the assumed correlation between calcium accumulation and increase in PP_i level.

In conclusion, all these data should be interpreted, to a certain extent, as favoring the participation of the membrane-bound mitochondrial pyrophosphatase in PP_i biosynthesis. However, so far this enzyme has been found only in bovine heart and rat liver mitochondria [28–30], and has not been investigated in detail as yet. It is believed, however, that its structural organization is considerably simpler than that of the ATP-synthetase complex [30].

The question of whether or not inorganic pyrophosphate might in some situations or in some organisms replace ATP remains unanswered. Recently, an expanding body of evidence indicates that the energy of PP_i serves to drive essential metabolic processes in microorganisms [31,32]. At present, a similar role for PP_i has been clearly demonstrated in some plant tissue [33,34]. With reference to these data, of major significance is the evidence for the transport of PP_i across the mitochondrial membrane in exchange for adenine nucleotides via the adenine nucleotide translocase [35–38].

With due regard for all data so far obtained, we firmly believe that the current PP_i metabolism concepts seriously understate the role of PP_i in the cell metabolism.

Acknowledgements

We are indebted to Mrs. E. Cymerska for her skillful technical assistance.

References

1 Baltscheffsky, H., Von Stedingk, L-V., Heldt, H-W. and Klingenberg, M. (1966) Science 153, 1120–1122
2 Baltscheffsky, H. and Von Stedingk, L-V. (1966) Biochem. Biophys. Res. Commun. 22, 722–728
3 Guillory, R.J. and Fisher, R.R. (1972) Biochem. J. 129, 471–481
4 Nishikawa, K., Hosoi, K., Suzuki, J., Yoshimura, S. and Horio, T. (1973) J. Biochem. 73, 537–553
5 Rao, P.V. and Keister, D.L. (1978) Biochem. Biophys. Res. Commun. 84, 465–473
6 Randahl, H. (1979) Eur. J. Biochem. 102, 251–256
7 Kondrashin, A.A., Remennikov, V.G., Samuilov, V.D. and Skulachev, V.P. (1980) Eur. J. Biochem. 113, 219–222
8 Shakhov, Y.A., Nyrèn, P. and Baltscheffsky, M. (1982) FEBS Lett. 146, 177–180
9 Nyrèn, P. and Baltscheffsky, M. (1983) FEBS Lett. 155, 125–130
10 Klungsoyr, L., King, T.E. and Cheldelin, H. (1957) J. Biol. Chem. 227, 135–138
11 Klungsoyr, L. (1959) Biochim. Biophys. Acta 34, 586–587
12 Mansurova, S.E., Ermakova, S.A., Zviagilskaya, R.A. and Kulaev, I.S. (1975) Mikrobiologia 44, 874–879
13 Mansurova, S.E., Shakhov, Yu. A., Belyakova, T.N. and Kulaev, I.S. (1975) FEBS Lett. 55, 94–98
14 Day, D.A. and Hanson, J.B. (1977) Plant Sci. Lett. 11, 99–104
15 Misra, H.P. and Fridovich, I. (1976) Anal. Biochem. 70, 632–634
16 Estabrook, R.W. (1967) Methods Enzymol. 10, 41–47
17 Drake, H.L., Goss, N.H. and Wood, H.G. (1979) Anal. Biochem. 94, 117–120
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

19 Hanes, C.S. and Isherwood, F.A. (1949) Nature 164, 1107–1112
20 Pomeroy, M.K. (1977) Plant Physiol. 59, 250–255
21 Hanson, J.B., Bertagnolli, B.L. and Shepherd, W.D. (1972) Plant Physiol. 50, 347–354
22 Batenburg, J.J. and Van den Bergh, S.G. (1972) Biochim. Biophys. Acta 280, 495–505
23 Earnshaw, M.J., Truelove, B. and Butler, R.D. (1970) Plant Physiol. 45, 318–321
24 Boyer, P.D. (1967) in Current Topics in Bioenergetics (Sanadi, D.R., ed.), Vol. 2, pp. 99–126, Academic Press, New York
25 Rosenberg, H. and Munk, N. (1969) Biochim. Biophys. Acta 184, 191–197
26 Okorokov, L.A., Sysuev, V.A. and Kulaev, I.S. (1978) Eur. J. Biochem. 83, 507–511
27 Otto, D.A. and Cook, G.A. (1982) FEBS Lett. 150, 172–176
28 Mansurova, S.E., Shakhov, Yu.A. and Kulaev, I.S. (1977) FEBS Lett. 74, 31–34
29 Volk, S.E., Baykov, A.A., Duzhenko, V.S. and Avaeva, S.M. (1982) Eur. J. Biochem. 125, 215–220
30 Volk, S.E., Baykov, A.A., Kostenko, E.B. and Abaeva, S.M. (1983) Biochim. Biophys. Acta 744, 127–134
31 Wood, H.G. (1977) Fed. Proc. 36, 2197–2205
32 Wood, H.G., O'Brien, W.E. and Michaels, G. (1977) Adv. Enzymol. 45, 85–155
33 Carnal, N.W. and Black, C.C. (1983) Plant. Physiol. 71, 150–155
34 Kowalczyk, S., Januszewska, B., Cymerska, E. and Masłowski, P. (1984) Physiol. Plant 60, 31–37
35 Asimakis, G.K. and Aprille, J.R. (1980) Arch. Biochem. Biophys. 203, 307–316
36 Asimakis, G.K. and Aprille, J.R. (1980) FEBS Lett. 117, 157–160
37 Kowalczyk, S. and Masłowski, P. (1981) Phytochemistry 20, 2611–2615
38 D'Souza, M.P. and Wilson, D.F. (1982) Biochim. Biophys. Acta 680, 28–32