SYNTHESIS OF INORGANIC PYROPHOSPHATE BY ANIMAL TISSUE MITOCHONDRIA

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1. Introduction

It is known that inorganic pyrophosphate (PP_i) is formed as a result of pyrophosphorolysis reactions (synthesis of nucleic acids, coenzymes, proteins, activation of fatty acids etc.). It was believed that, to shift the equilibrium of these reactions towards synthesis, PP_i should be hydrolyzed by inorganic pyrophosphatase, the energy of anhydride bonds being thereby spent [1].

But some facts have been accumulating, testifying that PP_i in some biochemical conversions may perform a function similar to that of ATP [2–8]. In 1942 Cori [9] reported to have observed accumulation of PP_i in oxidation of glutamate by rat liver homogenates and Green et al. [10], in 1949, by rabbit liver homogenates. Synthesis of PP_i as a result of oxidative phosphorylation was demonstrated to take place in *Acetobacter suboxidans* and yeast [11] and as a result of photophosphorylation, in *Rhodospirillum rubrum* [12].

The aim of this work was to elucidate the possibility of formation of PP_i and of its utilization for the ATP synthesis in rat liver mitochondria.

2. Materials and methods

Mitochondria were isolated by means of a modified method of Schneider [13]. Their Lardi-Welman respiration coefficient was 3.0 to 3.5. ATP synthesis was determined in an acid extract of mitochondria with hexokinase and glucose 6-phosphate dehydrogenase [14]. To avoid possible formation of ATP from ADP with participation of myokinase, the incubation medium was supplemented with AMP. Therefore, to compare the rates of PP_i synthesis and phosphorylation with the participation of the adenylic system, not only ATP increment but also ADP formation were to be taken into consideration. To determine ADP, it was converted into incubating it with PEP and pyruvate-kinase [15]. PP_i was determined colorimetrically in the presence of β-mercaptoethanol and metabisulphite after three-fold extraction of orthophosphate in the from of a phosphomolebdate complex [16]. Inorganic orthophosphate was determined by the method of Behrenblum and Chain as modified by Weil-Malherbe and Green [17]. To identify PP_i it was precipitated in the form of barium salts from the acid extract of mitochondria and subjected to t.l.c. on DEAE cellulose and paper.

3. Results and discussion

As is shown in fig.1, rat liver mitochondria in the oxidative phosphorylation conditions synthesize, in addition to ATP, PP_i. A similar synthesis was detected in beef heart mitochondria. In our experiments, the content of ADP exceeded that of ATP on an average 3–4 fold, and phosphorylation involving the adenylic system was 5–10 times as intensive as the PP_i synthesis.

One could suggest that PP_i synthesis is associated with the functioning of mitochondrial electron transport chain. To verify this, we have studied the effect on the PP_i biosynthesis of 2,4-dinithrophenol, an uncoupler, and electron transport inhibitors at the level of the second and third coupling sites. As is seen in fig.2, dinitrophenol (4×10^{-4} M), antimycin (2 μg/mg protein) and NaCN (10^{-3} M) inhibited PP_i

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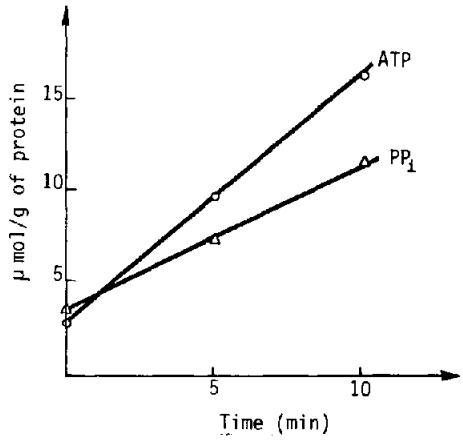


Fig.1. Synthesis of ATP and PP_i in rat liver mitochondria. Mitochondria were incubated at room temperature in the medium of the following composition: sucrose–0.25 M, Tris–HCl buffer pH 7.4–0.005 M, succinate–0.037 M, AMP–0.003 M, EDTA–0.0008 M, KH_2PO_4–0.0075 M, $MgSO_4$–0.03 M. The volume of the incubation medium –2 ml, protein–10-15 mg/ml. The reaction was interrupted by perchloric acid.

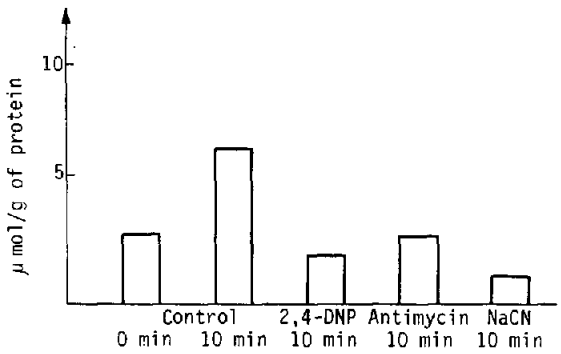


Fig.2. Effect of an uncoupler and respiratory chain inhibitors on PP_i synthesis. Incubation conditions are the same as in fig.1.

formation. In the mitochondria with uncoupled oxidation and phosphorylation (incubation in a sucrose-less medium), these compounds produced no effect on the pyrophosphatase and ATPase activities of mitochondria. The results obtained indicate that the synthesis of pyrophosphate is associated with the functioning of electron transport chain.

Two possible pathways of PP_i formation may be

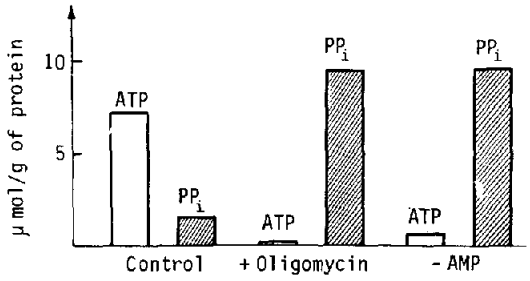


Fig.3. Synthesis of ATP and PP_i in the presence of oligomycin. Incubation conditions are the same as in fig.1. Time of incubation–10 min.

inferred from the above data. The first is hydrolysis of ATP to AMP and PP_i, and the second-an ATP-independent pathway. To choose between the two possibilities, the effect of oligomycin, and ATPase inhibitor, on PP_i synthesis was studied.

As is shown in fig.3, the action of oligomycin (1 μg/mg mitochondrial protein) induces stimulation of PP_i synthesis with the synthesis of ATP being inhibited. Similar results were obtained without AMP being added to the incubation medium. This testifies in favour of an independent formation of PP_i. The data obtained are in agreement with those of H. Baltscheffsky [12] on the effect of oligomycin on the PP_{in} synthesis in chromatophores. The data presented in fig.4 confirm this pathway. Preincubation of mitochondria in the presence of oligomycin, hexokinase and glucose in the absence of phosphate brought about disappearance of ATP and ADP and stabilization of the PP_i level. Further addition of phosphate stimulated ATP-independent PP_i synthesis.

In the laboratories of Baltscheffsky and Keister [1,3] it was demonstrated that inorganic pyrophosphatase is involved both in the biosynthesis and utilization of PP_i. We have detected inorganic pyrophosphatase to be present in a highly purified fraction of rat liver mitochondria. The activity was calculated per unit of cytochrome *a* which is known to be localized in mitochondria, and it was demonstrated that the pyrophosphatase activity of the cell is mostly localized in mitochondria. Electrophoresis in polyacrylamide gel revealed two isoenzymes to be present, of which at least one is firmly bound to the membrane. Similar data were previously reported by H. Baltscheffsky et al. [12] for pyrophosphatase

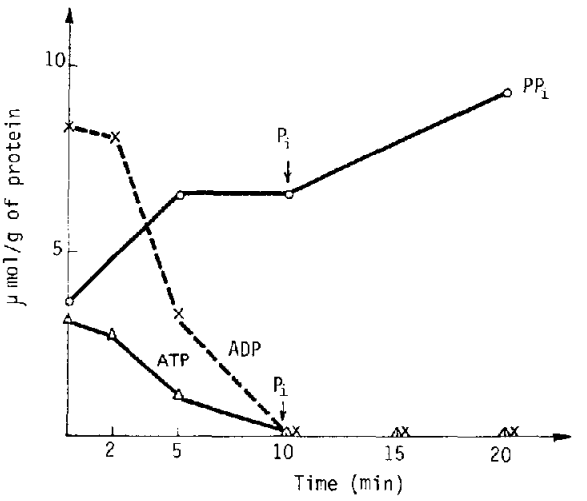


Fig.4. PP_i synthesis by ADP and ATP-exhausted mitochondria. The mitochondria were preincubated at room temperature with hexokinase (0.1 mg/ml medium), oligomycin (1 μg/mg protein) and glucose (4×10^{-2} M) in the medium of fig.1 but in the absence of phosphate. In 10 min KH_2PO_4 – 0.0025 M, was added.

from *R. rubrum* chromatophores and by M. Baltscheffsky [18] and Iria et al. [19] for pyrophosphatase from rat liver mitochondria.

A comparative analysis of the effect of various inhibitors on phosphatase and ATPase of mitochondria has shown that respiratory chain inhibitors (antimycin, sodium cyanide) produces no effect whatsoever of ATPase and pyrophosphatase. Oligomycin inhibited only ATPase and sodium fluoride at a concentration of

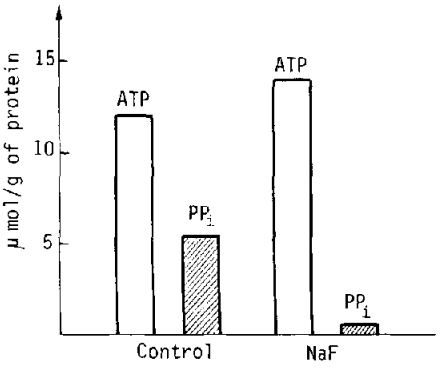


Fig.5. Effect of sodium fluoride on the mitochondrial ATP and PP_i synthesis. Incubation medium is the same as in fig.1. Concentration of NaF – 10^{-2} M. Time of incubation – 10 min.

10^{-2} M preferentially inhibited pyrophosphatase. It should be noted that NaF inhibits both ATPase and pyrophosphatase. But with 10^{-2} M NaF and 10–15 mg/ml mitochondrial protein pyrophosphatase is inhibited preferentially and ATPase- to a small extent. The pattern of inhibition of these two enzymes seems to be different. Our preliminary data may point to the fact that in the case of pyrophosphatase NaF not only inhibits the enzyme but also affects its interaction with the membrane. As is shown in fig.5, incubation of mitochondria in the conditions of oxidative phosphorylation with NaF at a concentration of 1×10^{-2} M selectively inhibits the synthesis of PP_i. The ATP synthesis is not thereby inhibited, it is even stimulated. These data should be interpreted as favouring the participation of pyrophosphatase of mitochondria in PP_i biosynthesis. It is appropriate to mention that Racker's coupling factor F_3 proved to display a pyrophosphatase activity [20].

Our data on the effect of oligomycin and NaF on the ATP and PP_i synthesis indicated that a common intermediate is involved in the biosynthesis of these compounds and the energy of electron transport may be consumed either for the synthesis of ATP or for that of PP_i.

To verify the possibility of ATP biosynthesis at the expense of PP_i, a series of experiments was performed in which the change in the ATP content in mitochondria was followed up during their incubation with and without PP_i. The relevant results are shown in fig.6. As is clear from the figure, in the presence of PP_i the intensity of ATP biosynthesis markedly increases. Special experiments were designed to demonstrate that an increased concentration of orthophosphate in the incubation medium did not affect the increment of ATP, and it was only the presence of PP_i that entailed an increase in the ATP concentration in the experimental conditions employed.

The respiratory chain inhibitor (NaCN) almost completely inhibited the ATP synthesis at the expense of oxidative phosphorylation. Addition of PP_i to the incubation medium in the same conditions led to an ATP synthesis. It is of interest that the difference in the amount of ATP synthesized in the presence and absence of PP_i and the quantity of ATP synthesized in the presence of PP_i and sodium

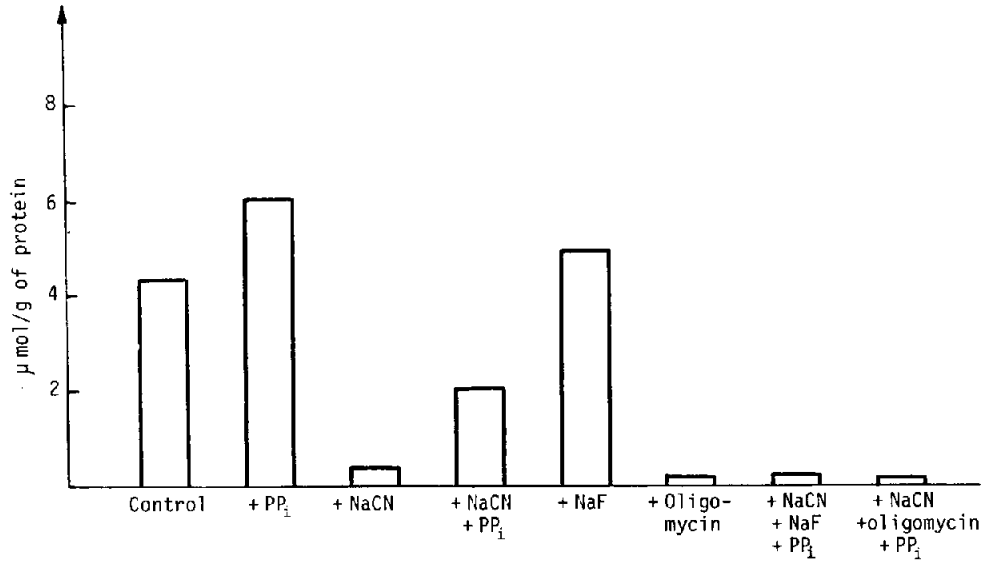
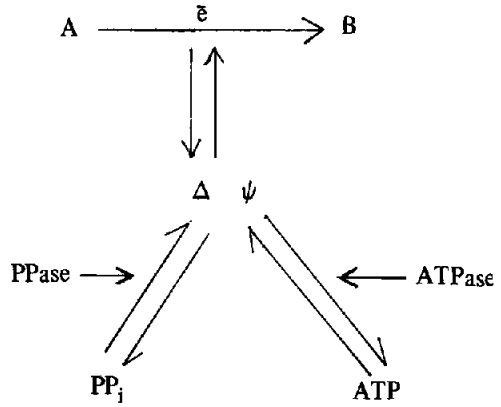


Fig.6. PP_i-dependent ATP synthesis. Incubation medium is the same as in fig.1. Concentration of added PP_i–1×10^{-3} M, NaCN–1×10^{-3} M, NaF–1×10^{-2} M, oligomycin–2 μg/mg protein. Time of incubation–10 min.

cyanide, i.e. at the expense of PP_i only, are rather close.

With the use of NaF and oligomycin against the background of cyanide, no PP_i-dependent ATP increment was observed. This means that in the PP_i-ATP reaction inorganic pyrophosphatase and ATPase are involved. There are grounds for believing that utilization of the PP_i energy for ATP synthesis is a process similar to that in *Rhodospirillum rubrum* chromatophores which proceeds according to the following scheme:



in which membrane electric potential should be the common source of energy [8,21].

Acknowledgements

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