MITOCHONDRIAL PYROPHOSPHATASE IS A COUPLING FACTOR OF RESPIRATION AND PYROPHOSPHATE SYNTHESIS

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1. Introduction

We have previously demonstrated that mitochondria of higher and lower eukaryotes are able to carry out synthesis of inorganic pyrophosphate (PP_i) coupled with electron transport [1–4]. The process has been shown to proceed concurrently with and independently of ATP synthesis and not to depend on ATPase activity.

The data on energy-dependent PP_i synthesis in mitochondria and light-dependent synthesis of this compound in *Rhodospirillum rubrum* chromatophores [5–7] suggest that the process involves membrane inorganic pyrophosphatase (EC 3.6.1.1).

In this work the reconstitution of PP_i synthesis system has been performed using pyrophosphatase-depleted beef heart submitochondrial particles and highly purified mitochondrial membrane pyrophosphatase. The data obtained suggest that membrane-bound pyrophosphatase is a coupling factor for electron transfer and PP_i synthesis in mitochondria.

2. Materials and methods

Beef heart mitochondria were isolated as described by Crane [8]. Submitochondrial particles capable of synthesizing PP_i were prepared according to Hansen and Smith [9] using milder sonication in an ATP-less medium [2]. ATP and PP_i synthesis were measured after incubation of mitochondria and submitochondrial particles (5–10 mg/ml) in a medium (2 ml) containing 0.25 M surcose, 5 mM Tris–HCl buffer, pH 7.4, 37 mM succinate 8 mM EDTA, 2.5 mM KH_2PO_4, 30 mM $MgCl_2$, for 10 min. To measure the rate of ATP synthesis, the medium was supplemented with 3 mM AMP. The reaction was stopped by addition of perchloric acid. ATP was determined using hexokinase and PP_i as described in [10].

The pyrophosphatase and ATPase activities were measured by orthophosphate release at 30°C in a medium containing 10 mM Tris–HCl buffer, pH 8.0 (for pyrophosphatase) and pH 7.5 (for ATPase), 4 mM $MgCl_2$, 1 mM $Na_4P_2O_7$ or ATP. The reaction was stopped by addition of perchloric acid. Inorganic phosphate was determined as described in [11], the protein content was measured by the Lowry procedure [12]. Submitochondrial particles were washed free of pyrophosphatase by suspending in 0.25 M sucrose solution, pH 7.5, and subsequent centrifugation.

Pyrophosphatase was extracted from acetone powder of mitochondria [13] by 10 mM Tris–HCl buffer, pH 7.4, containing 20 mM β-mercaptoethanol (standard buffer for isolation) using 1 ml buffer/20 mg powder. Further purification procedure is shown in table 3. Electrophoresis in 7.5% polyacrylamide gel was performed as in [14]. The samples were stained with amide black 10 B. To identify the bands with pyrophosphatase activities, the gels were placed in the above described medium and the orthophosphate formation was determined as described in [15]. The determination of the lipoprotein in the gel tubes was performed as suggested by Ressler et al. [16] for agar blocks.

3. Results

During the preparation of PP_i-synthesizing submitochondrial particles it was noted that pyrophosphatase is bound to the membrane less tightly than ATPase. The activity of pyrophosphatase in the

Table 1
The effect of sonication on PP_i synthesis and pyrophosphatase activity of submitochondrial particles

Time (min)	ATPase activity (μmoles/g protein·min)	Pyrophosphatase activity (μmoles/g protein·min)	PP_i synthesis (μmoles/g protein·min)
1	98	4.25	0.53
1.5	95	1.16	0.43
2	92	0.56	0.00

particles depends on the intensity and duration of sonication. Thereby a correlation was found to exist between the pyrophosphatase activity in submitochondrial particles and their ability to synthesize PP_i (table 1).

When particles were washed with 0.25 M sucrose their ATPase and ATP-synthesizing activities were retained, whereas the PP_i synthesis decreased parallel to the decrease in the pyrophosphatase activity (table 2).

The data presented in tables 1 and 2 indicate that mitochondrial pyrophosphatase participates in the biosynthesis of PP_i. This fact is supported by the data on the effect of NaF, a pyrophosphatase inhibitor (1×10^{-2} M) [3,4] on PP_i synthesis. A more specific inhibitor of the enzyme, imidodiphosphate [17,18] was without effect because it was rapidly hydrolysed by mitochondria phosphatases. To obtain a more conclusive result, a different approach was used, namely reconstitution of PP_i synthesis using submitochondrial particles with no pyrophosphatase activity ('washed' particles) and highly purified pyrophosphatase from beaf heart mitochondria.

For this purpose, pyrophosphatase was isolated and purified as shown in table 3. Electrophoresis of the mitochondrial homogenate in polyacrylamide gel showed it to contain two isozymes of pyrophosphatase. One of those could be readily solubilized (soluble, pyrophosphatase I) and the other could be extracted from mitochondria only after sonication or treatment with detergents (membrane, pyrophosphatase II). Treatment of mitochondria with acetone facilitated extraction of the membrane isoenzyme. Gel filtration on Sephadex G-150 (a 3.5 X 80 cm column, elution with a standard buffer) and ion-exchange chromatography at pH 7.4 and 6.7 (a 1.5 X 20 cm column, elution with a linear NaCl gradient in a standard buffer) gave two highly purified preparations of mitochondrial pyrophosphatases. Pyrophosphatase I was purified 200-times and pyrophosphatase II 246-times. On being developed with amide black 10 B produced one band.

Table 2
Dependence of PP_i synthesis by submitochondrial particles on the presence of pyrophosphatase

Activity (μmoles/g protein·min)	Control particles	'Washed' particles
ATPase	92.0	98.0
ATP synthesis	4.0	3.6
Pyrophosphatase	10.0	2.8
PP_i synthesis	0.8	0.0

Both isoenzymes were found to be extremely labile and their activities could be stabilized by SH-containing compounds. Both pyrophosphatases have a high specificities with respect to PP_i, have mol. wt 75 000 and activity optima at pH 8.0; they are both Mg^{2+}-dependent. It is interesting that all energy-generating cellular organelles are known to have two pyrophosphatases [5,19–22]. Rat liver mitochondria were also reported to have two pyrophosphatases with similar properties [19]. It seemed reasonable that one of these isoenzymes is involved in the energy-dependent PP_i synthesis. To verify this hypothesis, reconstitution of the PP_i synthesis system was performed using each of the enzymes obtained.

To reconstitute PP_i synthesis, the 'washed' particles were supplemented with purified preparations of mitochondrial pyrophosphatases (10–30 μg of enzyme protein were added to 1 mg particle protein). The particles and the enzyme were incubated in 0.25 M sucrose, pH 7.4, with succinate (1 mM) in a minimal volume for 20 min in the cold; the suspension was

Table 3
Purification of pyrophosphatases from beaf heart mitochondria

Fractions	Volume (ml)	Protein (mg)	Specific activity (μmoles/g protein·min)	Total activity (μmoles/min)	Degree of purity	Yield (%)
Mitochondria	200	12 000	50	600.0	1.0	100
Acetone powder extract	450	900	500	450.0	10	75
Precipitate (40–60% $(NH_4)_2SO_4$ saturation)	2.5	225	910	205.0	18.2	34
Gel-filtration G-150	87	61	1100	71.5	22	11.8
Ion-exchange chromatography DEAE-cellulose, pH 7.4	33	7.6	4900	37.0	98	6.35
Ion-exchange chromatography DEAE-cellulose, pH 6.7; Pyrophosphatase I	24	1.2	10 000	12.0	200	2.0
Pyrophosphatase II	15	2.0	4720	9.2	94	1.5
Gel-filtration G-150 Pyrophosphatase II	38	0.38	12 300	4.7	246	0.78

Table 4
Reconstitution of the PP_i synthesis system in submitochondrial particles of beaf heart mitochondria

Activity (μmoles/g protein·min)	Control particles	'Washed' particles	'Washed' particles + pyrophosphatase I	'Washed' particles + pyrophosphatase II	'Washed' particles + pyrophosphatase I succinate	'Washed' particles + pyrophosphatase II succinate
PP_i synthesis	0.27	0.05	0.07	0.37	0.04	0.49
Pyrophosphatase activity	10.7	2.5	2.7	3.8	2.8	3.9

then sedimented in a 10-fold volume of the medium. As seen from table 4, the incubation of the 'washed' particles with pyrophosphatase I and pyrophosphatase II following sedimentation, to remove the excess enzyme, resulted in the restoration of PP_i synthesis when pyrophosphatase II was added [23].

Reconstitution was more effective in the presence of succinate and was not observed in the presence of PP_i (1 mM). The reconstituted particles usually synthesized PP_i more intensively than the original ones.

It was evident from the data obtained that mitochondrial pyrophosphatases are similar in a number of properties, but differ in their ability to incorporate into the membrane. A possible explanation for this phenomenon is that pyrophosphatase II may have an additional component which facilitates incorporation of the enzyme into membrane. A special analysis showed that pyrophosphatase II, unlike pyrophosphatase I, is indeed a lipoprotein. Thus the ability of pyrophosphatase II to incorporate into membrane may well be due to the presence of a lipid component.

The conclusion following from the experimental data reported is that the membrane-bound pyrophosphatase can serve as a coupling factor for electron-transport driven PP_i synthesis in mitochondria.

References

[1] Mansurova, S. E., Shakhov, Yu. A. and Kulaev, I. S. (1973) Dokl. Akad. Nauk SSSR 213, 1207–1209.
[2] Shakhov, Yu. A., Mansurova, S. E. and Kulaev, I. S. (1974) Dokl. Akad. Nauk SSR 219, 1017–1019.
[3] Mansurova, S. E., Shakhov, Yu. A. and Kulaev, I. S. (1973) FEBS Lett. 55, 94–98.
[4] Mansurova, S. E., Ermakova, S. A., Zvyagilskaya, R. A. and Kulaev, I. S. (1975) Mikrobiologia 44, 874–879.
[5] Baltchefsky, M., Baltcheffsky, H. and van Stedinkg, L.-V. (1966) Brookhaven Symp. Biol. 19, 246–257.
[6] Keister, D. L. and Minton, N. J. (1971) Arch. Biochem. Biophys. 147, 330–338.
[7] Nishikawa, K., Hosoi, K., Suzuki, J., Yoshimura, S. and Horio, T. (1973) J. Biochem. 73, 537–553.
[8] Crane, F. L., Clenn, C. L. and Green, D. E. (1956) Biochim. Biophys. Acta 22, 475–487.
[9] Hansen, M. and Smith, A. (1964) Biochim. Biophys. Acta 81, 214–222.
[10] Grindey, G. B. and Nichol, C. A. (1970) Anal. Biochem. 33, 114–119.
[11] Berenbleem, J. and Chain, E. (1938) Biochem. J. 32, 295–298.
[12] Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Selvyn, M. L. (1967) Biochem. J. 105, 279–288.
[14] Davis, B. J. (1964) Ann. NY Acad. Sci. 121, 404–427.
[15] Sugine, J. and Migoshi, Y. (1964) J. Biol. Chem. 239, 2360–2364.
[16] Ressler, N., Springgate, R. and Kaufman, J. (1961) J. Chromatogr. 6, 409–415.
[17] Sperow, J. M., Moe, O. A., Ridlington, J. W. and Butler, L. G. (1973) J. Biol. Chem. 248, 2062–2065.
[18] Kelly, S. J., Feldman, F., Sperow, J. W. and Butler, L. G. (1973) Biochemistry 12, 3338–3343.
[19] Iria, M., Yabuta, A., Kimura, K., Shindo, Y. and Tomita, K. (1970) J. Biochem. 67, 47–58.
[20] Umnov, A. M., Egorov, S. N., Mansurova, S. E. and Kulaev, I. S. (1974) Biokhimia 39, 373–376.
[21] Gould, J. M. and Winget, C. D. (1973) Arch. Biochem. Biophys. 154, 606–613.
[22] Klemme, J.-H. and Gest, H. (1971) Proc. Natl. Acad. Sci. USA 68, 721–725.
[23] Mansurova, S. E. and Shakhov, Yu. A. (1976) Abstr. X. Intern. Congr. Biochem. Hamburg. p. 336.