BIOLOGIA PLANTARUM (PRAHA)

15 (4) : 241—249, 1973

Study of Oxidative and Phosphorylative Activity in Mitochondria from Cereal Seedlings

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Received June 16, 1972

Abstract. In the present paper the oxidative and phosphorylative activity of mitochondria isolated from rye, wheat, barley and corn seedlings are compared. Mitochondria from the shoots as well as from the roots of rye, wheat and corn oxidized succinate and in the presence of ATP or ADP exhibited the respiratory control which reached the values mostly of about 2. In the presence of ATP or ADP the decrease of inorganic phosphorus was contemporarily remarcable. The P/O ratio reached the values mostly of about 0.8 up to 1.0. The presence of ATP effected in some cases more favorable the respiratory control as well as the P/O ratio in comparison with ADP. With regard to the fact that a trapping hexokinase system was not added, the presence of endogenous hexokinase in mitochondria of experimental plants is presumed. The barley mitochondria exhibited the respiratory control as well, but instead of the decrease in inorganic phosphorus content in reaction mixture, an increase took place. It was caused by the presence of active ATP-ase which was not effectively inhibited by present NaF.

A series of papers is reported in contemporary literature dealing with isolation of mitochondria from plant tissues and with examination of their activity. The aim of many modifications in isolating methods is to obtain active, undammaged mitochondria characterised by tightly coupled oxidative and phosphorylative processes and displaying high respiratory control.

The results of many experiments show that the contemporary methods of isolation make it possible to obtain active mitochondria from some suitable plant tissues or plant organs — for instance from the tubers of white potatoes and sweet potatoes, from the pulp of some fruits, from the shoots of some seedlings *etc.* Many other plant tissues especially the more senescent ones are less suitable or unsuitable for isolation of highly active mitochondria by contemporary methods of isolation. This may be caused either by specific properties of plant tissues or by specific requirements in the isolating method. In our paper we are interested in verifying the isolating method of mitochondria from plant seedlings of some cereals and in comparing their oxidative and phosphorylative activity by succinate oxidation.

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Material and Methods

Plant Material

The seedlings of barley (*Hordeum distichon* L. cv. Branišovský), wheat (*Triticum vulgare* L. cv. Zlatka), rye (*Secale cereale* L. cv. Těšovské) and corn (*Zea mays* L. cv. Kočovská raná) were used in the experiments. The grains of the mentioned plants were germinated on moistened filter paper in Petri-dishes in the thermostat at 25 °C. After three days the shoots and the roots of the seedlings were cut, washed by distilled water and used for isolation of mitochondria.

Isolation of Mitochondria

Plant tissue (10 g) was ground in a mortar with quartz sand and with isolating medium (40 ml) in a cold room at 2 °C. As isolating medium 0.5 M sucrose in 0.1 M Tris-HCl buffer pH 7.3 with addition of 0.005 M EDTA and 0.1% serum albumine was used. Homogenat was squeezed through four layers of sterile gauze and centrifuged at 2 500 *g* for 5 min in a cooled centrifuge. The pellet was discarded and supernatant was again centrifuged in cooled ultracentrifuge at 20 000 *g* for 20 min. The pellet obtained was resuspended in 10 ml washing medium with the same composition as the mentioned isolating medium only with a small difference; the concentration of Tris-HCl buffer was lowered to 0.01 M. After a further centrifugation at 20 000 *g* for 15 min. the supernatant was discarded, the sediment was resuspended in the later medium and used in the experiments as mitochondrial fraction. The final sediment was every time resuspended in such volume of media that the amount of mitochondrial proteins reached 2 to 4 mg per ml of suspension (Raison and Lyons 1970).

Estimation of Oxidative and Phosphorylative Activity of Mitochondria

The oxidative activity of mitochondria was determined manometrically from O_2 consumption by succinate oxidation. The phosphorylative activity of mitochondria was determined from inorganic phosphorus decrease in reaction mixture. The complete reaction mixture contained 0.80 ml 1 M sucrose, 0.10 ml 1 M succinate, 0.06 ml 1 M glucose, 0.40 ml 0.1 M K-phosphate buffer at pH 7.3, 0.10 ml 0.1 M $MgSO_4$, 0.03 ml 1 M NaF, 0.10 ml ATP or ADP in concentration 20 mg/ml or their mixture, 0.10 ml serum albumine in concentration 10 mg/ml and 0.10 ml cytochrome C in concentration mg/ml. The final volume of reaction mixture was adjusted to 2.5 ml by distilled water. 0.2 ml 10% KOH solution was added separatelly into the center well for CO_2 absorption. The reaction was started at zero time by addition of 0.5 ml mitochondrial suspension containing 1 to 2 mg of proteins. The reaction took place for 60 min. at 25 °C and thereafter it was stopped by addition of 1.5 ml 10% TCA. Into the control vessels TCA was added before mitochondrial suspension. After stopping the enzyme reactions the content of inorganic phosphorus (Pi) was determined in reaction mixture. The oxidative and phosphorylative activity of mitochondria was expressed in µl O_2 consumption and µg Pi decrease per mg proteins during 60 min. The respiratory control (RC) was expressed as the ratio of mitochondrial respiration intensity in the presence of ATP, ADP or their mixture to respiration intensity in their absence. Oxidative phosphorylation was expressed by P/O ratio, it means by the ratio of inorganic phosphorus decrease in µatoms to O_2 consumption in µatoms per mg proteins during 60 min.

The ATP-ase activity was determined from the inorganic phosphorus amount released from ATP or ADP in reaction mixture. Mitochondrial fraction was in this case prepared by homogenisation of the tissue in 0.05 M Tris-HCl buffer at pH 7.6 and following centrifugation in the above mentioned way. The sediment of mitochondria was resuspended in the same buffer and the suspension was used for the determination of enzyme activity. The reaction mixture consisted of 0.5 ml buffer mentioned above, 0.1 ml 0.05 M $MgSO_4$ and 0.2 ml ATP or ADP solution in concentration 20 mg/ml. The reaction was started by adding 0.2 ml of mitochondrial suspension and took place for 30 min. at 25 °C. The enzyme activity was expressed in µg inorganic phosphorus released per mg mitochondrial proteins during 30 min.

The measurements of mitochondrial activity were carried out immediatelly after isolating procedure. Bidistilled water in glass was used for preparation of all solutions. Inorganic phosphorus was determined colorimetrically by the method of Skulatchev (1962). Protein content in mitochondrial suspension was determined also colorimetrically by the reaction with Folin reagent (Lowry *et al.* 1951). All measurements were done every time in three parallels at least and the results presented were verified by several repetitions of all experimental variants. The values in the tables are the averages of three repetitions at least.

Results

In the presence of ATP and succinate, mitochondria from rye seedlings showed oxidative and phosphorylative activity. Both processes were a little higher in mitochondria from overground parts than in mitochondria from roots. In the absence of ATP, the phosphorylative activity was not demonstrable and the oxidative activity decreased remarkable. In the absence of succinate, the phosphorylative activity was not demonstrable either and the oxidative activity reached a very small values. In the presence of succinate

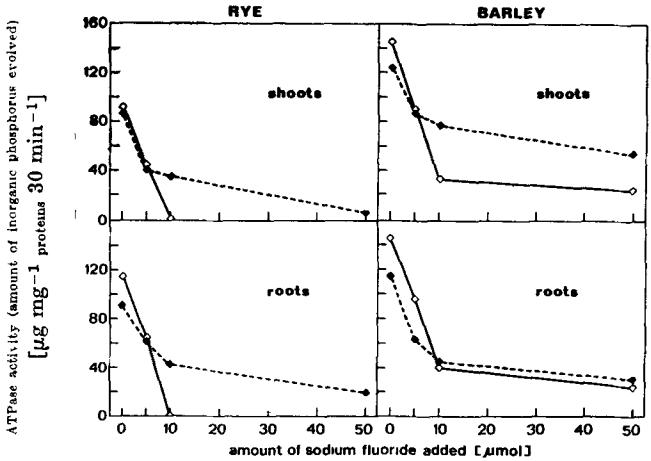

Fig. 1. Inhibition of activity of mitochondrial ATP-ase from overground parts and roots of barley and rye seedlings by NaF in the absence (dashed line) and in the presence (full line) of inorganic phosphorus in reaction mixture. Enzyme activity is expressed in μg inorganic phosphorus released per mg proteins during 30 min. Abscissa: amount of NaF added in μmoles. Ordinate: amount of inorganic phosphorus released in μg.

and ADP, oxidative and phosphorylative activity in mitochondria from both parts of rye was established a little lower than in the previous variant with ATP. In the presence of both substances — ATP and ADP — in reaction mixture, the established values of oxidative and phosphorylative activity were nearly the same as in the presence of ATP alone (Table 1).

Mitochondria from wheat seedlings exhibited in the presence of ATP and succinate an oxidative and phosphorylative activity similar to that of mitochondria from rye seedlings. If ATP in reaction mixture was replaced by ADP, the phosphorylative activity in shoots was established a little higher, in roots on the contrary a little lower. Oxidative activity changed in the same manner. In the presence of ATP and ADP in reaction mixture, the oxidative and phosphorylative activity of mitochondria from shoots increased a little more compared to both previous cases (Table 2).

Mitochondria from corn seedlings exhibited in the presence of succinate and ATP oxidative as well as phosphorylative activity. Both processes were approximately of the same intensity in the shoots and in the roots. Very similar results were obtained also in the case when ADP was used instead of ATP or when both substances were added into reaction mixture (Table 3).

TABLE 1

DETERMINATION OF OXIDATIVE AND PHOSPHORYLATIVE ACTIVITY of mitochondria from rye seedlings in dependence on the presence of succinate, ATP and ADP

Number of experiment	Treatment	O_2 consumption [µl/mg proteins]		Pi decrease [µg/mg proteins]		RC		P/O	
		Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
1	Succinate, ATP	114	60.5	265	135	2.10	2.01	0.84	0.81
	Succinate —	54.2	30.0	—	—	—	—	—	—
	— ATP	4.0	6.1	—	—	—	—	—	—
2	Succinate, ADP	80.1	39.3	195	68.5	1.76	1.46	0.88	0.63
	Succinate —	45.5	26.9	—	—	—	—	—	—
3	Succinate, ATP, ADP	102	56.1	256	130	1.97	2.04	0.91	0.84
	Succinate — —	52.0	27.6	—	—	—	—	—	—

TABLE 2

DETERMINATION OF OXIDATIVE AND PHOSPHORYLATIVE ACTIVITY of mitochondria from wheat seedlings in dependence on the presence of succinate, ATP and ADP

Number of experiment	Treatment	O_2 consumption [µl/mg proteins]		Pi decrease [µg/mg proteins]		RC		P/O	
		Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
1	Succinate, ATP	53.8	39.2	122	103	1.70	1.56	0.82	0.94
	Succinate —	31.6	25.4	—	—	—	—	—	—
	— ATP	2.4	9.4	—	—	—	—	—	—
2	Succinate, ADP	72.8	31.1	191	41.6	1.46	1.22	0.95	0.48
	Succinate —	49.9	25.4	—	—	—	—	—	—
3	Succinate, ATP, ADP	95.0	34.8	266	80.8	2.50	1.67	1.01	0.84
	Succinate — —	38.1	20.8	—	—	—	—	—	—

In the mitochondria of barley seedlings, the oxidative activity was established in the presence of ATP and succinate, the phosphorylative activity, however, was not proved. Inorganic phosphorus, on the contrary, increased in reaction mixture. In the absence of ATP, the content of inorganic phosphorus did not change and the oxidative activity slightly decreased. In the absence of succinate, the oxidative activity decreased markedly, inorganic phosphorus, however, increased in reaction mixture again. In the absence of NaF, very similar results were obtained only with the difference that the increase of inorganic phosphorus in reaction mixture in the variants where ATP was present was higher (Table 4). When ATP was supplied by means of ADP, analogous results to the previous ones were mostly obtained (Table 5).

By examining the inhibition effect of NaF on mitochondrial ATP-ase activity, it was established that not even a five time increase in NaF concentration above the used amount (10 μM) brought about the total inhibition of the activity of enzyme (Fig. 1). The effect of inhibition increased in the presence of phosphate added in the same concentration as in the case of determination of oxidative phosphorylation. Sodium fluoride forms in the presence of phosphate a complex of magnesium fluorophosphate (WARBURG and CHRISTIAN 1942). In the case of barley ATP-ase even then, however, the inhibition was not total.

In the mitochondria of barley and rye, the presence of enzymes liberating phosphate from ATP as well as from ADP was demonstrable. The activity of these enzymes was in barley plants significantly higher than in rye plants and it increased a little with the increasing amount of ATP or ADP present. By diminishing both substances in reaction mixture up to limit amount it was observable that mitochondria from barley, and in the case of ATP, mitochondria from rye as well, liberated higher amount of phosphorus than was the theoretically calculated amount for liberating one molecule of phosphate (Table 6).

Discussion

Mitochondria of all four species of cereals oxidized succinate. The oxidation increased foundamentally in the presence of ADP as well as ATP. Respiratory control reached the maximum values of about 2. In all cases, with the exception of barley mitochondria, a decrease of inorganic phosphorus in reaction mixture occured. This is evidence that isolated mitochondria were active and that the oxidation of succinate was coupled with phosphorylation.

The presence of ATP in reaction mixture had in comparison with ADP the same and sometimes even higher effect on oxidative phosphorylation of mitochondria. The presence of hexokinase is necessary for the conversion of ATP to ADP which is the phosphate acceptor by oxidative phosphorylation (BONNER and MILLERD 1953). With regard to the fact that hexokinase was not added into the reaction mixture, it is necessary to assume that mitochondria of tested plants contained an endogenous hexokinase system. Evidence of hexokinase present in plant mitochondria was given also by other authors (MILLERD *et al.* 1951, SALTMAN 1953). BONNER and MILLERD (1953) when studying hexokinase activity in mitochondria from bean hypocotyles have found that the enzyme activity was sufficiently high, so that it

TABLE 3

DETERMINATION OF OXIDATIVE AND PHOSPHORYLATIVE ACTIVITY of mitochondria from corn seedlings in dependence on the presence of succinate, ATP and ADP

Number of experiment	Treatment	O_2 consumption [µl/mg proteins]		Pi decrease [µg/mg proteins]		RC		P/O	
		Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
1	Succinate, ATP	71.1	66.6	174	175	1.79	1.78	0.88	0.95
	Succinate —	39.7	37.2	—	—	—	—	—	—
	— ATP	2.1	4.2	—	—	—	—	—	—
2	Succinate, ADP	61.9	75.5	188	169	1.71	1.76	1.10	0.81
	Succinate —	36.2	42.8	—	—	—	—	—	—
3	Succinate, ATP, ADP	76.8	73.6	186	172	2.05	1.65	0.87	0.84
	Succinate — —	37.4	44.6	—	—	—	—	—	—

TABLE 6

COMPARISON OF INTENSITY OF PHOSPHATE release from ATP and ADP by mitochondrial ATP-ase from overground parts of barley and rye seedlings. ATP and ADP were added to reaction mixture in increasing concentration. The release intensity is expressed in µg phosphorus released during 30 min. by an amount of 0.33 mg proteins of mitochondrial fraction

Added amount of ATP or ADP in µg	Theoretical amount of Pi in µg by releasing one molecule of phosphate from		ATP-ase activity of barley mitochondria µg Pi released from		ATP-ase activity of rye mitochondria µg Pi released from	
	ATP	ADP	ATP	ADP	ATP	ADP
4000	200	276	52.5	86.5	31.3	26.5
2000	100	138	48.2	63.7	34.5	23.4
1000	50	69	43.1	52.2	40.0	23.1
500	25	35	34.8	43.4	34.8	19.1

TABLE 4

DETERMINATION OF OXIDATIVE AND PHOSPHORYLATIVE ACTIVITY of mitochondria from barley seedlings in dependence on the presence of succinate, ATP and NaF. The increase of inorganic phosphorus in reaction mixture is indicated +

Number of experiment	Treatment		O_2 consumption µl/mg proteins		Pi change µg/mg proteins		RC	
			Shoots	Roots	Shoots	Roots	Shoots	Roots
1	NaF	Succinate, ATP	30.5	36.8	+35.1	+46.7	1.10	1.50
		Succinate —	27.4	23.7	—	—	—	—
		— ATP	2.8	5.9	+43.5	+59.0	—	—
2	—	Succinate, ATP	67.4	70.1	+80.0	+68.0	1.49	1.79
		Succinate —	45.1	39.1	—	—	—	—
		— ATP	3.8	4.5	+82.4	+82.4	—	—

TABLE 5

DETERMINATION OF OXIDATIVE AND PHOSPHORYLATIVE ACTIVITY of mitochondria from barley seedlings in dependence on the presence of succinate, ADP and NaF. The increase of inorganic phosphorus in reaction mixture is indicated +

Number of experiment	Treatment		O_2 consumption µl/mg proteins		Pi change µg/mg proteins		RC	
			Shoots	Roots	Shoots	Roots	Shoots	Roots
1	NaF	Succinate, ADP	28.0	32.1	+52.3	+48.4	0.93	1.09
		Succinate —	30.0	29.3	—	—	—	—
		— ADP	6.2	3.9	+63.2	+82.4	—	—
2	—	Succinate, ADP	50.2	67.2	+120	+114	1.62	1.64
		Succinate —	30.8	40.8	—	—	—	—
		— ADP	10.8	12.9	+116	+106	—	—

was in no case a limiting factor of oxidative phosphorylation. The same or even higher effect of ATP in comparison with ADP on succinate oxidation and on coupled phosphorylation is evidence that in mitochondria of plant tissues used in our experiments hexokinase in a sufficiently active state was present as well. A higher effect of ATP on succinate oxidation in comparison with ADP is explained by removing the inhibition effect of the endogenous oxalacetate on succinate oxidation (WISKICH and BONNER 1963, WISKICH 1967).

The respiratory control is one of the criteria of a convenient isolation of mitochondria because it shows the degree of the coupling of oxidative and phosphorylative processes (WISKICH and BONNER 1963, LEHNINGER 1965, RAISON and LYONS 1970). The value of respiratory control ratio for succinate in comparison with other substrates is relatively small. For mitochondria from a number of plant tissues (white potatoes, sweet potatoes, cucumber fruits, beet root, cauliflower buds and others) the value was determined mostly round about 2 to 3 (WISKICH and BONNER 1963, LYONS and RAISON 1970). The values of respiratory control determined in our experiments for mitochondria from cereal seedlings were similar or slightly lower. The P/O ratio determined in our experiments reached mostly the values round about 0,8 and less. In comparison with a theoretical value which reaches for succinate number 2, the values obtained were relatively small. The values of P/O or ADP/O ratio determined for mitochondria of a number of plant tissues are mostly lower in a different degree as against theoretical values, which is no doubt evidence of partial uncoupling of oxidative and phosphorylative processes (WISKICH and BONNER 1963, IKUMA and BONNER 1967, TIMASHOV 1968, LYONS and RAISON 1970).

In the case of barley mitochondria it was not possible to determine the P/O ratio at all, as the activity of present ATP-ase was not inactivated enough by the action of NaF added. Contrary to our results TIMASHOV (1968) established the values of P/O ratio in mitochondria from barley roots round about 1.0 to 1.6. As a particularity he used in this case isolating medium with 0.9 M sucrose solution. In our experiments we were not successful even with this isolating medium in the determination of oxidative phosphorylation in barley mitochondria. The explanation of these differences is possible to find perhaps in the specificity of metabolism in different varieties of barley.

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F. PLHÁK, Přírodovědecká fakulta University J. E. Purkyně v Brně: **Studium oxidační a fosforylační aktivity mitochondrií klíčních rostlin obilovin.** — Biol. Plant. **15** : 241—249, 1973.

V práci je porovnána oxidační a fosforylační aktivita mitochondrií klíčních rostlin žita, pšenice, ječmene a kukuřice. Mitochondrie z nadzemních částí i kořenů žita, pšenice a kukuřice oxidovaly jantaran a v přítomnosti ATP či ADP vykazovaly respirační kontrolu, která dosahovala hodnot většinou kolem 2. V přítomnosti ATP či ADP byl současně patrný úbytek anorganického fosforu. Vyčíslený P/O kvocient dosahoval hodnot většinou kolem 0,8 až 1,0. Přítomnost ATP měla v některých případech positivnější účinek na oxidační kontrolu i na P/O kvocient ve srovnání s ADP. Vzhledem k tomu, že do reakční směsi nebyl přidáván záložní hexokinázový systém, svědčí to o přítomnosti vlastní hexokinázy v mitochondriích testovaných rostlin. Mitochondrie ječmene rovněž vykazovaly respirační kontrolu, avšak místo úbytku anorganického fosforu v reakční směsi byl patrný jeho přírůstek. To bylo způsobeno přítomností aktivní ATP-ázy, která nebyla dost účinně inhibována přidaným NaF.