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The outer membrane of plant mitochondria contains a calcium-dependent protein kinase and multiple phosphoproteins

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Highly purified mitochondria from potato (*Solanum tuberosum* L. cv. Bintje) tubers were subfractionated into a matrix fraction, an inner membrane fraction and an outer membrane fraction with minimal cross-contamination. When the matrix and inner membrane fractions were incubated with [γ-^{32}P]ATP only one and three prominent phosphoproteins were detected after SDS-PAGE and autoradiography, respectively. In contrast, more than 20 phosphoproteins could be labelled in the outer membrane fraction, the main ones at 12, 18, 26, 43, 58, 60, 65, 74 and 110 kDa. Only one band, at 18 kDa, was detectable when the labelling was done in the presence of EGTA. We conclude that the outer membrane of plant mitochondria contains at least one Ca^{2+}-dependent protein kinase and more than 20 endogenous substrates.

Plant mitochondrion; Protein phosphorylation; Calcium; Outer mitochondrial membrane; Inner mitochondrial membrane

1. INTRODUCTION

Reversible protein phosphorylation by protein kinases/phosphoprotein phosphatases is a universal mechanism in eukaryotes for modulating the activity of intracellular proteins in response to extracellular signals. However, much information is still missing about how plant cells sense and transduce extracellular signals into physiological responses. Little is known about the regulation of protein phosphorylation in intact plant cells and in its subcellular compartments [1].

We have shown that plant mitochondria contain at least 3–4 protein kinases and multiple phosphoproteins [2–4], in agreement with the situation in yeast mitochondria [5] and possibly also mammalian mitochondria [6]. In this communication we show that Ca^{2+}-dependent protein kinase(s) as well as most of the phosphoproteins are specifically localized to the outer membrane of plant mitochondria. Very little is known about the outer membrane and its function in plant mitochondria. The only known enzyme marker is the antimycin A-insensitive NADH-cytochrome *c* reductase (CCR). Other markers for the outer membrane characteristic for mammalian mitochondria, such as monoamine oxidase and kynurenine hydroxylase, appear to be missing [7,8]. The outer membrane is normally considered to be freely permeable to all molecules smaller than 5–10 kDa, probably due to the presence of pores [9], and for that reason it is often ignored in studies on isolated mitochondria. However, the outer membrane clearly has a role in protein import [10] and if its permeability is regulated it could also have a profound influence on the mitochondrial metabolism. The presence of protein kinases and phosphorylatable proteins in the outer membrane opens up the possibility that outer membrane-localized events are regulated by protein phosphorylation and thus susceptible to regulation by intracellular messengers. This indicates that the mitochondrion is indeed part of the intricate regulatory network of the plant cell.

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Abbreviations: CCO, cytochrome *c* oxidase; MDH, NAD^+-dependent malate dehydrogenase; CCR, antimycin A-insensitive NADH-cytochrome *c* reductase.

2. MATERIALS AND METHODS

2.1. *Materials*

Carrier-free [^{32}P]phosphate was obtained from Amersham. [γ-^{32}P]ATP was synthesized according to Chang et al. [11]. Chemicals for SDS-PAGE were from Bio-Rad. All other chemicals were from Sigma or Boehringer Mannheim.

2.2. *Isolation of mitochondria and submitochondrial fractions*

Crude mitochondria were isolated from potato tubers (*Solanum tuberosum* L. cv. Bintje) essentially as in [12] and the purification was performed as in [13].

The outer membrane of the purified mitochondria was osmotically ruptured by quickly adding 6 ml of ice-cold dilution buffer (1 mM KH_2PO_4, 3 mM EDTA or 5 mM KH_2PO_4, pH 7.2) to 400 μl of concentrated (70–100 mg protein/ml) purified mitochondria resuspended in wash medium (0.3 M mannitol, 1 mM EDTA and 10 mM MOPS, pH 7.2). The final osmolarity was 20–25 mOsm. After 6 min the solution was centrifuged at 10,000 × *g* for 15 min. The (mitoplast) pellet, containing intact and broken mitochondria in addition to mitoplasts, was resuspended in wash medium and the supernatant was recentrifuged at 48,000 × *g* for 15 min to remove any residual broken mitochondria.

The matrix fraction was isolated by sonicating (MSE Sonicator,

5 × 5 s at amp 1, high power) the resuspended mitoplast pellet. The membranes were pelleted either at 105,000 × *g* or 300,000 × *g* for 60 min. The resulting supernatant constitutes the matrix fraction.

The outer membrane was isolated by centrifuging the mitoplast supernatant at 300,000 × *g* for 1 h. The resulting pellet was sonicated (3 × 5 s at amp 1, high power) and repelleted as above to remove any adsorbed proteins.

The inner membrane fraction was produced essentially as in [14]. The inner membrane was pelleted at 300,000 × *g* for 60 min instead of 105,000 × *g* for 60 min. The inner membrane pellet was further sonicated (3 × 5 s at amp 1, high power) and repelleted as above.

2.3. *Enzyme assays*

Cytochrome *c* oxidase (EC 1.9.3.1) and NAD^+-malate dehydrogenase (EC 1.1.1.37) activities and latencies were assayed according to Rasmusson and Møller [15] and Møller et al. [16], respectively, and antimycin A-insensitive NAD(P)H-cytochrome *c* reductase activity was measured essentially as in [17].

2.4. *Protein kinase analyses*

The protein kinase assay was as in [2] except that the buffer was 50 mM HEPES-KOH (pH 7.5). Phosphorylation of histones was performed in 50 μl containing (final concentrations): 100 mM HEPES-KOH (pH 6.5), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 0.2 mM [γ-^{32}P]ATP (500 cpm/pmol), 10 μg histone H1 (type III-S, Sigma) and 40–80 μg sample protein. Incubation time was 2 min at room temperature (22–23°C). The reaction was stopped and the filter paper washed as in [2].

2.5. *SDS-PAGE*

Samples were prepared as described by Sommarin et al. [2] and SDS-PAGE performed according to Laemmli [18] on a 10–16% T (C 2.7%) gradient gel.

2.6. *Protein determination*

Protein was determined by Coomassie brilliant blue binding according to the instructions from Bio-Rad using bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

When mung bean [19] or potato [20] mitochondria are suspended in a hypo-osmolar medium (20–40 mosM final concentration) they swell, the outer membrane ruptures and some of it is released into the medium. When these outer membranes are isolated as described in section 2, they contain minimal contamination by matrix, as shown by the absence of detectable MDH activity, and minimal contamination by inner membrane, as shown by low specific activity of CCO (Table I). The ratio of the outer membrane marker, CCR to CCO increases by 25- to 60-fold as compared to the mitochondria (Table I). The CCR in this outer membrane fraction is completely α-specific (K.M. Fredlund and I.M. Møller, unpublished results) consistent with the observation by Douce et al. [19] whereas all the NAD(P)H dehydrogenases in the inner membrane are β-specific ([21]; K.M. Fredlund and I.M. Møller, unpublished results). The soluble fraction also contains CCR activity (Table I) which is β-specific (results not shown). It is not detectable in intact mitochondria since cytochrome *c* does not pass an intact outer membrane [21a], but upon rupture of the outer membrane it is released and this accounts for the inflated recoveries of the CCR activity (Table I).

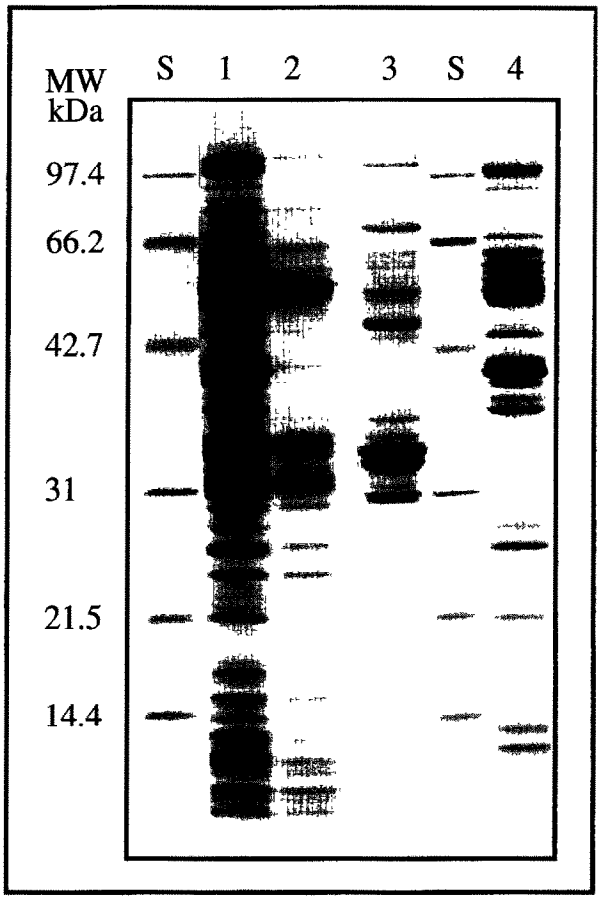

Fig. 1. Polypeptide pattern of intact mitochondria (lane 1), an inner membrane fraction (lane 2), an outer membrane fraction (lane 3) and a matrix fraction (lane 4). Intact mitochondria were purified from potato tubers, and the different mitochondrial subfractions obtained as described in section 2. S, molecular weight standards. The dots to the left of lane 3 indicate the most dominant polypeptides in the outer membrane. Lanes 1–4 contained 106, 47 and 56 and approx. 50 μg protein, respectively. Experiment from December 1992.

The outer membrane fraction does not have any antimycin A-insensitive NADPH-cytochrome *c* reductase activity, showing the absence of ER [22]. Also the matrix fraction and the inner membrane fraction are enriched in their respective markers and relatively uncontaminated by other markers (results not shown).

The polypeptide composition of the outer membrane is quite distinct from that of the inner membrane and the matrix (Fig. 1). Dominant polypeptides of 30, 31, 33, 34, 47, 48 and 74 kDa are strongly enriched in the outer membrane fraction. The presence of strong bands at 30–35 and 45–50 kDa is similar to what has been reported for the outer membrane of Neurospora crassa [23,24], yeast [25] and mung bean cotyledons [8].

The incorporation of ^{32}P from [γ-^{32}P]ATP into endogenous proteins is severalfold higher in the outer membrane fraction than in the other fractions tested

Table I

Distribution of marker enzymes in the mitochondria and the mitochondrial subfractions from potato tubers

Parameter	Fraction			
	Mitochondria	Mitoplasts	Outer membrane	300 K supernatant
Protein, mg	89 (100)	81 (91)	2.9 (3)	4.4 (5)
CCO, Total activity	349 (100)	332 (95)	0.5 (0.1)	0 (0)
Specific activity	3.9	4.1	0.2	0
Latency, %	99	69	92	–
Malate dehydrogenase				
Total activity	367 (100)	338 (92)	0 (0)	22 (6)
Specific activity	4.1	4.2	0	5.0
Latency, %	96	90	–	0
CCR, Total activity	6.2	5.9 (95)	0.23 (4)	11 (177)
Specific activity	71	73	79	2500
CCR/CCO	0.018	0.018	0.48	–

The fractions were obtained as described in section 2. Total activity is given in μmol · min^{-1}. Specific activity is given in μmol · (mg · protein)$^{-1}$ · min^{-1} for CCO and malate dehydrogenase and in nmol · (mg · protein)$^{-1}$ · min^{-1}for CCR. Yield in % of starting material (mitochondria) is given in parentheses. The results are from one experiment (6 kg peeled tubers), but similar results were obtained with two independent preparations except that the CCR/CCO ratio was > 1.0 in the outer membrane. The 300 K supernatant contains intermembrane space + matrix.

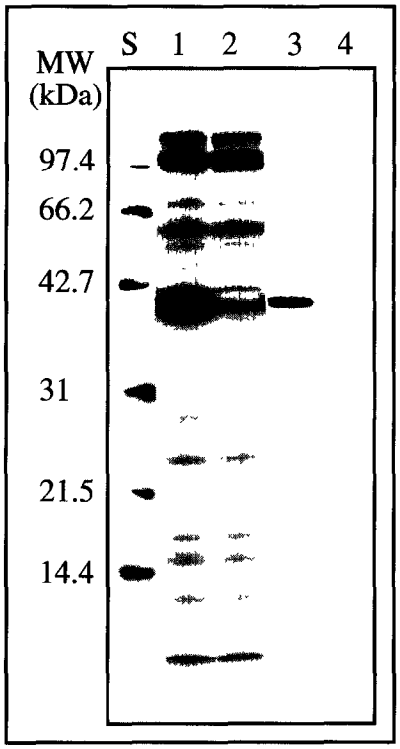

Fig. 2. Endogenous protein phosphorylation obtained with intact mitochondria (lanes 1 and 2, approx. 50 μg) and matrix fraction (lanes 3 and 4, approx. 40 μg) in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 10 mM pyruvate. Each sample was incubated with [γ-^{32}P]ATP, and the labelled proteins analysed by SDS-PAGE followed by autoradiography. S, molecular weight standards. The mitochondrial preparation was from October 1992, which gives different relative labelling of the phosphoproteins than later in the year [3].

(Table II). The exogenously added protein kinase substrate histone greatly enhances total incorporation in the intact mitochondria (Table II) indicating that the histones are accessible for mitochondrial protein kinase(s). This protein kinase(s) is Ca^{2+}-dependent since the phosphorylation of histones is abolished in the presence of EGTA (results not shown). The kinase is most likely located on the outer surface of the outer membrane since histones are too large to pass the outer membrane which is impermeable to molecules larger than 5–10 kDa [8]. Consistent with this conclusion, total incorporation is most enhanced by histones in the outer membrane fraction (Table II).

The phosphorylation pattern for intact potato tuber mitochondria is as reported earlier (Fig. 2) [2–4]. The prominent band at 41 kDa in the intact mitochondria and the only detectable band in the matrix fraction is the α-subunit of the pyruvate dehydrogenase complex as shown by the inhibition of the labelling by pyruvate (Fig. 2) and by atractylate [2], which prevents the entry of ATP into the matrix by inhibiting the ATP/ADP translocator [26]. The inner membrane fraction, consisting of inner membrane vesicles [14], contains three major phosphoproteins, one at 10 kDa and a double band at 16 kDa (Fig. 2), the labelling of which is abolished in the presence of EGTA, and is uninhibited by pyruvate and atractylate (Fig. 3). Thus, potato tuber mitochondria contain a Ca^{2+}-dependent protein kinase in the inner membrane.

The phosphoprotein pattern of the outer membrane is quite different from that of the matrix and the inner membrane. In many ways this is similar to that of intact mitochondria, however there is no band at 41 kDa

Table II

Comparison between endogenous and exogenous protein phosphorylation in intact mitochondria and in mitochondria subfractions

Fraction	Total incorporation (pmol · mg^{-1} · min^{-1})		Enhancement by histones (fold)
	Control	+ Histones	
Intact mitochondria	3.8	12.7	3.3
Matrix	0.8	0.99	1.2
Inner membrane	4.4	12.2	2.8
Outer membrane	12.9	60.4	4.7

The fractions were incubated with [γ-^{32}P]ATP and the incorporation of ^{32}P into proteins was measured at pH 6.5 as described in section 2. The results are from a typical experiment.

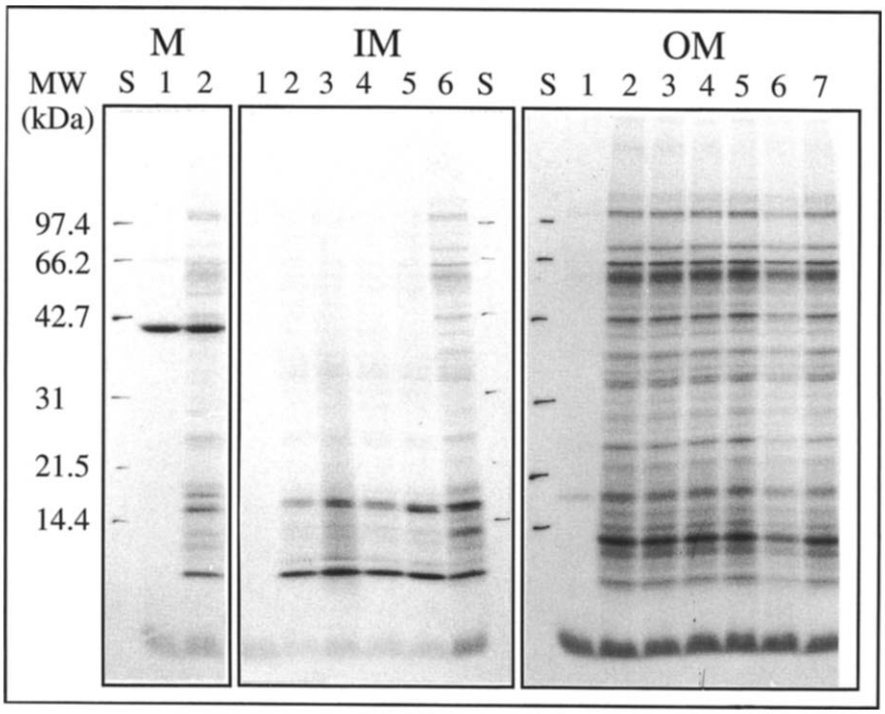

Fig. 3. Effect of different compounds on endogenous protein phosphorylation in intact mitochondria (M), an inner membrane fraction (IM) and an outer membrane fraction (OM). The fractions used were those whose polypeptide patterns are shown in Fig. 2. Polypeptides were labelled and analysed as in Fig. 2. S, molecular weight standards. Incubation was performed with (1) 2 mM EGTA; (2) no addition; (3) 10 mM pyruvate; (4) 100 μM $CaCl_2$; (5) 200 μM atractylate. IM6 contained a mixture of IM and OM (24 and 28 μg protein, respectively). OM6 and OM7 contained 1 and 10 μM $CaCl_2$, respectively. Mitochondria, inner and outer membranes were from the same preparation of mitochondria as in Fig. 1 and loading per lane was also the same.

which is inhibited in the presence of pyruvate (Fig. 3). Once more this indicates the absence of matrix contamination in the outer membrane fraction. More than 20 phosphoproteins are present in the outer membrane and the most prominent ones are at 12, 18, 26, 43, 58, 60, 65, 74 and 110 kDa. With the exception of the 18 kDa band, all these phosphoproteins disappear in the presence of EGTA. They all reappear at 1 μM Ca^{2+} but for some > 1 μM Ca^{2+} seems to be required for full labelling (Fig. 3). Since the cytoplasmic concentration of free Ca^{2+} is 0.1–0.3 μM in the normal undisturbed cell [27], it is possible that some of these phosphoproteins only appear under stress conditions.

The outer membrane fraction may contain inner membrane vesicles relatively low in CCO since CCO-rich and CCO-poor regions have been reported to be present in the inner membrane of both *Neurospora* [28,29] and plant mitochondria [16,30]. If such vesicles contributed to protein phosphorylation one might expect atractylate to reduce the labelling. No such effect was found (Fig. 3). When outer and inner membrane fractions are mixed, the phosphoprotein pattern is very similar to that of intact mitochondria with the exception of the 41 kDa band which is unique to the matrix fraction (Fig. 3).

The above results indicate that most of the phosphoproteins we previously localized to be outside the inner membrane are in fact in the outer membrane. This is consistent with the access of histones to protein kinase(s) in intact mitochondria (Table II). Particularly interesting is the observation that the very prominent polypeptides of 33 and 34 kDa (Fig. 1) are also phosphorylated (Fig. 3); they are probably porin since they are close to the molecular mass of the subunit of porin [8] and since they cross-react with antibodies raised against porin isolated from the outer membrane of mitochondria from *Dictyostelium discoides* [31]. Mannella [8] speculates that the pores in the outer membrane are open in State 4 (high ATP, low ADP) and closed in State 3 (low ATP, high ADP) due to changes in the mitochondrial structure bringing the two membranes in closer contact in State 3. However, an alternative possibility is that porin is phosphorylated and that this induces closure. We tested the latter hypothesis by incubating outer membranes with 200 μM ATP and different concentrations of ADP to cover the range from State 4 to State 3. No effect on total ^{32}P incorporation was observed with 5 μM ADP and a 50% decrease in total incorporation was found with 200 μM ADP. The labelling of all phosphoproteins appeared to be equally affected (results not shown). These results do not give any support to the hypothesis that porin phosphorylation is specifically involved in the State3/State 4 transition.

Since the only other known function of the outer mitochondrial membrane is in protein import, it is of great interest to investigate the possible involvement of protein phosphorylation in this process. With so many potential candidates for regulation via protein phosphorylation (Fig. 3) it seems likely that we will find that the outer membrane plays (an) important role(s) in the transduction of signals from the rest of the cell as well as from outside the cell to processes inside the mitochondrion.

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REFERENCES

[1] Ranjeva, R. and Boudet, A.M. (1987) Annu. Rev. Plant Physiol. 38, 73–93.
[2] Sommarin, M., Petit, P.X., Møller, I.M. (1990) Biochim. Biophys. Acta 1052, 195–203.
[3] Petit, P.X., Sommarin, M., Pical, C. and Møller, I.M. (1990) Physiol. Plant. 80, 493–499.
[4] Pical, C., Rémy, R., Sommarin, M., Møller, I.M. and Petit, P.X. (1992) in: Molecular, Bio-chemical and Physiological Aspects of Plant Respiration (Lambers, H. and van der Plas, L.H.W., Eds.) SPB Academic Publishing, The Hague, pp. 393–398.
[5] Müller, G. and Bandlow, W. (1987) Yeast 3, 161–174.
[6] Technikova-Dobrova, Z., Sardanelli, A.M. and Papa, S. (1993) FEBS Lett. 322, 51–55.
[7] Moreau, F. and Lance, C. (1972) Biochimie 54, 1335–1348.
[8] Mannella, C.A. (1985) Encycl. Plant Physiol., New Series 18, 106–133.
[9] Sorgato, M.C. and Moran, O. (1993) Crit. Rev. Biochem. Mol. Biol. 18, 127–171.
[10] Söllner, T., Griffiths, G., Pfaller, R., Pfanner, N. and Neupert, W. (1989) Cell 59, 1061–1070.
[11] Chang, K.J., Marcus, N.A. and Cuatrecasas, P. (1974) J. Biol. Chem. 249, 6854–6865.
[12] Neuburger, M., Journet, E.P., Bligny, R., Carde, J.P. and Douce, R. (1982) Arch. Biochem. Biophys. 217, 312–323.
[13] Struglics, A., Fredlund, K.M., Rasmusson, A.G. and Møller, I.M. (1993) Physiol. Plant. 88, 19–28.
[14] Rasmusson, A.G. and Møller, I.M. (1991) Physiol. Plant. 83, 357–365.
[15] Rasmusson, A.G. and Møller, I.M. (1990) Plant Physiol. 94, 1012–1018.
[16] Møller, I.M., Lidén, A.C., Ericson, I. and Gardeström, P. (1987) Methods Enzymol. 148, 442–453.
[17] Day, D.A. and Wiskich, J.T. (1975) Arch. Biochem. Biophys. 171, 117–123.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Douce, R., Mannella, C.A. and Bonner, W.D. (1973) Biochim. Biophys. Acta 292, 105–116.
[20] Mannella, C.A. and Bonner, W.D. (1975) Biochim. Biophys. Acta 413, 213–225.
[21] Rasmusson, A.G., Fredlund, K.M. and Møller, I.M. (1993) Biochim. Biophys. Acta 1141, 107–110.
[21a] Douce, R. (1985) Mitochondria in Higher Plants: Structure, Function, and Biogenesis, Academic Press, Orlando, FL.
[22] Møller, I.M. and Lin, W. (1986) Annu. Rev. Plant Physiol. 37, 309–334.
[23] Mannella, C.A. (1982) J. Cell Biol. 94, 680–687.
[24] Neupert, W. and Ludwig, G.D. (1971) Eur. J. Biochem. 19, 523–532.
[25] Riezman, H., Hay, R., Gasser, S., Daum, G., Schneider, G., Witte, C. and Schatz, G. (1983) EMBO J. 2, 1105–1111.
[26] Klingenberg, M. (1985) in: The Enzymes of Biological Membranes, Vol. 4, (Martonosi, A.N., ed.) Plenum Press, New York, pp. 511–553.
[27] Gilroy, S., Hughes, W.A. and Trewavas, A.J. (1989) Plant Physiol. 90, 482–491.
[28] Werner, S. and Neupert, W. (1972) Eur. J. Biochem. 25, 379–396.
[29] Brdiczka, D., Dölken, G., Krebs, W. and Hofman, D. (1974) Hoppe Seyler's Z. Physiol. Chem. 335, 731–743.
[30] Kay, C.J., Ericson, I., Gardeström, P., Palmer, J.M. and Møller, I.M. (1985) FEBS Lett. 193, 169–174.
[31] Troll, H., Malchow, D., Müller-Taubenberger, A., Humbel, B., Lottspeich, F., Ecke, F., Ecke, M., Gerisch, G., Schmid, A. and Benz, R. (1992) J. Biol. Chem. 267, 21072–21079.