Plant Cell Physiol. 40(12): 1271–1279 (1999)

Phosphoproteins and Protein Kinase Activities Intrinsic to Inner Membranes of Potato Tuber Mitochondria

André Struglics [1], Kenneth M. Fredlund [2, 3], Ian M. Møller [2] and John F. Allen [1, 4]

[1] *Plant Cell Biology, Lund University, Box 7007, S-220 07 Lund, Sweden*
[2] *Plant Physiology, Lund University, Box 117, S-221 00 Lund, Sweden*

Inside-out submitochondrial particles (IO-SMP) were isolated and purified from potato (*Solanum tuberosum* L. cv.) tubers. When these IO-SMP were incubated with [γ-^{32}P]ATP more then 20 proteins became labelled as a result of phosphorylation. The ^{32}P incorporation was stimulated by the oxidising reagent ferricyanide. Except for a 17 kDa protein which was phosphorylated only in the absence of divalent cations, the protein phosphorylation required Mg^{2+}. The time for half-maximum ^{32}P incorporation was 4 min for the 22 kDa phospho-F_1 δ'-subunit and 2 min for the 28 kDa phospho-F_o b-subunit of the proton-ATPase. The K_m for ATP for the detected phosphoproteins was between 65 μM and 110 μM. The pH optimum for protein phosphorylation in inner membranes was between pH 6 and 8, and for the F_1 δ'-subunit and the F_o b-subunit the pH optima were 6.5–8 and pH 8, respectively. A 37 kDa phosphoprotein was phosphorylated on a histidine residue while the remainder of the inner membrane proteins were phosphorylated on serine or threonine residues. Two autophosphorylated putative kinases were identified: one at 16.5 kDa required divalent cations for autophosphorylation, while another at 30 kDa did not. A 110 kDa protein was labelled only with [α-^{32}P]ATP, suggesting adenylylation.

Key words: Autophosphorylation — F_oF_1-ATPase — Inner membrane — Mitochondria — Potato (*Solanum tuberosum* L.) tubers — Protein phosphorylation.

Post-translational modification by reversible protein phosphorylation is a ubiquitous regulatory mechanism in cellular biochemistry, implicated in such processes as control of enzyme activity, protein degradation, gene expression and signal transduction (McEntyre 1994).

In mammalian mitochondria about 10 phosphoproteins have been detected by SDS-PAGE and autoradiography (Ferrari et al. 1990, Technikova-Dobrova et al. 1993). Plant mitochondria may be enriched in phosphoproteins compared with mammalian mitochondria, and about 30 phosphoproteins have been detected in intact plant mitochondria using SDS-PAGE and autoradiography (Danko and Markwell 1985, Sommarin et al. 1990, Pike et al. 1991). The regulation by reversible phosphorylation of the pyruvate dehydrogenase complex and the branched-chain α-ketoacid dehydrogenase complex are the only well-characterised mitochondrial protein phosphorylation-dephosphorylation events (Bradford and Yeamen 1986, Randall et al. 1989).

Previous studies on protein phosphorylation in plant mitochondria have been carried out with intact mitochondria, where inner membrane phosphoproteins are not easily distinguished from outer membrane phosphoproteins or from the strongly labelled α-subunit of pyruvate dehydrogenase located in the matrix compartment (Petit et al. 1990, Sommarin et al. 1990). With the exception of the highly active pyruvate dehydrogenase kinase, most protein kinase activities identified in intact plant mitochondria are localised in the outer membrane (Pical et al. 1993). To date, only two inner membrane phosphoproteins have been identified in plant mitochondria, the 22 kDa δ'-subunit and the 28 kDa b-subunit of the F_oF_1-ATPase (Struglics et al. 1998). In mammalian mitochondria an additional two inner membrane phosphoproteins have been identified, the 18 kDa (IP) AQDQ subunit of complex I (Papa et al. 1996), and the 17 kDa subunit IV of cytochrome *c* oxidase (Steenaart and Shore 1997). The general properties of protein phosphorylation in the inner mitochondrial membrane are unknown as is the number and location of the protein kinases involved.

Here we describe the kinetics, ATP-dependency and pH optima of protein phosphorylation for the 22 kDa δ'-subunit and the 28 kDa b-subunit of F_oF_1-ATPase as well as for other, as yet unidentified, phosphoproteins in the inner membranes of potato tuber mitochondria. We also report autophosphorylation of inner membrane proteins, suggesting the presence of a novel inner membrane protein kinase in plant mitochondria.

Abbreviations: Bis-tris, Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; IO-SMP, inside-out submitochondrial particles; MOPS, 3-Morpholinopropanesulfonic acid; NDPK, nucleoside diphosphate kinase; $t_{1/2}$, time for half-maximum ^{32}P incorporation; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; TLC, thin layer chromatography; Tween 20, Polyoxyethylene sorbitanmonolaurate.

[3] Present address; Novartis Seeds AB, Box 302, S-261 23 Landskrona, Sweden.
[4] Corresponding author: E-mail, john.allen@plantcell.lu.se; Fax, +46-46-2223684.

Materials and Methods

Isolation of mitochondria and inner mitochondrial membranes—Highly purified mitochondria free from plastids (amyloplasts) and peroxisomes were isolated from potato tubers (*Solanum tuberosum* L. cv. Bintje or Ukama) as described in Struglics et al. (1993). Inside-out submitochondrial particles (IO-SMP) were isolated according to Pical et al. (1993). Freshly prepared inner membranes were used for all experiments.

In vitro phosphorylation—The standard in vitro protein phosphorylation assay was carried out in a volume of 50 µl containing 140–250 µg inner membrane proteins. Final concentrations in the phosphorylation buffer (1 × R buffer) were; 0.3 M sucrose, 50 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 8–12 µCi [γ-^{32}P]ATP (AA0068, Amersham) and 0.2 mM ATP. In Figure 1 we also used [α-^{32}P]ATP (PB10200, Amersham) with the same specific activity as for [γ-^{32}P]ATP.

Phosphorylation experiments were carried out at room temperature (22°C) with incubation times from 20 s to 30 min as indicated in the text. The reactions were terminated by addition of sample buffer (Laemmli 1970) containing 0.1% SDS and then heat denaturated at 100°C for 3 min.

Reaction kinetics and ATP dependency—For the kinetic studies IO-SMP proteins (3 mg) were incubated in 1 × R buffer in the presence of 210 µCi [γ-^{32}P]ATP and 200 µM ATP in a total volume of 950 µl. 50 µl samples were withdrawn at different time points, and the reactions were stopped as described above.

To examine ATP concentration dependency, IO-SMP proteins (140 µg) were incubated in 1 × R buffer for 2 min in a total volume of 20 µl in the presence of different ATP concentrations: specific activity was maintained at 15 µCi nmol^{-1} ATP. The reactions were stopped as described above.

In situ phosphorylation—With a few modifications, the in situ phosphorylation assay is adapted from the method of San Agustin and Witman (1995). IO-SMP proteins (2.6 mg) were solubilised in SDS-containing sample buffer (Laemmli 1970), loaded in a 50 mm broad well, and separated by SDS-PAGE. The proteins were electrophoretically transferred to a PVDF membrane (PALL) as described in Struglics et al. (1998). The membrane was cut into 1 cm strips and incubated at room temperature for 15 min in 50 mM HEPES-KOH, 140 mM NaCl, pH 7.5 prior to incubation for 12 h at 8°C in renaturation buffer (10 mM HEPES-KOH, 140 mM NaCl, 2 mM EDTA, 2 mM DTT, 1% w/v BSA, 0.1% v/v Tween 20, pH 7.5). Following renaturation, the membrane strips were washed for 5 min in 50 mM HEPES-KOH, pH 7.5 and then incubated at room temperature for 45 min (up to 8 h) in 5 ml phosphorylation solution containing: (a) 50 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$ and 0.2 mM $CaCl_2$ or (b) 50 mM HEPES-KOH, pH 7.5 and 10 mM EDTA; both supplemented with 200 µCi [γ-^{32}P]ATP or [α-^{32}P]ATP. After in situ phosphorylation the PVDF membranes were washed as follows: 1 × 5 min then 2 × 20 min in 50 mM HEPES-KOH, pH 7.5; 1 × 10 min in 50 mM HEPES, 1 M KOH, 0.05% v/v Tween 20; 2 × 10 min in 50 mM HEPES-KOH, pH 7.5; 1 × 10 min in 1 M KOH and 1 × 10 min in distilled H_2O. The PVDF membranes were briefly stained with Coomassie blue (R250) and dried before exposure to either autoradiographic film or phosphorImager plates.

Acid and alkaline treatment of phosphorylated proteins—The acid-alkaline treatment of phosphorylated IO-SMP proteins is broadly based on the method of Oda and Hasunuma (1994). IO-SMP (3.2 mg protein) were phosphorylated according to the standard in vitro phosphorylation assay in a total volume of 625 µl. The proteins were loaded in a 70 mm broad well, and separated by SDS-PAGE and electrophoretically transferred to a Hybond-PVDF membrane (Amersham) as described in Struglics et al. (1998). The membrane was cut into 2 cm strips, incubated in 0.5 M HCl or 0.5 M NaOH or 50 mM HEPES-KOH pH 7.5 at 65°C for 2 h, dried and exposed to autoradiographic film or phosphorImager plates.

SDS-PAGE and detection of phosphoproteins—SDS-PAGE was performed according to Laemmli (1970), on a 10–15% T (C=2.7%) gradient gel with Protean II xi Slab Cell (BioRad) apparatus. Gels were stained with a Coomassie blue solution (0.4% w/v Coomassie Brilliant blue [R250], 50% v/v methanol, 10% v/v acetic acid), and destained in 35% v/v methanol, 15% v/v acetic acid before drying. Phosphoproteins were visualised by exposure for 3 to 4 d to a PhosphorImager plate (Molecular Dynamics) or to autoradiographic film (Hyper film MP, Amersham).

Other analytical methods—Protein concentration was determined according to Bradford (1976), using IgG as a standard. For quantification analysis, the stained and dried gels were exposed to PhosphorImager plates. The data were analysed with ImageQuant software (version 1.2), and the quantification was based upon total pixels in protein bands corrected for background signals.

Results

In vitro labelling using [γ-^{32}P]ATP and [α-^{32}P]ATP as phosphate donors—In protein phosphorylation catalysed by protein kinases, the γ-phosphate from ATP or GTP is transferred to the protein substrates. To determine whether protein radiolabelling in inner mitochondrial membranes results from covalent binding of P_i (phosphorylation) or if, instead, ATP (or ADP and AMP) is bound to the inner membrane proteins, we used [γ-^{32}P]ATP as a phosphate donor and compared the result with labelling obtained with [α-^{32}P]ATP.

Figure 1A shows SDS-PAGE separated inner membrane proteins stained with Coomassie blue. Figure 1B shows the corresponding PhosphorImage where inner membrane (IO-SMP) proteins have been labelled with [γ-^{32}P]ATP or [α-^{32}P]ATP. At least 20 labelled bands were detected when IO-SMP were incubated with [γ-^{32}P]ATP (Fig. 1B). Since none of these proteins were labelled when [α-^{32}P]ATP was used, we conclude that the labelling results from covalent binding of γ-phosphate groups to the proteins (Fig. 1B). Replacement of [γ-^{32}P]ATP with [γ-^{32}P]-GTP resulted in weak labelling and no specific GTP-dependent protein phosphorylation was observed in IO-SMP (data not shown). A 16 kDa protein was the most heavily labelled phosphoprotein in the IO-SMP, and the positions for the phosphorylated 22 and 28 kDa F_oF_i subunits are also marked in the figure. Incubation of IO-SMP proteins with [α-^{32}P]ATP resulted in labelling of only one protein, of 110 kDa (Fig. 1B). Since the 110 kDa protein is not labelled with [γ-^{32}P]ATP, the label probably originates from direct binding (adenylylation) between [α-^{32}P]AMP or [α-^{32}P]-ADP and the protein.

Effect of ferricyanide—The ^{32}P incorporation into the IO-SMP proteins was higher in the presence of the oxidis-

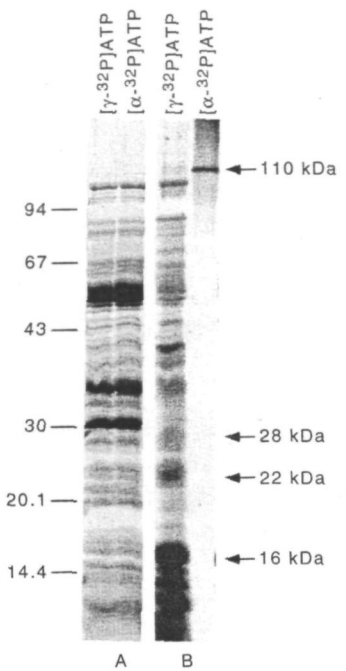

Fig. 1 Radio-labelling of IO-SMP proteins by [γ-^{32}P]ATP or [α-^{32}P]ATP. Inner mitochondrial membranes (140 μg protein) isolated from potato tubers were incubated with either [γ-^{32}P]ATP or [α-^{32}P]ATP in the presence of 200 μM ATP and 1 × R buffer for 10 min. The reactions were terminated by boiling in sample buffer and proteins separated by SDS-PAGE. (A) Coomassie blue-stained IO-SMP proteins. (B) PhosphorImage of radio labelled IO-SMP proteins. The positions of the 22 kDa F_1 δ'-subunit, the 28 kDa F_o b-subunit, the 16 kDa phosphoprotein as well as a 110 kDa protein discussed in the text are marked by arrows and the positions of molecular mass markers (kDa) are indicated.

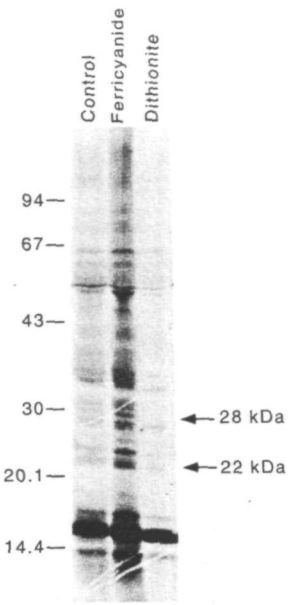

Fig. 2 IO-SMP proteins phosphorylated in the presence of redox reagents. Autoradiograph of IO-SMP proteins (250 μg) labelled with [γ-^{32}P]ATP/200 μM ATP in 1 × R buffer in the absence of redox reagents (control), or in the presence of 5 mM potassium ferricyanide, or in the presence of 5 mM sodium dithionite. Prior to addition of ATP the samples were preincubated for 3 min in the respective redox reagent. The phosphorylation reactions were terminated after 1 min by boiling in sample buffer and the proteins were separated by SDS-PAGE. The positions of the 22 kDa F_1 δ'-subunit, the 28 kDa F_o b-subunit discussed in the text are marked by arrows and the positions of molecular mass markers (kDa) are indicated.

ing reagent ferricyanide compared to in the presence of the reducing reagent dithionite or in the absence (called control) of any redox reagent (Fig. 2). The phosphorylation was found to be stimulated by low concentrations of ferricyanide (1 mM) and no further increase in ^{32}P incorporation could be detected at higher concentrations of ferricyanide, up to 10 mM (data not shown). In these experiments (Fig. 2) protein phosphorylation was carried out for only 1 min, which is within the linear phase of IO-SMP protein phosphorylation kinetics (see Fig. 4).

Effects of divalent cations—Figure 3 shows an autoradiograph of inner membrane proteins phosphorylated in the presence of EDTA or divalent cations. As a comparison with phosphorylation in the presence of Mg^{2+} or Ca^{2+} we also show in Figure 3 the standard assay for inner membrane protein phosphorylation (5 mM $MgCl_2$+0.1 mM $CaCl_2$).

Phosphorylation in the presence of EDTA abolished ^{32}P incorporation into all proteins, with the exception of a 17 kDa protein (Fig. 3). Phosphorylation of the 17 kDa protein was suppressed by divalent cations (Fig. 3). Except for phosphorylation of the 17 kDa protein, inner membrane kinase activity required both Mg^{2+} as shown in Fig. 3 and Ca^{2+} as shown by Pical et al. (1993). Mn^{2+} could replace Mg^{2+} (both tested over a range of 1 to 10 mM) without loss of kinase activity, but Zn^{2+} (5 mM) abolished all phosphorylation (data not shown). When phosphorylation was carried out in the presence of low Ca^{2+} concentrations ($\leq$0.2 mM), the ^{32}P incorporation into a 41 kDa protein was similar to that in the presence of Mg^{2+} (Fig. 3). At higher Ca^{2+} concentrations (>0.2 mM), the ^{32}P incorporation into the 41 kDa protein was suppressed, as for the rest of inner membrane proteins (results not shown).

Time-course—As an example of time-dependent protein phosphorylation in IO-SMP, we show the kinetics for four proteins. The half-maximum time of P_i incorporation ($t_{1/2}$) was approximately 4 min for the 16 kDa protein and the F_1 δ'-subunit (Fig. 4A), while it was 2 min for the 25 kDa protein and the F_o b-subunit (Fig. 4B). The $t_{1/2}$ for the different IO-SMP proteins varied between 0.5 and 5 min (data not shown). Maximal labelling was observed after 15–20 min (Fig. 4).

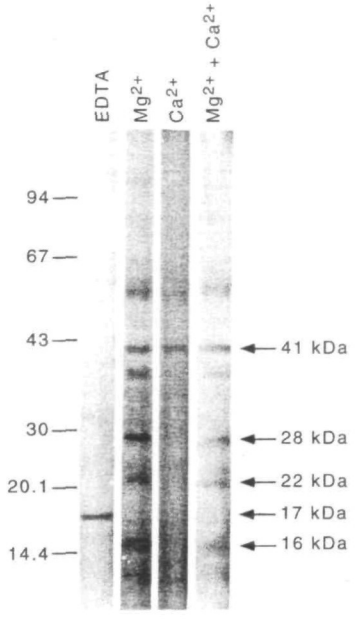

Fig. 3 Effect of divalent cations on protein phosphorylation in IO-SMP. Autoradiograph of inner membrane proteins, labelled with [γ-^{32}P]ATP/200 μM ATP. Prior to addition of ATP, 180 μg IO-SMP protein was incubated for 3 min in 0.3 M sucrose, 50 mM HEPES-KOH pH 7.5 in the presence of either 5 mM EDTA, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$ or 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The phosphorylation reactions were terminated after 5 min by boiling in sample buffer and the proteins were separated by SDS-PAGE. Proteins discussed in text are marked by arrows and the positions of molecular mass markers (kDa) are indicated.

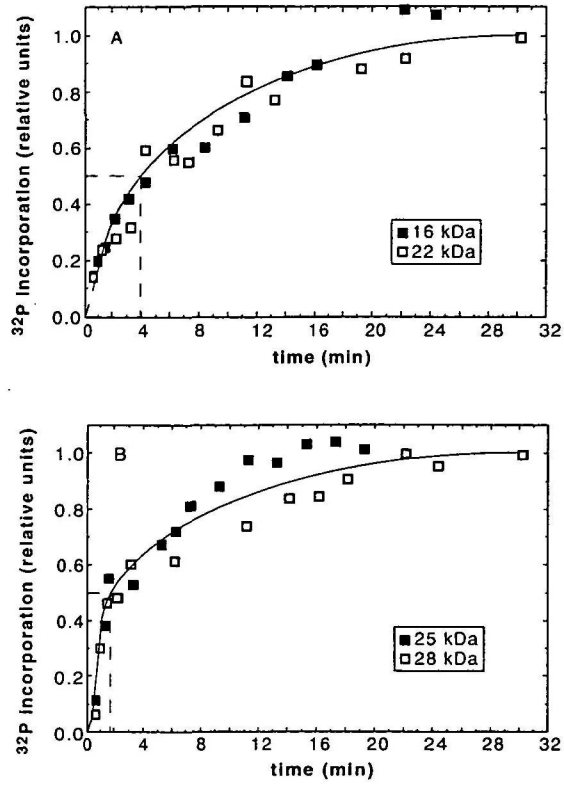

Fig. 4 Time-dependent phosphorylation of IO-SMP proteins. IO-SMP (3 mg protein) was incubated in 1 × R buffer in the presence of [γ-^{32}P]ATP/200 μM ATP. At different time points, samples of 50 μl (160 μg protein) were withdrawn and the reactions were terminated by boiling in sample buffer. IO-SMP proteins were separated by SDS-PAGE and the gel was Coomassie stained, dried and exposed to a PhosphorImager plate. The phosphoproteins were analysed using ImageQuant software, and relative units of ^{32}P incorporation was calculated where the incorporation after 30 min was set to 1. A, titration of phosphorylation of the 16 kDa and 22 kDa (F_1 δ'-subunit) proteins. B, titration of phosphorylation of the 25 and 28 kDa (F_o b-subunit) proteins. The broken line show the estimated of $t_{1/2}$ values.

Effect of ATP concentration—The apparent K_m for ATP of the protein kinase(s) responsible for phosphorylation of inner membrane proteins was between 65 to 110 μM ATP for all phosphoproteins including the 22 kDa F_1 δ'-subunit and the 28 kDa F_o b-subunit (Fig. 5). These results were obtained by phosphorylation for 2 min (Fig. 5) or for 12 min (data not shown). We have used 200 μM ATP as a standard in our phosphorylation assays, which will give close to maximum rates of IO-SMP protein phosphorylation.

pH dependence—Protein phosphorylation in inner mitochondrial membranes showed a broad pH optimum of pH 6.5 to 8, which is exemplified in Figure 6 by the pH-dependent phosphorylation of the 22 kDa F_1 δ'-subunit. The highly labelled 16 kDa had a sharper pH optimum at pH 6, and the 28 kDa F_o b-subunit showed a pH optimum at pH 8 (Fig. 6).

The pH in the cytosol of plant cells is around 7.5, and the pH in the mitochondrial matrix is thought to be slightly higher (Kurkdjian and Guern 1989). The pH of 7.5 in our standard assay was chosen to reflect physiological conditions, and as a compromise between the observed pH optima of phosphorylation for various inner membrane proteins (Fig. 6).

Autophosphorylation of inner membrane proteins—The renaturation and in situ phosphorylation of proteins separated by SDS-PAGE is a well established method for the detection of protein kinases (Geahlen et al. 1986, Ferrell and Martin 1991, Hutchcroft et al. 1991, San Agustin and Witman 1995, Race and Hind 1996), but it has never previously been used on plant mitochondria.

In the inner membranes, two autophosphorylated proteins with molecular mass of 16.5 and 30 kDa were identified by [γ-^{32}P]ATP phosphorylation after renaturation of proteins immobilised on PVDF membranes (Fig. 7, lane 2 and 3). For comparison with the migration of the 16.5 and 30 kDa proteins in SDS-gels, the position of the highly labelled 16 kDa phosphoprotein and the phos-

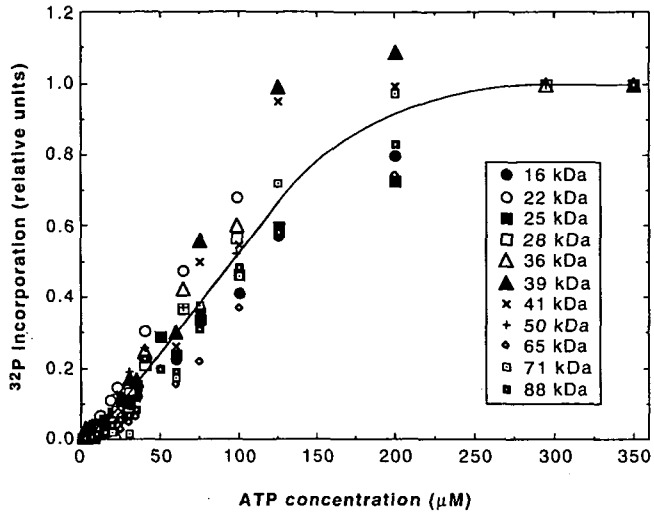

Fig. 5 ATP concentration-dependent phosphorylation of IO-SMP proteins. IO-SMP (140 μg protein) were incubated for 2 min in 1 × R buffer in the presence of different ATP concentrations where the specific activity was maintained at 15 μCi nmol^{-1} ATP. The reactions were terminated by boiling in sample buffer and proteins were separated by SDS-PAGE. After Coomassie staining, drying and exposure to a PhosphorImager plate, the phosphoproteins were analysed using ImageQuant software. Relative units of ^{32}P incorporation were calculated where the incorporation either at 200 or 350 μM ATP was set to 1.

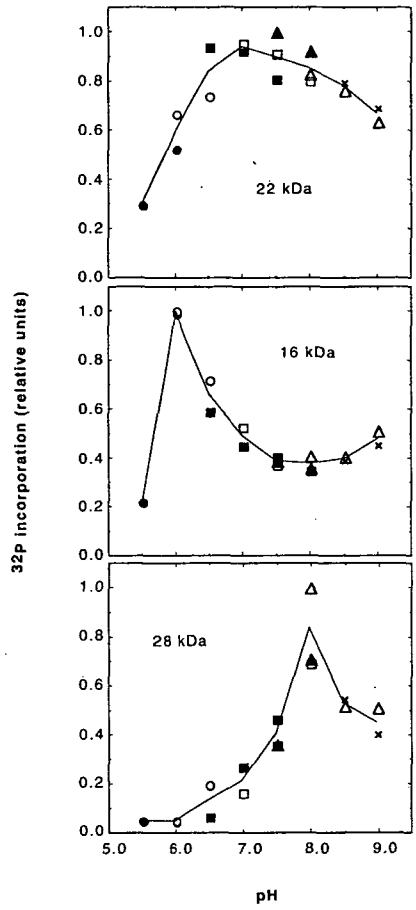

Fig. 6 pH-dependent phosphorylation of the 22 kDa F_1 δ'-subunit; the 16 kDa protein and the 28 kDa F_o b-subunit. IO-SMP proteins (140 μg) were phosphorylated with [γ-^{32}P]ATP/200 μM ATP for 5 min in the presence of 0.3 M sucrose, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 50 mM of the following buffers at different pH: ●, MES-KOH, pH 5.5 and 6; ○, Bis-tris-HCl, pH 6 and 6.5; ■, MOPS-KOH, pH 6.5, 7 and pH 7.5; □, TES-KOH, pH 7, 7.5 and pH 8; ▲, HEPES-KOH, pH 7.5 and 8; △, Tris-HCl, pH 8, 8.5 and pH 9; ×, Tricine-KOH, pH 8.5 and 9. The reactions were terminated by boiling in presence of sample buffer and proteins were separated by SDS-PAGE. Dried Coomassie stained gels were exposed to a PhosphorImager plate. The phosphoproteins were analysed using ImageQuant software, and relative units of ^{32}P incorporation were calculated where the maximum label was set to 1.

phorylated 22 and 28 kDa F_oF_1-ATPase subunits are also indicated in the figure (Fig. 7, lane 1). The 16.5 and 30 kDa proteins were not labelled when phosphorylation was carried out in vitro using [γ-^{32}P]ATP as a phosphate donor (Fig. 7, lane 1), which can be explained by rapid phosphate transfer from the enzymes (putative kinases) to available substrates. The 16.5 kDa protein was not labelled in situ when [α-^{32}P]ATP was used as phosphate donor and only a trace amount of isotope was associated with the 30 kDa protein (Fig. 7 lane 4). This indicates that these proteins have covalently bound phosphate groups. A difference in the in situ phosphorylation of the two proteins was observed in their requirement for divalent cations. The 16.5 kDa protein required divalent cations for autophosphorylation (Fig. 7, lane 3), while the 30 kDa protein showed autophosphorylation also in the presence of EDTA (Fig. 7, lane 2).

Pical et al. (1993), showed that the total rate of ^{32}P-incorporation into phosphoproteins of IO-SMP was stimulated when in vitro phosphorylation was carried out in the presence of histones (histone H1). In our in situ phosphorylation assay, the ^{32}P incorporation into the 16.5 and 30 kDa proteins were neither stimulated nor inhibited when labelling was carried out in the presence of histones or inner membrane peptides prepared by trypsin digestion (data not shown).

Acid/alkaline stability of phosphoamino acids in inner membrane proteins—The stability of the covalent bond between phosphate and protein depends on which amino acid is phosphorylated. This can be analysed by acid-alkali treatments of the phosphoproteins (Duclos et al. 1991).

When acid-alkali stability/lability measurements were applied to blotted ^{32}P-labelled inner membrane proteins (Fig. 8), none of the inner membrane phosphoproteins was stable under both acidic and alkaline conditions, which would have indicated phosphorylation on tyrosine residues

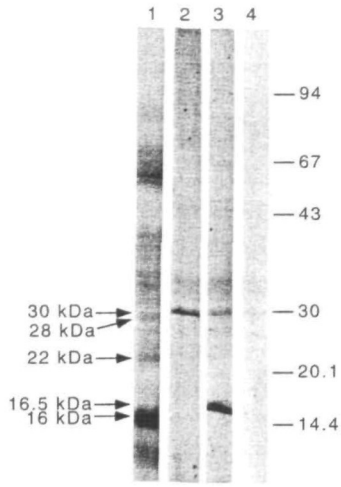

Fig. 7 Autophosphorylation of IO-SMP proteins. PhosphorImage of IO-SMP proteins blotted to PVDF membranes, phosphorylated either in vitro or in situ. Lane 1, IO-SMP proteins in 1xR buffer were phosphorylated for 10 min with [γ-^{32}P]ATP/ATP according to the standard in vitro assay prior to SDS-PAGE and transfer to membrane; Lanes 2 to 4, IO-SMP proteins renaturated and phosphorylated in situ on PVDF membranes. Lanes 2 and 3, proteins incubated with [γ-^{32}P]ATP. Lane 4, proteins incubated with [α-^{32}P]ATP. The in situ labelling was carried out for 8 h in 50 mM HEPES-KOH, pH 7.5 buffer supplemented with either 10 mM EDTA (lane 2) or 10 mM $MgCl_2$+0.2 mM $CaCl_2$ (lane 3 and 4). Phosphoproteins were visualised by PhosphorImaging. Proteins discussed in the text are marked by arrows and the positions of molecular mass markers (kDa) are indicated.

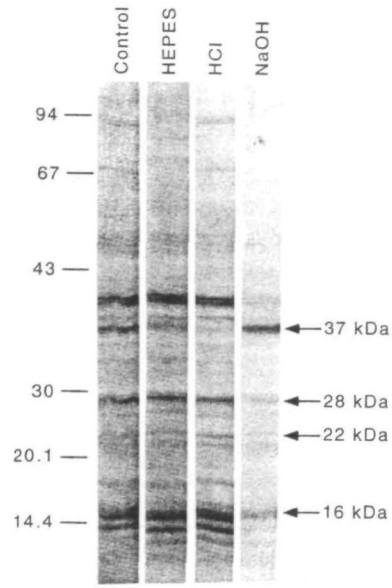

Fig. 8 Acid and alkali treatment of IO-SMP phosphoproteins. PhosphorImage of 16 min in vitro phosphorylated IO-SMP proteins separated by SDS-PAGE and transferred to PVDF membranes. Except for the PVDF membrane marked control, which was dried immediately, the rest of the PVDF membranes were incubated at 65°C for 2 h in either of the following solutions; 50 mM HEPES-KOH, pH 7.5 or 0.5 M HCl or 0.5 M NaOH. After incubation, the membranes were dried and exposed to a PhosphorImager plate. Proteins discussed in the text are marked by arrows, and the positions of molecular mass markers (kDa) are indicated.

(Duclos et al. 1991). A 37 kDa phosphoprotein was acid-labile and alkali-stable, suggesting phosphorylation on histidine residues. The remaining phosphoproteins in the inner membrane showed alkaline lability and acid stability (Fig. 8), indicating phosphorylation on serine or threonine residues.

Discussion

The phosphoproteins and kinase(s) are inner membrane proteins facing the matrix

IO-SMP prepared from potato tuber mitochondria consist of inner membrane vesicles depleted of matrix proteins (Lidén et al. 1987, Pical et al. 1993). Thus, protein phosphorylation in these membranes is catalysed by endogenous membrane-bound kinase(s) and all the substrates are membrane-bound proteins.

Both the kinase substrates and the active site of the protein kinase(s) are located on the matrix surface of the inner membranes since (1) the SMP are inside-out and sealed (Rasmusson and Møller 1991); (2) there is no ADP inside the IO-SMP vesicles to exchange with [γ-^{32}P]ATP on the ATP-ADP carrier.

The protein phosphorylation pattern of IO-SMPs (Fig. 1B) showed no similarity with the phosphorylation pattern of intact mitochondria, mitochondrial matrix proteins or mitochondrial outer membrane proteins (Pical et al. 1993; our unpublished results). Phosphorylation with [γ-^{32}P]ATP in intact mitochondria or IO-SMP in the presence of oligomycin (which inhibits both the synthesis and hydrolysis of ATP by the ATPase) changed neither the amount of ^{32}P incorporation nor the phosphorylation pattern compared with the control in intact mitochondria (Petit et al. 1990), or in the inner membranes (results not shown).

Properties of inner membrane protein phosphorylation

Although cAMP-dependent protein kinases have been reported in mammalian mitochondria (Papa et al. 1996 and ref. therein), no such protein kinases have been detected when phosphorylation was carried using intact plant mitochondria (Danko and Markwell 1995), or IO-SMP (our unpublished results).

The phosphorylation of the inner membrane proteins requires divalent cations. A notable exception is a 17 kDa protein which is phosphorylated only in their absence (Fig. 3). The phosphorylation takes 15–20 min to reach

maximum ^{32}P incorporation (Fig. 4), much longer than the 2–3 min reported for intact mitochondria (Sommarin et al. 1990). The K_m (ATP) varied between 65 and 110 μM for the various phosphoproteins and maximum ^{32}P incorporation was reached at an ATP concentration above 200 μM (Fig. 5). These results should not be compared directly with results obtained with intact mitochondria, where the activities of outer membrane and matrix kinases predominate (Pical et al. 1993).

Total ^{32}P incorporation into IO-SMP proteins had the same broad optimum at pH 6.5–8 (Fig. 6) as observed with intact mitochondria (Danko and Markwell 1985, Sommarin et al. 1990), whereas the optimum pH for labelling individual phosphoproteins varied from pH 6.0 to pH 8.0 (Fig. 6). As a comparison two mammalian membrane-bound protein kinases, purified from bovine heart mitochondria (Kitagawa and Racker 1982), and a inner membrane protein kinase(s) prepared from mouse liver (Vardanis 1976) showed sharp pH optima at pH 9, pH 7.5 and pH 8.5, respectively.

Protein phosphorylation of IO-SMP proteins, including the 22 and 28 kDa F_oF_1 subunits, was stimulated by the oxidising reagent ferricyanide (Fig. 2). Addition of ferricyanide to inner membranes lacking reduced substrates will oxidise the quinone pool, and this may trigger, directly or indirectly, an inner membrane kinase leading to an increased phosphorylation of inner membrane proteins. This is opposite to the effect of ferricyanide on protein phosphorylation in chloroplast thylakoid membranes (Allen et al. 1981).

Except for the 37 kDa phosphohistidine protein, the inner membrane phosphoproteins showed ^{32}P incorporation into serine and threonine residues (Fig. 8), which was also confirmed by TLC electrophoresis of the 22 and 28 kDa F_oF_1-subunit proteins (results not shown). Genistein inhibited tyrosine kinases in transformed plant roots (Rodríguez-Zapata and Hernández-Sotomayor 1998) and in chloroplast thylakoids whereas it had no effect on mitochondrial protein phosphorylation (Tullberg et al. 1998). None of the inner membrane phosphoproteins was stable under both acidic and alkaline conditions (Fig. 8), and their phosphorylation was unaffected by genistein (data not shown). All of this suggests that the detected mitochondrial inner membrane phosphoproteins are not labelled on tyrosine residues.

The individual phosphoproteins

The properties of a number of the phosphoproteins, identified by apparent molecular mass, are summarised in Table 1.

The 22 and the 28 kDa F_oF_1-subunits—The 22 kDa F_1-ATPase δ'-subunit and the 28 kDa F_o-ATPase b-subunit are known phosphoproteins (Struglics et al. 1998). The F_oF_1-subunits were both modified by phosphomonoester linkages (on serine or threonine), but the $t_{1/2}$, and the pH optima for phosphorylation of these proteins differ (see Table 1). This may mean that they are phosphorylated by two different protein kinases. Alternatively, one protein kinase has different affinities for the two F_oF_1-subunits.

The 16.5 and 30 kDa autophosphorylated proteins—Soluble autophosphorylated proteins have been identified in the matrix of mitochondria from mammals (Lambeth et al. 1997, Bradford and Yeamen 1986) and plants (Rubin and Randall 1977, Miernyk et al. 1992). One third of all detected/identified plant protein kinases showed autophosphorylation (Budde and Randall 1990). We suggest

Table 1 Features of ^{32}P labelling of specific inner membrane proteins of mitochondria

Mw	[α-^{32}P]ATP	[γ-^{32}P]ATP	Mg^{2+}	EDTA	$t_{1/2}$ (min)	pH[a]	Ser/Thr-P	His-P	Comments
16	−	+	+	−	4	6	yes	no	strongest labelled protein in IO-SMP[b]
16.5	−	+	+	−	nd	nd	nd	nd	autophosphorylated, putative kinase[b]
17	−	+	−	+	nd	nd	nd	nd	putative NDP K[b]
22	−	+	+	−	4	6.5–8	yes	no	F_1 δ'-subunit
28	−	+	+	−	2	8	yes	no	F_o b-subunit
30	−	+	+	+	nd	nd	nd	nd	autophosphorylated, putative protein kinase[b]
37	−	+	+	−	nd	nd	no	yes	acid labile protein
110	+	−	+	nd	nd	nd	no	no	adenylylated protein[c]

Summary of ^{32}P labelling of inner membrane proteins investigated in this study. +, stimulation and increased labelling; −, inhibition or no labelling; nd, not determined; Mw, apparent molecular mass in kDa.

[a] Optimum pH for labelling.

[b] See also text.

[c] AMP or ADP binding protein.

that the inner membrane 30 kDa autophosphorylated protein (Fig. 7) is a protein kinase, although it may also be the catalytic subunit of a larger multisubunit protein kinase. This would be consistent with the observation that the catalytic subunit of many protein kinases is approximately 30–45 kDa (Hanks et al. 1988).

The 16.5 kDa protein is autophosphorylated and therefore a putative kinase (Fig. 7). It is too small to agree with the current understanding of the size limitations of protein kinases (Hanks et al. 1988), and may therefore be a kinase for another class of substrate.

The 37 kDa phosphoprotein—The ^{32}P-labelled IO-SMP contain a 37 kDa phosphohistidine protein, as concluded from the acid-alkali stability measurements (Fig. 8). A 36 kDa phosphohistidine protein from rat mitochondria (Backer et al. 1986) and a 37 kDa phosphohistidine protein from pea leaf mitochondria (Håkansson and Allen 1995) have also been reported. Since phosphohistidine proteins are often involved in signal transduction pathways, and have been predicted to be present in the inner mitochondrial membrane (Allen 1993a), the 37 kDa inner membrane bound phosphohistidine protein may be a sensor kinase of the bacterial type two-component signal transduction pathway (Parkinson and Kofoid 1992).

The 17 kDa phosphoprotein—The 17 kDa inner membrane phosphoprotein was phosphorylated only in the absence of cations (Fig. 3). Noiman and Shaul (1995) showed that a 17 kDa NDPK protein was heavily phosphorylated in the presence of EDTA and Muhonen and Lambeth (1995) suggested that NDPK in rat liver mitochondria is membrane-bound. It is therefore possible that the 17 kDa EDTA-stimulated phosphoprotein (shown in Fig. 3) is a plant mitochondrial inner membrane bound NDPK.

The 16 kDa phosphoprotein—The 16 kDa phosphoprotein was the most heavily labelled protein in potato tuber inner mitochondrial membranes (Fig. 1). We can not exclude the possibility that this protein, in its unphosphorylated form, on SDS-PAGE migrates to an apparent size of 16.5 kDa and it may therefore be identical to the autophosphorylated 16.5 kDa protein (Fig. 7). It is interesting to note that Steenaart and Shore (1997) identified the most heavily labelled protein in their mitochondrial membrane fraction, a 17 kDa phosphoprotein, as cytochrome c oxidase subunit IV.

Perspectives

Protein phosphorylation and dephosphorylation are often involved in one or more steps in signal transduction pathways. These reactions are also ubiquitous as regulatory mechanisms which change the properties of target enzymes. The present paper describes the properties of a number of novel protein phosphorylation reactions in the inner membrane of plant mitochondria. The next step is to identify and characterize the kinases and phosphatases responsible for the phosphorylation/dephosphorylation. In parallel, the role of protein phosphorylation should be investigated by identifying the phosphoproteins and looking at possible changes in their properties or in the properties of the complexes in which they are subunits (e.g. the F_oF_1-ATPase subunits). Since the protein phosphorylation described here is redox-regulated (Fig. 2), it is tempting to speculate that some of the phosphoproteins are part of the respiratory chain and that electron transport is modified by the phosphorylation. Plant and fungal mitochondria contain specific enzymes, including the alternative oxidase (Vanlerberghe and McIntosh 1997) and alternative NAD(P)H dehydrogenases (Møller and Rasmusson 1998), and it would be of interest to know whether the protein phosphorylation events described here are specific to plant mitochondria, or of general importance in eukaryotic cells. In prokaryotes, two-component signal transduction pathways involve phosphorylation of a sensor kinase on histidine, and of a response regulator on aspartate (Parkinson and Kofoid 1992). The persistence, in evolution, of two-component redox signalling pathways (Allen 1993b) may be essential for the function of the genetic systems of mitochondria and chloroplasts (Allen 1993a, Race et al. 1999). The 37 kDa phosphohistidine protein identified here may be part of such a signal transduction pathway, allowing the respiratory chain to exert regulatory control over gene expression and other mitochondrial processes. Protein phosphorylation reactions such as those described here may thus be crucial for the integration of function, assembly and biogenesis of mitochondria.

We are grateful to Mrs Christina Nilsson and Miss Ineke de Jong for excellent technical assistance. This study was supported by grants from the Swedish Council for Planning and Coordination of Research (J.F.A.), the Swedish Natural Science Research Council (J.F.A. and I.M.M.) and The Royal Swedish Academy of Science (A.S.). The experiments were carried out by A.S. in collaboration with K.M.F. All authors provided ideas and interpreted results. The paper was written by A.S. with help of the other authors.

References

Allen, J.F. (1993a) Control of gene expression by redox potential and the requirement for chloroplast and mitochondrial genomes. *J. Theor. Biol.* 165: 609–631.

Allen, J.F. (1993b) Redox control of transcription: sensors, response regulators, activators and repressors. *FEBS Lett.* 332: 203–207.

Allen, J.F., Bennett, J., Steinback, K.E. and Arntzen, C.J. (1981) Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. *Nature* 291: 25–29.

Backer, J.M., Arcoleo, J.P. and Weinstein, I.B. (1986) Protein phosphorylation in isolated mitochondria and the effects of protein kinase C. *FEBS Lett.* 200: 161–164.

Bradford, A.P. and Yeaman, S.J. (1986) Mitochondrial protein kinases and phosphatases. *In* Advances in Protein Phosphatases. Edited by

Merlvede, W. and Di Salvo, J. Vol. 3, pp. 73–106. Leuven Univ., Belgium.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Budde, R.J.A. and Randall, D.D. (1990) Protein kinases in higher plants. *In* Inositol Metabolism in Plants. Edited by Moore, D.J. and Boss, W.F. pp. 344–351.

Danko, S.J. and Markwell, J.P. (1985) Protein phosphorylation in plant mitochondria. *Plant Physiol.* 79: 311–314.

Duclos, B., Marcandier, S. and Cozzone, A.J. (1991) Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. *Methods Enzymol.* 201: 10–21.

Ferrari, S., Moret, V. and Siliprandi, N. (1990) Protein phosphorylation in rat liver mitochondria. *Mol. Cell Biochem.* 97: 9–16.

Ferrell, J.E., Jr. and Martin, G.S. (1991) Assessing activities of blotted protein kinases. *Methods Enzymol.* 200: 430–435.

Geahlen, R.L., Anostario, M., Jr., Low, P.S. and Harrison, M.L. (1986) Detection of protein kinase activity in sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* 153: 151–158.

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 241: 42–52.

Hutchcroft, J.E., Anostario, M., Jr., Harrison, M.L. and Geahlen, R.L. (1991) Renaturation and assay of protein kinases after electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. *Methods Enzymol.* 200: 417–423.

Håkansson, G. and Allen, J.F. (1995) Histidine and tyrosine phosphorylation in pea mitochondria: evidence for protein phosphorylation in respiratory redox signalling. *FEBS Lett.* 372: 238–242.

Kitagawa, Y. and Racker, E. (1982) Purification and characterization of two protein kinases from bovine heart mitochondrial membrane. *J. Biol. Chem.* 257: 4547–4551.

Kurkdjian, A. and Guern, J. (1989) Intracellular pH: measurement and importance in cell activity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40: 271–303.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lambeth, D.O., Mehus, J.G., Ivey, M.A. and Milavetz, B.I. (1997) Characterization and cloning of a nucleoside-diphosphate kinase targeted to matrix of mitochondria in pigeon. *J. Biol. Chem.* 272: 24604–24611.

Lidén, A., Sommarin, M. and Møller, I.M. (1987) Lateral heterogeneity in the inner mitochondrial membrane. *In* Plant mitochondria: structural, functional and physiological aspects. Edited by Moore, A.L. and Beechey, R.B. pp. 139–142. Plenum Press, New York.

McEntyre, J. [Editor] (1994) Special issue on protein phosphorylation. *Trends Biochem. Sci.* 19: 439–518.

Miernyk, J.A., Duck, N.B., David, N.R. and Randall, D.D. (1992) Autophosphorylation of the pea mitochondrial heat-shock protein homolog. *Plant Physiol.* 100: 965–969.

Møller, I.M. and Rasmusson, A.G. (1998) The role of NADP in the mitochondrial matrix. *Trends Plant Sci.* 3: 21–27.

Muhonen, W.W. and Lambeth, D.O. (1995) The compartmentation of nucleoside diphosphate kinase in mitochondria. *Comp. Biochem. Physiol.* 110: 221–223.

Noiman, S. and Shaul, Y. (1995) Detection of histidine-phospho-proteins in animal tissues. *FEBS Lett.* 364: 63–66.

Oda, K. and Hasunuma, K. (1994) Light signals are transduced to the phosphorylation of the 15 kDa proteins in *Neurospora crassa*. *FEBS Lett.* 345: 162–166.

Papa, S., Sardanelli, A.M., Cocco, T., Speranza, F., Scacco, S.C. and Technikova-Dobrova, Z. (1996) The nuclear-encoded 18 kDa (IP) AQDQ subunit of bovine heart complex I is phosphorylated by the mitochondrial cAMP-dependent protein kinase. *FEBS Lett.* 379: 299–301.

Parkinson, J.S. and Kofoid, E.C. (1992) Communication modules in bacterial signalling proteins. *Annu. Rev. Genet.* 26: 71–112.

Petit, P.X., Sommarin, M., Pical, C. and Møller, I.M. (1990) Modulation of endogenous protein phosphorylation in plant mitochondria by respiratory substrates. *Physiol. Plant.* 80: 493–499.

Pical, C., Fredlund, K.M., Petit, P.X., Sommarin, M. and Møller, I.M. (1993) The outer membrane of plant mitochondria contains a calcium-dependent kinase and multi phosphoproteins. *FEBS Lett.* 336: 347–351.

Pike, C.S., Kopecek, K., Russel, J.P. and Sceppa, E.A. (1991) Regulation of phosphorylation in mitochondria of etiolated oat shoots. *Plant Physiol. Biochem.* 29: 565–572.

Race, H.L., Herrmann, R.G. and Martin, W. (1999) Why have organelles retained genomes? *Trends Genet.* 15: 364–370.

Race, H.L. and Hind, G. (1996) A protein kinase in the core of photosystem II. *Biochemistry* 35: 13006–13010.

Randall, D.D., Miernyk, J.A., Fang, T.K., Budde, R.J.A. and Schuller, K.A. (1989) Regulation of the pyruvate dehydrogenase complex in plants. *In* Alpha-Keto acid Dehydrogenase Complex: Organization, Regulation, and Biomedical Ramification. Edited by Roche, T.E. and Patel, M.S. Vol. 573, pp. 192–205. Annals of the New York Acad. Sci., New York.

Rasmusson, A.G. and Møller, I.M. (1991) NAD(P)H dehydrogenases on the inner surface of the inner mitochondrial membrane studied using inside-out submitochondrial particles. *Physiol. Plant.* 83: 357–365.

Rodríguez-Zapata, L.C. and Hernández-Sotomayor, S.M.T. (1998) Evidence of protein-tyrosine kinase activity in *Catharantus roseus* roots transformed by *Agrobacterium rhizogenes*. *Planta* 204: 70–77.

Rubin, P.M. and Randall, D.D. (1977) Regulation of plant pyruvate dehydrogenase complex by phosphorylation. *Plant Physiol.* 60: 34–39.

San Agustin, J.T. and Witman, G.B. (1995) Detection of flagellar protein kinases on polyvinylidene difluoride membranes following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *In* Methods in Cell Biology. Edited by Dentler, W. and Witman, G. Vol. 47, pp. 135–140. Academic press, London.

Sommarin, M., Petit, P.X. and Møller, I.M. (1990) Endogenous protein phosphorylation in purified plant mitochondria. *Biochim. Biophys. Acta* 1052: 195–203.

Steenaart, N.A.E. and Shore, G.C. (1997) Mitochondrial cytochrome *c* oxidase is phosphorylated by an endogenous kinase. *FEBS Lett.* 415: 294–298.

Struglics, A., Fredlund, K.M., Møller, I.M. and Allen, J.F. (1998) Two subunits of the F_oF_1-ATPase are phosphorylated in the inner mitochondrial membrane. *Biochem. Biophys. Res. Commun.* 243: 664–668.

Struglics, A., Fredlund, K.M., Rasmusson, A.G. and Møller, I.M. (1993) The presence of a short redox chain in the membrane of intact potato tuber peroxisomes and the association of malate dehydrogenase with the peroxisomal membrane. *Physiol. Plant.* 88: 19–28.

Technikova-Dobrova, Z., Sardanelli, A.M. and Papa, S. (1993) Phosphorylation of mitochondrial proteins in bovine heart. Characterization of kinases and substrates. *FEBS Lett.* 322: 51–55.

Tullberg, A., Håkansson, G. and Race, H.L. (1998) A Protein tyrosine kinase of chloroplast thylakoid membranes phosphorylates light harvesting complex II proteins. *Biochem. Biophys. Res. Commun.* 250: 617–622.

Vanlerberghe, G.C. and McIntosh, L. (1997) Alternative oxidase: from gene to function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 703–739.

Vardanis, A. (1976) Protein kinase activity at the inner membrane of mammalian mitochondria. *J. Biol. Chem.* 252: 807–813.

(Received July 5, 1999; Accepted October 13, 1999)