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# Two Subunits of the $F_oF_1$-ATPase Are Phosphorylated in the Inner Mitochondrial Membrane

André Struglics,* Kenneth M. Fredlund,†,[1] Ian M. Møller,† and John F. Allen*,[2]
*Plant Cell Biology, Lund University, Box 7007, S-220 07 Lund, Sweden; and
†Plant Physiology, Lund University, Box 117, S-221 00 Lund, Sweden

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**Inside-out submitochondrial particles from potato tuber mitochondria were incubated with [$\gamma$-$^{32}$P]ATP. More than 16 phosphorylated polypeptides were detected by autoradiography on an SDS-gel. Two phosphoproteins, migrating at 22 and 28 kDa, were excised from the SDS-gel, electroeluted, and purified further by anion chromatography. The phosphoproteins were N-terminally sequenced. Over the regions sequenced, the 22 and 28 kDa phosphoproteins had 100% sequence identity with potato proteins identified as the $\delta$'-subunit of the $F_1$-ATPase and the *b*-subunit of the $F_o$-ATPase, respectively. We suggest that phosphorylation of these proteins may control the interaction between $F_1$ and $F_o$ and regulate energy coupling in oxidative phosphorylation.** 

***Key Words:*** **mitochondria; inner mitochondrial membrane; protein phosphorylation; $F_oF_1$-ATPase.**

Except for the two well characterised protein phosphorylation systems of pyruvate dehydrogenase and branched-chain $\alpha$-oxoacid dehydrogenase (1), the role of protein phosphorylation in mitochondria is poorly understood. Phosphorylation of a number of proteins has been demonstrated in mammalian mitochondria. Of these, an 18 kDa phosphoprotein in bovine heart mitochondria was identified as a complex I subunit (2), and an 17 kDa phosphoprotein in rat heart mitochondria was identified as cytochrome c oxidase subunit IV (3), but the other phosphoproteins remain unidentified (4,5).

Protein phosphorylation in mitochondria from different plant species and tissues has also been reported (6–8), but the plant mitochondrial phosphoproteins are unidentified and their regulatory role is not known. Previous work on protein phosphorylation in plant mitochondria has been carried out on intact mitochondria prepared from potato tubers where inner membrane phosphoproteins are not easily distinguished from outer membrane phosphoproteins and from the strongly labelled $\alpha$-subunit of pyruvate dehydrogenase located in the matrix (7,9). After subfractionation of plant mitochondria, several phosphoproteins are detectable in the inner membrane (10).

In the present study two phosphoproteins were purified from inside-out submitochondrial (inner membrane) particles from potato tuber. N-terminal sequences revealed identity with two ATPase subunits, the $F_1$ $\delta$'-subunit in plants (also termed the $\epsilon$-subunit in bacteria) and the $F_o$ *b*-subunit.

## MATERIALS AND METHODS

Mitochondria were isolated and purified from potato tubers (*Solanum tuberosum L.* cv. Bintje) as in (11). Inside-out submitochondrial particles depleted of matrix proteins and respiratory substrates (12), were purified as in (10). After protein estimation using IgG as standard (13), a large batch (18.8 mg protein) of inside-out submitochondrial particles (SMP) was phosphorylated in order to provide starting material for the purification of phosphoproteins. The SMPs were incubated for 18 min with 0.2 mM ATP and 0.25 $\mu$Ci/$\mu$l [$\gamma$-$^{32}$P]ATP (AA0068 Amersham) in the presence of 0.3 M sucrose, 50 mM Hepes-KOH, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM $K_3Fe(CN)_6$ (pH 7.5) in a total volume of 3.25 ml. The reaction was stopped by diluting the suspension with ice-cold 50 mM Hepes-KOH, pH 7.5 and the membranes were pelleted at 248 000×g for 45 min. The proteins of the pelleted membranes were solubilised (14) and heated for 3 min at 100°C, samples were loaded on two 16 × 16 × 1.5 cm preparative SDS-polyacrylamide gels (T = 10-15%, C = 2.6%), both with wells 13.7 cm wide. The proteins were separated with a Protean II xi Slab Cell (BioRad) apparatus.

After a brief staining with Coomassie (R250), a 22 and 28 kDa phosphopolypeptide were cut out. The gel slices were placed inside 3.5 kDa cut off dialysis membranes (Spectrum) and electroeluted at 150 mA for 15.5 h with electroelution buffer (50 mM Tris-acetate, pH 7.8, 0.1% w/v SDS) (15). After electroelution the proteins were concentrated in 10 kDa microconcentrators (Filtron) and Triton X-

[1] Present address: Novartis Seeds AB, Box 302, S-261 23 Landskrona, Sweden.

[2] Corresponding author. Fax: +46-46-2223684. E-mail: john.allen@plantcell.lu.se.

Abbreviations used: Ig G, immunoglobulin G; FPLC, fast protein liquid chromatography; PVDF, polyvinylidene difluoride.

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100 was added to give a final concentration of 0.1% (w/v). The proteins were placed inside Spectra/Por 4 membranes (Spectrum) and dialysed against Buffer A (20 mM Tris-HCl, 0.1% (v/v) Triton X-100, pH 8 at 23°C). A portion (8% v/v) of the total amount of each electroeluted phosphoprotein was run on SDS minigels (BioRAD) and analysed by silver staining (Silver stain plus, BioRad) and autoradiography (Hyper film MP, Amersham). The remaining dialysed proteins were applied to a anion column (Mono Q HR 5/5, Pharmacia) using a FPLC system (Pharmacia), the column was first washed with 10 ml Buffer A and proteins were eluted with a linear 15 ml 0-0.35 M NaCl gradient in Buffer B (1 M NaCl, 20 mM Tris-HCl, pH 8 at 23°C), followed by a linear 25 ml 0.35-1 M NaCl gradient and a final wash of the column with Buffer B. The FPLC system was run at 8°C, 1 ml/min, and 1 ml fractions were collected. Mono Q fractions were desalted on PD10 columns (Pharmacia) and concentrated in a speedvac. A portion (15%) of the total volume from the different Mono Q fractions was run on SDS-minigels, the gels were silver stained, and after drying, put on autoradiographic film.

Mono Q fractions containing phosphoproteins [33-39 for the 28 kDa protein (see Fig. 3B), and fractions 21-22 for the 22 kDa protein (not shown)] were pooled and run on a 0.75 mm SDS-gel (T = 10-15%, C = 2.6%), and the proteins were electrophoretically transferred to a 0.2 $\mu$m PVDF membrane (PALL) using a Tris-glycine transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) and a Multiphore II NovoBlot unit (Pharmacia) with 1.6 mA/cm$^2$ for 2.5 h. The membrane was washed 5 × 20 min in double-destilled $H_2O$, dried and stained with sulforhodamine B [0.005% (w/v) sulforhodamine B (Sigma), 30% methanol, 0.2% acetic acid]. After exposure to a phosphorimager plate (Molecular Dynamics) the proteins were excised and N-terminally sequenced with an Applied Biosystem protein sequencer (Model 473A).

## RESULTS AND DISCUSSION

*Isolation of two phosphoproteins.* Highly purified intact mitochondria free from plastids (amyloplasts) and peroxisomes as shown in (11) were subfractionated, and inside-out inner membrane vesicles lacking any substrates or matrix proteins were prepared as in (10). Incubation with ATP for 18 min resulted in maximum phosphorylation of the inner mitochondrial membrane proteins (result not shown). By separating the proteins using a broad-well preparative SDS-gel, about 40 Coomassie-stained polypeptides of 14-104 kDa can be distinguished (Fig. 1A). The autoradiograph of the same gel allowed us to distinguish at least 16 phosphorylated polypeptides (Fig. 1B). None of the phosphoproteins was labelled when [$\alpha$-$^{32}$P]ATP was used instead of [$\gamma$-$^{32}$P]ATP in the assays (results not shown), which indicates that the labelling in Fig. 1B arises from covalently bound phosphate rather than to bound nucleotides. Pical et al. (1993) detected phosphorylation of only three polypeptides in inner membranes prepared from potato mitochondria. The more effective labelling reported here may be the result of longer phosphorylation time. The labelled band at about 16-17 kDa (Fig. 1) may correspond to subunit IV of cytochrome c oxidase, indicating that this protein is phosphorylated in both plant and animal mitochondria (3).

From the preparative gel (Fig. 1A) two phosphoproteins at molecular weights of 22 and 28 kDa were cut out, electroeluted, dialysed, and a small fraction of each

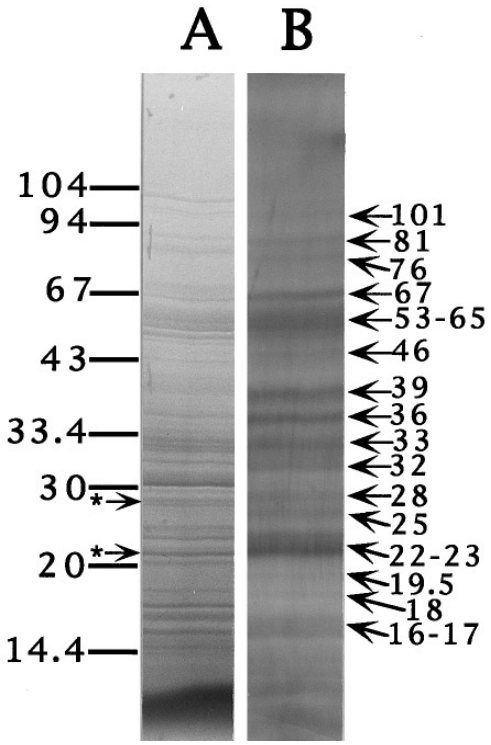


**FIG. 1.** A section of a preparative SDS-polyacrylamide gel with Coomassie blue stained inner mitochondrial membrane proteins from potato tubers (A) and corresponding autoradiograph with proteins labelled by incubation with [$\gamma$-$^{32}$P]ATP (B). The 28 and 22 kDa Coomassie stained proteins are marked with asterisks in A. The arrows in B indicate phosphoproteins in kDa, and the size of molecular weight markers are shown on the left.

protein was run on a second SDS-gel. From the silver-stained gel (Fig. 2A) and from the corresponding autoradiograph (Fig. 2B), it appears that the 28 kDa and possibly also the 22 kDa bands contain two polypeptides. We therefore carried out further purification.

The silver-stained SDS-gels and corresponding autoradiograph from the 28 kDa protein run on a Mono Q column, showed that the 28 kDa band seen in figure 2 contained two proteins (Fig. 3). Although two 28 kDa phosphoprotein-bands was seen in Fig. 2B, only one pure 28 kDa phosphoprotein was detected from the Mono Q column eluting in fractions 31-42 (Fig. 3A and 3B). The 28 kDa phosphoprotein was well separated from the unphosphorylated 28 kDa protein which eluted in fraction 19-30 (Fig. 3A and 3B).

The 22 kDa phosphoprotein from Fig. 2 was purified on the Mono Q column as described for the 28 kDa phosphoprotein and a similar pattern of an unphosphorylated protein and a phosphoprotein was observed (results not shown).

*Identification of the phosphoproteins.* The pooled Mono Q fractions containing phosphoproteins from each of the 22 and 28 kDa polypeptides were blotted

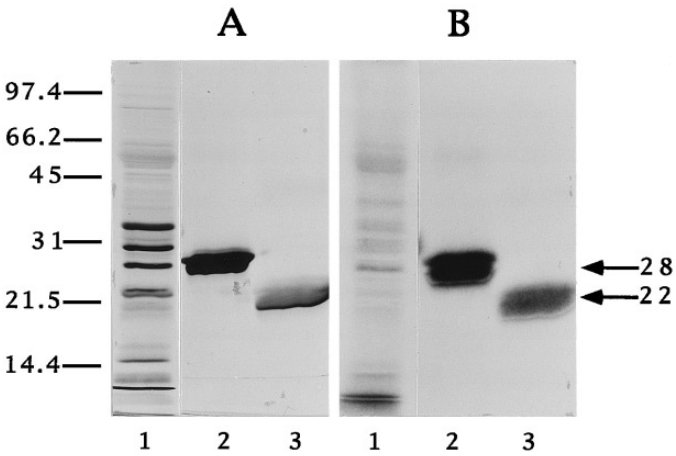


**FIG. 2.** Silver-stained SDS-polyacrylamide minigel (A) and the corresponding autoradiograph with proteins labelled by incubation with [$\gamma$-$^{32}$P]ATP (B). Lane 1, 25 $\mu$g inner mitochondrial membrane proteins; lane 2, 8% (v/v) of the total amount of electroeluted 28 kDa polypeptide; lane 3, 8% (v/v) out of the total amount of electroeluted 22 kDa polypeptide. Arrows indicate the positions of the 28 and 22 kDa proteins, and the molecular weight markers are shown in kDa on the left.

onto a PVDF membrane, and after staining with sulforhodamine B, the proteins were N-terminally sequenced.

The 22 and 28 kDa phosphoproteins both gave clear and homogenous 15 residue sequences, and these sequences were compared with other proteins in the SwissProt database (Figs. 4A and 4B). The 22 kDa and 28 kDa phosphoproteins both had a 100 % identity with a 19 kDa $F_1$ subunit and a 27 kDa $F_o$ subunit respectively from potato tubers (16).

The 37 amino acid N-terminal sequence of the 19 kDa potato $F_1$-subunit from Jänsch et al. (1996), has 46-70 % sequence identity to $F_1$ $\delta$'-subunits from other plant species (Fig. 4A). Mitochondrial $F_1$-ATPase purified from dicotyledons such as sweet potato (*Ipomoea batatis*), pea (*Pisum sativum*) and turnip (*Brassica napus*) consists of 6 subunits ($\alpha$, $\beta$, $\gamma$, $\delta$, $\delta$' and $\epsilon$), (17 and references therein). The $F_1$ $\delta$'-subunit from dicotyledons migrates with an apparent size of 22-23 kDa in SDS-gels, and the full-length $\delta$'-subunit amino acid sequence of sweet potato has 31 % and 36 % identity to the $\delta$-subunit of bovine and fungal mitochondria, respectively (17). We conclude that the 22 kDa phosphoprotein purified in the present paper is the $F_1$ $\delta$'-subunit of the ATPase in plant mitochondria corresponding to the $F_1$ $\delta$-subunit of mammalian $F_oF_1$-ATPase and to the $F_1$ $\epsilon$-subunit of bacterial $F_oF_1$-ATPase.

The 33 amino acid N-terminal sequence of the 27 kDa potato $F_o$-subunit from Jänsch et al. (1996), has about 60 % sequence identity with $F_0$ b-subunits from spinach and soybean (Fig 4B). The fractions containing the non-phosphorylated 28 kDa polypeptide (fractions 20-29 from the Mono Q column in Fig. 3) were also pooled and N-terminally sequenced. The sequence obtained (10 amino acids) was identical to that of the 28 kDa phosphoprotein (results not shown), and we therefore conclude that this is a non-phosphorylated form of the protein.

There is no N-terminal sequence similarity between the spinach 28 kDa $F_o$ protein and b-subunits from bacterial or mammalian $F_oF_1$-ATPases. However, both the spinach 28 kDa mitochondrial $F_o$ protein and a 27 kDa mitochondrial $F_o$ protein from potato tubers (probably identical to the 27 kDa protein of Jänsch et al. 1996) crossreact with antibodies raised against the b-subunit (also called PVP protein) of beef heart $F_oF_1$

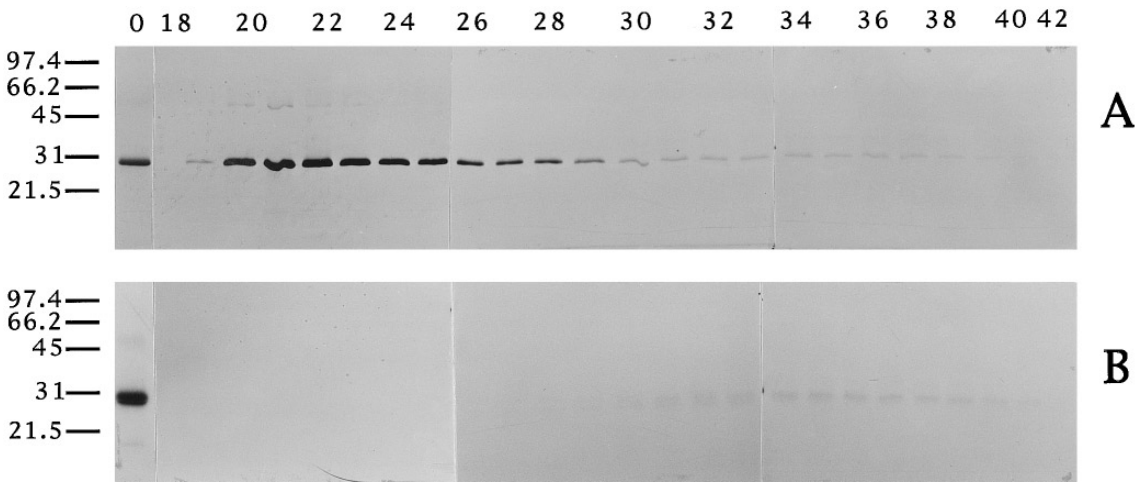


**FIG. 3.** SDS-polyacrylamide gel with fractions from the separation of the electroeluted 28 kDa polypeptide on a Mono Q column. 0, 4% of the total amount protein loaded on the column; fractions 18-42, 15% of the total amount protein of each fraction. A, silver-stained minigels; B, corresponding autoradiographs. Positions of the molecular weight markers are shown in kDa on the left.

**A**

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22 kDa phosphoprotein this study (1-15)          ATDLQAGYVADSNFT

                                                 1         10        20        30
19 kDa F1 protein from Solanum tuberosum (1-37)  ::::::::::::::EAWKKVVPNVDPPKTPSAFMXP
F1 δ'-subunit from Ipomoea batatis (1-37)        S:::P:ETA:::T:V:::::LI::::::::::Y:A:
F1 δ'-subunit from Arabidopsis thaliana (25-61)  :FSTELPSTL::T:V::::::A::M:::Q::::::K:
F1 δ'-subunit from Pisum sativum (18-55)         FSTDV:TPATN:S:V:::R::S::I::::::LE:LKT
F1 δ'-subunit from Brassica napus (1-30)         --XTEVPSTI::T:V::::::A::M:::Q:XI-----
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**B**

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28 kDa phosphoprotein this study (1-15)          AKEAAAPTTLKGDQV

                                                 1         10        20          30
27 kDa Fo protein from Solanum tuberosum (1-33)  ::::::::::::::LKDIFYEVKNKLETAIGV
ATP synthase subunit from Glycine max (32-64)    FAKES::PA::::EM::N::V:::::F:::::I
28 kDa Fo subunit from Spinacia olerecea (3-32)  XX::P::KG:::NEM::G::L:::K:F:::---
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**FIG. 4.** N-terminal alignment of the 22 kDa (A) and 28 kDa (B) inner mitochondrial membrane phosphoproteins with $F_1$ $\delta$'-subunits and $F_o$-subunits from plants. A colon represents an identical amino acid, X corresponds to unidentified amino acid, and bars indicates nonavailable sequence. References: 19 kDa $F_1$-subunit and 27 kDa $F_o$-subunit from *Solanum tuberosum* (16), *Ipomoea batatis* (23), *Arabidopsis thaliana* (24), *Pisum sativum* (25), *Brassica napus* (26), *Glycine max* (27), and *Spinacia olerecea* (18).

ATPase (18,19). We conclude that the 28 kDa phosphoprotein purified in the present paper is the $F_o$ b-subunit of the plant mitochondrial ATPase corresponding to the $F_o$ b-subunit of mammalian mitochondria and bacteria.

*Possible role of phosphorylation of ATPase subunits.* The structure of $F_1$-ATPase from bovine heart mitochondria has been resolved at 2.8 Å resolution, and the role of the individual subunits of the bacterial $F_oF_1$-ATPase are becoming clearer (20, 21). It is therefore of interest to look at the role of the $F_1$ $\epsilon$-subunit and the $F_o$ b-subunit in the catalytic process. The *E. coli* $F_1$ $\epsilon$-subunit interacts with the $F_o$ c-subunit, and the interaction is believed to be essential for energy coupling (21). The $F_o$ b-subunit of *E. coli* has both membrane-intrinsic and membrane-extrinsic domains and is thought to have a structural role in binding $F_1$ to $F_o$ (19, 21). Junge et al (1997) has suggested a model for the function of the $F_oF_1$-ATPase in which a central rotor is formed by a ring of c-subunits plus the subunits $\gamma$ and $\epsilon$, which rotate together within a stator composed of subunits a, $b_2$, $\delta$ and $(\alpha\beta)_3$ (22). We propose that the reversible phosphorylation of the $\epsilon$-subunit and the b-subunit described here controls the stability of the $F_o$-$F_1$ interaction and regulates energy coupling in the rotatory $F_o$-$F_1$ engine.

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