Identification of 14 new phosphoproteins involved in important plant mitochondrial processes

Natalia V. Bykova[1], Helge Egsgaard, Ian M. Møller*

Plant Research Department, Risø National Laboratory, Bldg. 301, P.O. Box 49, DK-4000 Roskilde, Denmark

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Abstract Protein phosphorylation is a very important post-translational modification the role of which is practically unexplored in mitochondria. Using two-dimensional gel electrophoresis followed by mass spectrometry, 14 new phosphoproteins are identified in potato tuber mitochondria, all household proteins also present in mammalian and fungal mitochondria. Seven of the new phosphoproteins are involved in the tricarboxylic acid cycle or associated reactions, four are subunits of respiratory complexes and involved in electron transport, ATP synthesis and protein processing, two are heat shock proteins and one is involved in defence against oxidative stress. These findings open up entirely new possibilities for the regulation and signal integration of mitochondrial processes.

Key words: Mitochondrion; Phosphorylation; Proteomics; Tricarboxylic acid cycle; Respiratory chain; Superoxide dismutase

1. Introduction

Phosphorylation of a protein can alter its behaviour in almost every conceivable way, including its intrinsic biological activity, subcellular location, half-life and interaction with other proteins. Therefore, it is an important factor in the integration of signals within the cell. Phosphorylation is the most common posttranslational modification of proteins and it is among the best studied processes involved in the regulation of cellular metabolism [1].

Surprisingly, only a few mitochondrial phosphoproteins have been identified to date and only in one case has the regulatory function of the phosphorylation been elucidated in mitochondria. The α subunit of the pyruvate dehydrogenase complex is reversibly phosphorylated – phosphorylation inactivates the enzyme complex and reduces the flow of carbon from glycolysis into the tricarboxylic acid cycle under conditions where the cell's requirements for ATP and carbon intermediates are satisfied [2].

In addition to the above protein, the following phosphoproteins have been identified in both mammalian and plant mitochondria: the b and δ′ subunits of the ATP synthase [3], the 70 kDa heat shock protein HSP70 [4], the small heat shock protein MTSHP [5] and nucleoside diphosphate kinase [6]. Phosphorylation of the E1α subunit of the branched-chain α-ketoacid dehydrogenase and the 18 kDa IP subunit of complex I [7] has so far only been detected in mammalian mitochondria. However, there are clearly more phosphoproteins present since labelling of intact mitochondria or submitochondrial fractions gives up to 30 labelled bands on one-dimensional (1D) gels [8–11].

It was the purpose of the present study to identify mitochondrial phosphoproteins by using two-dimensional (2D) gel electrophoresis to separate mitochondrial proteins followed by in-gel digestion and identification of the proteins by mass spectrometry (MS). We show that a number of well-known household proteins, involved in central metabolic processes such as the tricarboxylic acid cycle and ATP synthesis, are phosphorylated in potato tuber mitochondria.

2. Materials and methods

2.1. Chemicals, isolation and fractionation of intact mitochondria

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Mitochondria were isolated from potato tubers (*Solanum tuberosum* L.) [12] and subfractionated into inside-out inner membrane vesicles and a supernatant (the matrix fraction) as described [13].

2.2. Protein phosphorylation assay

Phosphorylation assays were carried out at 25°C for 10 min in a volume of 200 μl containing 1 mg protein, 0.3 M mannitol, 10 mM MOPS–KOH, pH 7.2, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM phenylmethylsulphonyl fluoride, 150 μCi [γ-^{32}P]ATP (AA0068, Pharmacia, Uppsala, Sweden) and 0.2 mM ATP. The reactions were terminated and excess [γ-^{32}P]ATP was removed either by addition of ice-cold reaction buffer (mitochondria and inner membranes) and centrifugation at 100 000 × *g* for 1 h at 4°C or by precipitation with 80% ice-cold acetone containing 35 mM mercaptoethanol (matrix fraction) followed by centrifugation at 10 000 × *g* for 20 min.

2.3. Construction and analysis of preparative 2D gels

In the first dimension either Blue Native (BN)–polyacrylamide gel electrophoresis (PAGE) [14], or isoelectric focusing (IEF)–PAGE was used, in both cases followed by gradient 10 to 15% Tricine sodium dodecyl sulphate (SDS)–PAGE in the second dimension. For BN–PAGE 1 mg protein was resuspended in 75 μl of 750 mM aminocaproic acid, 0.5 mM ethylenediaminetetra-acetate, 50 mM Bis-Tris, pH 7.0 supplemented with 15 μl of 10% (w/v) *n*-dodecyl maltoside. After centrifugation for 20 min at 100 000 × *g*, 15 μl of Coomassie

*Corresponding author. Fax: (45)-46 77 41 22.
E-mail address: ian.max.moller@risoe.dk (I.M. Møller).

[1] Present address: Department of Physics and Astronomy, University of Manitoba, 301 Allen Building, Winnipeg, MB, Canada R3T 2N2.

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; BN, Blue Native; FDH, formate dehydrogenase; HSP, heat shock protein; IEF, isoelectric focusing; SMP, inside-out submitochondrial particles

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blue solution (5% Serva Blue G, 750 mM aminocaproic acid) was added to the supernatant and loaded onto a 4.5–16% acrylamide gradient gel. The matrix fraction was supplemented with the same buffer and loaded directly onto BN gels. Electrophoresis was carried out as described [15].

The IEF was conducted according to the instructions in [16]. Mitochondrial and inner membrane proteins were solubilised in 300 μl rehydration solution containing 7 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), 2% (w/v) *n*-dodecyl maltoside, 20 mM dithiothreitol, 1% (v/v) IPG buffer pH 3.0–10.0 (Pharmacia, Uppsala, Sweden). For matrix proteins, *n*-dodecyl maltoside was omitted and 4% CHAPS used. Tricine SDS–PAGE was carried out as described [17]. The p*I* and molecular mass scales of the 2D maps were calibrated using the ImageMaster 2D Elite software (Amersham Pharmacia Biotech). The ^{32}P-containing phosphoproteins were visualised by phosphorimaging using Quantity One software (Bio-Rad).

2.4. Mass spectrometry for protein identification

Phosphoproteins were excised from the Coomassie-stained gels and MS/MS analysis of peptides generated by in-gel digestion [18] was performed on a Finnigan LCQ (Classic) quadrupole ion storage mass spectrometer (San Jose, CA, USA) equipped with a nano-electrospray source (Protana Engineering, Odense, Denmark). Prior to MS analysis samples were purified on reversed-phase POROS R2 or OligoR3 (20–30 μm bead size, PerSeptive Biosystems, Framingham, CA, USA) nanocolumns [19] and eluted directly to the precoated borosilicate nano-electrospray needles (Protana Engineering). Sequence tags were interpreted from the nano-electrospray ionisation tandem mass spectrometry (nano-ESI-MS/MS) spectra using Mascot MS-MS Ions Search engine (http://www.matrix-science.com) and the latest versions of the NCBI non-redundant database. Peptide mass mapping was performed by recording matrix-assisted laser desorption/ionization (MALDI) mass spectra on a Reflex IV time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany). MALDI mass spectra were analysed using the software package m/z (Proteometrics, New York, NY, USA).

3. Results and discussion

To identify phosphoproteins, isolated intact potato tuber mitochondria or subfractions thereof were labelled by incu-

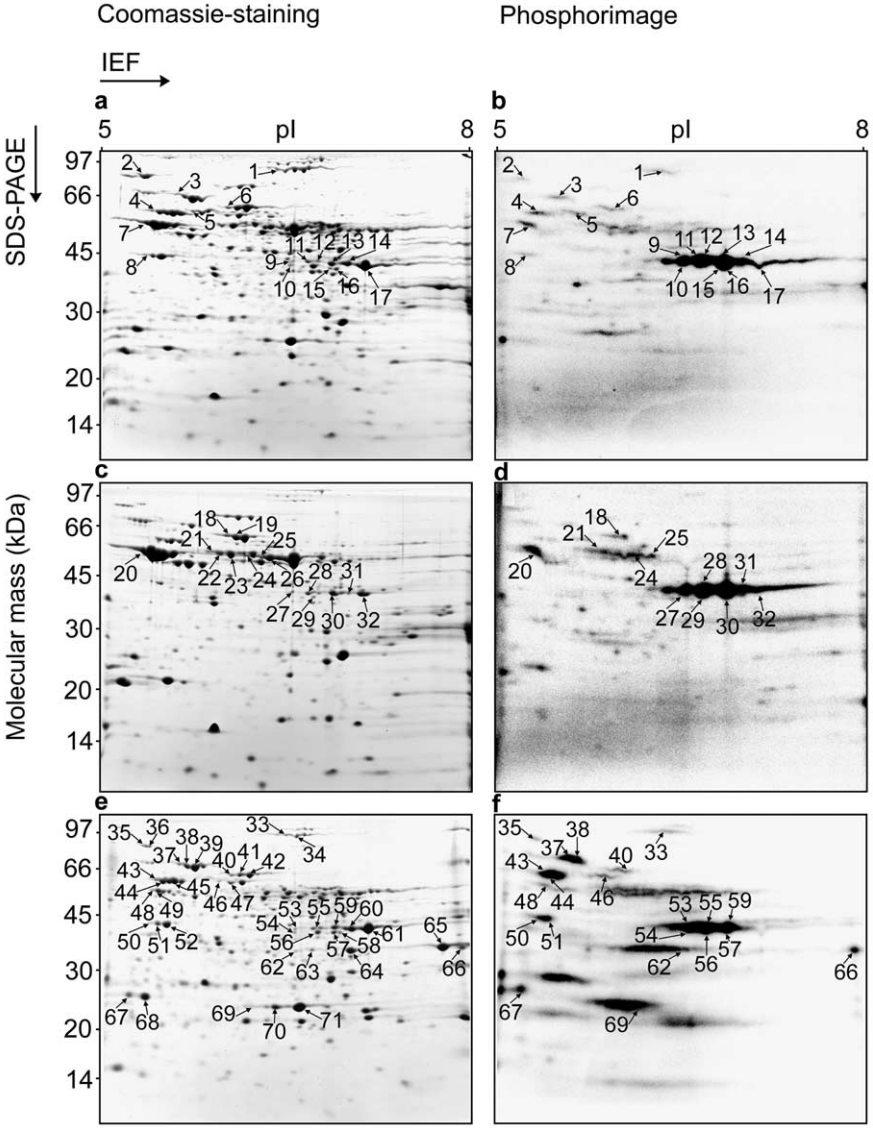

Fig. 1. 2D map of mitochondrial phosphoproteins from potato tubers resolved on IEF/SDS–PAGE 2D gels. Coomassie-stained total mitochondrial proteins (a) and corresponding phosphorimage (b). Coomassie-stained inner membrane proteins (c) and corresponding phosphorimage (d). Coomassie-stained matrix proteins (e) and corresponding phosphorimage (f). The numbers on the left refer to the molecular masses of the standard proteins (in kDa).

bating with [γ-^{32}P]ATP. After stopping the reaction, proteins in the samples were separated by two different types of 2D gel electrophoresis, proteins were visualised by Coomassie staining and phosphoproteins were visualised by phosphorimaging. Labelled and unlabelled spots were cut out and identified mainly by nano-ESI-MS/MS analysis. The gels are shown in Figs. 1 and 2 and the identity of the numbered protein spots shown in Table 1.

In these experiments, phosphorylation is dependent on the presence of kinases (and phosphatases) in the fractions. Since a number of labelled spots were observed on all the gels, kinases are clearly present and active not only in intact mitochondria (Fig. 1a,b), but also in the inner membrane fraction (Figs. 1c,d and 2a,b) and the matrix fraction (Figs. 1e,f and 2c,d) consistent with previous results obtained using 1D gels [8,10].

The 2D phosphoprotein patterns obtained by IEF–PAGE for the inner membrane and the matrix add nicely to give the pattern for intact potato tuber mitochondria (Fig. 1). The polypeptide pattern observed for the inner membrane fraction on BN–PAGE (Fig. 2a,b) is very similar to that reported [20], who identified the complexes as indicated. The polypeptide pattern for the matrix fraction on BN–PAGE (Fig. 2c,d) indicates that the matrix contains complexes that co-migrate under the conditions in the first dimension. Formate dehydrogenase (FDH; EC 1.2.1.2) and the E1α subunit of the pyruvate dehydrogenase complex (EC 1.2.4.1) can be seen to migrate in complexes of almost the same size (Fig. 2c,d, spots 79–80).

For most/all of the identified phosphoproteins, there is much less phosphorylated than unphosphorylated isoform. An example of this is FDH where the largest spot 61 was unlabelled (Fig. 1e,f). This could be due to phosphatases in the fractions so that the labelling we observe is the result of an equilibrium between phosphorylation and dephosphorylation.

The specific labelling of the phosphoproteins varies particularly on BN–PAGE where the phosphorylated and unphosphorylated forms of the proteins are not separated. For instance, the prominent spot 74 (which is known to contain only β-core subunit of complex III [20]) is only weakly labelled whereas the weak spot 75 is strongly labelled (Fig. 2a,b). The former protein may therefore have a low degree of phosphorylation under our experimental conditions. Alternatively, it was already phosphorylated before the incubation with [γ-^{32}P]ATP so that most phosphorylation sites were occupied by unlabelled phosphate. In fact, when unlabelled fractions

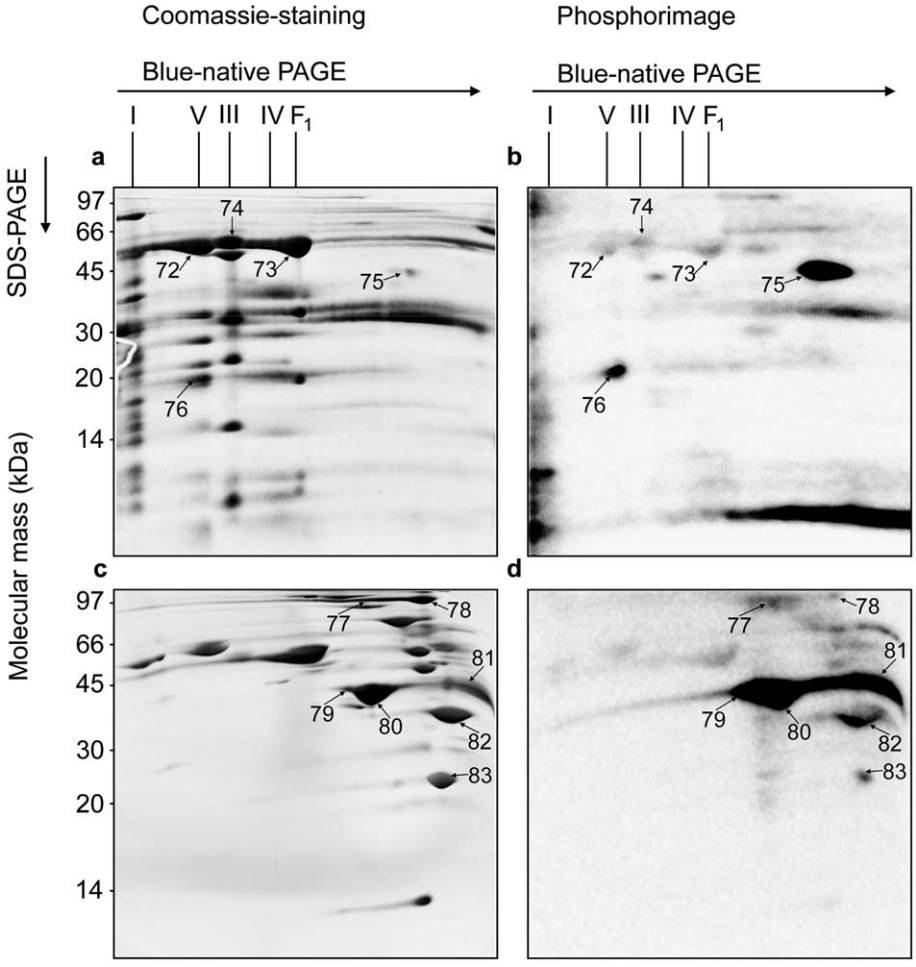

Fig. 2. 2D map of mitochondrial protein complexes resolved on BN/Tricine SDS–PAGE gels. Coomassie-stained inner membrane protein complexes (a) and corresponding phosphorimage (b). The designations on the top indicate the identity of the membrane protein complexes: I, NADH dehydrogenase; V, F_0F_1 ATP synthase complex; III, bc_1 complex; IV, cytochrome c oxidase; F_1, F_1 part of the ATP synthase complex [15]. Coomassie-stained matrix protein complexes (c) and corresponding phosphorimage (d). The sizes of standard proteins are given on the left.

Table 1A
Proteins identified by mass spectrometry: nano-ESI-MS/MS

Identity	Spot #	Localisation	Gel	DB #	Taxonomy	MW/p*I*	ST #	Cover %
(a) Tricarboxylic acid cycle and associated enzymes								
Pyruvate dehydrogenase E1α subunit	**9**, **11**, **12**	Mi	IEF	P52903	SOLTU	43/6.5–6.7	2, 4, 4,	6, 14, 14
	13, **27**, **28**	Mi/SMP	IEF			43/6.5–6.8	13, 2, 9	45, 10, 30
	30, **31**, **32**	SMP	IEF			43/6.8–7.1	13, 3, 13	45, 12, 46
	75, **79**	Ma	BN			43	1, 3	3, 11
Aconitate hydratase	**1**, **33**, 34	Mi/Ma	IEF	O04916	SOLTU	87/6.3–6.5	5, 2, 7	18, 8, 20
	77, **78**	Ma	BN			87	2, 3	8, 10
NAD-isocitrate dehydrogenase	**10**, **16**, **29**	Mi/SMP	IEF	T07167	LYCES	42/6.5–6.9	4, 7, 7	15, 25, 25
Succinyl-CoA-ligase α subunit	**66**	Ma	IEF	NP_197716	ARATH	35/8.0	3	15
Succinyl-CoA-ligase β subunit	**8**, **50**,	Mi/Ma	IEF	NP_179632	ARATH	45/5.3	3, 2	11, 6
	51, 52	Ma	IEF			44/5.4–5.5	3, 4	11, 14
NAD-malate dehydrogenase	**62**, 64	Ma	IEF	AAD56659	GLYMA	35/6.6–7.0	2, 6	9, 26
	63	Ma	IEF	T09286	MEDSA	35/6.7	4	17
	65	Ma	IEF	AAF69802	VITVI	35/7.8	4	24
	82	Ma	BN	T07167	GLYMA	35	3	14
NAD-malic enzyme, 62 kDa isoform	**5**, **46**	Mi/Ma	IEF	P37221	SOLTU	62/5.7–5.9	5, 5	12, 14
NAD-malic enzyme, 59 kDa isoform	**46**, 47	Ma	IEF	P37225	SOLTU	61/5.9–6.0	8, 11	25, 30
Formate dehydrogenase	**13**, **14**, **15**	Mi	IEF	CAA79702	SOLTU	43/6.8–7.0	7, 11, 7	33, 53, 31
	17, **31**, **32**	Mi/SMP	IEF			42/7.0–7.1	7, 7, 12,	36, 40, 57
	61	Ma	IEF			41/7.2	8	40
	79, **80**, **81**	Ma	BN			41–43	15, 12, 3	70, 60, 18
(b) Membrane respiratory complexes								
Succinate dehydrogenase flavoprotein	**6**, **18**, 19	Mi/SMP	IEF	NP_201477	ARATH	65/6.0–6.1	8, 4, 5	22, 9, 12
α subunit	**40**, 41, 42	Ma	IEF			64/6.0–6.2	4, 8, 6	12, 21, 16
bc_1 complex, β-MPP subunit	**21**, 22, 23	SMP	IEF	CAA56519	SOLTU	57/5.8–6.0	4, 4, 5	8, 12, 17
	74	SMP	BN			59	[15]	
ATP synthase α subunit	**24**, **25**, 26	SMP	IEF	P05495	NICPL	55/6.2–6.4	3, 5, 6	7, 14, 17
	72, 73	SMP	BN			55–57	[15]	
ATP synthase β subunit	**7**, **20**	Mi/SMP	IEF	P17614	NICPL	57/5.3–5.4	13, 10,	37, 29,
	48, 49	Ma	IEF			57/5.4–5.5	8, 10	21, 29
	72, 73	SMP	BN			55–57	[15]	
(c) Heat shock proteins								
Putative heat shock protein 90	**2**, **35**, 36	Mi/Ma	IEF	NP_187434	ARATH	82/5.3–5.4	2, 4, 3	3, 7, 5
Heat shock protein 70	**3**, **37**	Mi/Ma	IEF	Q08276	SOLTU	71/5.6	15, 7,	36, 16,
	38, 39	Ma	IEF			69/5.7–5.8	13, 16	28, 33
Chaperonin 60	**4**, **43**	Mi/Ma	IEF	Q05046	CUCMA	63/5.4	7, 6	19, 16
	44, 45	Ma	IEF			62/5.4–5.6	5, 7	13, 16
Heat shock protein MTSHP	**67**, 68	Ma	IEF	T04316	LYCES	25/5.2–5.3	2, 3	10, 22
(d) Oxidative stress defense								
Mn superoxide dismutase	**69**, 70, 71	Ma	IEF	P11796	NICPL	22/6.2–6.6	1, 1, 3	5, 8, 27
	83	Ma	BN			23	2	18

Table 1B
Proteins identified by mass spectrometry: MALDI-TOF MS

Identity	Spot #	Localisation	Gel	DB #	Taxonomy	MW/p*I*	MP #	Cover %
Pyruvate dehydrogenase E1α	**53**, **55**	Ma	IEF	P52903	SOLTU	42/6.6–6.8	5, 18	15, 30
subunit	**59**, 60	Ma	IEF			41/6.9–7.0	27, 8	44, 20
Formate dehydrogenase	**55**, **57**	Ma	IEF	CAA79702	SOLTU	41/6.7–6.9	13, 17	36, 64
	59, 60	Ma	IEF			41/6.9–7.0	13, 30	32, 80
NAD-isocitrate dehydrogenase	**53**, **54**	Ma	IEF	T07167	LYCES	41/6.6	5, 9	18, 26
	56, 58	Ma	IEF			40/6.8–7.0	9, 8	27, 25

Radiolabelled spots are shown in bold. Localisation of proteins in total mitochondrial fraction (Mi); matrix fraction (Ma); isolated submitochondrial particles (SMP). The type of 2D gel electrophoresis was either BN/Tricine SDS–PAGE (BN) or IEF/Tricine SDS–PAGE (IEF). The accession numbers represent protein entries in NCBI (DB #). Apparent molecular masses in kDa (MW) and isoelectric points (p*I*) for proteins from the gels in the figures are given. No. of sequence tags (ST) identified by nano-ESI-MS/MS analysis and Mascot MS/MS Ions Search engine matching at <1D and individual ion scores indicating identity or extensive homology. Minimum percentage of coverage for proteins with transit peptide intact was calculated. No. of matching tryptic peptides (MP) identified by MALDI-TOF covered $>15\%$ at 50 ppm of the precursor protein.

were separated by IEF–PAGE, the Coomassie-stained polypeptide pattern was very similar to that of labelled fractions (not shown) indicating that most of the labelled spots are already substantially phosphorylated in vivo. In a previous study we showed that only a 110 kDa protein was labelled when isolated submitochondrial particles (SMP) were incubated with [α-^{32}P]ATP [12]. Thus, none of the phosphoproteins identified in the SMP fraction could have been labelled by binding intact ATP.

Of the eight known mitochondrial phosphoproteins, four –

E1α subunit of the pyruvate dehydrogenase [2], δ′ subunit of ATPase [3], HSP70 [4] and MTSHP [5] – were also found in potato tuber mitochondria (Table 1). Of the other four, two have so far only been detected in mammalian mitochondria – E1α subunit of the branched-chain α-ketoacid dehydrogenase [2] and the 18 kDa IP subunit of complex I in mammalian mitochondria [7] – and we could not identify them in this study.

Table 1 lists 14 hitherto unknown mitochondrial phosphoproteins that were identified by MS using the Mascot search engine. They fall into four groups. (1) Seven enzymes belonging to or associated with the tricarboxylic acid cycle - aconitate hydratase (EC 4.2.1.3), the β subunit of succinate-CoA ligase (EC 6.2.1.5), NAD-isocitrate dehydrogenase (EC 1.1.1.41), malic enzyme (EC 1.1.1.39), the two subunits of NAD-malate dehydrogenase (EC 1.1.1.37) and FDH (EC 1.2.1.2). (2) Four subunits of the respiratory complexes – the flavoprotein of succinate dehydrogenase (EC 1.3.5.1), the β-MPP core subunit of complex III (EC 1.10.2.2) and the α and β subunits of the F_0F_1-ATPase (EC 3.6.3.14). (3) Two HSPs – HSP60 and HSP90. (4) One enzyme involved in defence against oxidative stress – superoxide dismutase (EC 1.15.1.1). All of these proteins are nuclear-encoded in mammalian mitochondria, and only the α subunit of the ATP synthase is mitochondrially encoded in plant mitochondria [21].

The mitochondrion contains many hundreds and possibly more than 2000 polypeptides [22]. Thus, a single radiolabelled spot on a gel can easily contain two polypeptides one of which is phosphorylated but not identified by MS because its amount is below the detection threshold whereas the other more abundant protein is unlabelled but identified by MS. For this reason we applied three stringency criteria in our identification of phosphoproteins: (i) The presence of a necklace of spots all containing the same protein with the most acidic one(s) labelled since phosphorylation will lower the isoelectric point of a protein. An example is superoxide dismutase found in spots 69–71 in the matrix fraction, where only spot 69 was labelled (Fig. 1e,f). There is, in fact, substantial labelling on the acidic side of spot 69, but no detectable Coomassie staining, so no identification was attempted. However, it is likely that this is also superoxide dismutase probably with several phosphate groups attached. (ii) Labelling was observed in at least two of the three samples, intact mitochondria, inner membrane vesicles and matrix. An example is spot 4 in intact mitochondria (Fig. 1a,b) and spot 43 in the matrix fraction (Fig. 1e,f) which are both labelled chaperonin 60. (iii) The labelled phosphoprotein was identified on both IEF and BN 2D gels that separate proteins according to quite different properties. IEF separates individual proteins according to their respective isoelectric point, whereas BN separates protein complexes according to their size. In both cases SDS–PAGE was used to separate individual polypeptides according to their size in the second dimension. It is therefore very unlikely that a given phosphoprotein will co-migrate with the same contaminating polypeptide on both gels. An example of a phosphoprotein satisfying this third stringency criterion is malate dehydrogenase, which was identified in the labelled spot 62 on IEF–PAGE gels (Fig. 1e,f) and in labelled spot 82 on BN–PAGE gels (Fig. 2c,d). In some cases this third criterion is not satisfied in our study simply because the protein is not part of a larger protein complex and is therefore not seen on BN–PAGE gels (Fig. 2) or because it has an isoelectric point below pH 5 or above pH 8 and is not seen on the IEF–PAGE gels (Fig. 1).

Of the 14 new phosphoproteins, 11 satisfied at least two of the three stringency criteria and can therefore be considered to be very firm identifications. The two malic enzyme subunits satisfied two stringency criteria between them. So at least one is labelled, although we cannot tell which one at the moment. Finally, one labelled protein – the α subunit of succinyl-CoA-ligase – satisfied none of the stringency criteria and its identification is tentative only.

Our results indicate that protein phosphorylation is involved in a number of important mitochondrial processes:

- Regulation of the tricarboxylic acid cycle, and therefore intermediary metabolism, in a number of different places.
- Activation of complex II in the respiratory chain. Complex II has been shown to be activated by ATP in intact mitochondria by an unknown mechanism [23].
- The relative activity of electron transport and protein processing in complex III. The β-MPP core subunit of complex III is also the mitochondrial processing peptidase that detaches signal peptides from proteins imported into the matrix of plant mitochondria [24].
- The activity of the mitochondrial ATP synthase through interaction with a 14-3-3 protein. This interaction is dependent on phosphorylation of the complex and results in a drastically reduced activity [25].
- The function of the mitochondrial HSPs. Two mitochondrial HSPs were previously reported to be phosphorylated [4,5], and we here extend the list by identifying HSP90 and chaperonin 60 as phosphoproteins.
- The detoxification of reactive oxygen species by superoxide dismutase. The mitochondrial electron transport chain is one of the major sites of production of reactive oxygen species in the cell and mitochondrial superoxide dismutase is one of the enzymes involved in the removal of reactive oxygen species [26].

The phosphoprotein pattern of mitochondria from the model plant *Arabidopsis thaliana* is very similar to that shown in Fig. 1b (H. Eubel and H.-P. Braun, personal communication). This indicates that the same mitochondrial proteins are phosphorylated in potato and *Arabidopsis* and that this is a conserved function. To what extent mitochondrial protein phosphorylation is also working in other organisms remains to be seen. An in silico analysis showed that plant mitochondria have considerably more proteins predicted to belong to the group 'signal transduction' than do fungal or mammalian mitochondria (J.L. Heazlewood and A.H. Millar, personal communication). Thus, plant mitochondria may contain more kinases and phosphatases, and therefore more phosphoproteins, than mitochondria from other organisms. However, the results presented here provide clear evidence that protein phosphorylation is likely to be one of the fundamental mechanisms for signal integration of the complex network of mitochondrial processes.

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