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# Identification of serine phosphorylation in mitochondrial uncoupling protein 1

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ABSTRACT

Native uncoupling protein 1 was purified from rat brown adipose tissue of cold-acclimated rats and rats kept at room temperature, in the presence of phosphatase inhibitors. The purified protein from cold-acclimated animals was digested with trypsin and immobilized metal affinity chromatography was used to select for phosphopeptides. Tandem mass spectroscopic analysis of the peptides derived from uncoupling protein 1, suggests phosphorylation of serine 3 or 4 and identified phosphorylation of serine 51. Furthermore, we were able to demonstrate that antibodies to phosphoserine detect full-length UCP 1 and that the proportion of phosphoserine on UCP1, purified from cold-acclimated rats, was significantly greater than that on UCP 1 from rats kept at room temperature (90±4% compared to 62±8%, $p=0.013$), respectively). We conclude that uncoupling protein 1 is a phosphoprotein and that cold-acclimation increases the proportion of UCP1 that is serine phosphorylated.



## 1. Introduction

Brown adipose tissue (BAT) is a site of heat production in mammals in response to cold stress. In BAT, dissipation of the proton electrochemical gradient across the mitochondrial inner membrane by uncoupling protein 1 (UCP 1; also known as UCP and thermogenin) results in a futile cycle of proton pumping and proton leak, a process that defines the molecular basis of non-shivering thermogenesis in mammals [1–3].

Evidence from work on isolated mitochondria and reconstituted native or expressed UCP 1 show that UCP 1 activity is fatty acid dependent and inhibited by binding of purine nucleotides [1–6]. Furthermore, our understanding of the UCP 1 function in isolated mitochondria appears to be paralleled in situ in cells. Evidence demonstrates that increased oxygen consumption by isolated brown adipocytes, following treatment with noradrenalin, results from a (cytoplasmic) protein kinase A triggered release of fatty acids from intracellular stores [1–3,7]. These fatty acids provide reducing equivalents for mitochondrial oxygen consumption, participate directly in UCP 1 function and release purine nucleotide inhibition of UCP 1 in situ. Interestingly, Bronnikov et al. [8] have shown that the stimulatory effect of noradrenalin, on brown adipocyte oxygen consumption, via the β-adrenergic receptor, is antagonized by the simultaneous binding of noradrenalin to the α-adrenergic receptor, via a calcium stimulated increase in phosphodiesterase (PDE) activity. Inhibition of the α-receptor augments the β-receptor response. These observations have been further interpreted by Lee et al. [9], who have demonstrated that dephosphorylation of tyrosine 304 on cytochrome oxidase subunit 1, increases cytochrome oxidase activity, presumably by calcium activated tyrosine phosphatases, and there are data that calcium-dependent dephosphorylation of mitochondrial proteins, in general, result in increased mitochondrial activity [10].

UCP1, like all mitochondrial inner membrane transporters characterized so far, is predicted to have a tripartite structure [4]. The sites of purine nucleotide and fatty acid binding to UCP 1 have been investigated. Photoaffinity studies suggest that residues in the third domain and the C-terminus are required for purine nucleotide binding [4], while chimeric studies have predicted that the central domain is required for fatty acid binding [11]. To date, there has been no evidence for covalent modification of UCP1 in the literature, however, there is in vitro evidence that polyunsaturated fatty acid oxidation products, such as 4-hydroxy-2-nonenals, can activate UCP function directly and presumably by covalent modification [12].

Our interest in investigating the possibility of covalent modification of UCP 1 stemmed from our discovery that thymocytes contain UCP 1 [13–16] and that thymocytes isolated from cold-acclimated animals have decreased oxygen consumption rates when compared to those from room-temperature animals [17]. Our premise was that

*Abbreviations:* ACN, acetonitrile; BAT, brown adipose tissue; BSA, bovine serum albumin; FBS, foetal bovine serum; HTP, hydroxyapatite; IMAC, Immobilized Metal Affinity Chromatography; Octyl-POE, octylpentaoctylethylene ether; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; UCP, uncoupling protein

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covalent modification of UCP 1 might affect function. Our starting point for the investigation was native UCP 1 from BAT, from which substantial amounts of the protein can be purified.

## 2. Materials and methods

### 2.1. Animal source

Wistar rats were bred and obtained from the Bioresources Unit, School of Biochemistry and Immunology, Trinity College, Dublin. Cold-acclimated rats (30) were housed in pairs and kept in a temperature controlled room set at 8±2 °C for 4 weeks prior to use. All animals were allowed free access to laboratory chow and water. All animals were sacrificed by $CO_2$ asphyxiation.

### 2.2. Isolation of brown adipose tissue mitochondria

BAT mitochondria were prepared by homogenization followed by differential centrifugation according to the method of Scarpace et al. [18]. To conserve the physiological phosphorylation status, the mitochondrial isolation buffer was supplemented with 10 mM KF and 1 mM sodium orthovanadate.

### 2.3. Purification of UCP 1 from BAT mitochondria

Purification of UCP 1 was performed using a hydroxyapatite (HTP) column chromatography procedure as described by Lin and Klingenberg [19] with slight modification. Intact BAT mitochondria (8–10 mg) suspended in STE buffer (250 mM Sucrose, 5 mM Trizma, 2 mM EGTA; 10 mM KF, 1 mM sodium orthovanadate; pH 7.4) were centrifuged at 22,600 ×g for 10 min at 4 °C. The mitochondrial pellet was solubilized in 13% (v/v) octylpentaoctylethylene ether (Octyl-POE) in STE buffer (total volume ~500 μl) and incubated on ice for 10 min prior to loading the solubilized mitochondrial proteins onto an hydroxyapetite (HTP) column. The HTP column was prepared by soaking 0.34 g of HTP in 10 ml of STE buffer (supplemented with 10 mM KF and 1 mM activated sodium orthovanadate) pH 7.4, at 4 °C, for 6 h prior to pouring it into a 1 ml column (Bio-Rad) (diameter 1 cm, length 6 cm). The soaking solution was removed by centrifugation at 800 ×g for 2 min at room temperature immediately prior to loading of the mitochondrial proteins. The HTP column, containing the solubilized mitochondria, was incubated at room temperature for 10 min (to denature the adenine nucleotide carrier), followed by a 25 min incubation at 4 °C. The column was then centrifuged at 800 ×g for 2 min to remove the HTP elute (UCP 1 enriched fraction). The protein concentration of this HTP elute was determined and the octyl-POE detergent was removed using a Biobead (Bio-Rad) column. The Biobead column was prepared by placing 2 ml of the Biobeads (previously suspended in distilled/deionised-$H_2O$), into a 2 ml syringe barrel and allowed to flow by gravity to pack the column bed. The $H_2O$ was removed from the Biobeads by centrifugation at 800 ×g for 2 min. The Biobeads were then equilibrated in ~2 ml of STE buffer (supplemented with 10 mM KF and 1 mM activated sodium orthovanadate), pH 7.4, for 30 min prior to use. Biobeads were further centrifuged at 800 ×g for 2 min to remove excess STE buffer, pH 7.4. The HTP elute was then loaded onto Biobeads, mixed gently, and incubated for two-hours at 4 °C with slight agitation using a vortex. The Biobead column was then centrifuged at 800 ×g for 2 min and the pure protein elute was collected. The protein concentration of this elute was determined [20].

### 2.4. SDS-PAGE and Western blot analysis

One-dimensional SDS-PAGE under reducing conditions was used to separate proteins prior to immunoblot analysis, as described by Cunningham et al. [21] Following SDS-PAGE, resolved proteins were transferred onto polyvinylidene difluoride membranes (Immobilin-$P^{SQ}$; Millipore) as described by Cunningham et al. (18). Commercial anti-UCP 1 (amino acids 145–159) was purchased from Calbiochem. Commercial anti-phosphoserine antibody was purchased from Chemicon International. The antibodies were all used at 1:1000 dilution. Following blocking with 5% bovine serum albumin and overnight primary antibody incubation, the blots

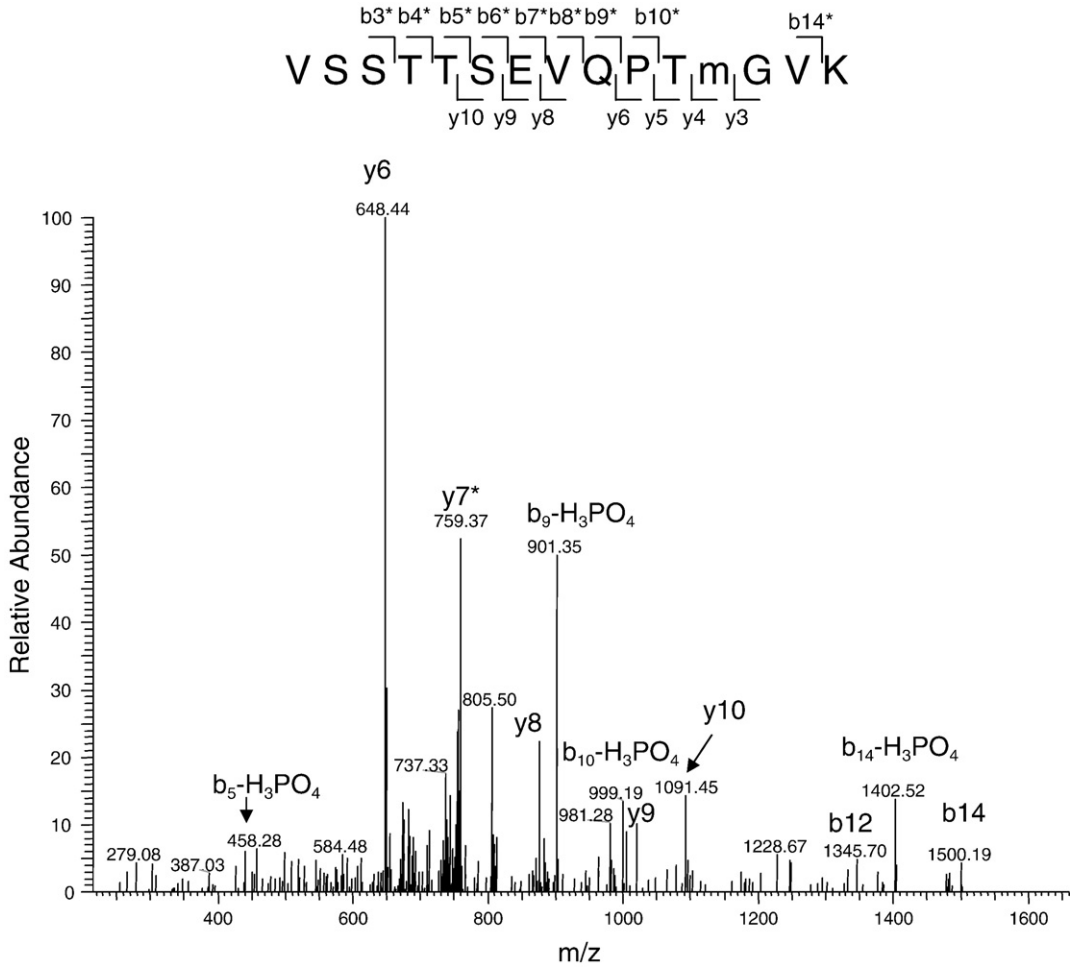


**Fig. 1.** Identification of serine phosphorylation at residue 3 or 4 of purified native rat UCP 1 by tandem mass spectroscopy. The data show the MS/MS spectrum for the peptide V(ss)TTSEVQPTmGVK (*m*/*z*=815.8754) equivalent to residues 2–16 of rat UCP 1 highlighting the phosphorylated serine site(s) at position 2 or 3 of the peptide, equivalent to residues 3 or 4 of UCP 1. s represents a phosphorylated serine and m represents an oxidised methionine residue. The fragment ions in the MSMS spectrum are highlighted and b* represents a b-$H_3PO_4$ ion and y represents a y ion.

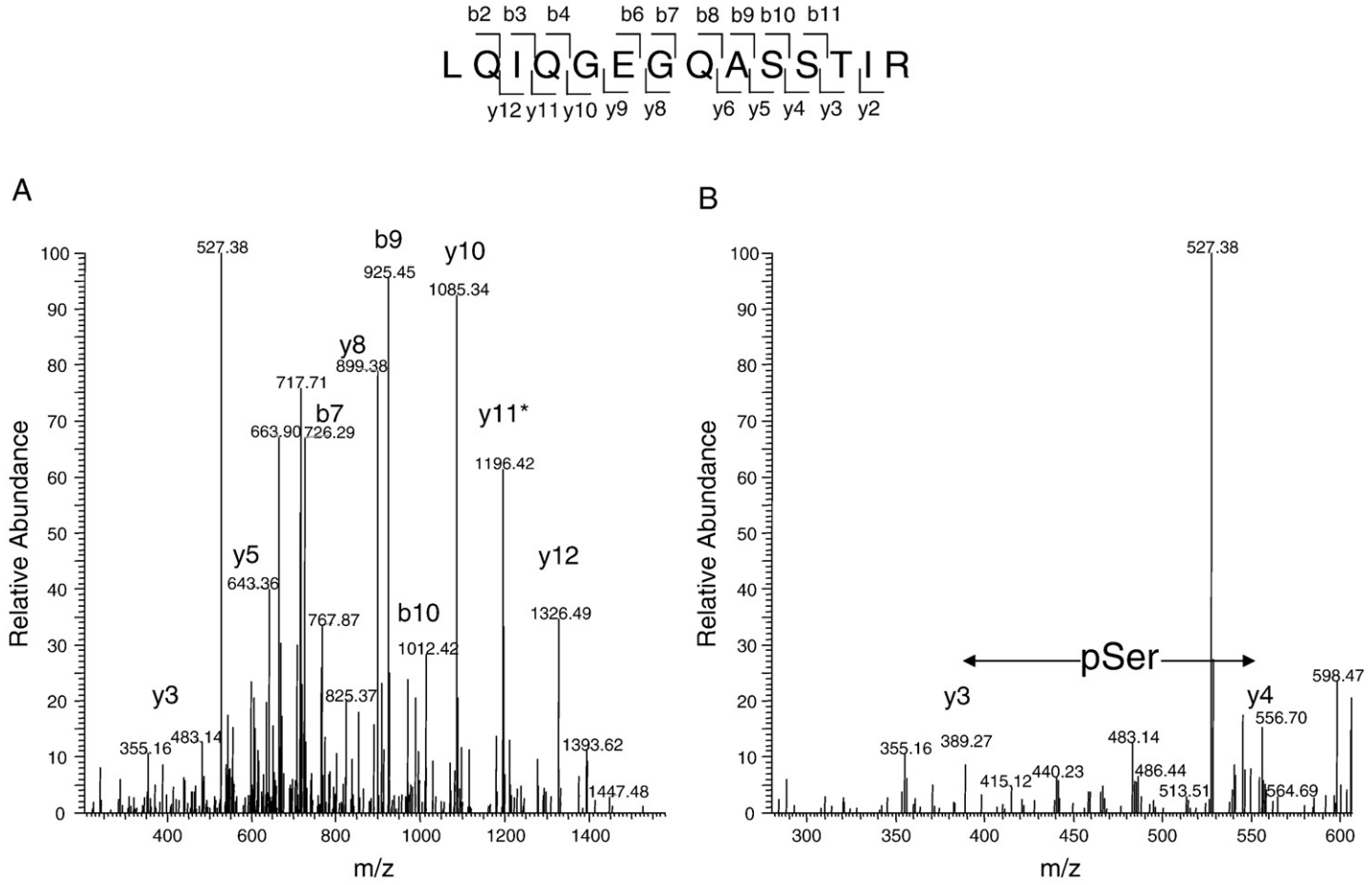


**Fig. 2.** Identification of serine phosphorylation at residue 51 of purified native rat UCP 1 by tandem mass spectroscopy. (A) The MS/MS spectrum for the peptide ion LQIQGEGQASpSTIR ($m/z$=784.3784) equivalent to residues 41–54 of rat UCP 1 highlighting the phosphorylated serine (pS) site at position 11 of the peptide, equivalent to residue 51 of UCP1. (B) The mass range 280–610 has been expanded to show the y4 and y3 ions pinpointing the site of phosphorylation to serine 51.

were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000) in Tris-buffered saline (TBS), 0.5% (v/v) Tween 20, 5% (w/v) bovine serum albumin for 1 h at room temperature. Blots were developed using an ECL detection system (Amersham Biosciences), and immunoreactions were visualized by exposure to Kodak X-Omat LS film.

### 2.5. Dephosphorylation of UCP 1

10 μg of purified UCP 1 protein was incubated with shrimp alkaline phosphatase (8 U, Promega) overnight at 37 °C.

### 2.6. Sample preparation for mass spectrometry

One-dimensional SDS-PAGE under reducing conditions was also used to examine UCP 1 purity after hydroxyapatite chromatography. Proteins were detected by staining gels with Coomasie Brilliant Blue R-250. This stain is compatible with the post-staining processing required for mass spectrometry. UCP 1 protein bands at 32.5 kDa were trimmed, excised and transferred to a fresh minifuge tubes. Gel pieces were washed for 3×30 min with 500 μl of 50% acetonitrile (ACN)/25 mM ammonium bicarbonate, pH 8. Following this, gel pieces were dehydrated in 500 μl 100% ACN for 5 min at room temperature. Gel pieces were dried in Speedvac (Savant Instruments Inc) for 30 min. Gel pieces were rehydrated with 10–15 μg/ml ice-cold trypsin solution (Promega Sequencing Grade Modified Trypsin) in 25 mM ammonium bicarbonate, pH 8 and left overnight in 37 °C water-bath. Gel slices were then soaked in ~30 μl of 50% ACN/5% trifluoroacetic acid (TFA) for 60 min with gentle agitation. The supernatant was transferred to a second minifuge tube. The gel was extracted again with another ~30 μl of 50% ACN/5% TFA for 60 min. The two extracts were combined and completely dried in Speedvac for 1 h. Dried extracts were stored at-80 °C prior to use.

### 2.7. Immobilized Metal Affinity Chromatography (IMAC)

The phosphopeptide content of UCP 1 protein were enriched using a PHOS-Select™ Iron Affinity Gel (Sigma). The Fe(III) chelate matrix provides high capacity affinity binding of molecules containing phosphate groups. The tryptic digested gel pieces were reconstituted with 250 mM acetic acid with 30% ACN. The PHOS-Select Iron Affinity Gel beads were mixed carefully and 80 μl of the 50% slurry (40 μl gel) were immediately added to a clean SigmaPrep Spin column (Sigma) that has been placed in a collection tube. The PHOS-Select Iron gel beads were washed three times with 500 μl of 250 mM acetic acid/30% ACN, vortexed and centrifuged at 8200 ×g for 30 s in an Eppendorf® 5417R microcentrifuge. 500 μl of the sample solution was added to the equilibrated gel column and incubated for 30 min at room temperature using end-over-end rotation (miniroller; LABNET). After incubation, the column was centrifuged at 8200 ×g for 30 s and the flow-through was discarded (containing unbound peptides). The column was then washed with 500 μl of 250 mM acetic acid/30% ACN, followed by a wash with 500 μl deionised/distilled water (to remove any residual washing solution) prior to elution. To elute enriched phosphopeptides, the column was incubated with 200 μl of 400 mM ammonium hydroxide solution for 5 min with mixing and centrifuged as above. The flow-through liquid was retained for phosphopeptide analysis. A similar protocol was essentially described by Lee et al [22].

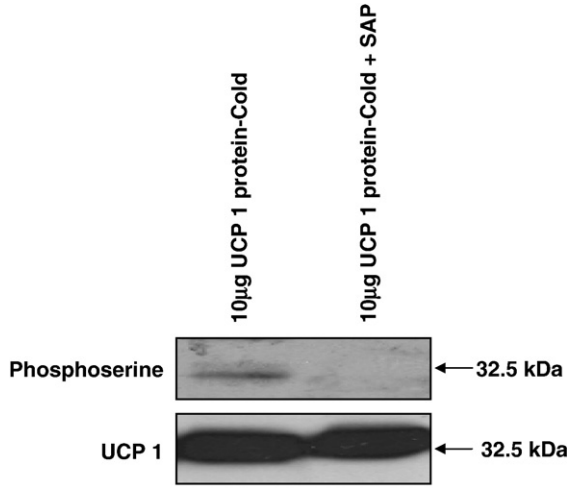


**Fig. 3.** Immunoblot detecting serine phosphorylation of purified native rat UCP 1. Sample immunoblot using an anti-phosphoserine antibody (Chemicon International) and anti-UCP 1 peptide antibody (Calbiochem) to UCP 1. Native UCP 1 protein has been purified from BAT mitochondria from cold-acclimated rats. Purified UCP 1 protein yields a positive signal with the anti-phosphoserine antibody, whereas overnight treatment with SAP (Shrimp alkaline phosphatase) eliminates that signal. Purified UCP 1 protein yields a positive signal with the anti-UCP 1 peptide antibody (Calbiochem) with and without SAP treatment.

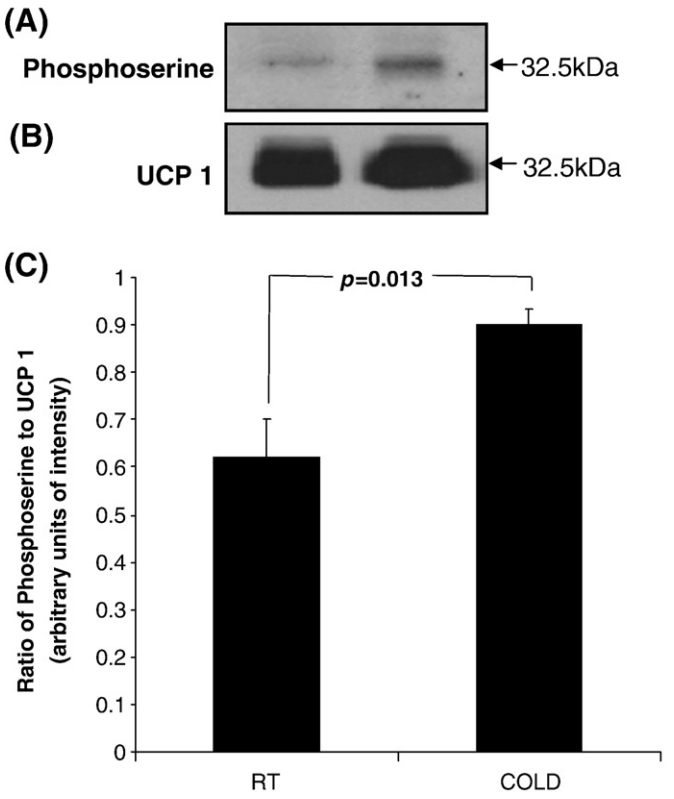


**Fig. 4.** Comparison of serine phosphorylation in purified UCP 1 (10 μg) from brown adipose tissue of cold-acclimated rats and rats kept at room temperature. Sample immunoblot of an anti-phosphoserine antibody (Chemicon International) (A), anti-UCP 1 peptide antibody (Calbiochem) (B) to purified UCP 1 protein isolated from room-temperature (RT) and cold-acclimated (cold) rats. The bar chart (C) shows the relative intensity of phosphoserine detection to UCP 1 detection, using both UCP 1 and phosphoserine antibodies, by densitometry for at least 5 separate preparations. The proportion of phosphoserine on UCP1, purified from cold-acclimated rats, was significantly greater than that on UCP 1 from rats kept at room temperature (90±4% compared to 62±8%, $p=0.013$), respectively). Data are expressed as mean±sem for $n=5$ separate experiments/purification for each condition.

### 2.8. Mass spectrometry analysis

An aliquot of the UCP1 tryptic digest prior to IMAC enrichment was analysed by LC–MS with precursor of 79 scanning on an Applied Biosystems 4000 Q-Trap system as described by Williamson et al [23]. After IMAC enrichment a further aliquot was analysed by LC–MS on a LTQ-orbitrap mass spectrometer system (ThermoElectron, Bremen, Germany) coupled to a Dionex 3000 nano-LC system. The peptide mixture was separated on an LC-Packings PepMap $C_{18}$ column (0.075×150 mm) equilibrated in 0.1% formic acid/water and eluted with a discontinuous gradient of acetonitrile/0.1% formic acid at a flow rate of 300 nl/min. The orbitrap was set to analyse the survey scans at 60,000 resolution and top 5 ions in each duty cycle, were selected for MSMS in the LTQ linear ion trap with multistage activation. The MSMS spectra were searched against SwissProt database using the Mascot search engine (Matrix Science) run on an in-house server using the following criteria; peptide tolerance=10 ppm, trypsin as the enzyme, carboxyamidomethylation of cysteine as a fixed modification with oxidation of methionine and phosphorylation of serine, threonine and tyrosine as a variable modification. Any MSMS spectra that could be assigned to a phosphopeptide were inspected manually using QualBrowser software (ThermoElectron, San Jose, CA).

## 3. Results

UCP-1 isolated from cold acclimated rats was separated by SDS-PAGE and digested with trypsin prior to analysis by LC–MS using precursor 79 scanning on a hybrid triple quadrupole linear ion trap mass spectrometer. This technique has been used extensively to identify phosphopeptides from phosphoprotein digests as described previously [23,24]. However the signals detected in our experiments were too weak to trigger MSMS events, suggesting the stoichiometry of phosphorylation of UCP1 was low (data not shown). A further

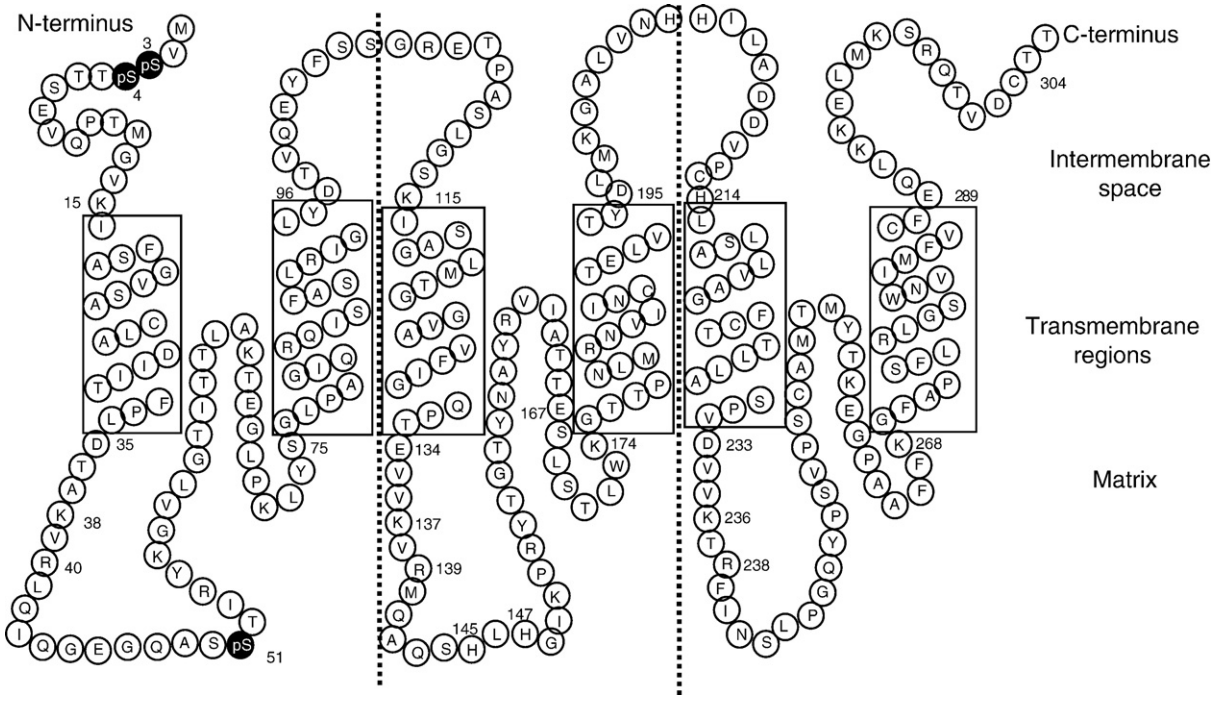


**Fig. 5.** Folding Diagram of uncoupling protein 1 from rat brown adipose tissue (BAT). The diagram represents a predicted organization of UCP 1 across the mitochondrial inner membrane (adapted from Klingenberg et al. [4]). The rectangles represent transmembrane regions. The "dotted-lines" help visualize the tripartite structure. The phosphorylated serines (residues 3/4 and 51) in native UCP 1 are highlighted (pS).

aliquot of the UCP-1 digest was purified by immobilised metal affinity chromatography on PHOS-select resin as described previously [25] and the purified sample analysed by LC–MS on a high resolution LTQ-orbitrap mass spectrometry system. To obtain the best MSMS spectra of phosphopeptide ions, multistage activation of the precursors was employed where the precursor ion [M] and the M-98/z ion are activated at the same time to yield a composite $MS^2/MS^3$ spectrum. This second activation is based on the mass of $H_3PO_4$ (98 Da) which is primarily lost from phosphoserine and phosphothreonine containing peptide ions with a multiple charge ($z$) after collisional activation.

From the Mascot database search, two phosphopeptides were detected, namely residues 2–16 and 41–54 both containing a single phosphorylation (Tables 1 and 2). The tandem MSMS spectrum for residues 2–16 is shown in Fig. 1 and this peptide was detected in its methionine oxidised form with a precursor mass of $m/z$=815.8754. The tandem MSMS spectrum lacks the b2 and b3 ions which could pinpoint the phosphorylation site to either serine 3 or 4. However the spectrum does contain the b4-98 and b5-98 ions that rule out either threonine 5 or 6 being phosphorylated and in conjunction with the y ions serine 7 or threonine 12 are also not phosphorylated. Fig. 2 shows the MSMS spectrum of residues 41–54 which was detected with a precursor mass of $m/z$=784.3784. The MSMS spectrum clearly shows that serine 51 is phosphorylated as the mass between the y4 and y3 ions is 167 Da which is the mass of phosphoserine (Fig. 2B). There is also a y4-98 ion which is not annotated in this spectrum, which is further evidence that serine 51 is phosphorylated. This analysis has been repeated on an independent experiment from purified UCP1 peptides confirming the phosphorylation at serine 51 and either serine 3 or 4.

Fig. 3 shows UCP 1 purified from mitochondria isolated from brown adipose tissue of cold-acclimated rats, in the presence of phosphatase inhibitors. UCP 1 is identified using a UCP 1 peptide specific antibody. Furthermore, the UCP 1 band is detectable using antibodies to phosphoserine, the detection of which is abolished on treatment of UCP 1 with alkaline phosphatase.

Fig. 4 compares the phosphorylation state of UCP 1 purified from BAT of cold-acclimated rats, with that of rats kept at room temperature. UCP 1 (10 μg) purified from BAT of cold-acclimated rats and rats kept at room temperature was run on a single dimension denaturing gel and an immunoblotting was performed using antibodies to phosphoserine (Fig. 4A) and to UCP1 (Fig. 4B). Collated data show that the proportion of UCP 1 that was detected by the phosphoserine antibody was significantly ($p$=0.013) greater in UCP 1 purified from cold-acclimated rats (90±4%) compared to UCP 1 purified from rats kept at room temperature (62±8%) (Fig. 4C). Data in Fig. 4A and B are single representative samples of the 5 separate experiments/purifications for each condition that provided the data in Fig. 4C.

**Table 1**
NCBI BLAST search of VSSTTSEVQPTMGVK

| Score | Mr (calc) | Delta | Sequence |
|---|---|---|---|
| 46.0 | 1645.727036 | 0.003986 | VSSTTSEVQPTMGVK |
| 46.0 | 1645.727036 | 0.003986 | VSSTTSEVQPTMGVK |
| 37.7 | 1645.727036 | 0.003986 | VSSTTSEVQPTMGVK |
| 36.2 | 1645.727036 | 0.003986 | VSSTTSEVQPTMGVK |
| 25.7 | 1645.727036 | 0.003986 | VSSTTSEVQPTMGVK |
| 10.3 | 1645.708344 | 0.022678 | ASLSLETSESSQEAK |
| 7.6 | 1645.708344 | 0.022678 | ASLSLETSESSQEAK |
| 5.6 | 1644.700714 | 1.030308 | VAMSHFEPSEYIR |
| 0.2 | 1645.708344 | 0.022678 | ASLSLETSESSQEAK |

**Table 2**
NCBI BLAST search of LQIQGEGQASSTIR

| Score | Mr (calc) | Delta | Sequence |
|---|---|---|---|
| 64.6 | 1566.740280 | 0.001908 | LQIQGEGQASSTIR |
| 55.4 | 1566.740280 | 0.001908 | LQIQGEGQASSTIR |
| 51.5 | 1566.740280 | 0.001908 | LQIQGEGQASSTIR |
| 4.7 | 1566.740250 | 0.001938 | NQSDADLEALRKK |
| 3.3 | 1566.765396 | −0.023208 | KLITSEEERSPAK |

## 4. Discussion

Several studies have been published on the sub-mitochondrial phosphoproteomes and have demonstrated serine, threonine and tyrosine phosphorylation of mitochondrial matrix and membrane proteins [22,26–31]. To date, UCP 1 has never been registered in the literature as a protein that is covalently modified in any way and this is confirmed by the lack of reported phosphorylation sites in the phosphoELM database (http://phospho.elm.eu.org/) which contains over 16,000 phosphorylation sites. Our mass spectroscopy data demonstrates, for the first time, that native UCP 1, purified from mitochondria of brown adipose tissue of cold-acclimated rats, is phosphorylated at a serine residue at position 51 (Fig. 2) and suggests serine phosphorylaton position 3/4 (Fig. 1). We were also able to show that full-length UCP 1 is phosphorylated using antibodies to phosphoserine (Fig. 3) and that the proportion of purified UCP 1 phosphorylated on serine, is greater in cold-acclimated rats, when compared to UCP 1 purified from rats kept at room temperature (Fig. 4).

Thus, our data suggest that increased phosphorylation of UCP 1 correlates with increased activity of UCP 1, with the obvious assumption being that UCP 1 is more active in cold-acclimated animals. Our data are thus consistent with reported phosphorylation events following noradrenalin stimulation of BAT cells [8], but at odds with data for calcium mediated dephosphorylation of electron transport chain protein complex subunits associated with activation of electron transport chain activity [9,10].

Predicted topology for UCP 1 places serine residues 3/4 in the intermembrane space side of the mitochondrial inner membrane, whereas serine residue 51 in located on the matrix side of the inner membrane [Fig. 5]. One possibility is that different kinases act at these two distinct sites. NetPhos analysis of the sequence around residue serine 51 of UCP 1, scores highly for a CDC2/CDK (cyclin dependent kinase 2) and protein kinase C [32]. The possibility of kinases unique to brown fat mitochondria may also be a possibility.

It should be noted that no site directed mutagenesis studies performed on UCP 1 focused on these residues [4]. Clearly, future endeavours will determine whether there is functional or regulatory significance to these covalent modifications.

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## References

[1] D.G. Nicholls, R.M. Locke, Thermogenic mechanism in brown fat, Physiol. Rev. 64 (1984) 1–64.
[2] B. Cannon, J. Nedergaard, Brown adipose tissue: function and physiological significance, Physiol. Rev. 84 (2004) 277–359.
[3] D.G. Nicholls, The physiological regulation of uncoupling proteins, Biochim. Biophys. Acta 1757 (2006) 459–466.
[4] M. Klingenberg, K.S. Echtay, M. Bienengraeber, E. Winkler, S.G. Huang, Structure–function relationship in UCP 1, Int. J. Obes. Relat. Metab. Disord. Suppl. 6 (1999) S24–S29.
[5] K.D. Garlid, M. Jabůrek, P. Ježek, M. Vařecha, How do uncoupling proteins uncouple? Biochim. Biophys. Acta 1459 (2000) 383–389.
[6] E.P. Breen, S.G. Gouin, A.F. Murphy, L.R. Haines, A.M. Jackson, T.W. Pearson, P.V. Murphy, R.K. Porter, On the mechanism of mitochondrial uncoupling protein 1 function, J. Biol. Chem. 281 (2006) 2114–2119.
[7] S.C. Souza, M.A. Christoffolete, M.O. Ribeiro, H. Miyoshi, K.J. Strissel, Z.S. Stancheva, Ni.H. Rogers, T.M. D'Eon, J.W. Perfield, H. Imachi, M.S. Obin, A.C. Bianco, A.S. Greenberg, Perilipin regulates the thermogenic actions of norepinephrine in brown adipose tissue, Lipid Res. 48 (2007) 1273–1279.
[8] G.E. Bronnikov, S.-J. Zhang, B. Cannon, J. Nedergaard, A dual component analysis explains the distinctive kinetics of cAMP accumulation in brown adipocytes, J. Biol. Chem. 274 (1999) 37770–37780.
[9] I. Lee, A.R. Salomon, S. Ficarro, I. Mathes, F. Lottspeich, L.I. Grossmann, M. Hütteman, J. Biol. Chem. 280 (2005) 6094–6100.
[10] R.K. Hopper, S. Carrol, A.M. Aponte, D.T. Johnson, S. French, R.F. Shen, F.A. Witzmann, R.A. Harris, R.S. Balaban, Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium, Biochemistry 45 (2006) 2524–2536.
[11] J. Jimenez-Jimenez, A. Ledesma, P. Zaragoza, M.M. Gonzalez-Barroso, E. Rial, Fatty acid activation of the uncoupling proteins requires the presence of the central matrix loop from UCP 1, Biochim. Biophys. Acta 1757 (2006) 1292–1296.
[12] K.S. Echtay, T.C. esteves, J.L. Pakay, M.B. Jekabsons, A.J. Lambert, M. Portero-Otín, R. Pamploma, A.J. Vidal-Puig, S. Wang, S.J. Roebuck, M.D. Brand, A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling, EMBO J. 22 (2003) 4103–4110.
[13] A.M. Carroll, L.R. Haines, T.W. Pearson, C.M. Brennan, E.P. Breen, R.K. Porter, Immunodetection of UCP 1 in rat thymus, Biochem. Soc. Trans. 32 (2004) 1066–1067.
[14] A.M. Carroll, L.R. Haines, T.W. Pearson, P. Fallon, C. Walsh, C.M. Brennan, E.P. Breen, R.K. Porter, Detection of a functioning UCP 1 in thymus, J. Biol. Chem. 280 (2005) 15534–15543.
[15] R.K. Porter, A new look at mitochondrial uncoupling protein 1, Biochim. Biophys. Acta 1757 (2006) 446–448.
[16] Adams, O. Hanrahan, D.N. Nolan, H.P. Voorheis, P. Fallon, R.K. Porter, Images of mitochondrial UCP 1 in mouse thymocytes using confocal microscopy, Biochim. Biophys. Acta 1777 (2007) 115–117.
[17] C.M. Brennan, E.P. Breen, R.K. Porter, Cold acclimation and oxygen consumption in the thymus, Biochim. Biophys. Acta 1757 (2006) 1463–1468.
[18] P.J. Scarpace, B.S. Bender, S.E. Borst, *Eschericia coli* peritonitis activates thermogenesis in brown adipose tissue: relationship to fever, Can. J. Physiol. Pharmacol. 69 (1991) 761–767.
[19] C.S. Lin, M. Klingenberg, Isolation of the uncoupling protein from brown adipose tissue mitochondria, FEBS Lett. 113 (1980) 299–303.
[20] M.A. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1978) 206–210.
[21] O. Cunningham, A.M. McElligott, A.M. Carroll, E.P. Breen, C. Reguenga, M.E.M. Oliveira, J.E. Azevedo, R.K. Porter, Selective detection of UCP 3 expression in skeletal muscle: effect of thyroid status and temperature acclimation, Biochim. Biophys. Acta 1604 (2003) 170–179.
[22] J. Lee, Y. Xu, Y. Chen, R. Sprung, S.C. Kim, S. Xie, Y. Zhao, Mitochondrial phosphoproteome revealed by an improved IMAC method and MS/MS/MS, Mol. Cell. Proteomics 6 (2007) 669–676.
[23] B.L. Williamson, J. Marchese, N.A. Morrice, Automated identification and quantification of protein phosphorylation sites by LC/MS on a hybrid triple quadrupole linear ion trap mass spectrometer, Mol. Cell. Proteomics 5 (2006) 337–346.
[24] O. Goransson, M. Deak, S. Wullschleger, N.A. Morrice, A.R. Prescott, D.R. Alessi, Regulation of the polarity kinases PAR-1/MARK by 14-3-3 interaction and phosphorylation, J. Cell Sci. 119 (2006) 4059–4070.
[25] M. Beullens, S. Vancauwenbergh, N.A. Morrice, R. Derua, H. Ceulemans, E. Waelkens, M. Bollen, Substrate specificity and activity regulation of protein kinase MELK, J. Biol. Chem. 280 (2005) 40003–40011.
[26] M. Salvi, N.A. Morrice, A.M. Brunati, A. Toninello, Identification of the flavoprotein of succinate dehydrogenase and aconitase as in vitro mitochondrial substrates of Fgr tyrosine kinase, FEBS Lett. 581 (2007) 5579–5585.
[27] D.J. Pagliarini, J.E. Dixon, Mitochondrial modulation: reversible phosphorylation takes center stage? Trends Biochem. Sci. 31 (2006) 26–34.
[28] M. Thompson, Evidence for the undiscovered cell regulatory mechanisms; phosphoproteins and protein kinases in mitochondria, Cell. Mol. Life Sci. 59 (2002) 213–219.
[29] B. Schulenberg, R. Aggeler, J.M. Beechem, R.A. Capaldi, W.F. Patton, Analysis of steady-state protein phosphorylation in mitochondria using novel fluorescent phosphosensor dye, J. Biol. Chem. 278 (2003) 27251–27255.
[30] A.M. Sardinalli, A. Signorile, R. Nuzzi, D. De Rasmo, Z. Technikova-Dobrova, Z. Drahota, A. Occhiello, A. Pica, S. Papa, Occurrence of A-kinase anchor protein and associated cAMP-dependent protein kinase in the inner compartment of mammalian mitochondria, FEBS Lett. 580 (2006) 5690–5696.
[31] M. Huttemann, I. Lee, L. Samavati, H. Yu, J.W. Doan, regulation of mitochondrial oxidative phosphorylation through cell signaling, Biochim. Biophys. Acta 1773 (2007) 1701–1720.
[32] N. Blom, S. Gammeltoft, S. Brunak, Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites, J. Mol. Biol. 294 (1999) 1351–1362.